

Sustainable Development and Biodiversity

Sumita Jha
Mihir Halder *Editors*

Medicinal Plants: Biodiversity, Biotechnology and Conservation



Springer

Sustainable Development and Biodiversity

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Editors

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Preface

Biodiversity of medicinal plants is the well-recognized resource of structurally and functionally diverse and versatile natural therapeutic phytochemicals utilized almost irreplaceably in traditional, herbal, and modern medicines to combat multiple human diseases and to sustain human healthcare from prehistoric times. Apart from their application in the traditional systems of medicine and folk medicine, these plant-derived natural bioactive phytochemicals are recognized as a central pillar of modern medicine as one-fourth of all prescribed medicines and about three-quarters healthcare of the world's population in developing countries directly or indirectly rely on medicinal plants. Medicinal plants may play an important role in finding solutions to unanswered problems related to diseases like COVID-19 via new drug discovery, design, and development in the pharmaceutical and biotechnology industries. Unmatched molecular diversity and novelties, potential of easy absorption and metabolism in the body despite structural complexity, superior bio-friendliness and drug-likeness than synthetic-derived drugs, and very limited success in production by modern chemical synthesis methods lead to increasing global demand for plant-based phytochemicals. Furthermore, cultivation of medicinal plants plays a significant role in the economy of rural people as well as in national and global economic development.

The biodiversity of medicinal plants has declined at an unprecedented rate worldwide, and several medicinal plants are being included under the threatened categories (critically endangered, endangered, and vulnerable) due to the cumulative consequences of human activities (enormous increase in demand, intensive resource overexploitation, habitat destruction, etc.), environmental factors (climatic change, pollution, etc.), and plant-specific factors (heterogeneous distribution, decreased fitness, reduced adaptability to changing environmental conditions, disease susceptibility, etc.). Several malpractices caused by modern socio-economic forces, inadequate public awareness and government policy, weakening of biodiversity protection laws, and lack of organized sector for supply of uniform material also impact negatively on the biodiversity and conservation of medicinal plants. The overall understanding of ecological, species, and genetic diversity of medicinal plants,

different threats and their impact on medicinal plants is crucial for sustainable utilization and conservation of medicinal plant biodiversity via integrated action of in situ and ex situ conservation strategies in the present scenario.

Multidimensional advancements of biotechnological techniques and approaches may be a game changer in sustainable use and conservation of medicinal plant biodiversity. Integration of different disciplines, such as ethnobotany, phytochemistry, biotechnology, genomics, transcriptomics, proteomics, and metabolomics; recent advancements in basic analytical techniques, instrumentation, and in situ analysis; and new methodologies for in vitro culture, multi-omics approaches, induction tactics for SMs production, digital databases construction, chemical structures and pharmacological property predicting tools and expansion of medicinal chemistry knowledge have the potential to revolutionize new drug discovery from medicinal plants and offer unique opportunities for evaluation of their pharmaceutical activity as well as to minimize the gap between the demand and supply of medicinal plants and medicinal plant-derived therapeutic molecules.

This book provides a comprehensive, in-depth, and subject-based knowledge on the current status/state-of-the-art information of medicinal plant biodiversity, its active ingredients, sustainable use and conservation along with special emphasis on current progresses, achievements, and future potentiality of different conventional and non-conventional biotechnological interventions for the protection of biodiversity for the future benefit of mankind.

In the first part, eight chapters are included on broader aspects of diversity of medicinal plants and their therapeutic phytomolecules. The impacts and consequences of major drivers of present population extinction and the biodiversity crisis of medicinal plants are discussed in detail along with comprehensive discussion of the present biodiversity and conservation status of threatened and endangered medicinal plants from different biodiversity rich areas in Asia, Brazil, Europe, etc. It also includes a comprehensive illustration of the structural and functional diversity of plant-derived therapeutic molecules and their importance in herbal, traditional, ethnobotanical, and modern medicine, which signify the need for harmonization between biodiversity, ecosystem, and human health, which is a prerequisite to sustain our life on this beautiful planet. The genetic diversity studies on endangered Brazilian medicinal plant species and the endophytic diversity in Solanaceous medicinal plants are also discussed in this section. Climatic change-related problems on plant biodiversity in general are a major concern today. This part also provides an inclusive account of the impact of climate change on the distribution and niche dynamics of medicinal plant diversity and its utilization.

The second part encompasses within its scope broader aspects of conservation of medicinal plants including as well their role in healthcare system and consists of 11 chapters. It includes chapters providing a vivid account on the role of scientific database in conservation of medicinal plants; current status of international trade of medicinal and aromatic plants; different methods, practices, and challenges associated with the conservation of biodiversity and sustainable use of threatened medicinal plants in general as well as some case studies, such as *Dactylorhiza hatagirea*, *Astragalus fridae* and some major naphthoquinones producing higher plants. This

part also highlights the relationship between medicinal plant biodiversity, healthcare system, and livelihood of rural people as well as a case study on the role of *Tinospora cordifolia* in healthcare. We have also dedicated a chapter highlighting the potential role of medicinal plant-derived traditional herbal medicines, and traditional herbal formulations to overcome SARS-CoV-2-induced health crisis are also highlighted.

In the third part (Conservation of Medicinal Plants by Biotechnology), ten chapters provide the progress, current status, challenges, and future aspects of various biotechnological interventions available currently for in vitro conservation of medicinal plants. It includes different biotechnological approaches, such as multiplication, artificial seed production, and cryopreservation technology for conservation of threatened and endangered medicinal plants, including cases studies such as orchids of Northeast Region of India. This part also provides insights into diverse biotechnological approaches and strategies, such as in vitro non-transformed cultures, and transgenic cultures of endangered threatened medicinal plant species (*Isatis tinctoria*, *Nasturtium officinale*, 29 medicinally important orchid species, *Verbena officinalis*, *Dendrobium fimbriatum*, *Coelogyne ovalis*) have been undertaken globally for the establishment of alternative platform for the synthesis of useful bioactive secondary metabolites to reduce the overexploitation of medicinal plants in wild habitats. Here, the exploration of metabolic engineering and large-scale cultivation in bioreactor to achieve maximum productivity of in vitro cultures have been presented, which indirectly ensure the protection of global biodiversity of medicinal plants providing alternative resources of therapeutic molecules. Future perspective of these emerging biotechnological approaches is also discussed in this part. Moreover, future prospects are predominant in this fascinating field as a very small fraction of the available medicinal plants are extensively studied so far for their therapeutic potential.

The chapters of this book are contributed by specialists from different countries, aiming to serve the endless opportunities and needs of graduate students, academicians, botanists, scholars, researchers, biotechnologists, policy makers, industrial scientists, and conservationists in the field of botany, medicinal plant biodiversity conservation, pharmacy, biotechnology, and phytochemistry.

Finally, we would like to gratefully acknowledge all our contributors who have made immense efforts to ensure the scientific quality of this book. We thank Professor Dr. K.G. Ramawat and Dr. J.M. Merillon for their valuable advice and encouragement.

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Part I
Biodiversity and Endangered Species
of Medicinal Plants

Chapter 1

The Current Status of Population Extinction and Biodiversity Crisis of Medicinal Plants



Mihir Halder and Sumita Jha

Abstract Loss of biodiversity or species extinction (the disappearance of existing species from the earth) and speciation (the origin of new species) are natural, fundamental, and irreversible biological processes that have shaped modern-day life on earth. The entire biodiversity on the planet, irrespective of plant, animal, or fungi, is presently undergoing an anthropogenic episodic extinction event in which they face a tremendous risk of reducing wild population and premature species extinction. The biodiversity of medicinal plants is also suffering the same crisis. Their therapeutic and commercial relevance frequently results in more significant anthropogenic pressure. The major drivers of the medicinal plant biodiversity crisis include the destruction of wild habitats, overexploitation of preferred wild species, the introduction of invasive alien species, conversion of land use, domestic and global biotrade, human-induced pollution and climate change, technological advancement, etc. The combined influence of these driving forces has resulted in a 100- to 1000-fold increase in species extinction rate compared to the average background extinction rate, which is expected to increase further during the next century. It will result in the irreversible loss of a high percentage of species from the earth in a short period if preventive measures are not implemented immediately. The risk of extinction is species-specific and influenced by their intrinsic properties, such as their growth and reproductive efficiency, range of distribution, competitiveness, adaptability, and extrinsic factors, such as the degree and severity of the driving force acting on them, environmental and climatic parameters, etc. In situ and ex situ conservation, cultivation conservation with good agriculture practices, minimizing human intervention in natural processes, and sustainable use of medicinal plants are major weapons to deal with the current biodiversity crisis. Although several preventive initiatives have already been implemented to tackle this issue, the outcomes still need improvement. The Convention on Biological Diversity has established

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20 Aichi Biodiversity Targets as part of its biodiversity strategy plan for 2011–2020. Despite significant improvements in a few categories, the biodiversity crisis still persists, and in most cases, neither the status nor the number of threatened plant species in nature is improving significantly in the last few decades. The solution to the current medicinal plant biodiversity crisis is not possible through a one-size-fits-all approach since it is a hugely challenging endeavour that demands the implementation of more constructive, effective, and equitable conservation and suitability strategies in the coming decade by taking the experiences of the previous decade as a baseline and addressing the lacunae and difficulties of the present strategies. It demands integrated, passionate cumulative global efforts of indigenous peoples; governmental, non-governmental, and public organizations at the local, national, and global levels; leaders; and ordinary people beyond the political and geographical borders.

Keywords Biodiversity crisis · Species extinction · Threatened species · Medicinal plant · Invasive alien species · Land use change · Habitat destruction

1.1 Introduction

Species are the fundamental building block of the incredible biodiversity of the earth, with their specific ecological niche and finite resource value in a range of very low to very high. They significantly impact the development of stable ecosystems through intricate interactions among the coexisting diverse community of life and their environment that ensure natural ecological and evolutionary processes. The estimates of the actual species diversity vary from time to time. According to the estimation of Mora et al. (2011), the current global biodiversity is $\sim 8.7 \pm 1.3$ million (Fig. 1.1), which is predominated by terrestrial biodiversity over the marine biodiversity by species number as only 25% of global biodiversity or $\sim 2.2 \pm 0.18$ million species lived in the marine environment. Global biodiversity encompasses variability among all forms of existing life on this earth, i.e., animals, plants, bacteria, archaea, chromists, fungi, and protists, along with their ecosystems. Later, Costello et al. (2013) estimated 5 ± 3 million species on the planet, of which only ~ 1.5 million have been recognized and named through the hard work of taxonomists over the last 250 years. According to the Global Biodiversity Information Facility data (<https://www.gbif.org>), over 2,568,139 species names have already been assigned, including 1,758,147 Animalia, 164,327, Fungi, 492,872 Plantae, 1,312,361 Arthropoda, 1,086,640 Insect, etc. If synonym, homotypic synonym, heterotypic synonym, doubtful, and pro parte synonym are included, the total number rises to 4,795,545. It implies that the majority of the biodiversity of this planet is still unknown. This biodiversity, which includes species diversity, genetic diversity, and ecosystem diversity, is precious as a resource for a variety of daily human requirements, financial opportunities, critical ecological functions and services, along with aesthetic and recreational importance via regulating natural ecosystem function and stability.

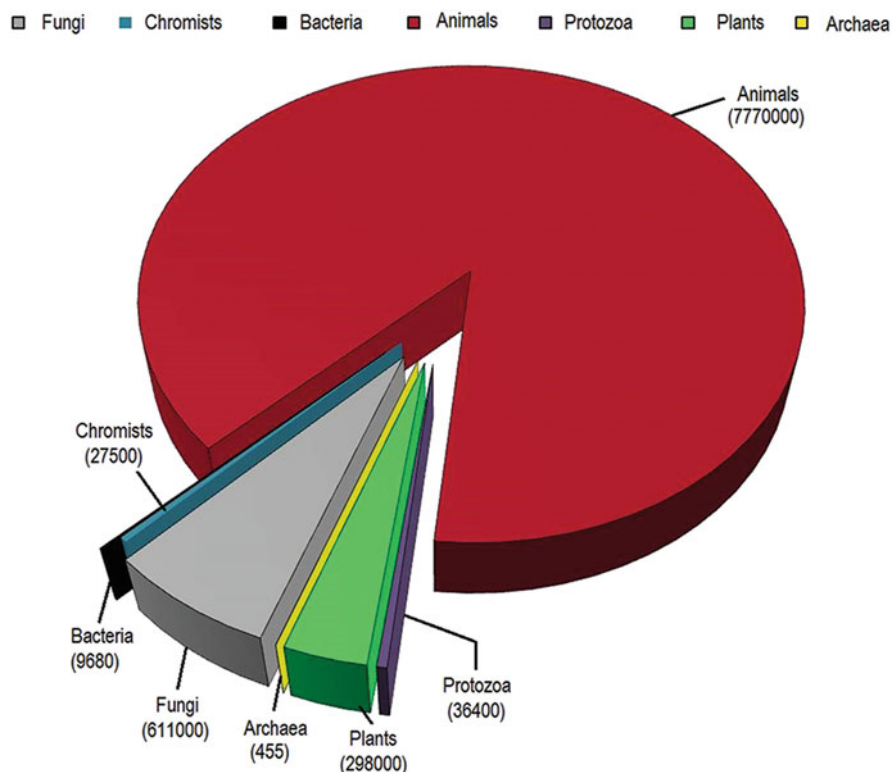


Fig. 1.1 Current predicted total species diversity of earth as per Mora et al. (2011)

Loss of biodiversity by species extinction (disappearance of existing species from the earth) is a natural, fundamental, irreversible, and necessary process of the planet's evolutionary history that continuously tries to balance process of speciation (origin of new species) and has shaped modern life on the earth. The balance between the rates of speciation and species extinction is a fundamental prerequisite for the evolutionary diversification process. Most species (99% of the 4×10^9 species) that have evolved on earth have already gone extinct (Barnosky et al. 2011) either through steady extinction (called background extinction) or episodic mass extinction events that occurred in the geologic era through non-anthropogenic reasons before the evolution of humanity in this planet.

The term background extinction is frequently applied to describe the natural extinction process that takes place at a steady slow rate (1–5 species each year) via natural selection, where ecological and biogeographical factors, such as competition, predation, diseases, habitat loss, climatic changes, dispersal, range shifts, etc., are the primary drivers, instead of the influence of modern human activities (Rull 2022). During the rare episodic extinction event, the rate of biodiversity extinction abruptly increases several folds to the background extinction rate due to specific reasons.

Such a circumstance results in a biodiversity crisis. If the driving factor continues to influence biodiversity, it may culminate in an extremely rare and worst mass extinction event. During mass extinction, the rate of species extinction grows considerably in vast geographic regions within a relatively very short period of geological time, causing drastic changes to the earth's biosphere. It shifts the evolution process and promotes the evolution of new organisms to fill the vacant niches. Based on fossil data, palaeontologists estimated that at least five unusual mass extinction events occurred during the geologic era (Rampino and Shen 2021). They are the end-Ordovician or Ordovician-Silurian extinction (443.8 Ma), the Late Devonian extinctions (372.2 Ma), the Permian extinction (251.9 Ma), end-Triassic extinction (201.4 Ma), and the end-Cretaceous or Cretaceous-Paleogene extinction (66 Ma) (Bambach 2006; Elewa and Abdelhady 2020). Various endogenous and exogenous biospheric factors are considered the primary driving forces (Rull 2022) of these Phanerozoic biodiversity mass extinctions during which only 1/4th or fewer species survived (Barnosky et al. 2011).

Medicinal plants are essential to plant biodiversity, providing a wide range of diverse secondary metabolites that have excellent potential and can be used as a drug or therapeutic molecule against several acute and chronic diseases, physical discomforts, and ailments (Halder and Jha 2021). Since prehistoric times, almost every civilization utilized the biodiversity of medicinal plants as a traditional wealth of therapeutic drugs in herbal remedies and health care to maintain and restore good health owing to their widespread acceptance and adoption, little or no adverse effects, and wide accessibility to the general public. The majority of potent active therapeutic agents and their raw ingredients or derivatives used in indigenous, traditional, ethnomedicine, and modern medicine are collected directly or indirectly from diverse medicinal plants grown in natural growing areas in developing and developed countries.

According to IPBES (2019), the earth's biodiversity, including medicinal plant diversity, presently faces a human-driven biodiversity crisis caused by land/sea use change, overexploitation of natural resources, habitat destruction/fragmentation, invasive alien species, and environmental/climate change. These primary drivers contributed to significant and irreversible ecological alterations like major landscape disturbances and the loss of several native species. These causes are currently contributing to a rapid, extensive, and continuous loss of biodiversity at a 100- or 1000-fold higher extinction rate than the natural background extinction rate of 10–7 species/species per year indicated from the fossil record (Singh 2002). Assuming that population extinction is a linear consequence of habitat loss, the population destruction of around 1800 per hour or 16 million populations annually in tropical forests was predicted (Hughes et al. 1997). In tropical forests, 2–5 species extinction occurs per hour. Many biologists anticipated that mother earth is on the verge of the sixth mass extinction (Shivanna 2019). If necessary measures are not taken instantly, this anthropogenic biodiversity crisis may lead to the magnitude of mass extinction in the near future, similar to those that occurred in past geologic times. A considerable number of the species and genetic and ecological diversity would be extinct

geologically in a short time in the near future due to the direct or indirect negative effects of human interventions.

A significant proportion of global medicinal plant species are becoming rare, threatened, or endangered and are on the verge of extinction. Moreover, many of them will be extinct within decades due to the fast expansion in the global human population, human interventions, and their interest in herbal medicine in the modern period, coupled with low productivity and environmental calamities. Additionally, geographical habitat fragmentation, heterogeneous distribution and availability throughout the world, weakening of biodiversity protection laws, and the tendency to undermine these laws by modern socio-economic forces are responsible for the loss of medicinal plant biodiversity at an alarming pace (Halder et al. 2021). This indisputable human-driven biodiversity crisis is frequently overshadowed by other environmental issues like climate change, despite the fact that they are inextricably linked to each other. Although the enormity and high extinction rate of the current biodiversity crisis demand conservation of each living species, this is almost impossible and unnatural, as extinction is an essential process for natural evolution.

Therefore, the assessment of extinction risk for conservation and appropriate planning for the conservation and management of biodiversity of traditional medicinal plant resources has become the need of the hour. The IUCN Red List of Threatened Species was created by the IUCN Species Survival Commission (SSC) to provide countries, communities, and individuals with the most comprehensive and scientifically credible information on the state of species conservation from a local to a global perspective. This information helps to make decisions about the conservation of plants at the most significant risk of extinction.

The present chapter deals with a comprehensive discussion on the current status of the global plant biodiversity crisis, with special emphasis on medicinal plants. It also includes direct and indirect drivers, their impacts on the biodiversity crisis, and the relationship between these drivers. The available conservational strategies, their major concern areas, and potential future goals to fight against the current biodiversity crisis are also discussed.

1.2 Importance of Medicinal Plants Biodiversity

Medicinal plants are the source of a diverse array of therapeutic molecules that are used in primary health care for around 65–80% of the population of developing countries. Between 50,000 and 80,000 flowering plants are used worldwide for medicinal purposes, as the International Union for Conservation of Nature and the World Wildlife Fund reported. According to an estimate, nearly 25% of globally recommended modern medicines are derived from different medicinal plants. The conservation of medicinal plant diversity and their suitable use is essential for supplying raw materials for existing plant-derived medicine and the development of new drugs in the future because both traditional herbal medicines and modern allopathic systems are greatly dependent on medicinal plant biodiversity.

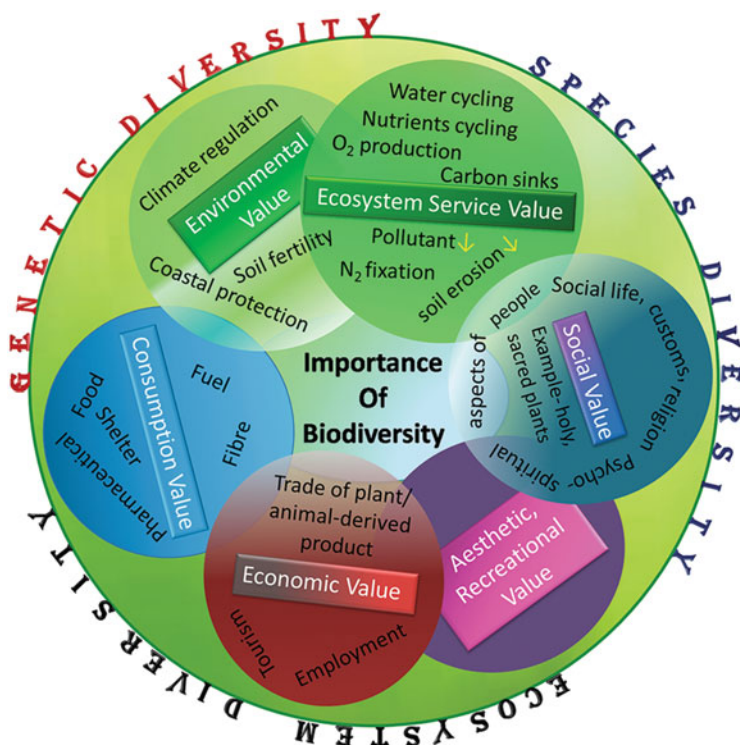


Fig. 1.2 The values of medicinal plant biodiversity

Furthermore, biodiversity-rich nations used them to produce various plant-derived phytotherapeutic supplements (nutraceuticals), cosmetics, and pesticides. 25% to 50% of pharmaceutical products are obtained from biological resources, and about 70% of cancer drugs, which include natural or semisynthetic drugs, are derived from natural molecules (Lebdioui 2022).

Apart from its medicinal values, medicinal plant biodiversity, which represents a small component of total biodiversity, also ensures normal ecological and evolutionary processes of nature (Fig. 1.2). These are very critical for the stability and sustainability of our planet as well as the survival of human beings via maintenance of air, water, and soil quality, controlling the climate, minimizing the impacts of natural disasters, etc. The aggregated value of biodiversity is very difficult to quantify because apart from its ecological significance, plant genetic diversity contributes various resources needed for every life on earth, starting from food to shelter, and used in aesthetic and recreational activities (Fig. 1.2). Floristic diversity acts as a buffer to a certain extent, reducing the adverse effect of natural calamities, climatic changes, and pollution. For example, marine and terrestrial ecosystems absorb grossly 5.6 gigatons of carbon per year as the only sinks for anthropogenic carbon emissions (IPBES 2019).

The impacts of the loss of medicinal plant biodiversity include lower access to conventional medications and fewer opportunities for drug research along with its environmental and ecological impacts. In developing countries, the socio-economic status of a substantial number of poor people is directly affected by these medicinal plant diversity losses. Sustainable use and preservation of these medicinal plants are crucial for economic stability. Thus, the conservation of medicinal plants and overall biodiversity cannot be put off during the crisis.

1.3 Biodiversity Crisis

According to the concept of planetary boundary, climate change, the rate of biodiversity loss, interference with the nitrogen and phosphorus cycles, ozone depletion, ocean acidification, global freshwater use, land use changes, chemical and other pollution, and atmospheric aerosol loading are the nine earth system processes that help regulate the planet. These processes have threshold limits beyond which they cannot withstand environmental change (Rockström et al. 2009). In other words, the planetary boundary concept defines the environmental limits within which humanity can safely operate (Steffen et al. 2015). The species extinction rates, which estimated the number of dead extinct species per million species each year, were used to calculate biodiversity loss within the context of the planetary boundary framework. According to fossil records, species extinction is a natural process that happens at a consistent rate. In the history of earth's evolution, mother earth has already experienced five mass extinctions, during which 75% or more of the existing species became extinct in a couple of million years or less (Barnosky et al. 2011). Endogenous and exogenous biospheric factors, such as meteorite impacts, global shifts in climate and/or atmospheric/oceanic biogeochemistry, recurrent marine transgressions coupled or not with eutrophication and deep-water anoxic events, and generalized increases in volcanism, uplift, and weathering episodes are considered as the main driving forces of these mass extinctions (Rull 2022).

Unfortunately, now the earth is undergoing an anthropogenic rare episodic extinction event that may lead to the sixth mass extinction. In this anthropogenic extinction event, the species extinction rate is enhanced 100- to 1000-fold compared to the natural average background extinction rate over the past several years. Moreover, this rate is predicted to increase tenfold during the next century. This anthropogenic extinction event results in the irreversible loss of a high percentage of species from the earth in a short period due to their inability to adapt fast enough to human-induced rapid environmental changes. Therefore, the severity of the biodiversity crisis is expected to worsen if proper steps are not taken immediately.

The IUCN Red List is the most reliable source to assess the magnitude and patterns of current anthropogenic extinctions, which have assessed 147,517 species since 1500 to date. According to their estimation, 902 species, including Animalia 778 and Plantae 124 (Fig. 1.3), were already extinct, representing 0.006% of all assessed species (IUCN Red List <https://www.iucnredlist.org/statistics> 1st October

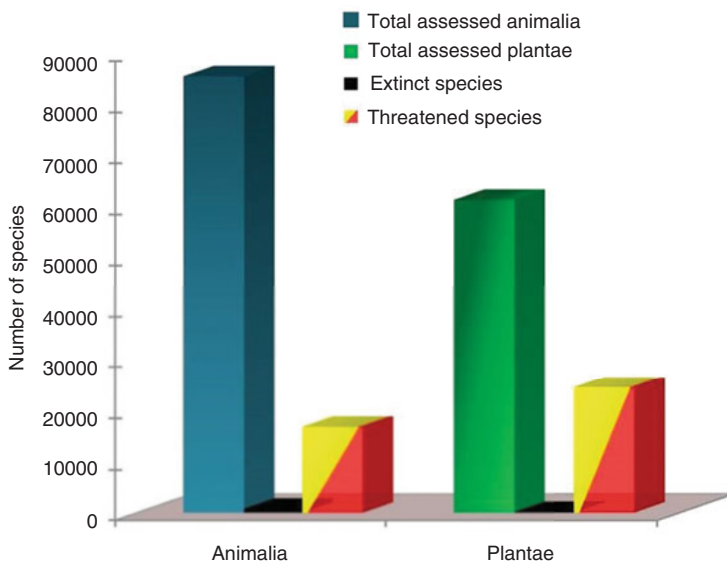


Fig. 1.3 The current state of the biodiversity crisis and species extinction risk includes total assessed, extinct, and threatened species in the Animalia and Plantae groups as per IUCN red list data of threatened species (accessed on 1st October 2022). Out of 85,534 species that belong to Animalia, 778 and 16,720 species are extinct and threatened, respectively, whereas among 61,371 assessed species of Plantae, 124 and 24,449 are extinct species and threatened species, respectively

2022). In addition, 82 species, including 38 Animalia and 44 Plantae, are extinct in the wild (EW), though very limited representatives are conserved in cultivation. The number of real extinction species is underestimated, as the total number of species that existed on the earth is still unknown, and only 7% of documented species have been reviewed. As a result, there is ample possibility that undiscovered species will become extinct before even anybody realizes they exist. Additionally, there will inevitably be more threatened species within the 93% of species that have yet to be evaluated.

The number of extinct species does not depict the entire picture of the biodiversity crisis. It represents only the tip of the iceberg. The degree and severity of driving pressure on existing species in their natural habitat should be considered to predict biodiversity crisis. According to the IUCN Red List, species that are still living on the planet but face extinction threats are grouped into three categories: critically endangered (CR), endangered (EN), and vulnerable (VU), which cumulatively form threatened groups. According to the IUCN Red List (IUCN Red List <https://www.iucnredlist.org/statistics> 1st October 2022), out of 147,517 species assessed, 41,459 species are threatened with extinction (Animalia 16,720, Plantae 24,449, Fungi 284, Chromista 6), which represent 28% of all assessed species (Fig. 1.3).

The comparative risk of species extinction of major plant and animal classes/kingdoms is shown in Fig. 1.4. As per the present data a maximum of 159 species of birds are extinct out of 11,162 species assessed, which represents 1.42%, followed

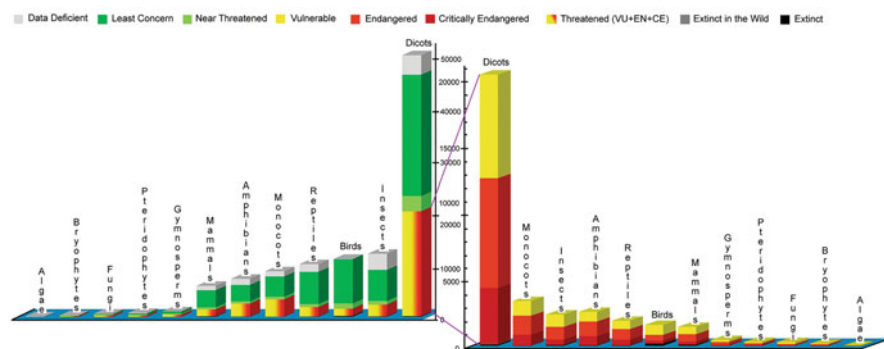


Fig. 1.4 Current global extinction risks of major groups based on IUCN red list category

by 104 dicots species (out of 50,442 species assessed), 85 species of mammals (out of 5969 species assessed), and 59 species of insects (out of 12,161 species assessed). Dicots species showed the highest threatened species by number (20,205 species), followed by monocots (3346 species) and amphibians (2515 species), whereas 58.5% bryophytes species (165 out of 282), 47.5% fungal species (284 out of 597), and 41.6% gymnosperm species (436 out of 1046) are included under the threatened category (Fig. 1.4).

The IUCN Red List (<https://www.iucnredlist.org/statistics>, accessed on 1st October 2022) includes only 61,371 plant species representing around 14% of the world's known plants. Category-wise, it contains 124 extinct species, 44 species of extinct in the wild, 5232 critically endangered species, 9996 endangered species, and 9221 vulnerable species (The IUCN Red List, Fig. 1.4). Among these species, 37,461 species are endemic. According to the IPBES (2019) reports, like animals, plant species are also losing at a faster rate than the average background rate of extinction over the past several million years.

Extinction threats on the plant kingdom are variable over the globe. It varies between different geographical regions (Fig. 1.5) and countries (Fig. 1.6) depending on the extinction pressure imposed by various drivers like habitat destruction, land and sea use change, agriculture expansion, etc., and the conservation strategies adopted and implemented. It is also influenced by regional biodiversity richness. The entire terrestrial habitats of the plant are categorized into 13 major regions – Antarctica, Caribbean islands, Europe, South America, Mesoamerica, North America, Oceania, sub-Saharan Africa, North Africa, West and Central Asia, North Asia, East Asia, and South and Southeast Asia. More than 31% of the total 9665 plant species evaluated from different countries of sub-Saharan Africa are threatened species, whereas more than 19% and ~17% of plant species in South America and South and Southeast Asia are threatened, respectively (Fig. 1.5). The regions of Mesoamerica and North Africa have only 0.33% and 0.40% of the world's threatened plant species as per the current data of IUCN Red List of threatened plant species (Fig. 1.5). Antarctica has the lowest threatened species because of very low

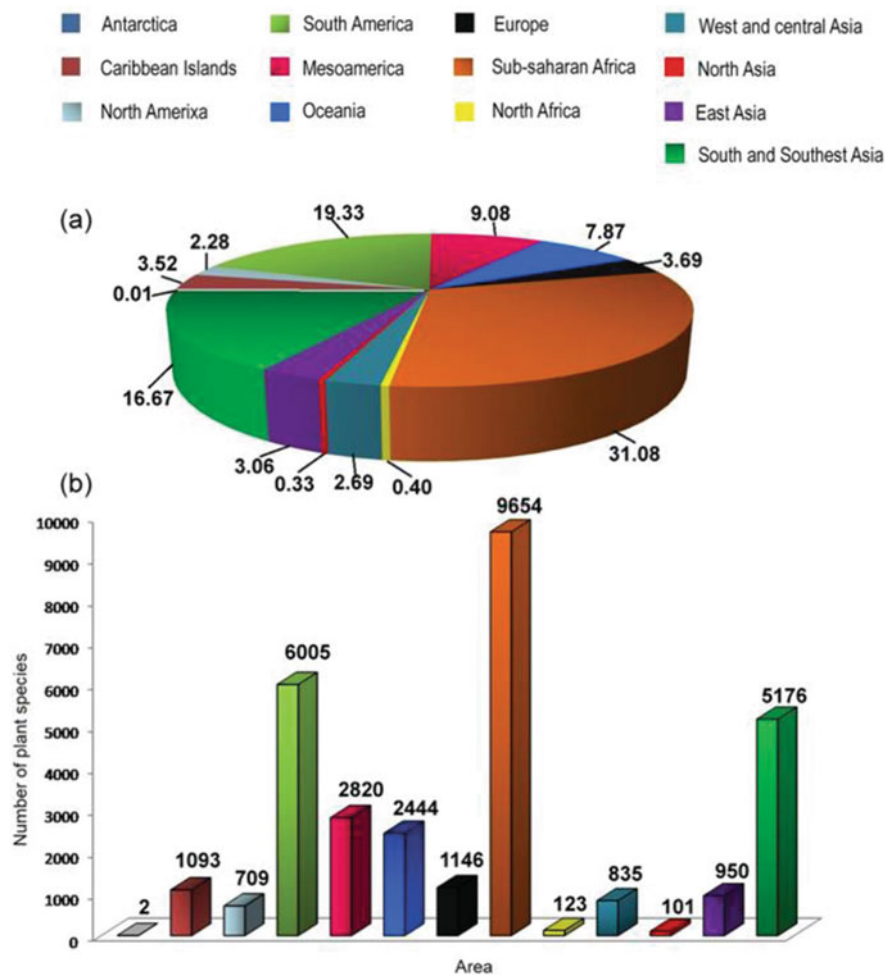


Fig. 1.5 Status of threatened plant species in (a) the percentage of total threatened species and (b) the absolute number of threatened plant species in major regions of the planet

biodiversity and least human influence in this region compared to other parts of the earth.

Based on IUCN Red List (<https://www.iucnredlist.org/statistics>, accessed on 1st October 2022), country-wise analysis of threatened species showed that Madagascar has highest approximately 61% of threatened species (11% of CR + 31% of EN + 19% of VU) out of 4844 assessed plant species, followed by ~41.5% in the Philippines (941 out of 2270), 37% in Ecuador (2045 out of 550), 34% in Malaysia (1350 out of 3963), and Mexico (Fig. 1.6). According to the total number of plant species evaluated, Brazil is the highest with 6890 species, followed by Colombia, Ecuador, Madagascar, and so on (Fig. 1.6).

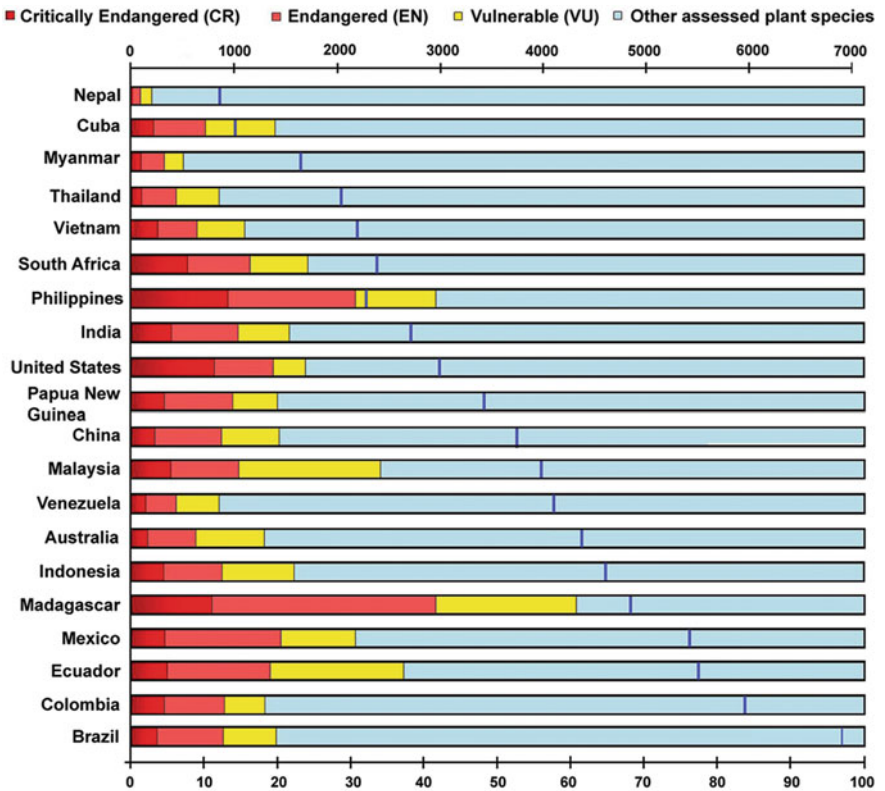


Fig. 1.6 The relative proportion of critically endangered, endangered, and vulnerable species category of IUCN red list with respect to other plant species and total assessed plant species in major biodiversity-rich countries based on the IUCN red list of threatened species (1/10/2022)

1.4 Biodiversity Crisis of Medicinal Plants

According to the IUCN Red List, 3544 of the 61,371 species of Plantae assessed still have documented reports of medicinal use. Table 1.1 provides an overview of the extinction risk of all these medicinally important species, which includes one extinct, seven extinct in the wild, 145 critically endangered, 333 endangered, 313 vulnerable, and 200 near-threatened species (IUCN 2022). Moreover, a gradual decline in the population of most critically endangered, endangered, and vulnerable medicinal plants was observed, which signifies that the extinction pressure is still acting on these species.

Table 1.1 Comparison of risk of extinction of all assessed plant species and medicinal plant species based on the IUCN Red List of threatened species

Category	Number of plantae species (<i>N</i> = 61,371)	Number of medicinal plant species (<i>N</i> = 3544)	Name of some medicinal plant species (family)
Extinct (EX)	124	1	<i>Angostura ossana</i> (Rutaceae)
Extinct in the wild (EW)	44	7	<i>Brugmansia arborea</i> , <i>B. aurea</i> , <i>B. insignis</i> , <i>B. sanguine</i> , <i>B. suaveolens</i> , <i>B. versicolor</i> , <i>B. vulcanicola</i> (Solanaceae)
Critically endangered (CR)	5232	145	<i>Aconitum chasmanthum</i> (Ranunculaceae), <i>Boesenbergia</i> <i>stenophylla</i> (Zingiberaceae), <i>Allophylus</i> <i>dasythyrsus</i> (Sapindaceae), <i>Chlorophytum borivillianum</i> (Asparagaceae), <i>Cinnamomum loheri</i> (Lauraceae), <i>Gentiana kurroo</i> (Gentianaceae), <i>Hazomalania voyronii</i> (Hernandiaceae), <i>Hydnocarpus</i> <i>cauliflorus</i> (Achariaceae), <i>Isoplexis</i> <i>chalcantha</i> (Scrophulariaceae), <i>Krapfia</i> <i>grace-servatii</i> (Ranunculaceae), <i>Lilium polyphyllum</i> (Liliaceae), <i>Nardostachys jatamansi</i> (Caprifoliaceae), <i>Saussurea costus</i> (Asteraceae), <i>Ternstroemia</i> <i>cameroonensis</i> (Pentaphragaceae), <i>Valeriana leschenaultii</i> (Caprifoliaceae), etc.
Endangered (EN)	9996	333	<i>Aconitum heterophyllum</i> (Ranunculaceae), <i>Aframomum atewae</i> (Zingiberaceae), <i>A. spirologulatum</i> (Zingiberaceae), <i>Aloe kilifiensis</i> (Asphodelaceae), <i>Anarrhinum</i> <i>pubescens</i> (Plantaginaceae), <i>Artemisia</i> <i>granatensis</i> (Asteraceae), <i>Atropa</i> <i>acuminata</i> (Solanaceae), <i>Cheirolophus</i> <i>junonianus</i> (Asteraceae), <i>Cinnamomum</i> <i>wightii</i> (Lauraceae), <i>Commiphora</i> <i>stocksiana</i> (Burseraceae), <i>Coptis teeta</i> (Ranunculaceae), <i>Fraxinus dimorpha</i> (Oleaceae), <i>Gymnema khandalense</i> (Apocynaceae), <i>Humboldtia vahliana</i> (Fabaceae), <i>Iphigenia stellata</i> (Colchicaceae), <i>Liquidambar orientalis</i> (Altingiaceae), <i>Okoubaka aubrevillei</i> (Santalaceae), <i>Phlomis aurea</i> (Lamiaceae), <i>Picrorhiza kurroa</i> (Plantaginaceae), <i>Taxus wallichiana</i> , <i>Taxus chinensis</i> (Taxaceae), <i>Tecomella</i> <i>undulata</i> (Bignoniaceae), <i>Trillium</i>

(continued)

Table 1.1 (continued)

Category	Number of plantae species (<i>N</i> = 61,371)	Number of medicinal plant species (<i>N</i> = 3544)	Name of some medicinal plant species (family)
			<i>govanianum</i> (Melanthiaceae), <i>Vatica diospyroides</i> (Dipterocarpaceae), etc.
Vulnerable (VU)	9221	313	<i>Artemisia argentea</i> (Asteraceae), <i>Anacyclus pyrethrum</i> (Asteraceae), <i>Garcinia indica</i> (Clusiaceae), <i>Hydrastis canadensis</i> (Ranunculaceae), <i>Brachystegia leonensis</i> (Fabaceae), <i>Aquilaria sinensis</i> (Thymelaeaceae), <i>Curcuma pseudomontana</i> (Zingiberaceae), <i>Aconitum violaceum</i> (Ranunculaceae), <i>Mentha gattefossei</i> (Lamiaceae), <i>Boswellia ovalifoliolata</i> (Burseraceae), <i>Aframomum mala</i> (Zingiberaceae), <i>Myristica argentea</i> (Myristicaceae), <i>Dipteryx alata</i> (Fabaceae), <i>Aframomum tchoutoui</i> (Zingiberaceae), <i>Ansellia africana</i> (Orchidaceae), <i>Prunus africana</i> (Rosaceae), <i>Cinnamomum macrocarpum</i> (Lauraceae), <i>trillium trillium</i> (Melanthiaceae), <i>Aframomum elegans</i> (Zingiberaceae), <i>Paris polyphylla</i> (Melanthiaceae), <i>Coptosperma madagascariense</i> (Rubiaceae), etc.
Near threat- ened (NT)	3668	200	<i>Intsia bijuga</i> (Fabaceae), <i>Galanthus nivalis</i> (Amaryllidaceae), <i>Pulsatilla vulgaris</i> (Ranunculaceae), <i>Sideritis scardica</i> (Lamiaceae), <i>Aegle marmelos</i> (Rutaceae), <i>Cyclopia genistoides</i> (Fabaceae), <i>Pistacia atlantica</i> (Anacardiaceae), <i>T. brevifolia</i> , <i>Pterocarpus officinalis</i> (Fabaceae), <i>Pterocarpus marsupium</i> (Fabaceae), <i>Pholidota chinensis</i> (Orchidaceae), <i>Senecio calvus</i> (Asteraceae), <i>Aconitum austrokoreense</i> (Ranunculaceae), <i>Melicope solomonensis</i> (Rutaceae), <i>Alstonia parvifolia</i> (Apocynaceae), <i>Echinops kebericho</i> (Asteraceae), <i>Gentianella nitida</i> (Gentianaceae), <i>Bursera karsteniana</i> (Burseraceae), <i>Guaiacum sanctum</i> (Zygophyllaceae), <i>Salix canariensis</i> (Salicaceae), <i>Knema stenocarpa</i> (Myristicaceae), <i>Telanthophora cobanensis</i> (Asteraceae), etc.

(continued)

Table 1.1 (continued)

Category	Number of plantae species ($N = 61,371$)	Number of medicinal plant species ($N = 3544$)	Name of some medicinal plant species (family)
Least concern (LC)	28,004	2414	<i>Wurfbainia longiligularis</i> (Zingiberaceae), <i>Capparis spinosa</i> (Capparaceae), <i>Crataegus laevigata</i> (Polygonaceae), etc.
Data deficient (DD)	4942	131	<i>Dioscorea sylvatica</i> (Dioscoreaceae), <i>Coscinium fenestratum</i> (Menispermaceae), etc.
Endemic	37,461	1186	<i>Aloe dorotheae</i> (Asphodelaceae), <i>Decalepis hamiltonii</i> (Apocynaceae), <i>Asarum gusk</i> (Aristolochiaceae), etc.

1.5 Major Drivers of Medicinal Plant Biodiversity Crisis

The decrease or extinction of a wide range of life forms at their biological organization, genetic constituents, and the patterns of natural occurrence level on the planet is generally termed biodiversity loss. At the moment, a significant proportion of biodiversity, including medicinal plant diversity, is at great risk of extinction owing to the adverse impacts of natural and anthropogenic drivers (Howes et al. 2020). Natural drivers include volcanic activities, cyclones, tsunamis, flooding, earthquakes, prolonged drought or cold periods, etc., beyond human control and not a result of anthropogenic activities. In contrast, the direct anthropogenic drivers include the destruction of wild habitats, the introduction of invasive alien species, overexploitation of preferred wild species, pollution, climate change, and land-use change mainly developed by human activities (Fig. 1.7). Figure 1.7 shows major drivers acting on global biodiversity and their impacts.

The individual drivers are interconnected (Fig. 1.8) and influenced by each other, compounded by their negative impacts on the wild biodiversity. For example, the drastic growth of the human population and worldwide civilization cause overexploitation of resources, habitat loss, pollution, etc., which in turn negatively impacts existing diversity and accelerates the extinction of several medicinal plants. Furthermore, pollution may causes climatic change, and climatic change may cause habitat destruction and biodiversity loss (Sharma et al. 2020).

1.5.1 Overexploitation of Preferred Species as a Potential Driver

Around 60,000 plant species are believed to be utilized globally for medical purposes out of approximately 390,000 plant species spread over the world, of which approximately 26,000 have well-documented usage (Timoshyna et al. 2020). In

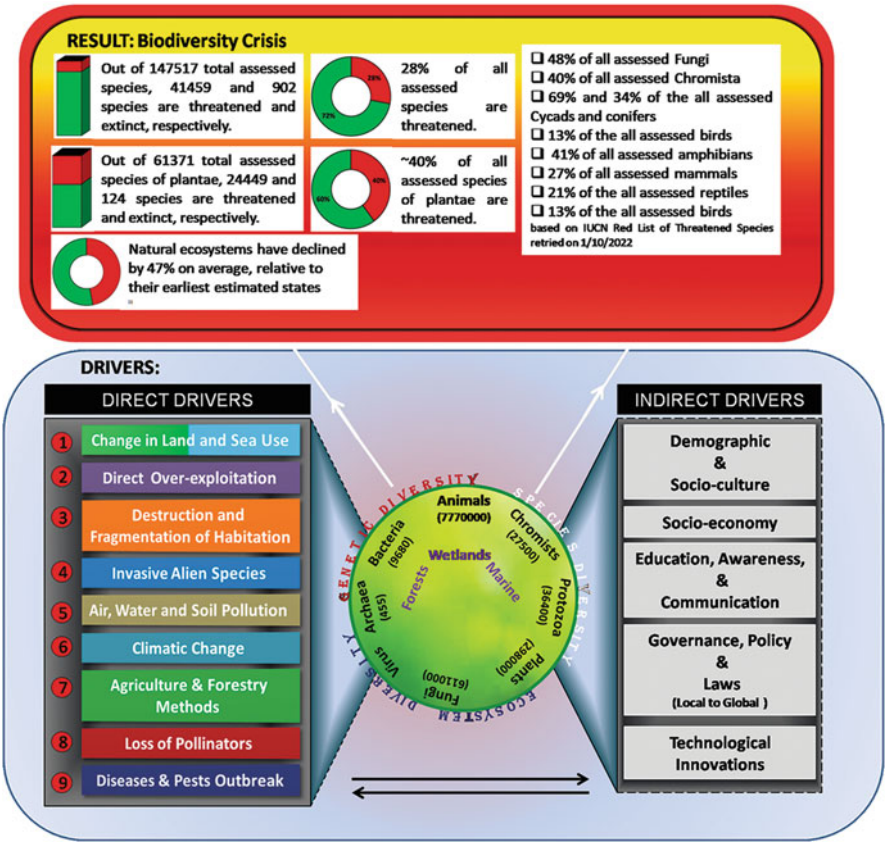


Fig. 1.7 Major direct and indirect driving forces and their impacts on global biodiversity resulting in declines in biodiversity

2018 the market value of therapeutic and aromatic plants sold globally was estimated at 131.4 billion, and it is predicted that it will touch 5 trillion US\$ by 2050 (Jan et al. 2020). Out of 26,000 well-documented medicinal plants, approximately 3000 species are traded internationally and based on geographies and sectors, of which 60–90% are collected from wild habitats, not in commercial cultivation (Jenkins et al. 2018). The wild population of medicinal plants is suffering an epidemic of biodiversity loss due to tremendous harvesting pressure enforced by the massive demand from pharmaceutical enterprises and the traditional health care system at the local or global level. Global average per capita demand for herbal medicinal products skyrocketed with the fast growth of the human population and the global trend of shifting their interest from the allopathic system to the traditional health care system or herbal medicine, along with their modern lifestyles.

Humans have the propensity to acquire commercially essential plant-derived natural resources in excess of real requirements, putting severe stress on global

1.5.2 Destruction of Habitat and Habitat Fragmentation as a Potential Driver

The total disappearance or the partial destruction of natural habitats of particular organisms to such an extent that they are unable to sustain those species and ecological communities is called habitat loss. The loss of natural habitat (terrestrial, freshwater, and marine) is one of the leading drivers associated with the drastic decline in the abundance and diversity of medicinal plants. It may happen either by natural phenomena like earthquakes, forest fires, flooding, etc., or by human interference, such as the change in land use and land cover due to industrialization, civilization, expansion of agricultural areas, unsustainable management practices on cropland, resource extraction (mining), infrastructure development, man-made pollution, and others (Nkonya et al. 2016).

Changes in land use and land cover (deforestation and clearance of native vegetation) are a pervasive, systemic phenomenon that happens at varying rates in all parts of the terrestrial world. Although the rate of land transformation has been slowed down or even reversed in developed countries in the recent past compared to the degree of earlier transformation, the rate of transformation is still very high in developing countries. Already, human interventions have extensively altered 75% of the land surface (IPBES 2019). Less than 10% of the earth's land surface in deserts, mountainous areas, tundra, and polar systems will remain substantially free of direct human impact by 2050 because it is unsuited for human use or settlement (IPBES 2018). The data of ~87% loss of global wetlands in the last 300 years and 54% loss since 1900 (IPBES 2018) depict the severity of human intervention on wetland ecosystems. 66% of the ocean area is experiencing rising cumulative effects (IPBES 2019).

The rate of anthropogenic natural habitat destruction and associated fragmentation, both aquatic and terrestrial, has been approaching and exceeding the critical resistance limit of most species and communities in different parts of the world. It has already shown pronounced effects on global plant diversity and ecological processes. High and rising per capita consumption is a crucial contributor underpinning increasing global habitat destruction in many parts of the world. The shift of global land-cover status is another reason of great concern since 2.3 million square kilometres of forest were lost globally during the 2000 to 2012 research period, which is equivalent to the loss of 68,000 soccer fields of forest per day or 50 soccer fields per minute (Sizer and Hansen 2013). Despite adding 0.8 million square kilometres of new forest, efforts to limit forest loss should be increased.

Habitat fragmentation occurs when the large wild ecosystems (habitats) are split into small isolated pieces by infrastructure development such as the construction of roads, housing, and dams; intensification of agriculture reduces genetic diversity and fitness. Small, isolated populations formed as a result of habitat fragmentation are more prone to genetic drift and inbreeding, putting many endangered species at risk of extinction (Frankham et al. 2019). Knowledge of genetic variation and reproductive biology is essential for protecting inter- and intra-species genetic variation,

interpreting the evolutionary aspect of a species, and identifying possible future risks to diversity via any conservation and management programme.

1.5.3 Invasive Species as a Potential Driver

An invasive species is either an animal, plant, fungus, or microorganism that naturally arrived or was introduced unintentionally (by land and water transportation, travel, and scientific research) or intentionally (by fish farming, pet trade, horticulture, biocontrol) by humans in the non-native ecosystems, followed by rapid expansion in their distribution compared to any other species leading to an imbalance in the pre-existing ecosystem. There is a great deal of concern about introducing invasive species due to its potential adverse effect on the health of local native biota, native ecosystems, and the socio-economic of the country. They may lead to a decrease in population or even extinction of native species by modification of habitat, imposing more competition for resources, alteration of fire regimen, and introduction of new diseases. Additionally, there is always a potential chance of hybridization with native species. Alterations in the composition and function of native ecosystems and nutrient cycling negative effects on native ecosystem health. The detrimental socio-economic cost of invasive species includes negative effects on food security, loss of billions of dollars in agricultural productivity, and increases in environmental expense (Haubrock et al. 2021; Liu et al. 2021; Cuthbert et al. 2022). Despite the knowledge of tremendous impacts on local plant diversity, the long-term effects of invasive plant species on local and global plant diversity are very scanty (Pyšek et al. 2012).

An increase in the human population and delimitation of biogeographical barriers by human-driven intensive global trade has facilitated the import of new invasive alien species into new regions since 1980. Human action has resulted in the naturalization of 13,168 plant species outside their natural geographical limits (van Kleunen et al. 2015). Prevention of entry of invasive species is the best solution to this problem since their eradication can be prohibitively expensive once they are established in the new area (Liu et al. 2021; Cuthbert et al. 2022). The impacts of invasive plant species like *Acacia saligna* (Tozzi et al. 2021), *Lantana camara* (Parveen et al. 2011), *Amorpha fruticosa* (Praleskouskaya and Venanzoni 2021), *Paspalum distichum* (Praleskouskaya and Venanzoni 2021), *Alternanthera philoxeroides* (Zhang et al. 2017), *Prosopis juliflora* (Tadros et al. 2020), *Parthenium hysterophorus* (Adkins and Shabbir 2014), and *Mikania micrantha* (Poudel et al. 2019) have been reported from different regions. All of these findings illustrate the urgency for globally coordinated efforts to regulate, control, and comprehend the spread of alien species.

1.5.4 Pollution as a Potential Driver

Various anthropogenic activities, such as excessive or inappropriate application of chemical fertilizers, pesticides, fossil fuels, and antibiotics and discharge of untreated wastewater into nature, use of plastic, disposal of non-biodegradable waste, mining, emission of greenhouse gases, etc., cause environmental (soil, air, and water) pollution. The primary cause of air pollution changes in the concentration of CO₂, CH₄, N₂O, SO₂, particulate matter, or aerosols, mainly caused by the burning of fossil fuels (Zvereva et al. 2008; Anand et al. 2022), whereas contamination with toxic chemicals and heavy metals (Mn, Cr, Pb, Fe, Cd, Co, Zn, Ni, and Hg) from the different sources is the main reason of water and soil pollution (Vardhan et al. 2019). Although types of pollution and their impact are variable in different regions of the earth, environmental pollution is considered an important challenge that has global trends to impact unprecedentedly on overwhelming medicinal plant diversity as well as human life, directly and indirectly. Many researchers have recently concentrated on detecting and eliminating various environmental pollutants to enhance the quality of air, soil, and water sources, which affect the quality of herbal plants (Long et al. 2021; Zamora-Ledezma et al. 2021; Karimi-Maleh et al. 2022). These pollutants have deleterious effects on medicinal plants at physiological, anatomical, biochemical, and genetic levels. Medicinal plants have different strategies for combating these pollutants, but only up to a certain plant-specific threshold level (Pruteanu and Muscalu 2014; Oksanen and Kontunen-Soppela 2021; Han et al. 2022).

1.5.5 Changes in Land Use as a Potential Driver

A significant amount of land conversion has occurred in the previous 50 years due to the expansion of infrastructures (expansion roads, construction of hydroelectric dams, and installation of power lines, oil, and gas pipelines), intensive agriculture, mining, industrialization, and urbanization. According to the recent global assessment report on biodiversity and ecosystem services, since 1992, urban areas have more than doubled as a result of land-use change (IPBES 2019). At present, about 12% and 25% of the world's ice-free land surface is devoted to crop and livestock production, respectively (IPBES 2019), to ensure the food security of the world population, and the percentage is increasing. Frequently these changes in terrestrial ecosystems due to land use are associated with high environmental and social costs that include deforestation, habitat fragmentation, wild plant diversity loss, and population displacement, putting many endangered species at risk of extinction (Aneva et al. 2020). 80% of deforestation is caused by the exploitation of forest land for agricultural and livestock activities, which hugely impacts plant biodiversity. Moreover, numerous wetlands are being drained to make way for new land, and

oceans are exploited for various commercial purposes, which significantly influencing aquatic ecosystems (Oliver and Morecroft 2014).

Besides these, land conversion and deforestation significantly negatively impact the population of many indigenous tribal/ethnic groups, who are a rich repository of practical-based knowledge concerning medicinal plant distribution and application. Current land conversion-mediated tribal population decline raises the risk of losing traditional knowledge of medicinal plants, their use, and conservation. Lack of well-documented knowledge on the distribution and use of medicinal plants at a spatial scale on a single platform also indirectly affects the biodiversity of endemic medicinal plants. Sustainable use, continuous practice, and safeguarding traditional knowledge are essential from a conservation planning and management perspective (Kunwar et al. 2022).

1.5.6 Biotrade as a Potential Driver

According to the recent UN COMTRADE data (<https://comtrade.un.org/db/default.aspx>), the global trade of medicinal and aromatic plant species has been enhanced almost threefold from USD1.3 billion in 1998 to USD3.3 billion in 2018 due to an enormous enhancement in consumption of herbal health-care formulations and dietary supplements over the past two decades. According to the World Health Organization (WHO) prediction, the global herbal market will be worth \$5 trillion by 2050 (Ekor 2014; Jan et al. 2020). China, India, Germany, the USA, and Hong Kong SAR are the world's top exporters, while the USA, Hong Kong SAR, Germany, and Japan are the leading importers, all based on the value of traded products and using combined trade data for 1998, 2008, and 2018 (Timoshyna et al. 2020). After China, India is the world's second-largest supplier of herbal drugs; these two countries combinedly account for ~70% of the world market for herbal medicine. Approximately 240,000 tons of medicines are exported annually, of which 200,000 tons are raw herbs, which account for 20% of the country's annual harvest (Helmut Kaiser Consultancy Studies 2017). In 2017–2018, India exported US\$ 330.18 million worth of herbs and US\$ 456.12 million worth of value-added extracts of medicinal herbs and herbal products, with a growth rate of 12.23% and 14.22% over the previous year, respectively (Madhavan and Soman 2022). Domestic demand and total consumption of raw herbal drugs in India have been estimated at 195,000 MT and 512,000 MT for 2014–2015, respectively (Bagade et al. 2022; NMPB 2019).

All these data suggest huge demand-based domestic and international trade of medicinal and aromatic plant materials. Although it is difficult to estimate the actual number of medicinal plants traded in the world market, the trade of ~2500 plant species worldwide for medicinal purposes was predicted based on a trade survey in Europe (Lange 1998) and considering Europe as a sink for medicinal and aromatic plant traded from all regions of the world (Schippmann et al. 2002). Generally, economically less-developed regions are the native home of the world's richest

biodiversity, and 60–90% of total internationally traded plant species are wild-collected and not in commercial cultivation, depending on geographies and sectors (Jenkins et al. 2018). Millions of wild harvesters in poor and marginalized areas across the world rely on this trade, which sometimes runs under complicated legality, with much of the trade being informal and under-reported. To fulfill the rapidly rising local and international demand for overexploitation of trade medicinal plants, especially rare, endangered, and endemic plant species face far greater threats to their survival.

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and governments control the collection and trade of these medicinally important plants. A total of 365 medicinal plant species belonging to 39 families have currently been listed in the CITES appendices, which include 351 taxa under appendix II, nine taxa under appendix I, and five taxa under appendix III (CITES 2017). Besides the legal trade, a significant amount of illegal trade of medicinal plants was reported. For example, 23% of all seizures reported by the EU Member States during 2018 were of medicinal plant and animal products and parts/derivatives for medicinal use, which includes 260,562 plant-derived medicinal items, including *Aloe maculate*, *Gastrodia elata*, and *Dendrobium nobile* and *Prunus africana* (Timoshyna et al. 2020; TRAFFIC 2020). In 2019, 1869 cases of seizure of medicinal (plant- and animal-derived medicinal) were reported by EU Member States out of a total of 6441 seizure records, which include 130,706 plant-derived medicinal items and 27,749 animal-derived medicinal items (TRAFFIC 2021). The most frequently reported species are roots of *Saussurea costus* and *Panax quinquefolius*. This type of unregulated, illegal, and uncontrolled biotrade of medicinal plants frequently results in biodiversity loss and ecological and socio-economic vulnerability as the collection and trading of CITES-listed medicinal plants from their wild habitats substantially supports regional economic growth and livelihoods of millions of rural dwellers. Increasing demand, complex trade chains, difficulties in traceability as most are traded as parts, derivatives, and mixed and processed products, lack of market awareness of sustainability, and lack of best practices and policy and legislative frameworks related to issues are major challenges associated with trade in wild plant ingredients.

1.5.7 Natural Disasters as a Potential Driver

Several reports pointed out that the severity and frequency of extreme weather events; natural calamities such as fires, floods, and droughts; and increases in average temperatures have become more frequent over the past 50 years, mainly due to the various activities of human beings. For example, an increase in the average temperature of 0.2 °C per decade and a rising average sea level of 3 mm per year have contributed to the disappearance of various islands and shoreline areas along with its wild plant diversity (IPBES 2019).

The diversity of medicinal plants grown in natural habitats, protected areas, botanical gardens, and public gardens are affected significantly by natural catastrophes, such as volcanoes, wildfires, floods, hurricanes, earthquakes, droughts, tsunamis, etc., from time to time in different counties (Souza 2022). In general, these natural calamities may cause a reduction in population size, shift in distribution, habitat destruction, and fragmentation (Chitale and Behera 2019; Braun et al. 2021), which in turn increase the extinction risk of medicinal plants. Medicinal plant species that are critically endangered and endemic or extinct in the wild have a maximum risk of prolonged natural disasters. Changes in land use, climate change, and natural disasters are all forming a nexus and amplifying the impact of each other. Thus, the frequency of occurrence and intensity of natural disasters is predicted to rise in the future. The occurrence of several natural disasters has been documented in various protected areas and biodiversity hotspot locations (Porwal et al. 2012; Tomas et al. 2021; Braun et al. 2021; de Magalhães and Evangelista 2022). Unfortunately, the majority of research focuses on the assessment of fauna diversity, and very little or no information is available on the impact on flora or medicinal plants (Silveira et al. 2016; Braun et al. 2021; Tomas et al. 2021).

A study on the impact of single and recurrent wildfires in a Brazilian Amazon Forest on biodiversity changes through multi-taxa evaluation has depicted that the frequency of fire had no influence on the beta diversity of ants, birds, and trees, and the effects of fire on faunal community structure were attributable to indirect effects, such as vegetation, rather than the fire itself (Silveira et al. 2016). Chitale and Behera (2019) predicted a significant reduction in the geographic distribution of the indicator species in a species-specific manner due to forest fires in the Himalayan biodiversity hotspot by a model-based approach using four endemic tree species on this site. The severity of the impact of natural disasters like earthquakes, tsunamis, etc., depends on topographic patterns and their distance from the epicentre. The tsunami of 26 December 2004 caused enormous damage and catastrophic losses to the ecosystem and biodiversity of the Andaman and Nicobar Islands (Porwal et al. 2012). Mangroves, littoral forests, beach forests, lowland swamps, and *Syzygium* wetlands were mostly affected by the tsunami (Porwal et al. 2012). A recent incident of wildfire (in 2020) in the Brazilian Pantanal, the largest wetland of earth located at the centre of South America in western Brazil's upper Paraguay River basin (de Magalhães and Evangelista 2022), resulted in a loss of more than 16.952 million vertebrates (Tomas et al. 2021). Abnormally hot summers have prevented reseeding of *Matricaria cutita* in Germany and Poland and severe flooding and reduced harvests of *Foeniculum vulgare* and *Pimpinella anisum* in Hungary (Pompe et al. 2008).

1.5.8 Loss of Pollinators as a Potential Driver

Pollinators are key agents of global biodiversity, concerning vital ecosystem services such as pollination and dispersal of seeds to crops and wild flora (Potts et al. 2010).

The significant decline in the population size of wild and domesticated pollinators, such as honeybees, has been reported from all the continents due to habitat loss, agricultural practices, climate change, and a common lack of knowledge among farmers (Christmann 2019). As 87% of all flowering plants depend on pollinators, their existence is important for maintaining the existing wild floral diversity, wider ecosystem stability, crop production, food security, and human welfare (Potts et al. 2010). With the decline of pollinators, parallel declines in the plants that rely upon them reported in recent times suggest their importance in maintaining the existing wild floral diversity.

1.5.9 Climate Change as a Potential Driver

Climate change refers to a long-term (many decades or more) change in the statistical features of the climate system that includes weather and related changes that occur in the oceans, terrestrial surfaces, and glaciers. Climatic changes such as an increase and decrease in atmospheric CO₂ concentration, temperature, or heavy rainfall, salinity in the soil, snowfall patterns, and alteration in seasonal events at the local or global level are natural phenomena that alter biodiversity and ecosystem functioning via extinction of pre-existing species and emergence/origin of new species, clearly suggesting a strong connection between climate change and biodiversity. In most cases, the prolonged natural climatic change allows species sufficient time to adapt and evolve gradually. In contrast, climatic changes caused by human interventions are relatively rapid, making native habitats hostile and leading to the loss of biodiversity.

These anthropogenic climatic changes and their consequences are complex and more pervasive, which is projected to become a key driver of biodiversity loss in the near future as it exacerbates the consequences of other drivers. As a result, the overall negative adverse impacts on marine, terrestrial, and freshwater ecosystems and human well-being are amplified many times. It may cause significant alterations in terrestrial biome borders, which impact the range of species distribution, population dynamics, community structure, shift in phenology, and ecosystem functions (Das et al. 2016; Sharma et al. 2020). It is anticipated that combating challenges associated with substantial climatic changes will be significantly more difficult than combating other drivers as they cannot be addressed very rapidly and simply. Thus, the assessment of its impacts on medicinal plants is necessary.

The impact of climate change on medicinal plants might be enormous, species-specific, and not fully known. It greatly depends on the acclimation capacity or evolutionary potential of each medicinal plant. Species must adapt, relocate, or die as a result of the present climate change (Corlett and Westcott 2013). It may reduce the yield and growth, flowering time, and quality and quantity of secondary metabolites (Sharma et al. 2020; Pant et al. 2021). Although shifting in distribution range by moving to a new phytogeographical region is adopted as a strategy to neutralize the adverse effect of the present climatic change, their ability to keep up with the faster

changes expected in the future is unclear (Corlett and Westcott 2013). Moreover, the risk of local extinctions has accelerated at the climatic margins due to anthropogenic climatic changes.

1.5.10 Diseases and Pests Outbreak as a Potential Driver

The ability of pathogens to cause diseases is usually species-specific. As a result, the majority of the diseases are restricted to certain species and rarely cause pandemics or epidemics. Thus, the impact of disease outbreaks induced by pathogens or pests on the loss of medicinal plant biodiversity is generally minimal compared to other factors, but there are potent drivers of plant diversity loss in a changing environment. Several studies on plant pests and disease outbreaks and their impacts on biodiversity and the delivery of ecosystem services have been evaluated (Freer-Smith and Webber 2017; Spence et al. 2020). Risk assessment, border inspection, and pest and disease control, surveillance, etc. are all recommended.

1.5.11 Monoculture as a Potential Driver

The monoculture of medicinal plants is relatively rare in natural ecosystems as a healthy natural ecosystem possesses a wide range of species, including different plants, fungi, microbes, vertebrates, insects, and other invertebrates. Plant monoculture refers to a common farming practice of cultivation of only one type of highly specialized and genetically homogeneous, economically important indigenous, or exotic plant species across a large field or in a forest under sophisticated monitoring and prediction systems against diseases rather than growing several types of plant species simultaneously. This is a centuries-old agriculture practice adopted by cultivars due to its several agro-economic benefits. Monoculture causes extremely reduced plant diversity as well as reduced microbes and fungal and animal diversity, which in turn adversely affects both the local and global environment by damaging and deteriorating ecosystem balance (Wu et al. 2013). Additionally, monoculture plantation has a negative impact on organic productivity and diminishes natural stability and complexity, resulting in the extinction of medicinal plants (Chandra 2016). Although these issues are not appearing as very devastating at the individual level, their combined effect might have a major detrimental impact on biodiversity.

1.5.12 Technological Innovations as a Potential Driver

In the present era, technological advancements occur at an incredible speed and scale. Application policy of a particular technology, awareness, and responsibility

are the key factors that determine its impacts on medicinal plant biodiversity. In terms of biodiversity, technological innovations can have positive or negative impacts. Nowadays, the application of several modern technologies, including a range of satellite-based and earth-based sensors, could provide valuable support to medicinal plants, conservation, and restoration of their diversity by helping in evidence-based decision-making, management, and surveillance of protected areas and biodiversity hotspots where limited human interference is allowed (Stephenson 2020). The Global Forest Watch is very effective in assessing the impact of humans on natural ecosystems by collecting data on deforestation, infrastructure, and trade in commodities development over time (<https://www.wri.org/initiatives/global-forest-watch>). Additionally, technology can be used in awareness development among neglected native, regional populations, and tourists regarding nature and their engagement in supporting the safeguarding of life on the planet.

On the contrary, many technological innovations, directly or indirectly leading to pollution, accelerate deforestation, and depletion of natural resources negatively affects biodiversity. For example, technological advancements have speeded up the discovery of active compounds in plants, their extraction, and purification that eventually increase yield causing the reduction in harvesting pressure in wild habitats. These discoveries may lead to the overexploitation of particular plant species causing threats to extinction.

1.6 Role of IUCN and IUCN Red List to Fight Against Biodiversity Loss

Since its establishment in 1948, the International Union for Conservation of Nature (IUCN) has served as a catalyst to protect species from extinction by providing critical information to the public, private, and non-governmental organizations about the threat status of different species, species prioritization for conservation, and the most suitable strategy for conservation programmes and dealing with other scientific, social, and political challenges associated with biodiversity conservation. IUCN is the world's largest and most extensive environmental network, having members from both government and civil society organizations, organized into six commissions dedicated to species survival, environmental law, protected areas, social and economic policy, ecosystem management, and education and communication (IUCN 2019; Bakar 2020).

The IUCN Red List of Threatened Species was created by the IUCN Species Survival Commission (SSC) to provide countries, communities, and individuals with the most comprehensive and scientifically credible information on the state of species conservation from a local to a global perspective. This information helps to make decisions about the conservation of plants that are at the greatest risk of extinction. As per IUCN classification, species are assigned the following ranked threat categories: extinct (EX), extinct in the wild (EW), critically endangered (CR),

endangered (EN), vulnerable (VU), near threatened (NT), least concern (LC), and data deficient (DD) and not evaluated via evaluation against quantitative criteria based on extinction risk factors (Gowthami et al. 2021). Despite several significant flaws in the IUCN Red List of Threatened Species, these categories and criteria are globally accepted to assess extinction risk, prioritize species for conservation, and determine conservation methods and policy-making (Gowthami et al. 2021). Although several national and international policies have been taken to address this burning issue, often a lack of detailed, accurate information and a complete inventory of these plants is the major challenge.

1.7 Conservation Strategies Available to Combat Current Biodiversity Crisis

On the verge of the current sixth mass extinction, the majority of the wild medicinal plant population is threatened with a high risk of extinction and erosion in the current and near future due to indiscriminate use and overexploitation by 7.6 billion human population, habitat loss, natural disasters, environmental change, lack of awareness and knowledge, weakening of customary rights, and/or the tendency for modern socio-economic forces to undermine these laws (Halder et al. 2021). The current rate of biodiversity loss can be reduced or halted through proper planning and management of the conservation of threatened medicinal plants. Biodiversity conservation is crucial for sustaining ecosystem resilience and protecting genetic diversity, species diversity, and ecosystem diversity that ensure sustainable direct and indirect benefits to humankind.

On-site ex situ conservation, off-site in situ conservation, and cultivation conservation of medicinal plants are the basic and effective scientific methods to prevent premature extinction by maintaining and sustainable utilization of rare, endangered, and threatened medicinal plant species. The strategy can be adopted depending on the type and severity of the risks, levels of endemism, or category of species threatened with extinction. In situ conservation is an eco-centric dynamic conservation approach to protect, maintain, and recover viable species along with all known and undiscovered components of the particular ecosystem available in their native niche or micro-climate without affecting the natural evolutionary processes. In contrast, ex situ conservation is concerned with safeguarding wild endangered species or genetic diversity by relocating them to a new wild area (in vivo) or within a human-controlled environment (in vitro) outside of their threatened native natural habitats. Although the best way of protecting species is to conserve their native habitats, which is not always feasible, the genetic diversity of wild populations needs a supplement. In such a situation, static, species-centric ex situ conservation of medicinal plants is an essential alternative or complementary method to in situ conservation. Ex situ conservation supplements in situ conservation by either

augmenting raw material supply that reduces pressure on wild habitats or providing a backup of plant germplasm that may be lost in their natural habitats.

In situ conservation of medicinal plants may be carried out in the forest-based protected areas, such as biosphere reserves, national parks, wildlife sanctuaries, wilderness areas, nature reserves, and non-forest public protected areas such as conserved community areas, sacred groves, wild nurseries, and traditional agricultural lands. Wild nurseries and traditional agricultural lands of medicinal plants are some explicitly demarcated areas as 'Medicinal Plant Conservation Reserves'. A biosphere conservation and biodiversity hotspot programme have a significant impact on wildlife conservation as it is a more effective way of protecting the entire ecosystem with a large number of species on a small amount of land. According to the IUCN and UN Environment's World Conservation Monitoring Centre, there are 253,419 terrestrial and inland water protected areas, covering almost 21.3 million square kilometres or 15.79% of the world's land (<https://www.protectedplanet.net/en> accessed on 30.10.2022). The incorporation of indigenous tribal communities and their ethnobotanical expertise in the protection and management process of the protected area and natural resources may be very effective, and it may prevent or reduce illegal wildlife trafficking and crime. Sacred groves are culturally and traditionally protected small or large forests or forest patches, which are a rich repository of diverse flora, including medicinal plants (Behera et al. 2015; Chanda and Ramachandra 2019). The establishment of species-oriented wild nurseries of endemic, endangered, and high-demand medicinal plants in a protected area, in natural habitat, or within their natural distribution range can provide an effective approach to in situ conservation of medicinal plants (Li and Chen 2007; Chen et al. 2016).

Ex situ conservation of medicinal plants includes collection from wild habitats, transfer, cultivation, and maintenance of selected individual species in botanical gardens, ethno-medicinal plant gardens, nurseries, or storage of plant propagules (seeds, tissues, organs, genes) in seed banks, gene banks, DNA storages, field gene banks, in vitro tissue culture repositories, and cryopreservation (Halder et al. 2021). Over 3758 botanical gardens distributed worldwide (BGCI 2022) are playing a significant role in scientific research and ex situ conservation of rare and endangered medicinal plant species of native and exotic origin and serving as repositories of limited individuals of diverse plant germplasm which are available to the public for exhibition and education purposes (Chen and Sun 2018). For example, Acharya Jagadish Chandra Bose Indian Botanic Garden (India) has more than 200 medicinal plants in Charak Udyan. The Botanical Survey of India is actively involved in the ex situ conservation of plants through its network of botanic gardens and research works. Similarly, databases and service tools of Botanic Gardens Conservation International (BGCI) help in ex situ and in situ conservation of plants at the local, regional, and global scales (BGCI 2022). Different countries, such as India, established a network of regional and sub-regional ethno-medicinal plant gardens that act as important regional repositories of knowledge and medicinal plants used by various ethnic communities. For example, the FRLHT effort has established a network of 15 medicinal plant gardens in three South Indian states. These

ethno-medicinal plant gardens also play a significant role in medicinal plant conservation. Medicinal plant nurseries also play an important role in medicinal plant conservation by serving as a key source of supply of plants and seed material, which may then be propagated by various user groups.

Seed banks are also a key strategy for ex situ conservation of diverse seed-producing wild medicinal plants by collecting seeds of selected plant species from wild populations, storing and maintaining seeds under suitable conditions through constant and meticulous surveillance, and testing of seed viability that makes them available for future use. The germplasm of medicinal plants can be conserved for hundreds of years in seed banks. A number of seed banks have been established around the entire world, including Millennium Seed Bank (over 2.4 billion seeds), Svalbard Global Seed Vault, and Indian Seed Vault (over 10,000 seeds) with the geographic area-, the taxonomical group-, forestry-, and ecosystem-specific specialized collections. They complement the in situ conservation process via the germination of seeds stored in seed banks and reintroduction into the wild for in situ conservation if the situation demands it.

Field gene banks (clonal repository) exploit biotechnology technologies, such as in vitro conservation by slow growth on sterile plant tissue culture medium, plantlet on nutrient medium, and cryopreservation in liquid nitrogen (Chandra 2016; Kadam and Pawar 2020). For example, the National Bureau of Plant Genetic Resources (New Delhi), Central Institute of Medicinal and Aromatic Plants (Lucknow), and Tropical Botanical Gardens Research Institute (Kerala) are three national gene banks that have been established in India for ex situ conservation of medicinal and aromatic plants (Chandra 2016). Cryopreservation-mediated ex situ conservation is a reliable and popular practiced method that involves long-term backup storage of tissues, any organs, plantlets, pollen, seeds, embryos, and cells of medicinally essential plants at ultra-low temperatures, usually in liquid (at -196°C) and vapour phase (at -165°C to -170°C) nitrogen (Kundu et al. 2018; Rohini et al. 2021). Plant materials are generally treated with suitable high molecular weight cryoprotectants or vitrification mixtures to avoid problems of intracellular freezing injury. Only limited metabolic processes can occur in plant materials stored at such an extremely low temperature. Factors like genotype of plant, age and size of explants, cell density, growth phase of explants, method of cryopreservation applied, nature of cryoprotectants, etc., significantly affect cryopreservation and viability of explants. Several medicinal plants, such as *Astragalus membranaceus* (Yin and Hong 2015), *Petiveria alliacea* (Gonçalves et al. 2021), *Stevia rebaudiana* (Benelli et al. 2021), *Rauwolfia serpentina* (Kisku et al. 2020), *Panax ginseng* (Le et al. 2019), and *Dioscorea deltoidea* (Sharma et al. 2022), are evaluated for the efficacy for cryopreservation.

Tissue culture-based ex situ conservation utilizing the totipotency of plant cells at various degrees in micropropagation, organogenesis (direct or indirect development of plant organs), morphogenesis, and callogenesis to ensure survival, rapid mass multiplication, and germplasm conservation of medicinal plants (Máthé et al. 2015, Niazian 2019, Lemma et al. 2020, Moraes et al. 2021,). In addition, secondary metabolites can also be produced and improved in in vitro cell suspension culture, callus culture, organ culture, whole plant culture, and transgenic culture in a

relatively short period and space under controlled nutritional and environmental conditions irrespective of the season and weather on a year-round basis. It can be used as an alternative to the wild production system that has the potential to reduce harvesting pressure in the wild and helps in plant conservation.

The cultivation conservation approach involves the inclusion of economically highly valued medicinal plants into cultivation systems which provides conservation-based socio-economic benefits to the rural farmer. Large-scale medicinal plant cultivation is a profitable agribusiness with a very moderate investment that provides fantastic opportunities to eradicate or reduce rural farmer poverty by boosting socio-economic growth as well as assuring an alternate way of generating additional medicinal plant-based raw materials that reduce anthropogenic pressure on the natural medicinal plant resource pool. It could be a game-changer in terms of protection against premature loss of wild biodiversity, biodiversity restoration, and sustainable supply of desired medicinal plant-based products (Van Wyk and Prinsloo 2018; Nwafor et al. 2021). Different government, private, and public organizations are recommending medicinal plant cultivation to fulfil the rapidly expanding demand for herbal products in national and global markets. In recent years, the cultivation of medicinal plants has started to gain momentum in different countries like India, China, and South Africa (Van Wyk and Prinsloo 2018, Nwafor et al. 2021). Government, researchers, and policy-makers should resolve or reduce possible constraints regarding agronomic, agro-ecological, socio-economical, and socio-cultural issues, such as lack of knowledge about ideal soil, local sources of medicinal plants, growing practices, and disease and pest management, low pricing, inadequate research, insufficient herb marketing, unfavourable legislation, lack of suitable policy frameworks, and poor extension service of medicinal plant cultivation faced by farmers (Nwafor et al. 2021) to encourage their active participation. For example, the implementation of subsidies from municipal, state, and national governments can motivate the cultivation of medicinal plants (Van Wyk and Prinsloo 2018).

1.8 Conclusion

The biodiversity of the earth's biota, including medicinal plants, is invaluable in terms of their environmental, resource, aesthetic, and recreational aspects. Medicinal plant biodiversity is a repository of species, and its genetic and molecular richness serves as a buffer against potential health-related issues, severe environmental changes, and economic reforms. Unfortunately, on the verge of sixth mass extinction, a significant percentage of global medicinal plant diversity continues to decline in terms of genes, species, and habitats across all regions at a rate unprecedented in human history and suffering the risk of premature extinction. Human activities are the main driving force that directly or indirectly triggers other driving forces such as overexploitation, pollution-mediated climatic change, habitat loss, etc. The cumulative effect of all the driving forces ultimately creates the present biodiversity crisis by

accelerating the loss of medicinal plants in the near future. The interactions of these driving forces and their consequences on nature and the natural world are extremely complicated and interwoven. The magnitude of the effects of a few drivers is often species-specific and regional, which reduces the effectiveness of the implemented solution strategies.

Many medicinal plants will be lost unless crucial decisive steps are adopted and implemented to combat major driving forces. Various strategies have been adopted in different parts of the world at local as well as global scales in order to mitigate the current biodiversity crisis of economically significant species, including in situ and ex situ conservation, implementation of laws and policies, etc. Science-based conservation planning involves conducting the scientific research study, collection of information, and analysis of information to prioritize conservation activities and enable a better knowledge of the status and protection measurement of global biodiversity. The effectiveness of species-centric conservation of rarest and most threatened wild medicinal plant species may reduce due to the socio-economic background of local communities. Thus, community-based scientific planning, management, and decision-making strategies are recommended to combat the present biodiversity crisis that strikes a balance between the economy of indigenous peoples and environmental goals. Safeguarding nature and cultural resources, improving livelihoods, and sustainable economic development may be possible together by promoting business, reducing extinction risk, and securing supply chains via sustainable uses of medicinal plants in their natural habitats. Proper strategies for their restoration in the wild and recovery of entire ecosystems are also highly recommended. Although most governments have enacted a variety of legislative, regulations, and administrative procedures to achieve the goal of effective conservation of inter- and intra-specific genetic variation in distinct biogeographic regions by regulating, protecting, and managing natural habitats and biodiversity of medicinal plants, more national and global policies and laws regarding protection and sustainable utilization of biodiversity are still recommended.

The mission of conservation of medicinal plants and their sustainable use is often affected by a lack of education and public awareness on the present biodiversity crisis and its consequences and communication gaps between indigenous tribal groups and policy-makers. Public education and awareness regarding the potential ecological and economic worth of medicinal plant biodiversity, driving factors of the biodiversity crisis, conservation methods, cultivation practices, and sustainable and equitable consumption is greatly required. The prevention of erosion of local indigenous knowledge related to medicinal plants held by many native and tribal communities is critical in biodiversity conservation, which may make possible through the establishment of communication bridging with them and the prevention of the loss of their traditional habitat.

Under the Strategic Plan for Biodiversity 2011–2020, the Convention on Biological Diversity includes 20 Aichi Biodiversity targets that attempt to address the fundamental causes of biodiversity loss; reduce direct pressures on biodiversity and encourage sustainable usage; improve the biodiversity status by safeguarding ecosystems, species, and genetic diversity; enhance the benefits to all from

biodiversity and ecosystem services; and enhance implementation through participatory planning, knowledge management, and capacity building. Since 2010 when 20 Aichi Biodiversity targets were adopted to address the climate and biodiversity crises, there has been a great proliferation of protected and conserved areas worldwide as well as knowledge and tools to promote their efficacy and thousands of billions of dollars have been spent every year for the implementation of these goals. Unfortunately, still, the biodiversity crisis continues. They are not so effective in terms of conservation and suitability as neither the status nor the number of these threatened plant species in nature has been improving significantly in the last few decades. There are several gaps and problems associated with the aim of replenishing the lost biodiversity. The protection of biodiversity is a hugely challenging task, and the solution to the current biodiversity crisis is not possible through a one-size-fits-all approach. It demands integrated, passionate cumulative global efforts of indigenous peoples; governmental, non-governmental, and public organizations at local, national, and global levels; leaders; and ordinary people beyond the political and geographical boundaries. In order to achieve a more constructive, effective, and equitable area system in the coming decade, the experiences of the previous decade should be taken into consideration as a baseline for implementing a new set of goals of the post-2020 framework for global biodiversity.

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Chapter 2

Medicinal Plants and Bioactive Phytochemical Diversity: A Fountainhead of Potential Drugs Against Human Diseases



Mihir Halder and Sumita Jha

Abstract The diversity of bioactive phytochemicals in plants of medicinal importance is the most precious gift of nature. The floral and phytochemical diversity of earth has been exploited as a primary source of life-supporting plant-derived bioactive phytochemicals to fight against common ailments and disorders in traditional-, herbal-, and ethnomedicine since the beginning of human civilization. Despite certain challenges associated with plant-derived drugs, modern medicine also is keenly interested in exploring this phytochemical diversity for the discovery of new bioactive molecules or unique templates or scaffolds for the development of novel synthetic or semi-synthetic drugs due to the inability of alternative drug discovery strategies, the huge structural diversity of plant-based therapeutic phytochemicals, their superior quality, higher curative properties with fewer adverse side effects, safety, affordability and acceptance across multiple cultures, and ethnicities in contrast to synthetic chemical drugs. Nations with a wealth of biodiversity have been an eagle's eye for the global pharmaceutical sector to identify new therapeutic agents or develop new drugs to manage specific chronic diseases throughout the last two centuries. The discovery of new phytochemicals from medicinal plants is a complicated, challenging, and/or time-consuming scientific task, usually involving the selection and collection of biota, extraction of phytochemicals, isolation, and purification of each or targeted compound, structure elucidation and identification of innovative phytochemicals, bioactivity tests (biochemical and pharmacological tests) using various techniques according to the structural variety, and stability. A comprehensive and in-depth systematic phytoanalysis and phytopharmacological investigation of the full spectrum of phytochemical diversity of medicinal plants should be performed to achieve the dreams of plant-derived drug discovery via exploration of recent advances in technologies, instruments, and integration of different disciplines, such as ethnobotany, phytochemistry, analytical chemistry,

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biotechnology, genomics, transcriptomics, proteomics, metabolomics, and pharmacology. These will help to overcome challenges associated with phytochemical discovery, screening, isolation, separation, purification, detection, identification, and structural characterization in the realm of phytochemicals. Simplification in comprehensive and effective regulatory governing systems of biodiversity-rich countries for the accessibility of plant resources and techniques and conservation of threatened, endangered plants may motivate phytochemical-based drug research programs and many pharmaceutical and biotechnology companies.

Keywords Medicinal plant · Phytochemical diversity · Alkaloid · Terpene · Modern medicine · Ethnomedicine

Abbreviations

ADMET	Absorption distribution metabolism excretion and toxicity
ASE	Accelerated solvent extraction
CapNMR	Capillary NMR
CCE	Counter-current extraction
CE	Capillary electrophoresis
CETSA	Cellular thermal shift assay
DARTS	Drug affinity responsive target stability
DESI	Desorption electrospray ionization
DNA	Deoxyribonucleic acid
EAE	Enzyme-assisted extraction
FT-IR	Fourier transform infrared
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HPLC-FTIR	High-performance liquid chromatography-Fourier transform infrared spectroscopy
HPTLC	High-performance thin-layer chromatography
HRMS	High-resolution mass spectrometry
HSCCC	High-speed-counter current chromatography
IR spectroscopy	Infrared spectroscopy
ITS	Internal transcribed spacer
LAESI	Laser ablation electrospray ionization
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
NIR	Near-infrared
NMR	Nuclear magnetic resonance
NPOT	Nematic protein organization technique
PEFE	Pulsed electric field extraction

QTOF	Quadrupole time-of-flight
SCAR	Sequence characterized amplified region
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SILAC-PP	Stable Isotope labeling with amino acids in cell culture and pulse proteolysis
SPE	Solid-phase extraction
TLC	Thin-layer chromatography
TPP	Thermal proteome profiling
UHPLC	Ultra-high-performance liquid chromatography
UPLC-PDA-HRMS/MS	Ultra-performance liquid chromatography-photodiode array-high-resolution tandem mass spectrometry
UV	Ultraviolet

2.1 Introduction

Nature is the finest combinatorial chemist that has created a diverse spectrum of multi-dimensional chemical structures using specific enzymes within the storehouse of living organisms, including microbes, fungi, lichens, animals, and higher plants. Plant diversity acts as a central pillar of resource for this molecular diversity. Therapeutic phytochemicals are a special group of natural phytomolecules with important, diverse, and versatile pharmacological properties. They evolved under evolutionary pressure under strict environmental, developmental, and genetic control. They are utilized irreplaceably in treating multiple diseases and sustaining human healthcare.

The phrase “medicinal plants” refers only to a wide section of plants, predominated by herbs with a significant number of therapeutic phytochemical constituents that help to combat human illnesses from prehistoric times. These medicinal plants are the gift of nature. Since the dawn of human civilization, they have been acknowledged as an essential source of active ingredients in traditional, herbal, and complementary medicines. The endless chemical and structural diversity of potential therapeutic phytochemical components of medicinal plants has a significant alleviating role attributed to traditional medicine and folk use by boosting their therapeutic efficacy. A substantial amount of data demonstrates that the use of raw plant materials in the form of dried powders, extracts, or mixtures of products is the foundation of the traditional systems of medicine, such as Ayurvedic medicines or formulations. In addition to these systems, several multi-cultural tribal communities and economically backward populations of several nations have a rich heritage of ethnobotanical use of medicinal plants.

Indeed, medicinal plant-derived drugs are the prime life-supporting systems on modern earth, as one-fourth of all prescribed medicines contain compounds obtained directly or indirectly from plants. Medicinal plants have received considerable

attention globally with enormous financial relevance in the pharmaceutical and biotechnology industries for critical drug discovery, design, and development of modern medicines. Historically, they have served as the foundation for discovering many drugs that are still now accessible on the market. For example, aspirin and Taxol originated from plant-derived phytochemicals, salicin from *Salix*, and Taxol from *Taxus* plants. Furthermore, these plant-derived phytochemicals and their derivatives have played a pivotal role in finding and developing pharmaceuticals and are expected to continue to do so.

This chapter deals with the phytochemical diversity of higher plants and the procedure of isolation, purification, and identification of phytochemicals, emphasizing recent developments and their impacts on traditional medicine and ethnomedicine. Additionally, the effects, advantages, and challenges of phytochemicals on modern medicine in terms of discovering new therapeutic chemicals or designing novel drugs in the present scenario have been discussed as it has enormous potential in the future, and the chemical structures, sources, and bioactivity of certain well-known phytochemicals are discussed here.

2.2 Distributions and Biodiversity of Medicinal Plants

The earth has plant-dominated life, in which plants account for 82% of total biomass, in contrast to the contribution of only 0.4% and 0.01% of global biomass by the animal kingdom and humans, respectively (Ritchie and Roser 2021). The World Conservation Monitoring Centre (WCMC) of the United Nations Environment Programme (UNEP) has identified 17 countries, namely, Australia, Brazil, China, Colombia, the Democratic Republic of the Congo, Ecuador, India, Indonesia, Madagascar, Malaysia, Mexico, Papua New Guinea, Peru, Philippines, South Africa, United States, and Venezuela, as mega-biodiversity centers, which are native repositories of the bulk of the world's species, including medicinal plants (Rajeswara et al. 2012). In addition, 35 biodiversity hotspots (Kapoor and Usha 2020) have been identified with high levels of species endemism (>1500 species) and biodiversity depletion (70% of native habitat destroyed), which are the home to rich native medicinal plants (Máthé and de Sales Silva 2018; Banerjee et al. 2019; Mehta et al. 2021). Although the actual floral biodiversity on the earth is still unknown, according to an estimation 265,000 flora are present on this planet (Sharif et al. 2018), and only a small proportion of it has been yet investigated for their medicinal properties and phytochemical composition (Sharif et al. 2018). Out of the total floral biodiversity of the earth, more than 70,000 plant species (Alamgir 2017) were utilized by 80% and 60% of the population of developing countries and developed countries, respectively, throughout the world to fight against different diseases through traditional medicines (Dwivedy et al. 2019). According to Chen et al. (2016), around 1/10th of the world's plant species has been used for medicinal purposes.

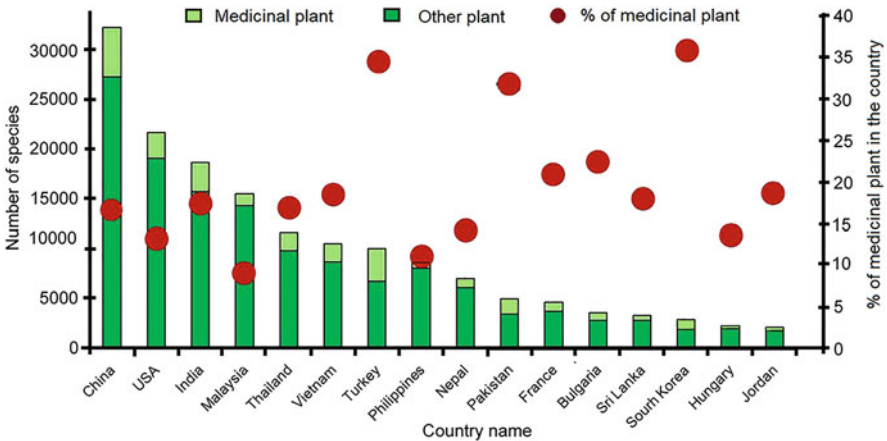


Fig. 2.1 Number and percentage of medicinal plant species in proportion to total plant species in selected biodiversity-rich countries. The bar represents the number of plant species, and the circle represents the percentage of medicinal plants compared to the total plant species. (Data source—Schippmann et al. 2006)

The worldwide distribution, species richness, and abundance of plants, including medicinal plants, are not uniform (Fig. 2.1, Table 2.1). The tropical regions, primarily in developing countries, have the most diversified and distinctive ecosystems, with approximately 2/3rd of the biological diversity of the world and the most significant number of endemic species. The Mediterranean region, the Alps and the Pyrenees, the Massif Central in France, the Balkan Peninsula, the Crimean Peninsula, and the Carpathian Mountains sub-regions in Europe have high medicinal plant species richness (Allen et al. 2014).

Ethnobotanical information regarding medicinal plant applications is critical for ensuring the health requirements of current and future generations, which are unevenly distributed and gradually disappearing with the loss of indigenous tribal and ethnic groups. A few years ago, the WHO also attempted to identify medicinal plants existing in the world. The present data about medicinal plants available from different countries are inspiring and given in Table 2.1.

China is one of the world's mega-diverse countries, with 35,873 specific and infra-specific taxa of angiosperms (belonging to 270 families and 3227 genera), 311 taxa of gymnosperms (10 families and 45 genera), 3167 taxa of bryophytes (belonging to 157 families and 599 genera), and 2336 taxa lycophytes and ferns belonging to 41 families and 181 genera (Xie et al. 2021) along with agricultural, forest, inland water, marine and coastal, dry land and semi-arid, mountain, and island ecosystems. In addition, half of the 30,000 plants found in this country are also endemic. China is the repository site of 10,608 diverse higher medicinal plant species and serves as a significant resource of medicinal raw materials for both traditional medicine systems, such as traditional Chinese medicine (TCM) and the pharmaceutical industry (Chi et al. 2017).

Table 2.1 Number of total plant species and medicinal plant species in selected biodiversity-rich countries

Countries	Number of higher plant species	Number of medicinal plant species	References
China	35,873	10,608	Chi et al. (2017)
United States	28,000	2500	Máthé (2020)
India	17,000–18,000	6000–7000	http://nmpb.nic.in/ ; Nautiyal et al. (2020)
Mexico	23,314/30,000	3000–5000	Villaseñor (2016), Alonso-Castro et al. (2017)
Malaysia	12,500–15,000	1200	Saw and Chung (2015), Tan et al. (2020)
Philippines	13,500	1500	Suba et al. (2019)
Pakistan	5700	2000	Ullah (2017)
Thailand	15,000	2187	Singh et al. (2021), Phumthum and Balslev (2019)
Nepal	5309	819	
Bulgaria	4102	950	
Brazil	45,000	1500	Dutra et al. (2016)
Bangladesh	5700	500	
Bhutan	4567	600	WHO (2009)
Indonesia	30,000–40,000	5408	
Sri Lanka	4143	1430	WHO (2009)
DPR Korea	5308	900	Sixth National Report to the United Nations Convention on Biological Diversity
Africa, all African countries combined	45,000	5000	
Myanmar	11,800	500	WHO (2009)
Australia	19,324	1511	

Nature has endowed India with an immense abundance of biodiversity that represents ~8% of total global diversity, including over 49,000 plant species, of which 4900 are endemic (Nautiyal et al. 2020; Table 2.2). This rich floral diversity in India is dispersed within its 15 agro-climatic zones with diverse ecological habitats, representing only 2.4% of the world's total land. It contains 17,000–18,000 species of flowering plants, of which 6000–7000 are documented in different systems of Indian medicine like Ayurveda, Siddha, Unani, and Homeopathy (<http://nmpb.nic.in/>; Nautiyal et al. 2020). Among the 12 mega-diverse countries worldwide, it ranked 6th, and it has 4 global biodiversity hotspots, namely, the Himalayas, the Indo-Burma, the Sundaland, and the Western Ghats, among 35 global biodiversity hotspots (Jaisankar et al. 2018; Kapoor and Usha 2020). India also boasts a great reservoir of endemic flora, which contains over 4045 flowering plant species

Table 2.2 Floral biodiversity (including algae, fungi, and lichens) of the world and India with endemic and threatened species

Type	Number of known species		% of occurrence in India in respect to global species	% of total Indian flora	Number of endemic species ^c	Number of threatened species ^c
	World ^a	India ^b				
Virus and bacteria	11,813	1239	2.47	10.49	–	–
Algae	40,000	7434	14.86	18.59	1924	Not known
Fungi	98,998	15,447	30.9	15.60	ca. 4100	ca. 580
Lichens	17,000	2917	5.83	17.16	ca. 520	Not known
Bryophytes	16,236	2786	5.57	17.16	629	ca. 80
Pteridophytes	12,000	1307	2.61	10.89	66	414
Gymnosperms	1021	82	0.16	8.03	12	7
Angiosperms	268,600	18,800	37.6	7.00	4303	1700
Total	465,668	50,012	100	–	11,554	2781

^a Schippmann et al. (2006)
^b Annual Report 2019–20, Botanical Survey of India
^c Implementation of India’s National Biodiversity Action Plan—An Overview (2019)

belonging to 141 genera and 47 families (Rajpurohit and Jhang 2015). The entire Eastern Himalaya has around 9000 flowering plant species, 3500 (39%) of which are endemic. In contrast, the Indian portion of the Eastern Himalayas possesses roughly 5800 plant species, of which 2000 (36%) are endemic (Singh and Singh 2016). Out of 7388 flowering plant species found in the Western Ghats, 5584 species are indigenous, 377 are exotic naturalized, and 1427 are cultivated or planted. There are 2242 Indian endemics, and 1261 species are the Western Ghats endemics among the 5584 indigenous species (Singh and Singh 2016). Furthermore, India is widely renowned for its unique ancient heritage of coexisting conventional codified medicine (Ayurveda, Siddha, Unani, Homeopathy, Naturopathy, and so on) and various area- and community-specific folk medicine distributed across the country. The domestic herbal industry in India is represented by 8610 licensed herbal units (Ayurveda 7494, Unani 421, Siddha 328, and Homeopathy 367) distributed across the nation (Goraya and Ved 2017).

Mexico has the world’s fourth highest floristic richness, represented by 23,314/30,000 native vascular plants, 50% of which are endemic (11,600) (Villaseñor 2016). Indigenous peoples of Mexico employ nearly 3000–5000 plant species (Villaseñor 2016; Alonso-Castro et al. 2017) to solve health issues (Palma-Tenango et al. 2017). Most medicinal plants are gathered from the wild, and only 15% are cultivated. There is no specific number of endemic species with medicinal and aromatic uses, although 3000 species are reported with medicinal uses in Mexican traditional medicine (Palma-Tenango et al. 2017). It is mentioned that 1549 are used in the Mayan culture, 816 in the Nahuas, and 3059 in the Zapotecs (Heinrich et al. 1998). Several important medicinal species are found in this region, such as *Agastache mexicana*, *Montanoa tomentosa*, *Lippia graveolens*, *Erythrina* spp.,

Hintonia latiflora, *Salvia hispanica*, *Bursera fagaroides* var. *fagaroides*, *Jatropha neopauciflora*, *Eysenhardtia platycarpa*, etc. (Palma-Tenango et al. 2017). *Smilax aristolochiifolia*, *Chrysactinia mexicana*, *Lippia graveolens*, *A. adstringens*, and *H. inuloides* are exported to the United States, Japan, and Germany (Palma-Tenango et al. 2017).

Thailand is well known for its vast biodiversity in Southeast Asia. It has 15,000 plant species (Singh et al. 2021) distributed in 2 biogeographical regions, the Indo-Chinese Region in the north and the Sundaic Region in the south. There were 964 threatened plant species divided into 737 vulnerable, 207 endangered, and 20 critically endangered species or 8.76% of the total plant species classified in Thailand. *Vanda coerulescens* and *Amherstia nobilis* are now extinct in the wild. According to Phumthum and Balslev (2019), 2187 species belong to 206 families that form part of the medicinal flora and are used in 121 villages inhabited by 26 ethnic groups in Thailand. *Chromolaena odorata*, *Blumea balsamifera*, and *Cheilocostus speciosus* are the most frequently used medicinal plants (Phumthum et al. 2018).

Pakistan is blessed with a rich flora and medicinal plant diversity of over 5700 species and ~2000 species due to its climatic conditions, ecological zones, and topography (Ullah 2017), and it ranks as the 7th producer of medicinal plants in Asia (Kanwal and Sherazi 2017; Shah et al. 2020). Out of 5700 plant species in Pakistan, around 700 are endangered, including 64 medicinal plants (Ullah 2017). However, Shinwari (2010) reported the use of more than 10% of the national flora of Pakistan (600–700 plant species) for medicinal purposes.

Vietnam is also known across the world for its tremendous biodiversity of flora and wildlife. According to Vietnam's Sixth National Report to the Convention on Biological Diversity, the country has 20,000 terrestrial and aquatic plant species and 10,267 angiosperms (Nguyen et al. 2003–2005) with 464 overall endangered plant species (Viet Nam Red Book 2007). The expected number of angiosperms is much higher than it is because more than 50 new species are recorded each year. For example, 136 new plant species were discovered in this area from 2014 to September 2015. Some previously reported threatened plant species, like *Cupressus torulosa* and *Panax bipinnatifidus*, are now critically endangered, whereas *Paphiopedilum vietnamense* is now extinct.

The Malaysian rainforest is not only acknowledged as the world's oldest rainforest but also one of the 12th mega-biodiverse countries in the world and 4th on the list of biodiversity hotspots in Asia after India, China, and Indonesia (Ahmed 2021). According to Saw and Chung's (2015) reports, it has 15,000 species of vascular plants along with 291 species diversity of dipterocarps in the Sabah and Sarawak. Malaysia has an estimated 12,500 species of seed plants, with about 1200 medicinal plants used by the Malaysian herbal industry and multi-ethnic cultures offering a unique combination of folk and traditional medication (Tan et al. 2020).

The Philippines is often regarded as one of the world's most species-rich and ethnically diverse countries. The recent quantitative ethnopharmacological documentation study coupled with molecular confirmation of medicinal plants confirmed the ethnopharmacological use of 122 medicinal plant species belonging to

108 genera and 51 families (Dapar et al. 2020). However, about 1500 medicinal plants out of approximately 13,500 plant species are known in the Philippines (Suba et al. 2019).

Brazil has one of the richest flora diversities, with nearly 40,989 species, of which 18,932 are endemic (Forzza et al. 2012). According to the Brazilian List system 2015, there are 32,086 native angiosperms (belonging to 2740 genera and 224 families) and 23 native gymnosperms (belonging to 6 genera and 5 families) in Brazil, with 57.4% of endemism in seed plants (Zappi et al. 2015). The Brazilian Red List (Martinelli and Moraes 2013) includes 1974 species (1772 endemic and 202 not endemic to Brazil) that were currently listed under one of these threat categories: critically endangered CR, endangered EN, and vulnerable VU. According to Máthé and de Sales Silva (2018), 55,000 native species are distributed over 6 major biomes of Brazil, namely, Amazon (30,000), Cerrado (10,000), Caatinga (4000), Atlantic rainforest (10,000), Pantanal (10,000), and subtropical forest (3000). *Mikania glomerata*, *Bauhinia forficata*, *Psychotria ipecacuanha*, *Pilocarpus microphyllus*, *Cordia verbenacea*, *Euphorbia tirucalli*, *Mandevilla velutina*, *Euterpe oleracea*, *Hypericum caprifoliatum*, *Trichilia catigua*, *Ocotea odorifera*, *Erythrina verna*, *Pfaffia paniculata*, etc., are some common medicinal plants in Brazil (Dutra et al. 2016; Máthé and de Sales Silva 2018).

Medicinal plant diversity, including species, genetic, and ecosystem diversity, is a global asset for current and upcoming generations. This tremendous diversity strengthens the potential power of the chemical and structural variety of phytochemicals in higher plants. Unfortunately, plant diversity faces the enormous pressure of extinction enforced by certain human activities and climate change. Currently, two in five plant species are threatened with extinction (Pironon et al. 2020).

2.3 Phytochemical Diversity in Medicinal Plants

Phytochemical diversity means the richness and abundance of phytomolecules synthesized by plants. They are broadly classified into primary metabolites like carbohydrates, proteins, fats, nucleic acids, Krebs cycle intermediates, etc., and secondary metabolites like alkaloids, polyphenols, terpenoids, saponins, phytosterols, sterols, etc. Primary metabolites are essentially required to sustain the fundamental life process, whereas secondary metabolites are not vital for the normal growth and development of the plant. Plant secondary metabolites are a wide variety of low molecular weight molecules synthesized from the primary metabolites via secondary metabolic pathways in a tissue-, organ-, or developmental stage-specific manner (Halder et al. 2019). These secondary metabolites enable plants to survive against biotic and abiotic factors and act as attracting and/or stimulating agents during pollination, seed dispersal, and symbiotic association of nitrogen-fixing bacteria and mycorrhiza, and for adaptation and environmental resilience (Guerriero et al. 2018; Halder et al. 2019; Sharma and Sharma 2022). Apart from these, they

play a significant role in therapeutic usefulness in traditional medicine, folk medicine, and modern medicine.

The fitness and adaptability of the plant and the services they provide to the ecosystem and humanity are heavily reliant on phytochemical diversity. There are currently about 2,140,000 known phytochemicals, divided into numerous classes based on their chemical structure, function, and biosynthetic diversity, such as polyphenols (~8000), terpenoids, alkaloids (~20,000), phytosterols, polyketides, etc. (Thirumurugan et al. 2018). It represents a relatively small proportion of the full spectrum of phytochemicals because the majority of plants have still not been explored. Despite its relevance in drug development, knowledge about the full spectrum of phytochemical diversity and its functions, distributions, and evolutionary origins among different plant families is not available due to the lack of systematic phytochemical analyses from different plant groups. The majority of phytochemical data are scattered and fragmented since most of phytochemical analyses have been carried out utilizing pre-existing ethnomedical information or randomized sampling. Additionally, several reports proved that there was variability in phytochemical diversity across ecosystems.

2.3.1 Alkaloids

Alkaloids are a large cluster of a chemically diverse group of secondary metabolites with heterocyclic tertiary nitrogen-containing organic bases (exceptions are colchicine and caffeine). They predominate in the plants of the Apocynaceae, Annonaceae, Amaryllidaceae, Berberidaceae, Boraginaceae, Gnetaceae, Liliaceae, Leguminosae, Lauraceae, Loganiaceae, Magnoliaceae, Menispermaceae, Papaveraceae, Piperaceae, Rutaceae, Rubiaceae, Ranunculaceae, and Solanaceae families (Dey et al. 2020). Higher plants are considered the richest source of a vast range of bioactive alkaloids represented by the most widely recognized morphine (analgesics), reserpine (antihypertensive), camptothecin (anticancerous), ajmaline (antiarrhythmic), quinine (antimalarial), vinblastine (anticancerous), vincristine (anticancerous), colchicine (gout suppressant), berberine (anti-inflammatory), scopalamine (sedative), tubocurarine (muscle relaxant), sanguinarine (antibiotic), etc. They are frequently commercially exploited as diet ingredients, supplements, medicines, or therapeutic agents in pure form or plant extracts. Between 2014 and 2020, 27,683 alkaloids were added to the Dictionary of Natural Products, with 990 hits of newly reported or reinvestigated alkaloids from nature (Heinrich et al. 2021).

They are biosynthetically derived from different L-amino acids, such as phenylalanine, tyrosine, tryptophan, anthranilic acid, ornithine, lysine, etc., or transamination (Coqueiro and Verpoorte 2015). Alkaloids are often classified according to their molecular skeleton, such as indole alkaloids (>4000 compounds), tropane alkaloids (~300 compounds), quinoline alkaloids, isoquinoline alkaloids (>4000 compounds), steroidal alkaloids (~450 compounds), imidazole alkaloids, pyridine alkaloids (~250 compounds), pyrrolizidine alkaloids (~570 compounds), etc. (Coqueiro

and Verpoorte 2015; Dey et al. 2020). Several reviews on alkaloids have been published that include their extraction, purification, fractionation, identification, and quantification processes and biological activities (Dey et al. 2020; Rosales et al. 2020). Alkaloids continue to hold considerable promise for revealing new compounds with diverse pharmacological properties, and the synthesis of novel semi-synthetic or synthetic molecules with potential superior efficacy compared to parent phytochemicals.

2.3.2 Phenolic Compounds

Plant phenolic compounds or phenols are also abundant, structurally versatile, and ubiquitous natural SMs that contribute to plant growth, development, and protection against biotic and abiotic stresses and the color, taste, and flavor of plant products. They are produced mainly through pentose phosphate, shikimate (from L-phenylalanine and L-tyrosine), or phenylpropanoid pathways in plants. There are several reviews on types of phenolic compounds, extraction and analysis methods, and their potential biological activities and limitations (Tungmunthum et al. 2018; Ofosu et al. 2020; Albuquerque et al. 2021). Based on their chemical structure, they are classified into simple phenols or phenolic acids, flavonoids, stilbenes, tannins, lignans, lignins, etc. They are strong natural antioxidants and free radical scavengers with one or more hydroxyl groups attached directly to an aromatic ring.

More than 8000 phenolic compounds have been identified, including *p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid, syringic acid, rosmarinic acid, chlorogenic acid, ferulic acid, caffeic acid, *p*-coumaric acid, sinapic acid, aesculetin, hydroxycoumarin, furocoumarin, isofurocoumarin, pyranocoumarin, biscoumarin, dihydroisocoumarin, umbelliferone, scopoletin, silybin, genistein, daidzein, etc. They showed a variety of biological and pharmaceutical properties, including anti-inflammatory (quercetin), anticancer, antimicrobial, antihepatotoxic, antiallergic, antiviral, antithrombotic, phytoestrogenic, and hepatoprotective activity, and as food additives, signaling molecules, and many more.

Coumarins (over 1300 natural coumarins have been isolated) are a unique and versatile class of phytochemicals derivative of benzo- α -pyrone with oxygen-containing (at position 7) heterocyclic structure (lactone of *O*-hydroxycinnamic acid), usually found both in the free and glycosylated states in various parts (seeds, fruits, flowers, roots, leaves, and stems) of more than 150 plant species from more than 40 families, such as Apiaceae, Rutaceae, Asteraceae, Fabaceae, Oleaceae, Moraceae, and Thymelaeaceae.

More than 2000 flavonoids have been identified, which may be further sub-divided into anthocyanins, flavones, and flavonols depending on the oxidation status of the central ring, predominantly found in members of the Polygonaceae, Rutaceae, Fabaceae, Umbelliferae, and Asteraceae. Several plants are listed in the British Pharmacopeia and United States Pharmacopeia due to their flavonoids with several bioactivities. Tannins are a group of phenolic compounds with various

biological activities, including antimicrobial, antiparasitic, antiviral, antioxidant, anti-inflammatory, immunomodulation, etc. (Ullah et al. 2020). Vegetables, fruits, and nuts of plants belonging to Asteraceae, Pinaceae, and Rutaceae families produce lignans by oxidative dimerization of two molecules of phenylpropene, which also showed specific therapeutic values.

Stilbenes are a relatively small group of phytochemicals, widely and heterogeneously distributed among plant species such as *Vitis vinifera*, berries, *Arachis hypogaea*, etc. Resveratrol, pterostilbene, and 3'-hydroxypterostilbene are well-known stilbenes with anti-inflammatory, anticarcinogenic, antidiabetic, and antidyslipidemic properties (Singh et al. 2019; Gómez-Zorita et al. 2021).

2.3.3 Terpenes

Terpenes, also known as terpenoids (about 25,000), are a diverse category of plant SMs mostly found in the flowers, fruits, and leaves of Lamiaceae, Pinaceae, Rutaceae, Apiaceae, Theaceae, etc. They are derived from isopentenyl diphosphate (C_5H_8), a 5C- gaseous hydrocarbon precursor unit. Terpenes are classified based on the number of isoprene units into hemiterpene (C_5H_8 , made up of a single isoprene unit), monoterpene ($C_{10}H_{16}$, made up of two isoprene units), sesquiterpene ($C_{15}H_{24}$, made up of three isoprene units), diterpene ($C_{20}H_{32}$, made up of four isoprene units), sesterterpene (made up of five isoprene units), triterpene ($C_{30}H_{48}$, made up of six isoprene units), sesquaraterpene ($C_{35}H_{56}$, made up of seven isoprene units), tetraterpene ($C_{40}H_{64}$, made up of eight isoprene units), etc. (Ninkuu et al. 2021). They facilitate inter- and intra-species interactions by attracting pollinating insects and beneficial mites, acting as chemical messengers for biotic stressors, modulating the expression of genes involved in plant defense mechanisms in response to abiotic and biotic stressors, acting as thermoprotectants, and mediating pigments and flavors. Besides these, terpenes are also used in traditional and allopathic medicine since they possess analgesic, antibacterial, antifungal, anti-inflammatory, antineoplastic, anticancer, anti-itching, antiprotozoal, anthelmintic, and vasodilation activities (Cox-Georgian et al. 2019, Tetali 2019).

2.4 Discovery of Phytochemicals

The discovery of phytochemicals from higher plants is a complicated scientific task. It usually follows the selection and collection of biota, extraction of phytochemicals, isolation and purification of each or targeted compound, structure elucidation and identification of innovative phytochemicals, bioactivity tests (biochemical and pharmacological tests) using various techniques according to the structural variety, and stability, which can be challenging and/or time-consuming.

Generally, parallel and sequential approaches are utilized to discover bioactive phytochemicals. The parallel approach is used for the isolation of bioactive molecules from crude extracts of selected traditionally or ethnopharmacologically recognized medicinal plants, whereas the sequential approach is primarily utilized for randomly selected plants whose bioactivity is unidentified. A parallel approach involves the determination of the primary bioactivity of different solvent extracts, followed by sub-fractions(s) of the most active extract(s). Then the bioactivity of sub-fraction(s) is determined, and the most active sub-fraction(s) is subsequently subjected to phytochemical purification using various purification procedures. In the subsequent step, the purified phytochemicals are tested for additional confirmation of bioactivity, and the chemical structure of phytochemicals have the highest biological activity is analyzed utilizing cutting-edge methods.

Discovered new phytochemicals may be employed as a promising, robust, and viable therapeutic agents or lead candidates for drug development. The entire process necessitates knowledge and experience. Further, disease-related *in vitro*, *in vivo*, and clinical safety tests are carried out with the selected promising phytochemicals or lead compounds prior to their release as drugs. Once they have all the positive results, they may be considered potential drug candidates. In some cases, structural modifications of the lead compounds lead to enhanced efficacy for their target molecules according to the expected structure-activity relationships.

2.4.1 Selection of Candidate Plants for Screening

Choosing suitable candidate plants for the extraction of phytochemicals, especially active principles, and screening their biological activity is a critical step in finding plant-based novel bioactive chemicals. Candidate plants may be selected for this purpose based on two broad approaches: (a) plants with previous knowledge of a survey and literature in terms of their therapeutic qualities and (b) plants that have no prior knowledge of their healing properties. According to Najmi et al. (2022), a candidate plant can be chosen using a random approach, a taxonomic approach, a chemotaxonomic approach, a pre-existing ethnopharmacological knowledge-based approach, or a traditional system of medicine-based approach.

In the random approach, candidate plants are selected and collected simply in a random, unbiased manner from all plants in local and national areas based on their availability, without any previous knowledge or experience of taxonomic, chemotaxonomic, and pharmacological values. Then they are subjected to screening for target bioassays and identification. This approach offers an outstanding possibility of finding new bioactive phytochemicals with novel structures and unique biological activities from biodiversity-rich areas. It may be further used for both general and focused pharmacological screening.

In the taxonomic approach, the taxonomic knowledge, such as scientific name, systematic position, and distribution, is known for the selected plant. However, ethnomedicinal knowledge and experience are missing, whereas in the

chemotaxonomic approach, the plant is selected based on some phytochemical understanding along with their taxonomic knowledge.

The potential plant is chosen on the basis of historical experiences and knowledge about our ancestors' usage of ethnic medicine or ethnomedicine in an ethnopharmacological knowledge-based approach. Evaluating bioactive phytochemicals using this plant is rational and might be a game-changer in treating and recovering from some medical demands. For example, several bioactive phytochemicals such as andrographolide and morphine were isolated through this approach from *Andrographis paniculata* and *Papaver somniferum* that are utilized in traditional and ethnomedicine.

Apart from the more ancient and less documented ethnobotanical approach, which involves the application of crude extracts and the empirical experiences of a localized small fraction of the community, several countries have a history of the codified system of folklore/traditional medicine like Ayurveda, Siddha medicine, Unani, ancient Iranian medicine, traditional Chinese medicine, acupuncture, Irani medicine, Kampo medicine, traditional Korean medicine, Muti medicine, Ifa medicine, traditional African medicine, Islamic medicine, etc. (Singh et al. 2020). Scientifically unexploited plants that have been used in traditional systems of medicine are selected as potential plants for undertaking an investigation to identify bioactive chemicals and validate their medicinal values. In comparison to a random method, the ethnopharmacological approach has a considerably higher possibility of discovering novel bioactive chemicals since there is previous knowledge about the bioactivity connected with the plant. Thus, numerous government and private companies, such as the CSIR in New Delhi, have already been involved in the validation of hundreds of formulations for various operations. For example, active compounds bacosides and reserpine were discovered from *Bacopa monnieri* and *Rauwolfia serpentina*, respectively, based on the prior information of their application as memory enhancers and antihypertensive agents, respectively, in the codified system of medicine.

2.4.2 Authentication of Plant or Plant Parts

Identification and authentication of the investigated plants or parts are the fundamental and essential steps towards discovering bioactive compounds. Researchers may employ a single or a combination of more than one method, such as morphological, anatomical, microscopic, chromatographic (TLC, HPTLC, HPLC, LC, GC), spectroscopic (NMR, MS, NIR, FT-IR), chemometric, immunoassays, and molecular analysis (DNA fingerprinting, internal transcribed spacer (ITS) sequences, random amplified polymorphic DNA markers, sequence characterized amplified region (SCAR) markers, high-resolution melting analysis) to accomplish this goal depending on their needs and availability (Najmi et al. 2022). In addition to the primary way of authentication by macroscopic morphological features, microscopic techniques may be needed to determine anatomical features at tissue and cellular

levels to help distinguish and identify quite similar medicinal plants. Nowadays, a reliable method called DNA barcoding, which uses a short DNA segment (DNA barcode) for accurate species-level identification of plants, authenticating products, and controlling quality, is widely applied. Additionally, herbal raw material authentication is vital to the safety and efficacy of herbal medicines.

It is recommended that a voucher specimen of the selected plant be prepared and identified by experts. Subsequently, they are retained locally and deposited in a major herbarium like the botanical survey of India.

2.4.3 Pre-extraction Preparation of Plant Samples

Extraction is the most crucial step in phytochemical research and plays a significant role in the isolation, characterization, and identification of specific phytochemicals. Some pre-extraction steps such as washing, grinding, powdering, and drying may be applied to improve overall phytochemical extraction efficiency in terms of yield and purity.

Grinding or powdering of samples breaks samples into uniform smaller coarse particles using traditional mortar and pestle, electric blenders, or mills, causing a reduction in particle size. It increases the availability of interacting surface area between sample(s) and extraction solvent(s), which improves the analytic extraction kinetics and extraction efficiency. Usually, less than 0.5 mm particle size is recommended for efficient extraction, especially enzyme-assisted extraction.

The application of fresh plant material is recommended immediately after harvest to minimize the enzymatic and microbial degradation of phytochemicals and plant tissue. Alternatively, plant tissues are also air-dried under room temperature, heat-dried using sunlight, oven at optimum temperature ($<50^{\circ}\text{C}$), or freeze-dried (lyophilized based on the principle of sublimation) at -60 to -80°C before extraction as quickly as possible to minimize thermolabile chemical degradation. Lyophilization (freeze-drying) of plant tissue is also recommended, especially for volatile or thermolabile phytochemical isolation. It has been reported that phytochemicals are more abundant in fresh plant materials compared to dried plant materials.

2.4.4 Extraction of Phytochemicals

The separation of different desired phytochemicals in the form of a mixture of other pre-existing soluble biomolecules from the insoluble inert cellular matter of plant materials using selective solvents through reliable and effective standard techniques is called extraction.

During solid-liquid extraction, the solvent penetration inside the plant materials is followed by mass diffusion of dissolved phytochemicals outside the plant tissue as a crude extract in a concentration gradient-dependent process. In contrast, the

insoluble inert cellular matter is left behind as residue. The polarity of the solvent, the particle size of the raw material, plant parts used, solvent-to-solid ratio, extraction temperature, and extraction duration greatly influence the efficiency and efficacy of the applied extraction method and the composition of an extract (Zhang et al. 2018).

A suitable solvent(s), extraction method, and extraction procedures should be carefully selected through a meticulous review of the pre-existing literature to produce extract rich in targeted phytochemicals. The nature and diversity of phytochemicals being extracted, their solubility and stability in extraction solvents and applied method, water content of the plant material, the potential toxicity of the extracting solvent, cost, and desired outcome of extraction should also be taken into account as these parameters greatly influence the ultimate outcome. Polar solvents such as methanol, ethanol, or ethyl-acetate are used to extract hydrophilic phytochemicals, whereas nonpolar solvents are used for more lipophilic compounds. An excellent extraction solvent may have a high rate of extraction, evaporation at a suboptimal temperature, preservative action, easy absorption by cells, and no toxic effects.

According to the extraction principle, several extraction methods, such as solvent extraction, distillation method, pressing, and sublimation, have been reported in the literature (Zhang et al. 2018). A number of conventional (classical) methods such as, maceration, percolation, Soxhlet extraction, tincture, reflux extraction, decoction, infusion, lixiviation (elution), steam distillation and hydrodistillation, serial exhaustive extraction, etc., and non-conventional (innovative) methods such as ultrasound-assisted extraction or sonication extraction (Roriz et al. 2021), turbo-extraction or turbo-distillation extraction or turbolysis (Zhang et al. 2018), microwave-assisted extraction (Bagade and Patil 2021), pulsed electric field (PEF) extraction (Liu et al. 2018), pressurized liquid extraction or pressurized fluid extraction or accelerated solvent extraction or pressurized solvent extraction (Wianowska and Gil 2019; Pereira et al. 2019), enzyme-assisted extraction (Das et al. 2021), counter-current extraction (Li et al. 2022), supercritical fluid extraction (Cid-Ortega et al. 2021), and solid-phase extraction (SPE) are employed to accomplish this purpose (Zhang et al. 2018). The basic extraction procedures, applications, advantages, and disadvantages of these conventional and modern extraction procedures involved in natural bioactive compounds research from plants have recently been reviewed by Zhang et al. (2018) and Stéphane et al. (2021).

2.4.5 Isolation and Purification of Phytochemicals

Isolation and purification of phytochemicals is a difficult task that involves further separation of phytochemicals from the complex mixture of heterogenous unknown soluble phytochemicals in the crude extract to acquire pure phytochemicals utilizing their variability in physical or chemical properties such as polarity, adsorption affinities, partition coefficient, and molecular size. Their plant organ-specific or

developmental-specific biosynthesis and the large number and extremely low concentrations in extract often make their isolation and purification process very challenging.

Several methods like TLC, HPTLC, HPLC, and CE are extremely useful for isolating and purifying phytochemicals, and their qualitative and quantitative analysis from the crude extract (Najmi et al. 2022). The most often utilized approaches are very briefly discussed below.

TLC is a simple, qualitative, and semiquantitative technique readily utilized for separating low molecular weight metabolites. It operates on the principle of adsorption chromatography, relying on the interaction of a thin layer of adsorbent (silica gel, aluminum, cellulose powder, starch, etc.) connected to the plate and solvent (Koparde et al. 2019).

HPTLC is a much more sophisticated and efficient separation technique compared to TLC. It works based on the planar chromatography principle for the separation of sample components, along with a quantitative analysis and visualization system in the form of the chromatogram. A high-performance TLC scanner can generate TLC fingerprint profile data that includes information about the absorption spectrum, λ max, R_f value, and chromatogram of a given sample. These data could be used to determine the authenticity via ensuring the quality of the given sample.

Displacement chromatography is one of three operating modes (elution chromatography, displacement chromatography, and frontal chromatography) of column chromatography that provides preparative-scale or process-scale nonlinear multicomponent separations as an alternative to elution chromatography. All of the molecules present in a sample are initially retained on a chromatographic column (loading phase), and then a displacer is employed to displace additional molecules adsorbed into the stationary phase.

Different variants of chromatographic methods, such as adsorption column chromatography, gel filtration chromatography, size exclusion chromatography, ion-exchange chromatography, counter-current chromatography, HSCCC, and centrifugal partition chromatography, are popularly utilized for the separation of phytochemicals, particularly during the initial separation stage.

In adsorption column chromatography, phytochemicals are separated based on adsorption affinities towards the surface of used adsorbents such as silica gel (an amorphous and porous form of silicon dioxide), alumina or aluminum oxide, and macroporous adsorptive resins (synthetic porous cross-linked polymer beads), polyamides (having both acryl and amide groups), charcoal, Florisil (e.g., magnesium silicates), and diatomaceous earth. However, in partition chromatography, the components are separated based on their partition coefficients in two distinct immiscible liquids.

HPLC is a versatile and extensively exploited analytical technique built around the concept of reversed-phase partition chromatography for separation, identification, and fingerprinting analysis of phytoconstituents at both analytical and preparative scales. It is usually operated in either isocratic (with a constant flow rate of a single mobile phase) or gradient elution modes (with alteration in the proportion of mobile phase components during the chromatography) using UV (majority) and/or

visible detectors for separation of non-volatile and heat-labile molecules. Altering the stationary phase (particular column) and mobile phase can improve the degree of separation. According to the need for separation, a new mobile phase can be developed using the trial-and-error method together with the previous knowledge of separation.

Sephadex is employed in the manufacture of size-exclusion chromatography, which separates phytochemicals according to their molecular size or hydrodynamic volume, i.e., smaller molecules are retained for more extended periods than large molecules. Phytochemicals are separated using ion-exchange resin in ion-exchange chromatography depending on their variations in net surface charge.

Supercritical fluid chromatography (SFC) uses supercritical fluid as a mobile phase instead of liquid or gas in liquid chromatography and gas chromatography, respectively. It helps in fast and efficient separation of nonpolar, non-volatile, or thermolabile phytochemicals compared to GC and HPLC. Low viscosity, high diffusivity, and high dissolving capability of supercritical fluid compared to liquid fluid are the major advantages of this method.

In the research, pharmaceutical, and food industries, varieties of membrane filtration methods such as microfiltration, ultrafiltration, and nanofiltration are used as strong tools to separate, concentrate, and eliminate contaminants depending on their molecular sizes using a semipermeable membrane (Zhang et al. 2018). A semipermeable membrane permits the passing of small compounds and the retention of larger molecules. A combined membrane filtration process is sometimes recommended for better results. Phytochemicals with high molecular weight and thermosensitivity can be separated by molecular distillation, which is performed under a vacuum at temperatures significantly lower than their boiling points (Zhang et al. 2018).

2.4.6 Identification and Characterization of Phytochemicals

Classical techniques such as ultraviolet absorption spectroscopy, infrared spectroscopy, high-resolution mass spectrometry, single-crystal X-ray analysis, and nuclear magnetic resonance spectroscopy have been used in metabolite profiling of phytoextract and identification and/or chemical structure elucidation of natural phytochemicals. All of these existing analytical approaches do not provide a complete coverage of the plant phytochemicals due to their advantages and shortcomings.

The arena of drug development has been hampered by the limits of existing analytical chemistry and computational technology in identifying bioactive phytochemicals due to their insufficient amounts, absence of bioactivity, and the rediscovery of known compounds in the complicated plant extracts.

The integrated use of multiple analytical techniques, such as HRMS-NMR, LC-HRMS, GC-MS, HPLC-NMR, microcoil HPLC-NMR, capillary NMR (CapNMR), HPLC-FTIR, FIA-NMR, and HPLC-NMR-MS is a sensible option to increase specificity, sensitivity, depth of coverage, and dynamic range of

phytochemical analyses, which eventually provide a “high-fidelity” snapshot of the constituents in the extract, which ultimately facilitate dereplication and confidence annotation of unknown metabolite analogues and new phytochemical scaffolds based upon multiple orthogonal data types.

HRMS is a modern technology routinely applied for qualitative and quantitative metabolite profiling and the determination of precise molecular mass. In spite of continuous overwhelming tremendous advances in analytical instrumentation and computational tools of metabolomic analysis over the last two decades, problems related to data mining and large-scale confident identification of metabolites based on MS- or NMR-derived spectral characteristics in most untargeted metabolomics experiments were experienced (Ghosh et al. 2021).

According to the metabolomics standards initiative (MSI) guidelines, the confident identification of a metabolite is based upon a minimum of two orthogonal parameters such as retention time, accurate mass, MS/MS fragmentation pattern, collision cross-section, NMR chemical shifts, etc., relative to an authentic standard (Schymanski et al. 2014; Ghosh et al. 2021). A credible and quick method for compound annotation may be possible through spectrum matching with data from authentic standards hosted in mass spectral databases such as MassBank, NIST, METLIN, etc., and NMR databases, e.g., BMRB, COLMAR, etc. (Ghosh et al. 2021). In addition, the integration of numerous analytical data sources improves the possibility of more accurate phytochemical identification and potentially reduces the likelihood of false-positive identification by increasing the degree of confidence in the identification process.

Recent improvements, such as community-wide contributions to data annotation (Wang et al. 2016) and molecular networking, are leading to dramatic changes in quick identification and discovery of phytochemicals in complicated mixtures. The Global Natural Products Social Molecular Networking is the largest web-based open-access mass spectrometry repository and data analysis platform for fast chemical identification using MS/MS data visualization (Aron et al. 2020). Moreover, several *in silico* computational tools, such as MetFrag CL, CFM-ID, MAGMa+, MS-FINDER, etc. and construction of databases, such as PubChem and ChemSpider which have *in silico* predicted spectral information of nearly 100 million known chemicals provide alternate metabolite identification abilities (Blaženović et al. 2017; Milman and Zhurkovich 2017; Djoumbou-Feunang et al. 2019; Wolfender et al. 2019).

Modern techniques, such as MALDI-MS, DESI-MSI, and LAESI-MS have expanded the potential of *in situ* sample analysis (Howes et al. 2020). MADByTE is an amazing novel tool for detecting and identifying specific phytochemicals in complex mixtures, and it can detect both known and unknown natural products utilizing two-dimensional NMR spectra (Caesar et al. 2021). SpaceM, an open-source single-cell metabolomics technology that combines light microscopy and MALDI-MSI methods, can now identify >100 compounds from >1000 unique cells per hour *in situ* (Rappez et al. 2021).

NMR spectroscopy is a versatile non-invasive, non-destructive, highly repeatable quantitative analytical technology routinely utilized for biomedical metabolomics

and structural characterization of unfractionated phytochemicals or fractions obtained with effective isolation strategy investigation for qualitative assessments of both known and unknown compounds (Emwas et al. 2019). With the introduction of improved and integrated UHPLC-QTOF-MS/MS-SPE-NMR methods with greater magnetic field strengths and sensitivity, it is possible to detect and confidently identify numerous phytochemicals at nanomole quantities (Bhatia et al. 2019).

X-ray diffraction and NMR-mediated structure confirmation or absolute structure elucidation often face problems of insufficient availability of purified phytochemicals due to their low content in native sources; the process is lengthy and complex and required the expertise of skilled researchers for data interpretation. Application of attractive and powerful diffraction-based EM techniques, such as microcrystal electron diffraction (MicroED) and electron cryo-microscopy (cryo-EM) has shown great promise for rapidly determining 3D structures of microcrystalline (<1 mg) samples from heterogeneous mixtures of natural products (Nannenga 2020; Danelius et al. 2021). Furthermore, the application of a droplet probe in conjunction with UPLC-PDA-HRMS/MS system is able to chromatographically separate micro-extracts without causing damage to herbarium samples (Kao et al. 2018). Recent advancements in the coupling of different analytical platforms, like ultra-high-performance liquid chromatography (UHPLC)-tandem mass spectrometry (MS/MS)-solid-phase extraction (SPE) and NMR, enable greater throughput empirical metabolite identifications (Ghosh et al. 2021). MicroED incorporation with UHPLC-MS/MS-SPE-NMR system can provide quicker and more straightforward identification of SMs (Ghosh et al. 2021).

The identification of molecular targets of bioactive compounds could be clarified via the development of techniques such as the nematic protein organization technique (NPOT), drug affinity responsive target stability (DARTS), stable isotope labeling with amino acids in cell culture and pulse proteolysis (SILAC-PP), the cellular thermal shift assay (CETSA), and an extension known as thermal proteome profiling (TPP) (Atanasov et al. 2021).

2.5 Impacts of Phytochemicals on Ethnomedicine and Traditional System of Medicine

Since prehistoric times, higher plants have been exploited to gather fundamental therapeutic molecules to fight against common ailments and disorders in traditional systems of medicine, such as traditional Chinese medicine, Ayurveda, Unani, and ethnomedicine. An archeological study carried out by Shanider in Iraq proved the application of medicinal plants, such as *Ephedra*, *Achillea millefolium*, and *Althea officinalis*, for healing purposes by Neanderthals approximately 60,000 years ago. A large number of plant species with therapeutic properties are highly appreciated for their healing power against different diseases and livelihood support. Currently,

different plant extract or plant-derived active principle-based traditional medicine, ethnomedicine, and herbal medicines are the principal weapons in the fight against sickness for 80% of the world's population. For these purposes, medicinal plants are mostly collected from natural sources; rarely, they are cultivated from cultivated areas.

The existence of less toxic and more effective, valuable bioactive molecules (secondary metabolites) justifies their various therapeutic capabilities and prospective application as natural drugs. Many ancient tribes and communities around the world have exploited these bioactive phytochemicals in traditional medicines and ethnomedicines as a therapeutic agent in the crude form of extract, decoction, paste, juice, infusion, and powder, as prescribed by traditional practitioners and herbalists or for self-medication based on traditional knowledge, without knowing their mechanism of bioactivity and molecule targets.

Despite the fact that the pharmacological activities of medicinal plants employed in folk medicine lack scientific proof and rely on public faith, this knowledge has the potential to be useful in drug development because it is based on empirical knowledge gained over many years and often reached by trial and error. These traditional medicines are considered safer compared to allopathic treatment and comprise of either a single ingredient or a complex mixture of multiple bioactive ingredients. They have a wide range of pharmacological activities and are used to treat and/or prevent the majority of common human diseases, such as cancer, peptic ulcer, and infectious, respiratory, digestive, and cardiovascular disease. The therapeutic qualities of the herbal formulations and crude extracts are the results of pharmacological and toxicological effects of all the possible synergistic or antagonistic therapeutic benefits of the mixture's components. The major concern in this approach is the lack of appropriate government registration or documentation of traditional healers and herbal medicine dealers and government regulations for quality assurance and control of the herbal practice.

In this context, ethnobotany, which deals with the study of human interactions with plants and cultural and ecological relationships between plants and human communities, may play an immense role in understanding biodiversity conservation, documentation, and maintenance of indigenous and local botanical knowledge and systematic assessment of traditional medicinal plants. Indigenous healers, traditional healthcare practitioners, and tribal communities have gathered a vast amount of knowledge about indigenous medicinal plants and herbal drugs through offering services to communities over time. The majority of this ethnobotanical knowledge is restricted to a specific niche of human communities in a scattered and unorganized form as transmitted verbally from one generation to another. Although this ethnobotanical knowledge has been neglected for several decades, its importance is now realized. Now, this ethnological knowledge is considered a part of national heritage and its conservation should be done as it may supply important clues for plant-derived drug discovery and development.

Thus, it is critical to adopt appropriate procedures to retrieve such information and appropriately preserve all information in such a way that it may be accessed from all over the world for drug discovery. Several scientific databases, such as

NeMedPlant (Meetei et al. 2012), ADNCD (Khatoon et al. 2018), IMPPAT (Mohanraj et al. 2018), Phytochemica, ETM-DB (Bultum et al. 2019), DIA-DB (Perez-Sanchez et al. 2020), the Sistemax (Costa et al. 2021), and MPDB 2.0 (Hussain et al. 2021) have been established in recent times throughout the world, which includes basic information about chemical constituents of different medicinal plants and their therapeutic applications. They prevent erosion of indigenous knowledge linked to medicinal plants as well as encourage conservation and sustainable management of medicinal plants. Additionally, the integration of ethnobotanical knowledge into national development programs should be done.

Modern medicine research is thus keenly interested in exploring pharmacological screening and isolation, identification, and structural elucidation of these chemical constituents of the plant extract. These chemical constituents may serve as the resources of new phytochemicals that may be utilized directly as natural therapeutic molecules and lead molecules for the development of synthetic or semi-synthetic drugs. Since the first discovery of active constituent morphine from the opium poppy in the early 1800s, numerous other crucial life-saving active ingredients, such as quinine, ephedrine, omacetaxine mepesuccinate, cocaine, codeine, and digitoxin have been isolated from different plants, and some are still used to treat a variety of disorders. However, a variation in the qualitative and quantitative composition of crude extracts with the environment and location changes. The absence of appropriate standardization, quality, safety, and efficacy assessment techniques is the major obstacle to the optimum efficiency of conventional medicine.

2.6 Impact of Phytochemicals on Modern Drug Discovery

Before the twentieth century, only natural pharmaceuticals obtained from natural resources like plants, animals, microbes, and minerals have been employed for illness treatment. All pharmaceuticals available today on the market may be categorized as synthetic drugs, semi-synthetic drugs, or natural drugs depending on the origin of their constituents. In contrast to natural drugs predominantly derived from living organisms, synthetic drugs, such as arterolane used as antimalaria, are artificially created by humans. However, semi-synthetic drugs, such as paclitaxel, are a combination of natural and synthetic sources that are often created by converting natural ingredients into new end products through chemical processes.

About 35% of the annual global market of medicine is derived from natural products or related drugs, which include plants (25%), followed by microorganisms (13%) and animal (3%) sources (Calixto 2019). The compelling tale of the therapeutic capacity of plant-derived ingredients against various ailments in traditional medicine systems and ethnobotany always serves as motivation to find promising pharmaceutical drugs or lead candidates. Plants have an enriched pool of structurally diverse and complex natural phytochemicals with bioactivity. These natural phytochemicals are either directly used as therapeutic agents, lead compounds (with specific biological activity) for the development of more potent analogs, or

compounds whose structures may provide novel pharmacophores. These phytochemicals may be converted to druggable compounds or chemical entities using expansion of novel drug discovery methods (high-throughput screening, automated separation methods, and combinatorial chemistry), synthetic chemistry, and combinatorial chemistry through the rearrangement of chemical entities or structural isomers of naturally occurring products (Mathur and Hoskins 2017).

Nations with a wealth of biodiversity have been an eagle's eye for the global pharmaceutical sector in identifying new therapeutic agents or developing new drugs to manage specific chronic diseases throughout the last two centuries. However, a considerable decrease in financing and shifting of focus from phytochemical-based drug research programs by many pharmaceutical and biotechnology companies has been observed in the last few decades due to the complexity or absence of comprehensive and effective regulatory governing system in biodiversity-rich countries for the accessibility of plant resources, technical barriers, higher time and cost associated with traditional plant-derived drug research, and the emergence of other advanced alternative chemical synthesis techniques like combinatorial chemistry. A number of obstacles associated with the acquisition of a sufficient amount of biological material to isolate and characterize new phytochemicals and complications connected with intellectual property rights for natural products (unmodified) with pharmaceutical value compared to patent-protected synthetic products also act as obstacles. Additionally, the complexity associated with the regulations of advantage sharing with countries of origin of the biological material imposed in the United Nations 1992 Convention on Biological Diversity and the Nagoya Protocol (Atanasov et al. 2021) also should be addressed. In certain circumstances, unavailability of defined culture methods for candidate species and their inability to biosynthesize specified phytochemicals in alien or non-natural habitats complicates the system.

However, natural product research is again getting its pace to find solutions by discovering the most promising new phytochemicals to answer challenging problems posed by several diseases. For example, several researches have been conducted to find out plant-derived drug development against COVID-19. The application of unmodified natural plant-derived phytochemicals in drug research has several clear advantages, including unmatched chemical diversity and novelities and capability of easy absorption and metabolism in the body in spite of their structural complexity. Moreover, plant-derived phytochemicals also have a superior bio-friendliness and drug-likeness than synthetic-derived drugs due to their evolutionary optimized structure for specific biological functions and their prior experience of successful and safe administration in traditional medicine. Sometimes natural products are chemically modified into semi-synthetic drugs to improve efficacy or ADMET properties.

In addition to plant biodiversity and their enormous range of enduring chemical structure diversity, recent advancements in basic analytical techniques and in situ analysis, new methodologies for in vitro culture, multi-omics approaches, induction tactics for SM production, digital database construction, chemical structures and pharmacological property predicting tools, and expansion of medicinal chemistry

knowledge have been reinvigorating. These improvements in drug discovery techniques offer unique opportunities for more accessible genomic, chemical, and spectral studies and act as major driving forces in reinstating phytochemicals as a primary resource of drug development. It has the potential to revolutionize the process of discovery of natural phytochemicals from medicinal plants and the evaluation of their pharmaceutical activity.

Newman and Cragg (2020) extensively addressed and updated the importance of natural products as modern drugs or their role in creating novel pharmaceutical drugs in their reviews. According to their most current review, which is mainly based on all approved therapeutic agents by the US Food and Drug Administration (USFDA) in the time frame 1981 to 2019, 929 out of a total of 1881 newly approved drugs are of natural origin, including biological macromolecules, unaltered natural products, botanical drugs, or derivatives of natural products or vaccines, while the other 952 drugs are classified as synthetic drugs, synthetic drugs with natural product pharmacophores, or drugs that mimic natural products (Newman and Cragg 2020).

In 2020, the USFDA approved 53 drugs, including 40 new chemical entities (NCEs) and 13 biologic drugs (biologics), which is the second highest together with 1996 in terms of the number of drugs accepted, followed by 59 pharmaceutical agents (42 NCEs and 17 biologics) in 2018 (de la Torre and Albericio 2021).

2.7 Some Natural Bioactive Phytochemicals

The discussion of all the natural bioactive phytochemicals in a single paper is not possible. Thus, some selected phytochemicals are discussed here.

Acacetin ($C_{16}H_{12}O_5$, M.W. 284.26 $gmol^{-1}$; Fig. 2.2a) is plant-derived natural mono-methoxy-flavone found in a few members of Scrophulariaceae, Fabaceae, and Passifloraceae, and it has an array of pharmacological potentials (Singh et al. 2020).

The natural alkaloid ajmaline ($C_{20}H_{26}N_2O_2$, M.W. 326.4 $gmol^{-1}$; Fig. 2.2b) found in the roots of several *Rauwolfia* species, such as *R. serpentine*, *R. yunnanensis*, and *R. balansae*, has significant and distinct pharmacological effects like class 1-A antiarrhythmic and antihypertensive properties (Yang et al. 2018). Ajmaline has been used with considerable success to treat atrial fibrillation in person with Wolff-Parkinson-White syndrome and well-tolerated monomorphic ventricular tachycardias. It is an effective drug in acute intravenous therapies due to their sodium channel blocking capabilities and short half-life.

Different members of the amaryllidaceae, such as *Allium sativum*, *A. schoenoprasum*, etc., possess allicin ($C_6H_{10}OS_2$, M.W. 162.3 $gmol^{-1}$; Fig. 2.2c), an allyl sulfur compound with antibacterial, antifungal, and anticarcinogenic activities. Chen et al. (2018) reported the use of allicin in a trial study of follicular lymphoma treatment for its effects on cholangiocarcinoma.

Alpinumisoflavone ($C_{20}H_{16}O_5$, M.W. 336.3 $gmol^{-1}$; Fig. 2.2d) is a natural prenylated pyranisoflavone found in members of Fabaceae such as *Derris eriocarpa*, *Ulex parviflorus*, and *Erythrina suberosa*, and it has a multitherapeutic

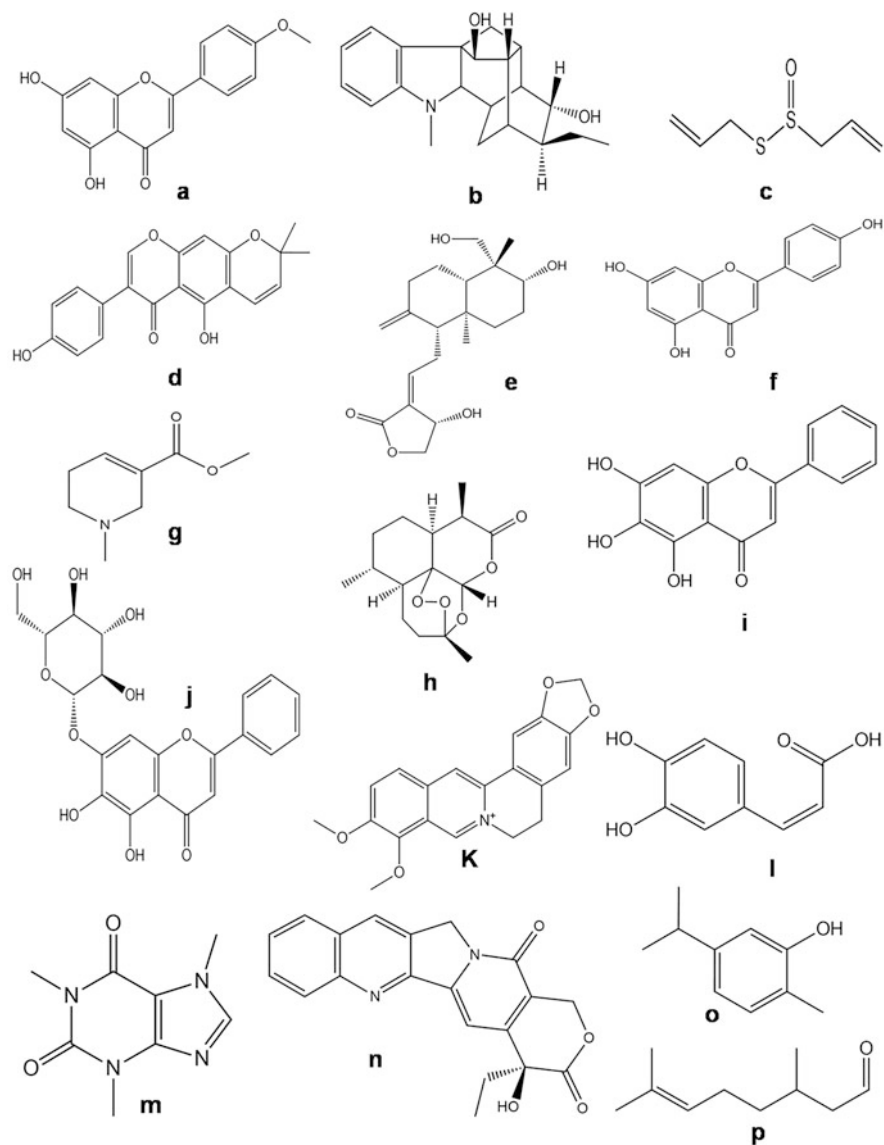


Fig. 2.2 Chemical structure of (a) acacetin, (b) ajmaline, (c) allicin, (d) alpinumisoflavone, (e) andrographolide, (f) apigenin, (g) arecoline, (h) artemisinin, (i) baicalein, (j) baicalin, (k) berberine, (l) caffeic acid, (m) caffeine, (n) camptothecin, (o) carvacrol, and (p) citronella

potential with anti-osteoporotic, antioxidant, anti-inflammatory, antimicrobial, anti-cancer, estrogenic and antiestrogenic, antidiabetic, and neuroprotective properties (Ateba et al. 2019).

The leaves and roots of *Andrographis paniculata* (Acanthaceae) have a bicyclic diterpenoid lactone known as andrographolide ($C_{20}H_{30}O_5$, M.W. 350.4 $gmol^{-1}$; Fig. 2.2e), which has different medicinal uses such as anti-HIV, anti-inflammatory, anti-platelet aggregation, and antineoplastic properties (Islam et al. 2018). Additionally, as part of anticancer activity of andrographolide, several mechanisms have been proposed, like blocking tumor adaptation to hypoxic conditions by inhibiting hypoxia-inducible factor (HIF)-1 α activity and its upstream PI3k/AKT/mTOR pathway (Li et al. 2015; Choudhari et al. 2020).

Apigenin ($C_{15}H_{10}O_5$, M.W.: 270.24 $gmol^{-1}$; Fig. 2.2f) is a naturally occurring trihydroxyflavone, and its derivatives obtained from the fruits and vegetables of plants belonging to Asteraceae, Fabaceae, and Lamiaceae showed effectiveness in the treatment of diabetes, amnesia, Alzheimer's disease, depression, insomnia, and cancer in in vivo research (Salehi et al. 2019).

Arecoline ($C_8H_{13}NO_2$, M.W. 155.19 $gmol^{-1}$; Fig. 2.2g) is a 1,2,5,6-tetrahydropyridine alkaloid found in the fruit of *Areca catechu* and *Piper betle* and acts as an agonist of muscarinic and nicotinic acetylcholine receptors.

The discovery of a sesquiterpene lactone, artemisinin ($C_{15}H_{22}O_5$, M.W. 282.33 $gmol^{-1}$; Fig. 2.2h) from the root and shoot of *Artemisia annua*, dramatically changed the worldwide treatment of malaria caused by *Plasmodium falciparum* (Su and Miller 2015). The use of artemisinin derivatives (dihydroartemisinin, artemether, arteether, and artesunate) in artemisinin-based combination therapies is recommended by the World Health Organization (2009). Artesunate (dihydroartemisinin-12- α -succinate) is a semi-synthetic artemisinin derivative, currently used in combination with selected active antimalarial drugs as a more potent drug (Khanal 2021).

Atropine ($C_{17}H_{23}NO_3$, M.W. 289.4 $gmol^{-1}$) is a tropane alkaloid initially isolated from the *Atropa belladonna* (Solanaceae) but present in other members of Solanaceae such as *Datura innoxia*. It is available in the market under the brand name of Atropen and used for the treatment of the symptoms of anesthesia premedication. It is a racemate composed of equimolar concentrations of (S)- and (R)-atropine with muscarinic antagonist properties. A 2-year clinical trial study on 438 children showed the effectiveness of atropine in lowering myopia progression (Yam et al. 2020).

Several bacosides and bacopa saponins such as bacoside A3 ($C_{47}H_{76}O_{18}$, 929.1 $gmol^{-1}$), bacoside A ($C_{41}H_{68}O_{13}$, 769 $gmol^{-1}$), bacoside B ($C_{41}H_{68}O_{13}$, 769 $gmol^{-1}$), bacoside A2 ($C_{46}H_{74}O_{17}$, 899.1), bascopasaponin C ($C_{46}H_{74}O_{17}$, $gmol^{-1}$), and bacopasaponin A ($C_{40}H_{64}O_{12}$, 736.9 $gmol^{-1}$) have been isolated from *Bacopa monnieri* and showed potential use against several neurological disorders and as a memory enhancer (Charoenphon et al. 2016; Banerjee et al. 2021).

Baicalin ($C_{21}H_{18}O_{11}$, M.W. 446.4 $gmol^{-1}$; Fig. 2.2i) is a natural glycosyloxyflavone, i.e., 7-O-glucuronide of baicalein, found in the root of *Scutellaria baicalensis* and *S. alpina* (Lamiaceae). Several diseases are treated with baicalin, and its aglycone, baicalein, as they act as non-steroidal, anti-inflammatory, neuroprotective, antineoplastic, cardioprotective, antiatherosclerotic,

antioxidant, antibacterial and RNA-directed RNA polymerase inhibitor (Dinda et al. 2017; Sowndhararajan et al. 2018; Yang et al. 2021).

Baicalein ($C_{15}H_{10}O_5$, M.W. 270.24 $gmol^{-1}$; Fig. 2.2j) is a natural 5,6,7-trihydroxyflavone with antioxidant, anti-inflammatory, antineoplastic, antifungal, and antibacterial properties found in the roots of *Scutellaria alpina* and *Scutellaria squarrosa* (Verma et al. 2021). It also acts as a hormone antagonist, prostaglandin antagonist, arachidonate 12-lipoxygenase inhibitor, apoptosis inducer, angiogenesis inhibitor, ornithine decarboxylase inhibitor, and prolyl oligopeptidase inhibitor (Sowndhararajan et al. 2017; Verma et al. 2021). Recent studies showed baicalein has potential as an anticoronaviral agent via SARS coronavirus proteinase inhibition (Lin et al. 2021). Baicalein and baicalin showed downregulation of ZIKV replication and may be used for the treatment of Zika virus (Oo et al. 2019).

Berberine ($C_{20}H_{18}NO_4^+$, M.W. 336.4 $gmol^{-1}$; Fig. 2.2k) is a benzyl-tetra isoquinoline alkaloid with potential antineoplastic, radiosensitizing, anti-inflammatory, hypoglycemic, antineoplastic, antilipidemic, antifungal, antioxidant, and antidiabetic activities found in *Coptis chinensis* (Ranunculaceae), *Berberis* sp., and *Mahonia aquifolium* (Berberidaceae) (Song et al. 2020). Its preclinical effectiveness against several cancer cells, digestive illnesses, nonalcoholic fatty liver disease, and hypertension was also demonstrated (Abrams et al. 2019; Song et al. 2020).

Caffeic acid ($C_9H_8O_4$, M.W. 180.16 $gmol^{-1}$; Fig. 2.2l) is a plant-derived hydroxycinnamic acid with both phenolic and acrylic functional groups that may exist in cis- and trans-forms (Khan et al. 2021). Caffeic acid and its several derivatives have potential antioxidant, anti-inflammatory, and antineoplastic properties (Espíndola et al. 2019; Khan et al. 2021).

Caffeine ($C_8H_{10}N_4O_2$, M.W. 194.19 $gmol^{-1}$; Fig. 2.2m) is a naturally existing trimethylxanthine alkaloid with psychotropic and anti-inflammatory activities, predominantly present in the seeds of *Coffea arabica* (Rubiaceae) and leaves of *Camellia sinensis* (Theaceae), *Ilex paraguariensis* (Aquifoliaceae), *Theobroma cacao* (Malvaceae), and *Senegalia polyacantha* (Fabaceae). It functions as a central nervous system stimulant via adenosine receptor antagonists owing to its structural similarities to adenosine and also enhances neurotransmitter release. The FDA authorized the use of caffeine to treat apnea of prematurity and bronchopulmonary dysplasia in premature infants (Evans et al. 2021). It is also under investigation for its efficacy in treating depression and neurocognitive declines (Evans et al. 2021).

The pyranoindolizinoquinoline alkaloid, camptothecin ($C_{20}H_{16}N_2O_4$, M.W. 348.4 $gmol^{-1}$; Fig. 2.2n), is well known for its antineoplastic activity and used in the treatment of various types of cancer. This anticancerous phytochemical can be extracted from the bark of *Camptotheca acuminata* (Nyssaceae). During DNA replication, it inhibits the formation of DNA topoisomerase I-mediated single-strand DNA breaks by stabilizing topoisomerase I-DNA covalent complexes (Martino et al. 2017).

Canadine ($C_{20}H_{21}NO_4$, M.W. 339.4 $gmol^{-1}$) is a berberine alkaloid found in *Glaucium squamigerum* and *Hydrastis canadensis*.

Carvacrol ($C_{10}H_{14}O$, M.W. $150.22 \text{ g mol}^{-1}$; Fig. 2.2o) and its derivatives are natural phenolic monoterpenoids commonly found in essential oils of different species of *Thymus*, *Origanum*, *Thymbra*, and *Lepidium flavum*. It has a broad spectrum of potential therapeutic applications, including antimicrobial, antioxidant, and anticancer activities (Sharifi-Rad et al. 2018; Kachur and Suntres 2020).

Chlorogenic acid ($C_{16}H_{18}O_9$, M.W. $354.31 \text{ g mol}^{-1}$), a polyphenolic cinnamate ester derived from caffeic acid and quinic acid, has recently received significant reorganization because of their many pharmacological roles, which include antioxidant, antimicrobial, hepatoprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, and anti-hypertension activity (Naveed et al. 2018). Beans of *Coffea arabica* and leaves of *Camellia sinensis* are enriched sources of different types of chlorogenic acid, including caffeoylquinic acid, di-caffeoylquinic acid, and feruloylquinic acid.

Citronella oil has citronellal ($C_{10}H_{18}O$, M.W. $154.25 \text{ g mol}^{-1}$; Fig. 2.2p), a monoterpenoid, as a main secondary metabolite that is responsible for its unique lemon aroma with an antifungal agent.

Cocaine ($C_{17}H_{21}NO_4$, M.W. $303.3529 \text{ g mol}^{-1}$; Fig. 2.3a) is a tropane alkaloid extracted primarily from the leaves of coca species (Erythroxylaceae), including *Erythroxylum coca* and *E. novogranatense*. The drug is primarily applied as an analgesic and psychostimulant for local anesthetic and vasoconstrictor in the eye, ear, nose, and throat. Cocaine is used as a narcotic for recreational and euphoria purposes due to its significant complex multifactorial influence on the central nervous system, which is caused by several processes on brain catecholaminergic neurons. Cocaine addiction can lead to cocaine use disorders and illnesses influenced by hereditary and environmental factors (Fernández-Castillo et al. 2021).

Codeine or *methymorphine* ($C_{18}H_{21}NO_3$, M.W. 299.4 g mol^{-1} ; Fig. 2.3b) is a natural plant alkaloid found in *Papaver somniferum* (Papaveraceae) and is commonly used as an opioid analgesic to decrease mild-to-moderate degrees of pain without impairing consciousness or altering other sensory functions (Chidambaran et al. 2017). Additionally, codeine is utilized as a sedative, hypnotic, antinociceptive, antitussive, and antiperistaltic agent.

Alkaloid colchicine ($C_{22}H_{25}NO_6$, M. W. 399.4 g mol^{-1} ; Fig. 2.3c) is a bioactive phytochemical of *Colchicum autumnale* and *Gloriosa superba*, which is utilized in the treatment of gout and inflammatory diseases (Paul et al. 2013). Colchicine showed anti-mitotic activity via interference in tubulin polymerization process (Paul et al. 2013).

A bioactive sesquiterpene lactone, costunolide ($C_{15}H_{20}O_2$, M.W. $232.32 \text{ g mol}^{-1}$; Fig. 2.3d) was identified from different species of *Magnolia*. Several preclinical types of research have proven it has multiple potential therapeutic properties, such as antioxidative, anti-inflammatory, antiallergic, bone remodeling, neuroprotective, hair growth-promoting, anticancer, and antidiabetic activities (Kim and Choi 2019).

Curcuma longa (Zingiberaceae) was exploited in traditional Chinese and Ayurvedic medicine due to its main bioactive ingredient, curcumin ($C_{21}H_{20}O_6$, M.W. 368.4 g mol^{-1} ; Fig. 2.3e), a polyphenolic compound having anti-inflammatory, neuroprotective, immunomodulatory, and hepatoprotective

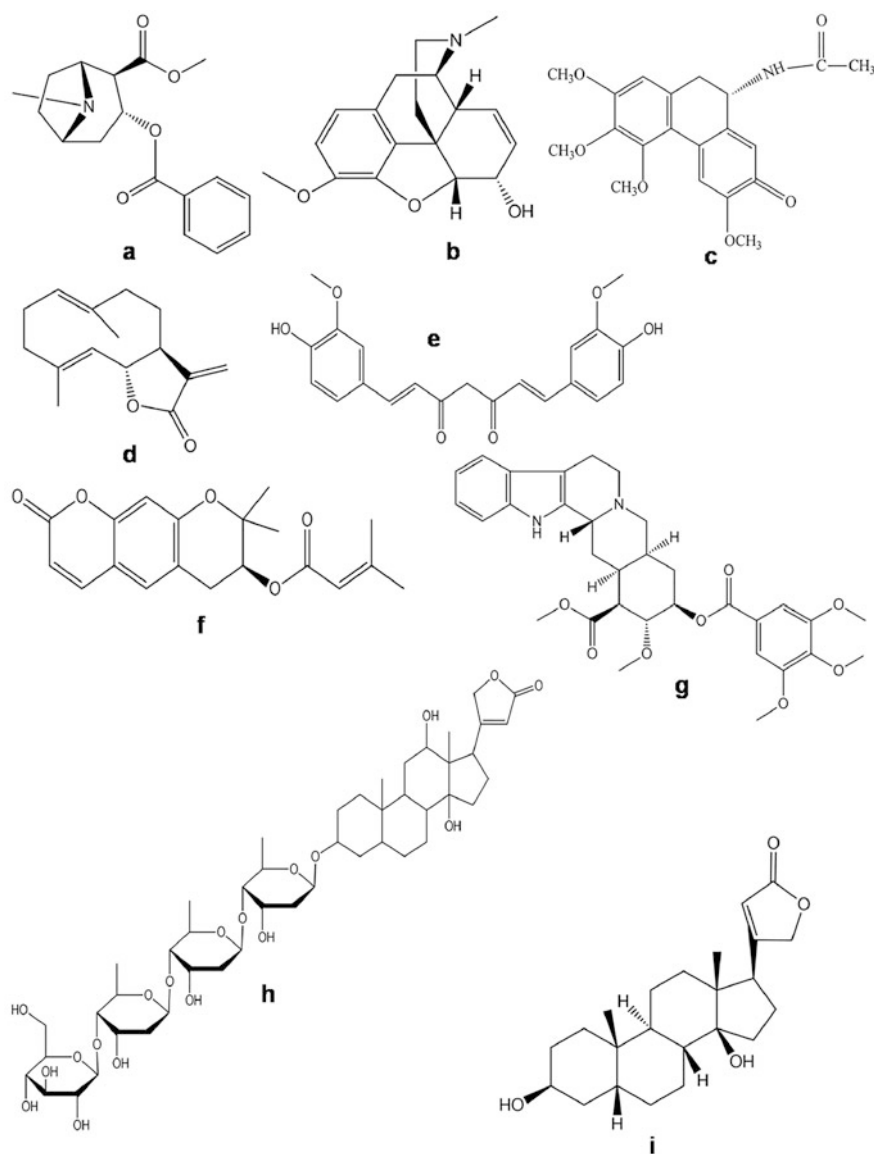


Fig. 2.3 Chemical structure of (a) cocaine, (b) codeine, (c) colchicine, (d) costunolide, (e) curcumin, (f) decursin, (g) deserpidine, (h) deslanoside, and (i) digitoxigenin

properties. Several studies in recent years have shown that curcumin has chemopreventive and chemotherapeutic properties in several cancer cells (Ashrafizadeh et al. 2020; Mansouri et al. 2020). It has anti-inflammatory properties because it inhibits

multiple enzymes involved in inflammation and interrupts cell signal transmission through different methods, including the suppression of protein kinase C.

Decursin ($C_{19}H_{20}O_5$, M.W. 328.4 $gmol^{-1}$) and decursinol angelate ($C_{19}H_{20}O_5$, M.W. 328.4 $gmol^{-1}$; Fig. 2.3f) are pyranocoumarins present in the roots of *Angelica gigas* (Shehzad et al. 2018). They are used for the prevention of cancer, rheumatoid arthritis, diabetic retinopathy, hepatic fibrosis, osteoclastogenesis, allergy, and Alzheimer's disease via multiple molecular targets (Shehzad et al. 2018).

Deserpidine ($C_{32}H_{38}N_2O_8$, M.W. 578.7 $gmol^{-1}$; Fig. 2.3g) is alkaloid, extracted from *Rauwolfia canescens* (Schlittler et al. 1955), and used to manage high blood pressure via competitive inhibition of angiotensin-converting enzyme and relieve psychotic behavior.

The leaves of *Digitalis lanata* have cardiac glycoside deslanoside or deacetyl lanatoside C ($C_{47}H_{74}O_{19}$, M.W. 943.1 $gmol^{-1}$; Fig. 2.3h) applied in traditional herbal medicine for the treatment of congestive heart failure and supraventricular arrhythmias and block Na-K-ATPase pump to manage ventricular rate in chronic atrial fibrillation. Recently, Liu et al. (2021) reported its antineoplastic properties in prostate cancer cells by modulating multiple signaling pathways.

Digitoxigenin ($C_{23}H_{34}O_4$, M.W. 374.5 $gmol^{-1}$; Fig. 2.3i) is an aglycon of digitoxin found in several members of the Apocynaceae (*Beaumontia grandiflora*, *Nerium oleander*, *Mandevilla pentlandiana*) and Plantaginaceae (*Digitalis grandiflora*, *Digitalis purpurea*, etc.).

Digitoxin ($C_{41}H_{64}O_{13}$, M.W. 764.9 $gmol^{-1}$; Fig. 2.4a) is a lipid-soluble cardenolide glycoside derived from *D. purpurea* and *D. lanata* (Plantaginaceae) that aids in the treatment of cardiac, cancer, and viral ailments (Patel 2016). It induces apoptosis and prevents the proliferation of various cancer cells via inhibition of plasma membrane Na-K-ATPase (Patel 2016; Gan et al. 2020).

Digoxigenin ($C_{23}H_{34}O_5$, M.W. 390.5 $gmol^{-1}$; Fig. 2.4b) is a cardenolide that is the aglycon of digoxin found in *D. purpurea*, *Digitalis orientalis*, and *D. lanata*.

Digoxin ($C_{41}H_{64}O_{14}$, M.W. 780.9 $gmol^{-1}$; Fig. 2.4c) is C-12 β -hydroxylated digitoxin. It is cardenolide glycoside isolated from *D. lanata*, used as a cardiotonic drug in patients with heart problems with a very narrow therapeutic window due to its cardiotoxic effect (Patocka et al. 2020). It enhances cardiac contractility via an increase in intracellular calcium through inhibition of the Na-K-ATPase pump. Digoxin induces apoptosis of tumor cells via a pathway involving mitochondrial cytochrome c and caspases 8 and 3 (Choudhari et al. 2020).

Emetine ($C_{29}H_{40}N_2O_4$, M.W. 480.649 $gmol^{-1}$; Fig. 2.4d) is one of the pyridoisoquinoline and isoquinoline alkaloids found in the roots of *Cephaelis ipecacuanha* (Rubiaceae), *Hedera helix*, *Alangium salviifolium*, and *Alangium longiflorum*. It is exploited as an antiprotozoal drug to cure amoebic dysentery and induce vomiting in toxicity-related therapy, regardless of its serious negative impacts, including cardiotoxicity and renal dysfunction. Interestingly, a few preliminary reports highlighted their potential role in the treatment and prevention of COVID-19 (Bleasel and Peterson 2020; Fan et al. 2021).

The chloroform extract of *Glycyrrhiza inflata* roots was used to isolate and structurally elucidate seven retrochalcones, namely, echinatine and

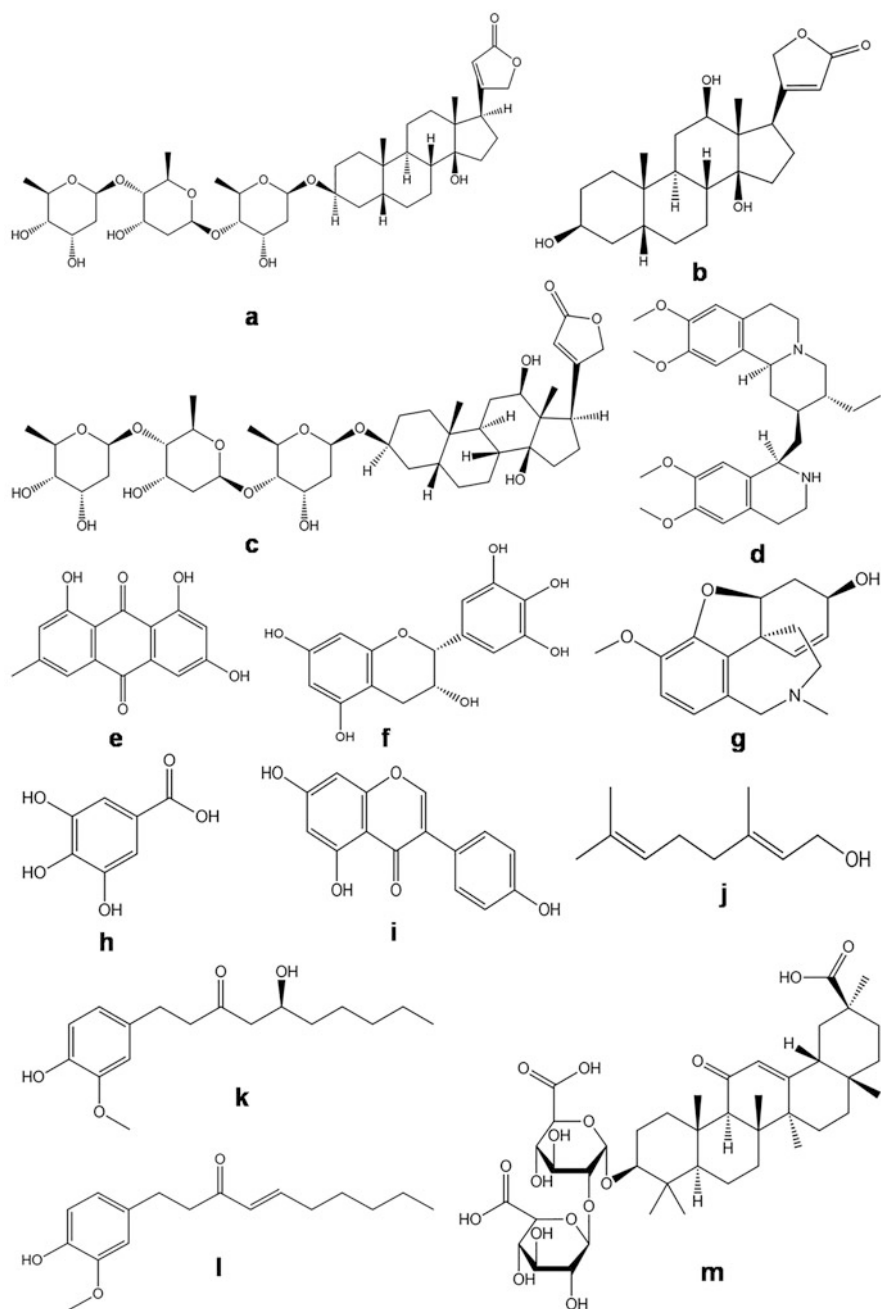


Fig. 2.4 Chemical structure of (a) digitoxin, (b) digoxigenin, (c) digoxin, (d) emetine, (e) emodin, (f) epigallocatechin, (g) galantamine, (h) gallic acid, (i) genistein, (j) geraniol, (k) 6-gingerol, (l) 6-shogaol, and (m) glycyrrhizin

licochalcones A, C, D, E, K, and L (Ali et al. 2021). Licochalcone A ($C_{21}H_{22}O_4$, M.W. 338.4 gmol^{-1}) is a phenol chalconoid derivative also found in the roots of *Pogostemon cablin*, *Glycyrrhiza glabra*, and others with potential anti-inflammatory, antibacterial, and anticancer activities (Wu et al. 2018). Licochalcone A promotes Sirt-1/AMPK pathway in mice fed a high-fat diet, which has preventive functions towards ameliorating obesity and nonalcoholic fatty liver disease (Liou et al. 2019). Several bioactivities of licochalcones C, D, E, K, and L were also reported.

The roots and rhizomes of *Rheum palmatum*, *Polygonum multiflorum*, *P. cuspidatum*, and *Aloe vera* are sources of a trihydroxyanthraquinone emodin ($C_{15}H_{10}O_5$, M.W. 270.24 gmol^{-1} ; Fig. 2.4e) (Ya et al. 2020). Cui et al. (2020) reported that this phytochemical has a high potential for use as a preventative and therapeutic drug for cancer, myocardial infarction, atherosclerosis, diabetes, acute pancreatitis, asthma, periodontitis, fatty liver, and neurodegenerative disease based on its fundamental properties.

(-)-Epigallocatechin ($C_{15}H_{14}O_7$, M.W. 306.27 gmol^{-1} ; Fig. 2.4f) is a catechin found in *Camellia sinensis* (Theaceae), *Acacia binervata*, and *Uncaria tomentosa*. It is a highly promising chemopreventive agent with antioxidant activity. Besides these, green tea contains lots of catechins, including catechin ($C_{15}H_{14}O_6$, M.W. 290.27 gmol^{-1}), epicatechin ($C_{15}H_{14}O_6$, M.W. 290.27 gmol^{-1}), epicatechin-3-*O*-gallate ($C_{22}H_{18}O_{10}$, M.W. 442.4 gmol^{-1}), and epigallocatechin-3-gallate ($C_{22}H_{18}O_{11}$, M.W. 458.4 gmol^{-1}), which are associated with several health-related benefits such as antioxidant, anticancer, anti-inflammatory, anti-collagenase, and antifibrosis effects (Chu et al. 2017).

Galantamine ($C_{17}H_{21}NO_3$, M.W. 287.35 gmol^{-1} ; Fig. 2.4g) is a tertiary alkaloid commonly found in the bulbs and flowers of different members of the maryllidaceae, including *Galanthus nivalis*, *Narcissus*, *Lycoris*, *Leucojum*, etc. (Janssen and Schäfer 2017). The FDA authorized the use of this drug in Alzheimer's disease in 2001. It is also used in several mental diseases, including moderate cognitive impairment in schizophrenia, bipolar disorder, and autism. It functions as an allosteric modulator of the nicotinic receptor as well as a cholinesterase inhibitor that blocks acetylcholine breakdown in the synaptic cleft, enhancing cholinergic neuron function and signaling.

Gallic acid ($C_7H_6O_5$, M.W. 170.12 gmol^{-1} ; Fig. 2.4h) is a trihydroxy-benzoic acid frequently extracted from the leaves of *Camellia sinensis*, *Malus domestica*, *Vitis vinifera*, *Fragaria* $\times$ *ananassa*, and pineapple, which exhibits antioxidant, antineoplastic, neuroprotective, and cyclooxygenase 2 inhibition activities (Kahkeshani et al. 2019).

Several traditional Chinese and Ayurvedic formulations have naturally occurring isoflavone genistein ($C_{15}H_{10}O_5$, M.W. 270.24 gmol^{-1} ; Fig. 2.4i) due to their significant beneficial effects on osteoporosis, cancer, cardiovascular disease, and postmenopausal problems (Jaiswal et al. 2019). Genistein is a phytoestrogen present in *Glycine max* with tyrosine and topoisomerase inhibition ability (Jaiswal et al. 2019).

Geraniol ($C_{10}H_{18}O$, M.W. 154.25 gmol^{-1} ; Fig. 2.4j) is an acyclic isoprenoid monoterpene found within many essential oils of aromatic plants, such as *Cinnamomum tenuipilum* and *Valeriana officinalis*, which have several bioactivities such as antimicrobial, anti-inflammatory, antioxidant, anticancer, hepatoprotective, cardioprotective, and neuroprotective properties (Lei et al. 2019).

Zingiber officinale extract has various types of polyphenolic gingerols, including 6-gingerol ($C_{17}H_{26}O_4$, M.W. 294.4 gmol^{-1} ; Fig. 2.4k), 6-shogaol ($C_{17}H_{24}O_3$, M.W. 276.4 gmol^{-1} ; Fig. 2.4l), 8-gingerol ($C_{19}H_{30}O_4$, M.W. 322.4 gmol^{-1}), 10-gingerol ($C_{21}H_{34}O_4$, M.W. 350.5 gmol^{-1}), zingerone ($C_{11}H_{14}O_3$, M.W. 194.23 gmol^{-1} ; Fig. 2.7i), 6-dehydrogingerone ($C_{17}H_{22}O_4$, M.W. 290.4 gmol^{-1}), and gingerone-A ($C_{21}H_{24}O_5$, M.W. 356.4 gmol^{-1}) with several bioactivities.

In folk medicine, glycyrrhizin ($C_{42}H_{62}O_{16}$, M.W. 822.9 gmol^{-1} ; Fig. 2.4m), a plant glycoside isolated from the roots of *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, and *Glycyrrhiza inflates*, has been utilized to treat bronchitis, gastritis, jaundice, stomach ulcers, constipation, viral hepatitis, etc. It has anti-inflammatory and antioxidant properties as well as the ability to promote endogenous interferon synthesis. Interestingly, the glycemic index remains unchanged when it is used as a sweetener since it is 30–50-fold sweeter than sucrose.

Hispidulin ($C_{16}H_{12}O_6$, M.W. 300.26 gmol^{-1} ; Fig. 2.5a) is a promising 4',5,7-trihydroxy-6-methoxyflavone with diverse antineoplastic properties (Liu et al. 2020) isolated from *Saussurea involucrea* (Asteraceae), *Achillea ageratum*, and *Arnica discoides*. It has a role as an apoptosis inducer, an anti-inflammatory agent, an antioxidant, and an anticonvulsant.

Hyoscyamine ($C_{17}H_{23}NO_3$, M.W. 289.3694 gmol^{-1} ; Fig. 2.5b), the levorotatory form of racemic atropine, is a valuable tropane alkaloid produced in high concentrations in the members of Solanaceae such as *Hyoscyamus niger* (Kohnen-Johannsen and Kayser 2019). Hyoscyamine is marketed in the United States without FDA approval. It is an anticholinergic drug to treat nausea, vomiting, motion sickness, smooth muscle spasms, and allergic rhinitis that competitively inhibits the binding of the acetylcholine to the muscarinic acetylcholine receptor (Kohnen-Johannsen and Kayser 2019).

Kaempferitrin ($C_{27}H_{30}O_{14}$, M.W. 578.5 gmol^{-1} ; Fig. 2.5c) is a natural glycoside of kaempferol found in aerial parts of *Vicia faba* and *Prunus cerasifera* (Wu et al. 2019; Patel 2021). It has a role as an antioxidant, anti-osteoporotic, hypoglycemic, immunomodulatory, anti-inflammatory, and antineoplastic agent (Patel 2021).

Kaempferol or kaempferide ($C_{15}H_{10}O_6$, M.W. 286.24 gmol^{-1} ; Fig. 2.5d) is a flavonoid component found in many common plants like *Spinacia oleracea*, *Brassica oleracea* var. *sabellica*, *Camellia sinensis*, *Brassica oleracea* var. *italic*, *Phaseolus vulgaris*, etc. (Dabeek and Marra 2019). Kaempferol is employed to address a variety of acute and chronic inflammation-related disorders, including postmenopausal bone loss, cancer, liver damage, obesity and diabetes, and acute lung injury (Ren et al. 2019).

Linalool ($C_{10}H_{18}O$, M.W. 154.25 gmol^{-1} ; Fig. 2.5e) is another monoterpene alcohol significantly found in essential oils of lavender and coriander. The

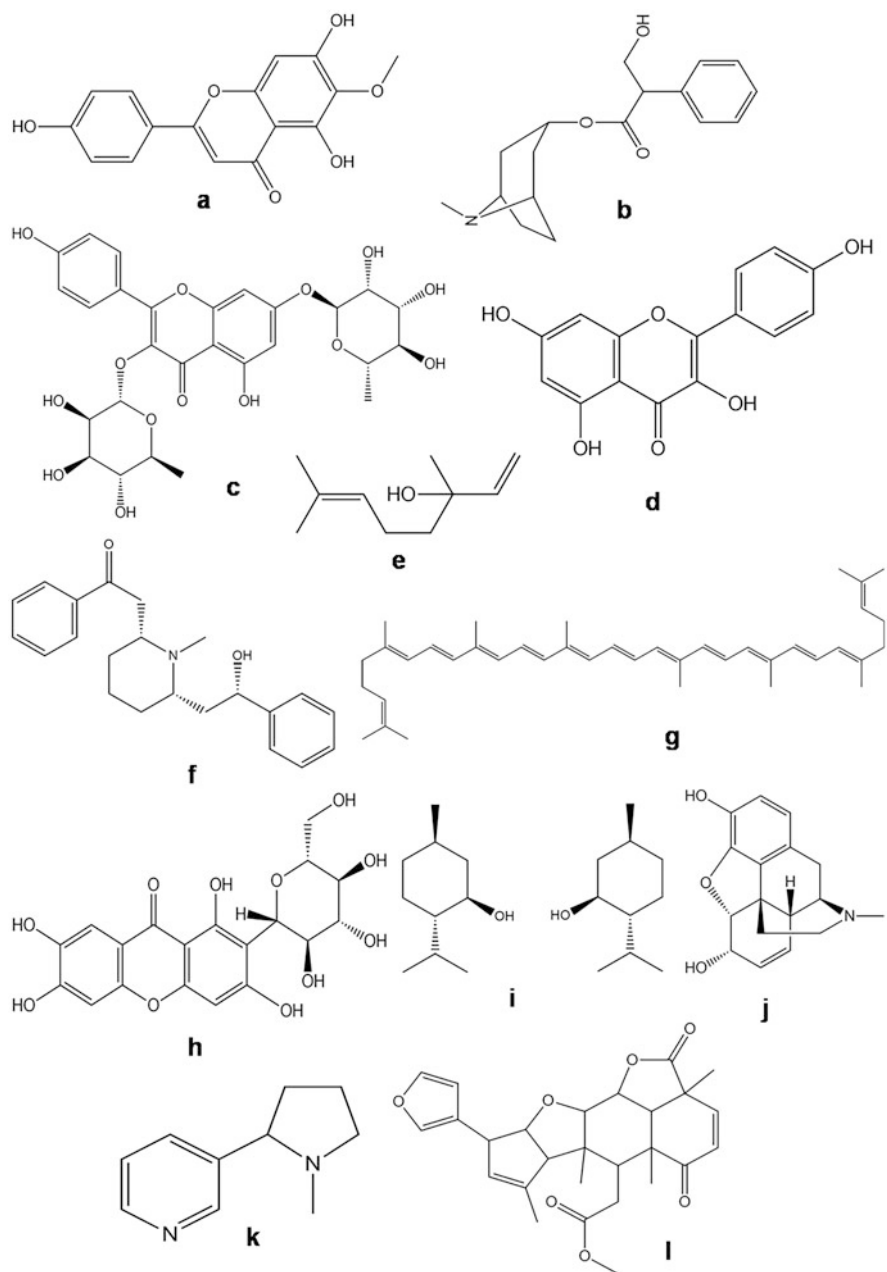


Fig. 2.5 Chemical structure of (a) hispidulin, (b) hyoscyamine, (c) kaempferitrin, (d) kaempferol, (e) linalool, (f) lobeline, (g) lycopene, (h) mangiferin, (i) menthol, (j) morphine, (k) nicotine, and (l) nimbolide

anti-inflammatory, antineoplastic, anti-hyperlipidemic, antibacterial, antinociceptive, analgesic, anxiolytic, antidepressive, and neuroprotective effects of linalool have been demonstrated in various in vitro and in vivo investigations (Pereira et al. 2018).

Lobeline ($C_{22}H_{27}NO_2$, M.W. 337.5 $gmol^{-1}$; Fig. 2.5f) is an optically active piperidine alkaloid derived from plants *Lobelia inflata* and *Lobelia sessilifolia*. It showed agonist and antagonist activity on nicotinic acetylcholine receptors and antiepileptic activity in mice via increasing the GABA release.

Lycopene ($C_{40}H_{56}$, M.W. 536.9 $gmol^{-1}$; Fig. 2.5g) is a naturally occurring linear, unsaturated hydrocarbon comprised of eight isoprene units responsible for the color of fruits and vegetables, abundantly found in different members of the Solanaceae, including *Solanum lycopersicum*. It is a potent antioxidant with several health benefits, including slowing the aging process and decreasing the risk of different chronic diseases such as cardiovascular diseases, obesity, cancer, type 2 diabetes, and neurodegenerative disorders (Saini et al. 2020).

Mangiferin ($C_{19}H_{18}O_{11}$, M.W. 422.3 $gmol^{-1}$; Fig. 2.5h) is a bioactive SM found in different parts of *Mangifera indica* with potential multifactorial and pharmacological effects such as antioxidant, antimicrobial, antidiabetic, antiallergic, anticancer, hypocholesterolemic, and immunomodulatory properties (Imran et al. 2017).

Menthol ($C_{10}H_{20}O$, M.W. 156.26 $gmol^{-1}$; Fig. 2.5i) is naturally found in peppermint and other mint plants used in multiple industries including pharmaceuticals, pesticides, and flavoring agents. Menthol and other peppermint oils are extensively utilized as ingredients in traditional medicine for the treatment of nasal decongestant and cough and inhalant to cure common cold.

Morphine ($C_{17}H_{19}NO_3$, M.W. 285.34 $gmol^{-1}$; Fig. 2.5j) is an ancient natural drug that belongs to the alkaloid group isolated from *Papaver somniferum* (Brook et al. 2017). Morphine is a powerful opiate analgesic psychoactive pain-relieving drug that operates directly on the central nervous system (CNS). It also has a significant potential for addiction as a narcotic.

A well-known natural addictive central nervous system stimulant is nicotine ($C_{10}H_{14}N_2$, M.W. 162.23 $gmol^{-1}$; Fig. 2.5k) belonging to the alkaloid group of compound and is the predominant ingredient of *Nicotiana tabacum*. It is a nicotinic cholinergic receptor agonist that causes the release of various neurotransmitters, including acetylcholine, beta-endorphin, dopamine, norepinephrine, serotonin, and ACTH.

Nimbolide ($C_{27}H_{30}O_7$, M.W. 466.5 $gmol^{-1}$; Fig. 2.5l) is a triterpene derived from the leaves and flowers of *Azadirachta indica*. Accumulating evidence from various in vitro and in vivo studies indicates that nimbolide is a promising candidate for the anticancer drug arsenal to fight against several types of cancer, which inhibits cancer cell growth and metastasis by inducing apoptosis (Pooladanda et al. 2018; Nagini et al. 2021).

Oleandrin ($C_{32}H_{48}O_9$, M.W. 576.7 $gmol^{-1}$; Fig. 2.6a) is a lipid-soluble cardiac glycoside derived from *Nerium oleander* (Apocynaceae) that may be used to treat heart diseases, cancer, and viral diseases (Zhai et al. 2022). Despite certain toxicity concerns, it has been studied as a chemotherapeutic agent to treat lung cancer. It

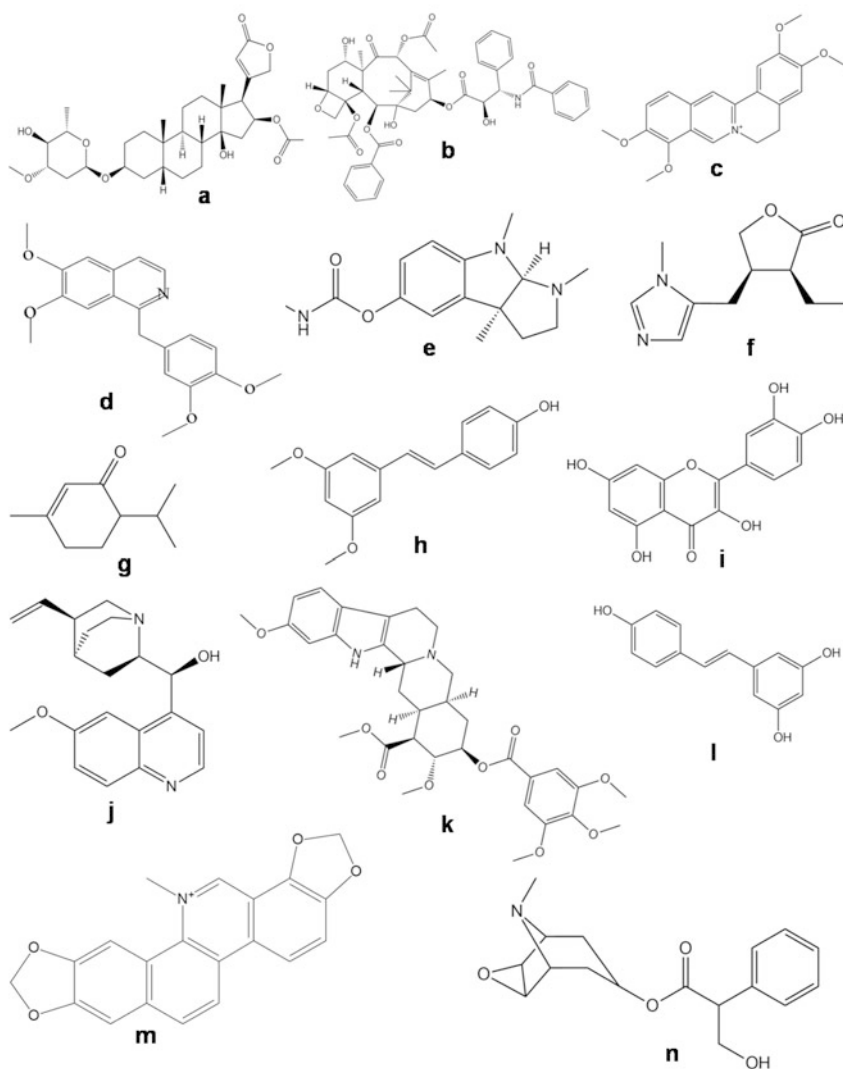


Fig. 2.6 Chemical structure of (a) oleandrin, (b) paclitaxel, (c) palmatine, (d) papaverine, (e) physostigmine, (f) pilocarpine, (g) piperitone, (h) pterostilbene, (i) quercetin, (j) quinidine, (k) reserpine, (l) resveratrol, (m) sanguinarine, and (n) scopolamine

promotes apoptosis in human cancer cells by inhibiting the $\alpha 3$ subunit of the Na/K-ATPase pump, Akt phosphorylation, NF- κ b activation, and FGF-2 export (Kanwal et al. 2020).

Paclitaxel ($C_{47}H_{51}NO_{14}$, M.W. 853.9 $gmol^{-1}$; Fig. 2.6b) is a tetracyclic diterpenoid with antineoplastic activity extracted from the bark of *Taxus brevifolia*.

It is also known as Taxol, which has a large legitimate market as a chemotherapeutic agent against a wide variety of cancers, including ovarian, breast, and lung cancer (Gallego-Jara et al. 2020). Paclitaxel binds to tubulin and stabilizes microtubules, causing cell division inhibition.

Palmatine ($C_{21}H_{22}NO_4$, M.W. 352.4 $gmol^{-1}$; Fig. 2.6c) is a berberine alkaloid and an organic heterotetracyclic compound.

Papaverine ($C_{20}H_{21}NO_4$, M.W. 339.4 $gmol^{-1}$; Fig. 2.6d) is a benzyloisoquinoline alkaloid derived from *Papaver somniferum* that has anti-smooth muscle relaxant, vasodilatory, and anticancer properties and inhibits calcium channel and phosphodiesterases (Sajadian et al. 2015).

Zhang et al. (2016) reported four new phorbol derivatives from the seeds of *Croton tiglium*, which are tetracyclic diterpenoids with antitumor activity.

Physalis pubescens (Solanaceae) contains a steroidal lactone-type phytochemical physapubescin B ($C_{30}H_{42}O_8$, M.W. 530.6 $gmol^{-1}$), which is effective against various types of cancer cells (Zhao et al. 2017; Dai et al. 2020) via different mechanisms.

Indole alkaloid physostigmine ($C_{15}H_{21}N_3O_2$, M.W. 275.35 $gmol^{-1}$; Fig. 2.6e), also called eserine, is a *N*-monomethyl carbamate with a narrow therapeutic index, isolated from *Physostigma venenosum* (Batiha et al. 2020). Physostigmine is utilized as a cholinergic agent via reversible inhibition of acetylcholinesterase (AChE) in mammalian peripheral tissues, and the brain increases the concentration of ACh at cholinergic transmission sites. It has a short half-life and the power to cross the blood-brain barrier. It has recently been recognized for treating glaucoma, myasthenia gravis, and intoxication produced by tricyclic antidepressant overdoses, antihistamines, antipsychotics, and benzodiazepines (Batiha et al. 2020).

The leaves of *P. jaborandi* and *P. racemosus* (Rutaceae) contain pilocarpine ($C_{11}H_{16}N_2O_2$, M.W. 208.26 $gmol^{-1}$; Fig. 2.6f) as a major alkaloid with muscarinic cholinergic parasympathomimetic agonist activity and smooth muscle contraction in the bronchi, urinary tract, biliary tract, and digestive tract. This alkaloid is pharmacologically used to treat xerostomia (dry mouth) and Sjogren syndrome and to reduce intraocular pressure in primary open-angle glaucoma by miosis that stimulates aqueous humor outflow (Carlson and Kraus 2021).

Piperitone ($C_{10}H_{16}O$, M.W. 152.23 $gmol^{-1}$; Fig. 2.6g) is a natural product found in *Eucalyptus fasciculosa* and *Eucalyptus intertexta*.

Plants like *Vitis vinifera* (Vitaceae), *Pterolobium hexapetalum*, and *Pterocarpus santalinus* possess pterostilbene ($C_{16}H_{16}O_3$, M.W. 256.3 $gmol^{-1}$; Fig. 2.6h), a natural stilbenoid with potential antioxidant, anti-inflammatory, pro-apoptotic, anti-neoplastic, and cytoprotective activities (Chan et al. 2019). Several reports also highlighted its beneficial effects on type 1 diabetes, insulin resistance, and type 2 diabetes in animal models, liver steatosis, and dyslipidemia (Gómez-Zorita et al. 2021).

Quercetin ($C_{15}H_{10}O_7$, M.W. 302.23 $gmol^{-1}$; Fig. 2.6i) is a ubiquitous polyphenolic flavonoid in plant-derived food with potential chemopreventive, anti-inflammatory, antioxidant, antibacterial, anti-allergy, and antiparasitic activity

(Ay et al. 2021). It can modulate EGFR or estrogen-receptor-mediated signal transduction pathways and inhibits lipoxygenase and cyclooxygenase pathways.

The bark of *Cinchona* tree contains different natural cinchona alkaloids, such as quinidine ($C_{20}H_{24}N_2O_2$, M.W. 324.4 gmol^{-1} ; Fig. 2.6j), quinine ($C_{20}H_{24}N_2O_2$, M.W. 324.4 gmol^{-1}), cinchonine ($C_{19}H_{22}N_2O$, M.W. 294.4 gmol^{-1}), and cinchonidine ($C_{19}H_{22}N_2O$, M.W. 294.4 gmol^{-1}) with a variety of pharmacologic properties (Boratynski et al. 2019). Quinine is a potent alkaloid used for the treatment of malaria and idiopathic muscle cramps for relaxation of muscles (Gachelin et al. 2017; El-Tawil et al. 2015). It is also used as a non-narcotic analgesic. Quinidine is a stereoisomer of quinine and has been used to treat atrial and ventricular arrhythmias for its class 1A antiarrhythmic activity (Song et al. 2018). Its antimalarial activity is mediated by its intra-erythrocytic schizonticide activity, in which the production of the heme polymerase enzyme (hemozoin) is inhibited within the acidic food vacuole of the parasite. Additionally, it acts as an alpha-adrenergic antagonist, a muscarinic antagonist, a sodium channel blocker, a potassium channel blocker, and a P450 inhibitor.

Reserpine ($C_{33}H_{40}N_2O_9$, M.W. 608.7 gmol^{-1} ; Fig. 2.6k) is one of the very old lipid-soluble FDA-approved (1955) indole alkaloids applied as an antihypertensive and antipsychotic drug derived from the roots of *Rauvolfia serpentina* and *R. vomitoria* (Li et al. 2020). This phytochemical is used in combination with thiazides to manage and treat hypertension (Shamon and Perez 2016). In central and peripheral adrenergic neurons, it binds and inhibits catecholamine pumping, leading to a reduction in alpha-adrenergic transmission and subsequently a decrease in catecholamines and serotonin. They are also used as diuretics or vasodilators.

Resveratrol ($C_{14}H_{12}O_3$, M.W. 228.24 gmol^{-1} ; Fig. 2.6l) is a polyphenolic nutraceutical found in several plant species like *Polygonum cuspidatum* (Polygonaceae) and *Vitis vinifera* used as a potential pharmaceutical agent in diabetes mellitus, obesity, and different types of cancer, cardiovascular diseases, kidney diseases, inflammatory diseases, and rhinopharyngitis (Singh et al. 2019).

Sanguinarine ($C_{20}H_{14}NO_4^+$, M.W. 332.3 gmol^{-1} ; Fig. 2.6m) is a benzophenanthridine and belongs to benzyisoquinoline alkaloid mostly found in plants from the Fumariaceae, Papaveraceae, and Rutaceae families (Laines-Hidalgo et al. 2022). Several reports revealed its role as antioxidant, anti-inflammatory, and anticancer agent (Fu et al. 2018).

Scopolamine ($C_{17}H_{21}NO_4$, M.W. 303.35 gmol^{-1} ; Fig. 2.6n) is a sedative and anticholinergic class of tropane alkaloid derived specifically from *Datura stramonium*, *Hyoscyamus niger*, and *Atropa belladonna* (Solanaceae) (Riad and Hithe 2021). The FDA approved its use to control and treat postoperative nausea, vomiting, and motion sickness (Riad and Hithe 2021). It is responsible for muscarinic receptor-mediated cholinergic antagonism due to its structural similarity to acetylcholine.

A steroidal alkaloid sapogenin, solasodine ($C_{27}H_{43}NO_2$ M.W. 413.6 gmol^{-1}), found in Solanaceae members, is used as a precursor in the synthesis of complex steroidal compounds such as contraceptive pills. It has antifungal, antioxidant, antipyretic, cardiotonic drug, and apoptosis induction properties.

Strychnine ($C_{21}H_{22}N_2O_2$, M.W. 334.419 g mol^{-1} ; Fig. 2.7a) is a strychnidine having a keto substituent at the 10-position and belongs to the monoterpene indole alkaloid derived from the seeds of *Strychnos nux-vomica* (Loganiaceae) and a few related trees native to Southeast Asia and Australia. It is primarily used as a pesticide, especially a rodenticide. This SM acts as a competitive antagonist for the neurotransmitter glycine receptor, leading to increased neuronal activity and excitability, which contributes to muscle contraction (Patocka 2020).

Sulforaphane ($C_6H_{11}NOS_2$, M.W. 177.3 g mol^{-1} ; Fig. 2.7b) is a dietary isothiocyanate group of organosulfur compounds synthesized from a precursor found in cruciferous vegetables. It exhibits antioxidant, antitumor, anti-inflammatory, and antimicrobial activities in mammals (Houghton 2019; Vanduchova et al. 2019).

Thymol ($C_{10}H_{14}O$, M.W. 150.22 g mol^{-1} ; Fig. 2.7c) is a natural monoterpene derivative of *p*-cymene found in different species of *Thymus*. It has antimicrobial activity against a broad spectrum of microorganisms and has anticancerous, antiseptic, analgesic, and anti-inflammatory properties (Dheer et al. 2019). De La Chapa et al. (2018) reported that intratumor injection of thymol decreased tumor volume while decreasing cell proliferation and inducing mitochondrial membrane depolarization-mediated apoptosis in oral squamous cell carcinoma Cal27- and HeLa-derived mouse xenografts.

Thymoquinone ($C_{10}H_{12}O_2$, M.W. 164.2 g mol^{-1} ; Fig. 2.7d) is a 1,4-benzoquinone isolated from the seed oil of *Nigella sativa* (Ranunculaceae), *Monarda fistulosa* (Lamiaceae), and *Ayapana triplinervis* as an active constituent which has demonstrated promising anti-inflammatory, antioxidant, immunomodulatory, hepatoprotective, nephroprotective, and chemotherapeutic activity (Darakhshan et al. 2015).

Polar hydrophilic alkaloid trigonelline ($C_7H_7NO_2$, M.W. 137.14 g mol^{-1} ; Fig. 2.7e) commonly found in *Trigonella foenum-graecum*, *Allium sepapea*, and *Lycopersicon esculentum* plant species, which showed promising results against hyperglycemia, hypercholesterolemia, cancer, and nervous and hormonal disorders (Mohamadi et al. 2018).

Tubocurarine ($C_{37}H_{41}N_2O_6^+$, M.W. 609.7 g mol^{-1}) is a benzyloquinoline alkaloid with nicotinic antagonist and muscle relaxant activities, predominantly found in plants belonging to Menispermaceae.

Turmerone ($C_{15}H_{22}O$, M.W. 218.33 g mol^{-1} ; Fig. 2.7f) is a naturally occurring sesquiterpene isolated from *Curcuma longa* that inhibits proliferation and induces apoptosis in cancer cell lines.

The roots and leaves of *Tylophora indica* (Asclepiadaceae) have several alkaloids, including tylophorine ($C_{24}H_{27}O_4N$, M.W. 393.5 g mol^{-1} ; Fig. 2.7g), tylophorinine ($C_{23}H_{25}O_4N$, M.W. 379.4 g mol^{-1}), and tylophrinidine ($C_{22}H_{22}O_4N$, M.W. g mol^{-1}) having immunosuppressive, antitumor, antifeedant, antibacterial, antifungal, antiamebic, diuretic, and hepatoprotective activities (Cyriac et al. 2020).

Plants like *Uncaria tomentosa* and *Salvia nemorosa* have a natural pentacyclic triterpene known as ursolic acid ($C_{30}H_{48}O_3$, M.W. 456.7 g mol^{-1} ; Fig. 2.7h) with a wide range of potential pharmacologic activities, including anti-inflammatory, antioxidative, antiviral, serum lipid-lowering, and anticancer activities. Prasad

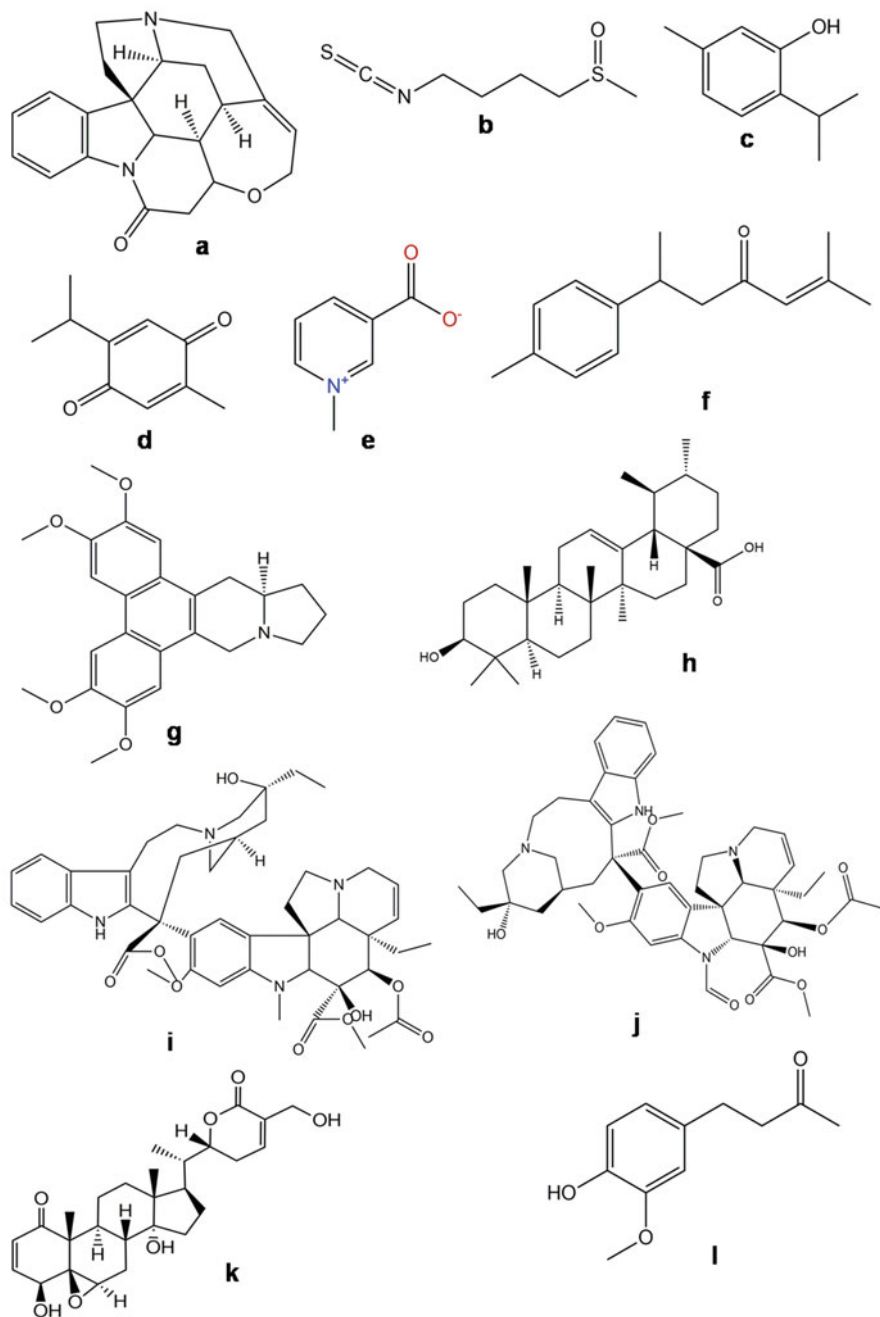


Fig. 2.7 Chemical structure of (a) strychnine, (b) sulforaphane, (c) thymol, (d) thymoquinone, (e) trigonelline, (f) turmerone, (g) tylophorine, (h) ursolic acid, (i) vinblastine, (j) vincristine, (k) withaferin A, and (l) zingerone

et al. (2018) reported its involvement as a cancer chemosensitizer to standard chemotherapeutic drugs, whereas Wang et al. (2021) reported more effective tumor growth inhibition and apoptosis by the use of ursolic acid nanoformulations.

The natural alkaloids vincristine ($C_{46}H_{56}N_4O_{10}$, M.W. 825 gmol^{-1} ; Fig. 2.7i) and vinblastine ($C_{46}H_{58}N_4O_9$, M.W. 811 gmol^{-1} ; Fig. 2.7j) are asymmetric dimeric alkaloids of vindoline and catharanthine commonly found in *Catharanthus roseus* leaves (Kumar et al. 2018). Vinblastine and vincristine are structurally similar, except vinblastine has a methyl group instead of an aldehydic group linked to the nitrogen of the core indole moiety in vincristine. Vincristine is available on the market under multiple commercial brand names, such as Marqibo and Vincasar. Their microtubule destabilization-mediated antineoplastic action leads to cell cycle arrest via inhibition of mitotic spindle formation, which makes these phytochemicals effective in treating different types of cancers, including breast cancer, testicular cancer, and neuroblastoma (Kumar 2016). Additionally, they are used in the treatment of Hodgkin's and non-Hodgkin's lymphoma, acute erythraemia, acute pancytopenia, etc.

W. somnifera (Solanaceae) is a traditional medicinal herb in Ayurveda for the treatment of cardiac failure, diabetes, arthritis, epilepsy, gout, and skin diseases. Different withanolides with steroidal lactone, such as withanolide A ($C_{28}H_{38}O_6$), withanolide D ($C_{28}H_{38}O_6$), anaferrine ($C_{13}H_{24}N_2O$), and withaferin A ($C_{28}H_{38}O_6$, M.W. 470.6 gmol^{-1} ; Fig. 2.7k), are extracted from roots and leaves of the Solanaceae including *W. somnifera*, *Vassobia breviflora*, *Physalis longifolia*, etc. (Bhasin et al. 2019). Withaferin A exhibits cytotoxic activity in in vivo studies and may be used as an antineoplastic agent and an apoptosis inducer (Hassannia et al. 2020).

2.8 Phytochemical-Derived Semi-synthetic Drugs

Apomorphine (brand names: Kynmobi, Apokyn; $C_{17}H_{17}NO_2$, M.W. 267.32 gmol^{-1}) is an aporphine alkaloid, a subcutaneously administered dopamine receptor agonist used predominantly in the therapy of hypomobility in advanced Parkinson's disease.

Artemotil or β -arteether (brand name: Alfabet; $C_{17}H_{28}O_5$, M.W. 312.406 gmol^{-1}) is a semi-synthetic derivative of artemisinin used as an antimalarial medicine specifically indicated for the treatment of chloroquine-resistant *Plasmodium falciparum* malaria and cerebral malaria cases.

Dicumarol (brand names: Coumadin, Sintrom; $C_{19}H_{12}O_6$, M.W. 336.3 gmol^{-1}) is a 4-hydroxycoumarin-derived natural anticoagulant via competitive inhibition of vitamin K epoxide.

Rivastigmine (brand names: Exelon, Exelon Patch; $C_{14}H_{22}N_2O_2$, M.W. $250.3367\text{ gmol}^{-1}$) is a derivative of physostigmine from Calabar beans and a reversible cholinesterase inhibitor used in the treatment of Alzheimer's or Parkinson's disease.

Tiotropium (Spiriva Respimat, Spiriva HandiHaler; $C_{19}H_{22}NO_4S^{2+}$, M.W. 392.5 g mol^{-1}) is a synthetic anticholinergic agent that is used as an inhalant for the treatment of acute bronchospasm due to chronic bronchitis or emphysema.

Vindesine (brand name: Eldisine; $C_{43}H_{55}N_5O_7$, M.W. 753.9 g mol^{-1}) is a semi-synthetic vinca alkaloid derived from vincaleukoblastine showing vincristine-like antitumoral activity against different types of cancer, including leukemia, melanoma, and breast cancer, by inhibition of the polymerization of tubulin.

Vinflunine (brand name: Javlor; $C_{45}H_{54}F_2N_4O_8$, M.W. 816.9 g mol^{-1}) is a bi-fluorinated derivative of the semi-synthetic vinca alkaloid vinorelbine available on the market for the treatment of advanced or metastatic transitional cell carcinoma with antitubulin, antineoplastic, and antiangiogenic activities.

Vinorelbine (Navelbine; $C_{45}H_{54}N_4O_8$, M.W. $778.947 \text{ g mol}^{-1}$) is a semi-synthetic third-generation vinca alkaloid used for the treatment of cancer.

Vinpocetine (brand name: Neurovin, Cognitol; $C_{22}H_{26}N_2O_2$, $350.45 \text{ g mol}^{-1}$) is a synthetic ethyl ester derivative of apovincamine, a vinca alkaloid used to treat neurological symptoms of cerebrovascular disease.

2.9 Harmonization Between Biodiversity, Ecosystem, and Human Health

According to the fossil record, the therapeutic relationship between humans and plant biodiversity is traced back at least 60,000 years. Despite significant advancements/growth in modern medicine over the last 200 years, it continues to rely greatly on the unevenly distributed plant biodiversity and phytochemical diversity of the planet for less toxic and more effective valuable therapeutic phytochemicals to cure human diseases and the enhancement of immunity or mental health. The relevance of plant-derived unrefined active ingredients (crude plant extract or herbal formulation) in traditional, ethno-, and herbal medicine in the past, present, and future health issues is self-evident. Furthermore, plant-derived active chemicals can be employed as drugs or lead molecules, which may be released as drugs after structural modification by synthetic chemists in modern pharmaceutical enterprises. Furthermore, apart from medical care issues, human health is more deeply intertwined with the ecosystem because it also supplies food, drink, fresh air, shelter, and climate, all of which can impact human health and their existence on earth. Thus, the loss of biodiversity and natural ecosystems poses a significant threat to modern medicine, traditional medicine, and human healthcare.

The long-held public misconception that plants are an abundant and inexhaustible natural gift should be changed via awareness programs. The biodiversity of MPs is more prone to biodiversity loss as a result of growing demand and commercialization-driven overexploitation for medicinal compounds. Several anthropogenic activities and environmental changes, such as pollution, global

warming, and synthetic chemical toxicity, increase the chance of plant diversity extinction.

Re-evaluating the crucial relationship between human health, MPs, and ecosystems and restoring the planet's ecosystem by making reasonable efforts to mend this bonding are urgently needed since the fate of the other impacts both. Economic benefits should be evaluated in light of environmental effects. Natural plant diversity conservation and their sustainable exploitation are also important for pharmaceutical and biotechnological industries for their plant-derived drug developmental research. It is vital to adopt biodiversity and ecosystem conservation legislations, ethnomedicinal knowledge documentation, shared benefits and information, and awareness campaigns to improve biodiversity worldwide.

Additionally, the cultivation of high-demand medicinal plants using controlled and scientific methods capable of producing high-quality MPs reduces exploitation pressure from natural populations. The conservation of MP diversity will assist in the sustainability of ecosystems and might be used to discover unique pharmaceutical and biological compounds.

2.10 Future Perspective

In spite of the sharp fall in the predominant use of plant-derived phytochemicals as therapeutic agents in modern medicine during the early twenty-first century due to certain challenges associated with plant-derived drugs, plant-derived phytochemicals serve as the foundation for rational medicine creation by providing clinical candidates and a variety of unique templates/substructures or scaffolds for the development of novel synthetic drugs or analogs for the pharmaceutical and biotechnology industries.

Spite of some current inability of alternative drug discovery strategies, the huge plant biodiversity, along with the structural diversity of plant-based therapeutic phytochemicals in individual plants, is the greatest blessing of nature to the human population. Plant-based therapeutic phytochemicals have become more popular due to their superior quality, higher curative properties with fewer adverse side effects, safety, affordability, and acceptance across multiple cultures and ethnicities, in contrast to synthetic chemical drugs, which are derived with the help of computer-based research and numerous chemical methods. Furthermore, the development and utilization of plant-based therapeutic drugs are encouraged since they generally have low molecular weight, higher molecular rigidity and interactivity towards different biomolecules, low partition coefficient, and fewer heavy metals compared to synthetic substances (Mathur and Hoskins 2017).

Currently, phytomedicines perform an important part in the healthcare systems of more than 80% of the human population. The potential role of plant and phytochemicals in the discovery of pharmaceutically active biomolecules cannot be underestimated, as only a small fraction of the existing plants have been chemically and pharmacologically examined fruitfully as yet. Handa (1991) reported that drug

discovery is still in its infancy because only 6% of the 4 lakh plant species have been explored for their activities, and <20% of plants have been investigated phytochemically. Fabricant and Farnsworth (2001) also revealed that only 15% and 6% of the 250,000 higher plant species had been evaluated for phytochemical screening and bioactivity, respectively. Thus, existing knowledge on phytochemical diversity and bioactivities is only the tip of the iceberg, and several treasures are still hidden within the pool of phytochemicals.

Exploration and acquisition of comprehensive knowledge about the chemical and structural diversity of phytochemical constituents of plants, particularly medicinal plants, is a dynamic research field. In comprehensive and in-depth modern research, a continuous thrust is required to discover structure-activity relationships of natural phytochemical ingredients to better understand their versatile pharmacological value and therapeutic applications. It utilizes for drug development across the world to counter different medicinal challenges.

The highly competitive field of modern pharmaceuticals has begun to move away from traditional activity-guided natural phytochemical discovery to the exploration of uncharted chemical space. It demands adaptation of recent advances in the fields of modern techniques, instrumentation, and automation, and integration of different disciplines such as ethnobotany, phytochemistry, analytical chemistry, biotechnology, genomics, transcriptomics, proteomics, metabolomics, and pharmacology, to improve the efficiency of screening, isolation, separation, purification, detection, identification, and structural characterization in the realm of phytochemicals.

Such strategic adaptations can result in a drastic increase in the repository of phytomolecules and the expansion of knowledge frontiers, paving the foundation for future discovery of pharmaceutical therapeutic molecules. Additionally, the natural product community will need to collaborate constantly to create approaches for effectively analyzing and successfully assessing natural product biosynthetic gene clusters to accelerate speed discovery efforts.

Nature is constantly producing biological diversity via trial and error over millions of generations, allowing for the development of new anti-disease weapons enabling discovery of novel anti-disease weapons. In biomedicine, progress in determining how structural confirmations or functionalities influence molecular performance is fundamental for drug discovery. To uncover bioactive phytochemicals and bring them forward into clinically and therapeutically relevant useful medicines, drug formulation, stability, effectiveness, pharmacokinetics, and metabolic engineering investigations are required (Mathur and Hoskins 2017). Large-scale extraction or biotechnology manufacturing is necessary for the clinical feasibility of any possible lead compounds.

However, constraints in data accessibility and its rational usage are the major hurdles due to the lack of a composite database of phytochemicals that need to be solved to facilitate the identification of novel phytomolecules (lead molecules) for plant-based drug discovery worldwide to fight against various pharmacological targets and their secondary complications. Thus, a comprehensive phytoanalysis and phytopharmacological investigation of the various bioactive fractions should be performed via recent technological and scientific advances to overcome challenges

associated with phytochemical discovery and achieve the dreams of herbal drug discovery.

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Chapter 3

Threats and Opportunities for Sustainable Use of Medicinal Plants in Brazilian Atlantic Forest Based on the Knowledge of Indigenous Peoples and Local Communities



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Abstract The Brazilian Atlantic Forest is the second largest humid tropical forest in Brazil and is known due to its high biodiversity. Several human groups live within this biome and have developed unique wisdoms and knowledge about the native flora. Based on a literature review, in this study we investigate the richness of plants known and used as medicinal in the Brazilian Atlantic Forest biome by Indigenous peoples and local communities (IPLCs), to understand the threats and opportunities for the sustainable use of these plants. We analyzed 162 articles, which comprise information about 1686 species of medicinal plants. Of the total species, 54% are native to Brazil, 25% are exotic species, and 7% are naturalized. Among the native species, 165 are on the national red list in different categories of threat. When comparing the location of the studies in relation to the presence of protected areas and traditional territories, it became evident that the wealth of plants known and used by human populations in the Atlantic Forest is not legally protected in their natural environments. In this sense, it is essential to expand and regularize the areas of traditional territories and strengthen the protected areas (conservation units, in Brazil) for sustainable use in the Atlantic Forest region, in order to protect the knowledge and practices of the IPLCs and to support their health and well-being.

Keywords Ethnobotany · Local ecological knowledge · Health · Brazil

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3.1 Introduction

Medicinal plants are one of the main foci of ethnobotanical studies (Oliveira et al. 2009). Beyond their importance as a source of active ingredients or molecules for bioprospection, under an ethnopharmacological approach (Heinrich et al. 2006), the issue of medicinal plants is a subject of ethnobotanical hypothesis and theories on to how people select plants (including demographic traits of plants, phytochemistry, and social dynamics and human traits of users affecting plant selection; Gaoue et al. 2017). In this chapter, we highlight the central role medicinal plants have to the health and well-being of rural, urban, traditional, and nontraditional peoples, expanding the perspective to understand the role of medicinal plants not only as curative resources but as important elements for ecosystem health and for the resilience of socio-ecological systems (Zank et al. 2015, 2019a, 2022).

Within this broader context, the value of ethnobotanical information on medicinal plants has implications in the debate on biodiversity conservation and on the recognition of traditional territories. Although these both implications are intrinsically related, we are still far from meeting the global objectives of the Convention on Biological Diversity (Hanazaki et al. 2018). Issues such as plant harvesting and extraction and the impacts of the exploration of non-timber forest products facing growing urban markets are only the tip of the iceberg of discussions with implications to local livelihoods, sustainable use of resources, and social and environmental justice. In the post-2020 biodiversity agenda, Indigenous peoples' and local communities' rights and agency may have a central role, supporting both conservation targets and their own livelihoods, rights, and territories (Reyes-García et al. 2022). Understanding broader scenarios of ethnobotanical knowledge on medicinal plants can support these debates, highlighting the importance of Indigenous peoples' and local communities' being at the forefront of the discussions on the threats and potentials for sustainable use of medicinal plant resources.

In Brazil, Indigenous peoples and local communities (or IPLCs, following IPBES definition, see McElwee et al. 2020) include both Amerindian groups, descendants from people who inhabited the area currently called Brazil since before European colonization in the sixteenth century. It also includes several other traditional groups of mixed origin. For example, Quilombolas or Maroons are descendants of people who were forcibly enslaved and transported by Europeans to Brazil, from several parts of Africa. Slavery in Brazil was abolished in the end of the nineteenth century and left deep marks and inequalities in Brazilian society. Other traditional peoples are defined due to their characteristics of being "culturally differentiated groups that recognize themselves as such, that have their own forms of social organization, that occupy and use territories and natural resources as a condition for their cultural, social, religious, ancestral and economic reproduction, using knowledge, innovations and practices generated and transmitted by tradition" (Brasil 2007). These groups include (but are not restricted to) Quilombolas, artisanal fishers, farmers, extractivists, people of African-Brazilian religions, some groups of descendants of European migrants, and more than 300 different ethnicities of Amerindians. The

IPLC concept also includes other local communities that do not have this self-identification as indigenous or traditional, but that also maintain knowledge and uses associated with biodiversity.

The Brazilian law recognizes the paramount importance of the relationship of IPLCs with ancestral territories, which are a place for traditional practices, for supporting their worldviews, and for providing natural resources such as medicinal plants. Traditional health practices are also intertwined with the existence of traditional territories and IPLCs' traditional knowledge. The guarantee of access to territories contributes to the autonomy of communities in obtaining plants for health care and well-being, as well as in the dynamic of transmission, innovation, and production of this traditional knowledge. Plants are one of the main resources used in local medical systems, both in urban and rural areas (Vandebroek et al. 2004; Zank and Hanazaki 2017), and are used as medicines and in symbolic and ritualistic contexts providing health and well-being (Zank et al. 2016; Diniz 2019). These resources are usually extracted from the forest environments or cultivated in areas close to the residences. However, with the increasing urbanization and the loss of traditional territories, they are also being obtained through purchase in markets and fairs.

Several research efforts accessed the diversity of medicinal plants from the Brazilian Atlantic Forest (Begossi et al. 2002; Di Stasi et al. 2002; Liporacci et al. 2017). To update these efforts, and going beyond the role of medicinal plants as a source of remedies but understanding them in the whole context of traditional health practices, we aim to investigate the richness of plants known as medicinal in the Brazilian Atlantic Forest biome, to understand the threats and potential for the sustainable management of these plants. Our focus is especially on IPLCs' traditional knowledge, but in our review we also included studies which specified other social groups who were the knowledge holders. In this sense, we analyzed the richness of known medicinal plants (native, exotic, and naturalized), the species that are threatened for conservation, the exotic species that can bring risks due to their invasive potential, and the opportunities of protected areas and traditional territories for the sustainable use and conservation of medicinal species in the Atlantic Forest.

3.2 Study Area: The Brazilian Atlantic Forest

The Brazilian Atlantic Forest is a wide region that extends from the east coast of the state of Rio Grande do Norte to the state of Rio Grande do Sul, with different plant typologies (IBGE 2012), and covers a latitudinal range from about 3° latitude S to 33° latitude S and a total of 17 Brazilian states. After the Amazon, it represents the second largest humid tropical forest in Brazil and is considered to harbor the second largest species biodiversity of Brazilian fauna and flora. However, this biome is highly fragmented due to human occupation. The Brazilian Atlantic Forest is a hotspot for biodiversity conservation, with an estimated richness of 20,000 species

of vascular plants, of which 8000 are endemic (Mittermeier et al. 2004; Stehmann et al. 2009).

The process of fragmentation of the Brazilian Atlantic Forest has occurred since European colonization, starting in the sixteenth century. The intensive extraction of forest resources associated with economic cycles and urban expansion has increasingly degraded this biome. Currently, the major Brazilian cities are located within the Atlantic Forest domain and also the highest demographic densities in the country. The current forest cover of the Atlantic Forest is less than 12.5% of the original cover (<https://www.sosma.org.br/>).

The Brazilian Atlantic Forest, in addition to being an extremely urbanized environment, is home to several Indigenous peoples and traditional communities that have settled in these regions with historical land use practices, contributing to the conservation of natural resources over time (Oliveira 2007). The conservation and use of these resources includes, mainly, medicinal species, which are the most used by communities in general (Bortoli et al. 2021). However, despite the existence of some integrated databases (e.g., Plataforma de Territórios Tradicionais, MPF 2019), the precise number of how many Indigenous peoples and traditional communities and family farmers that inhabit the original area of the Atlantic Forest, and their population size, is still an unresolved issue.

The Atlantic Forest, in its borders, makes contact with three other Brazilian biomes: the *Caatinga* (located mainly in northeastern Brazil in a region with a semi-arid climate), the *Cerrado* (shelters savanna-type vegetation and occurs mainly in the Brazilian central plateau), and the *Pampa* (located in the extreme south of Brazil and comprises mainly grassland vegetation). The different plant typologies (IBGE 2012) of the Atlantic Forest domain include dense ombrophilous forest in the areas with a strong influence of the humidity of the Atlantic Ocean, mixed ombrophilous forest (with the remarkable presence of *Araucaria angustifolia*) in higher altitudes, semi-deciduous seasonal forests, restingas (vegetation associated to sandy soils), and mangroves near the coast.

3.3 Methods

We did a systematic literature review using the databases Web of Science and Scopus, using the search key “Ethnobotany” OR “Plants use” OR “Plants used” OR “Uses of plants” OR “Knowledge of plants” OR “Ethnobotanical works” OR “Ethnobotanical work” OR “Useful Plants” OR “Local Knowledge” OR “Traditional Knowledge” OR “Ethnoecology” OR “Ethnobiology” AND “Brazil”, considering article title, keywords, and abstracts and for articles published until 2021. The list of publications about medicinal plants in the Atlantic Forest before (and including) 2014 was based on the review of Liporacci et al. (2017), but complemented in this new search. The literature search was done between November 2021 and March 2022.

This search key returned 746 indexed articles. After removing duplicates, an automatic filter for “medicine” OR “medicinal” OR “healing” OR “health” OR “remedy” OR “remedies” resulted in the exclusion of 525 articles. The remaining 221 articles were downloaded, read, and analyzed, and another 59 articles were further excluded.

We used as inclusion criteria the following: (1) if the study was done in the Atlantic Forest (for this, in some cases when not informed, we checked the municipality where the study was done and crossed this information with Brazilian distribution of biomes, using the original cover of the Atlantic Forest as a reference (<https://www.sosma.org.br/>), (2) if the knowledge or use of medicinal plants was reported in the study (some studies although focusing on food plants, for example, may have also mentioned medicinal plants), and (3) if the study identified whose knowledge has been investigated (e.g., Indigenous people, farmers, interviewees in a given city or community). If the study meets criteria 1 and 2 but not 3 (e.g., a study in hospitals or fairs/markets), we analyzed if it could be included as diffuse knowledge.

We excluded from our list the studies that (4) mentioned the use of medicinal plants but did not mention the identified botanical species, (5) review articles (except reviews of gray literature, or reviews of theses/dissertations, or reviews of non-indexed articles), (6) studies on medicinal plants in historical documents without a link with contemporary users, (7) studies on archeology/archaeoethnobotany, and (8) studies that had an essentially pharmacological focus, without addressing the ethnobotanical knowledge or the knowledge holders.

From each article, we extracted the metadata describing the study and the data about the plants used and organized this information in spreadsheets. The metadata included the bibliographic reference, DOI, details on data collection, sampling, ethic authorizations, latitude, longitude, city/municipality, and state. The data about the plants used included, for each species, the botanical name, local name, varieties, uses reported, parts used, users (including if they belong to an Indigenous people or traditional community, or family farmers), voucher numbers, and if the study was related to a protected area (or conservation unit, in Brazil). For spreadsheet formatting and standardizations, we used Useflora (2021) as a reference.

The list of plant species was revised with Kew Names Matching Service (<http://namematch.science.kew.org/api>) and Flora do Brasil 2020 (currently Flora e Funga do Brasil 2022), using the app Plantminer which is based in the R package ‘flora’ (Carvalho 2017; <http://www.plantminer.com/>). The data about the origin (native, exotic, naturalized) and conservation status for the plants present in CNCFlora (2022) was extracted from Flora do Brasil 2020 (Flora e Funga do Brasil 2022), although there are different interpretations on how to classify a species as native or exotic.

The Brazilian ethical and legal guidelines for access to traditional knowledge associated with biodiversity from secondary sources were followed. The research was registered at SisGen -National System for the Management of Genetic Heritage and Associated Traditional Knowledge (A5E6088). Free and informed consent was requested to communities representative of knowledge holders and the project was registered on the SISGEN.

3.4 Results and Discussion

3.4.1 *Traditional and Local Knowledge on Medicinal Plants in Atlantic Forest*

We analyzed 162 articles,¹ which reported field studies conducted in 151 municipalities of 16 Brazilian states (Alagoas, Bahia, Espírito Santo, Goiás, Mato Grosso do Sul, Minas Gerais, Paraíba, Paraná, Pernambuco, Piauí, Rio de Janeiro, Rio Grande do Norte, Rio Grande do Sul, Santa Catarina, São Paulo, and Sergipe). Only one state that is in the scope of the Brazilian Atlantic Forest biome had no study (state of Ceará, whose original cover of the Atlantic Forest is only 6%). Regarding the distribution of studies by geographic region, 37% were in the northeast region, 32% in the southeast region, 31% in the south, and 1% in the midwest region, where the Atlantic Forest coverage is small. These percentages reflect the study efforts in ethnobotany and human ecology in different regions of Brazil. Additionally, it is important to highlight that a large part of the northeastern region shares its cover with the *Caatinga* biome, and the midwest with *Cerrado*. Only in the south and southeast we find states in which their full area was originally covered by the Atlantic Forest (Espírito Santo and Rio de Janeiro, in the southeast, and Santa Catarina in the south).

Through the analysis of the articles, we recorded 7094 citations of medicinal plants, totaling 1686 species identified, of which 38 appeared in at least 20% of the studies (Table 3.1). Of the total species, 895 were cited in only 1 study, so it is a knowledge that probably is restricted to a locality (or more salient in a given community). Furthermore, as knowledge about the use of medicinal plants is often restricted to a few people in a community, events such as the death of elders or moving to other regions could increase the risk of loss of this knowledge. Some studies highlight this concern with the loss of traditional knowledge of plants in recent decades, which may worsen in the future, affecting the health care of communities (Bussmann et al. 2018; Reyes-García et al. 2013). Specifically regarding medicinal plants, Cámara-Leret and Bascompte (2021) concluded that 75% of medicinal plant services in the world are restricted to a single language, which is the situation of many of the knowledge of Brazilian Indigenous peoples.

Of the total species, 54% are native to Brazil, 25% are exotic species, 7% naturalized, and 14% could not be classified as native, exotic, and naturalized (Fig. 3.1). Although there is some inconsistency in the use of these concepts and in the categorization of plants as native, exotic, naturalized, and even invasive (Moro et al. 2012), this data highlights the prominence of native Brazilian flora in ethnobotanical medical repertoires. However, as much as native species stand out from the general total, among the 38 species that appeared in at least 20% of the studies, 24 are exotic (63%), 11 are native (29%), and 3 are naturalized (8%), demonstrating

¹The full list of 162 articles is available upon request.

Table 3.1 List of medicinal plant species that were registered in at least 20% of the studies ($N = 162$). Threat status according to the National Flora Conservation Center, in which NE = not evaluated and LC = least concern

Botanical name	Family	Native (Brazil)	Threat status	Frequency in the articles (%)
<i>Cymbopogon citratus</i> (DC.) Stapf	Poaceae	Exotic	NA	43
<i>Lippia alba</i> (Mill.) N.E.Br. ex Britton & P.Wilson	Verbenaceae	Exotic	NA	39
<i>Plectranthus barbatus</i> Andr.	Lamiaceae	Exotic	NA	39
<i>Mentha</i> sp. L.	Lamiaceae	Exotic	NA	37
<i>Bidens pilosa</i> L.	Asteraceae	Native	NA	36
<i>Eugenia uniflora</i> L.	Myrtaceae	Native	NA	36
<i>Psidium guajava</i> L.	Myrtaceae	Exotic	NA	33
<i>Ruta graveolens</i> L.	Rutaceae	Exotic	NA	32
<i>Rosmarinus officinalis</i> L.	Lamiaceae	Exotic	NA	30
<i>Baccharis crispa</i> Spreng.	Asteraceae	Native	NA	30
<i>Schinus terebinthifolia</i> Raddi	Anacardiaceae	Native	NA	30
<i>Persea americana</i> Mill.	Lauraceae	Exotic	NA	28
<i>Foeniculum vulgare</i> Mill.	Apiaceae	Exotic	NA	28
<i>Ocimum basilicum</i> L.	Lamiaceae	Exotic	NA	28
<i>Citrus aurantium</i> L.	Rutaceae	Exotic	NA	27
<i>Gymnanthemum amygdalinum</i> (Delile) Sch.Bip. ex Walp.	Asteraceae	Native	NA	27
<i>Matricaria chamomilla</i> L.	Asteraceae	Exotic	NA	27
<i>Punica granatum</i> L.	Lythraceae	Exotic	NA	27
<i>Melissa officinalis</i> L.	Lamiaceae	Exotic	NA	25
<i>Plantago major</i> L.	Plantaginaceae	Naturalized	NA	25
<i>Zingiber officinale</i> Roscoe	Zingiberaceae	Exotic	NA	25
<i>Petiveria alliacea</i> L.	Phytolaccaceae	Exotic	NA	24
<i>Aloe vera</i> (L.) Burm.f.	Asparagaceae	Exotic	NA	23
<i>Phyllanthus niruri</i> L.	Phyllanthaceae	Native	NA	23
<i>Dysphania ambrosioides</i> (L.) Mosyakin & Clemants	Amaranthaceae	Naturalized	NA	23
<i>Maytenus</i> sp. Molina	Celastraceae		NA	23
<i>Ocimum gratissimum</i> L.	Lamiaceae	Exotic	NA	23
<i>Achyrocline satureioides</i> (Lam.) DC.	Asteraceae	Native	NA	22
<i>Stachytarpheta cayennensis</i> (Rich.) Vahl	Verbenaceae	Native	NA	22
<i>Mikania glomerata</i> Spreng.	Asteraceae	Native	LC	21
<i>Alternanthera brasiliana</i> (L.) Kuntze	Amaranthaceae	Native	NA	21
<i>Artemisia absinthium</i> L.	Asteraceae	Exotic	NA	21
<i>Mentha pulegium</i> L.	Lamiaceae	Exotic	NA	21
<i>Achillea millefolium</i> L.	Asteraceae	Exotic	NA	20
<i>Ageratum conyzoides</i> L.	Asteraceae	Native	NA	20

(continued)

Table 3.1 (continued)

Botanical name	Family	Native (Brazil)	Threat status	Frequency in the articles (%)
<i>Carica papaya</i> L.	Caricaceae	Exotic	NA	20
<i>Dysphania ambrosioides</i> (L.) Mosyakin & Clemants	Amaranthaceae	Naturalized	NA	20
<i>Plectranthus amboinicus</i> (Lour.) Spreng.	Lamiaceae	Exotic	NA	20

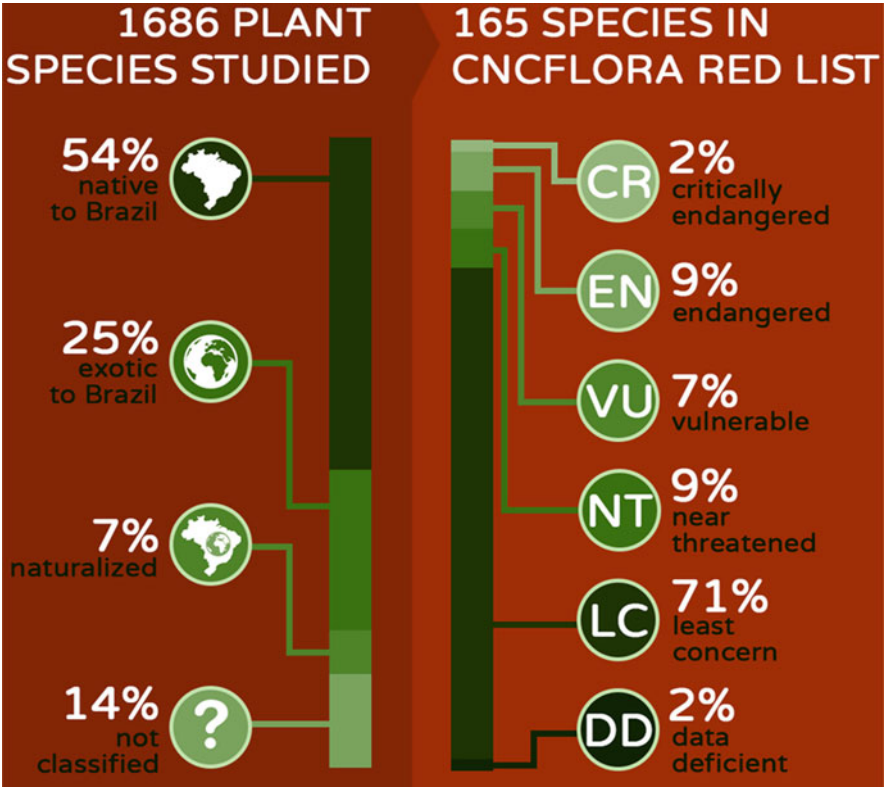


Fig. 3.1 Profile of the 1686 botanical species analyzed in the review of 162 studies on ethnobotany and medicinal plants in the Brazilian Atlantic Forest. Classifications of origin and conservation status were based on the database Flora e Funga do Brasil (2022) and CNCFlora (2022)

a predominance of exotic species among the most known species in the Atlantic Forest. The native species in Table 3.1 are species that are usually cultivated or that have a wide distribution occurring in backyards and altered areas, such as roadsides and brushwood, among others. The relevance of medicinal exotic species could be expected, following the pattern observed by Bennett and Prance (2000) in much

more remote regions (including the Amazon area in the northern South America) than the Brazilian Atlantic Forest.

3.4.2 Threatened Species

Of the 1686 species recorded, 165 are on the national red list of threatened flora organized by the National Center for the Conservation of Flora, of the Botanical Garden of Rio de Janeiro (Brazil) (CNCFlora 2022). Of these, 60% were cited in only one study. Thirty of these species are considered threatened species and are in the “Critically Endangered” (CR), “Endangered” (EN), or “Vulnerable” (VU) categories (Fig. 3.1). However, a few of these species, although being native from Brazil, are not native from the Atlantic Forest. This is the case of species native from Cerrado, which are the most endangered biome in Brazil (Mittermeier et al. 2004) and were included in some ethnobotanical studies conducted in areas of transition between the Atlantic Forest and Cerrado.

In addition, there are 134 species that do not yet fall into a threat category but need attention, for example, 14 species as “Near Threatened” (NT) (species that are close to or likely to be qualified in a threat category in the near future), 117 species as “Least Concern” (LC) (abundant and widely distributed species, which do not qualify as threatened at this moment), and 3 “Data Deficient” (DD) (species that do not have sufficient information for their categorization of extinction risk based on distribution and/or population status).

In the “Critically Endangered” category, which includes species that are facing an extremely high risk of extinction in the wild, three species were found: *Lychnophora brunioides* Mart., *Lantana canescens* Kunth, and *Struthanthus flexicaulis* (Mart.) Mart. These three species are endemic of Minas Gerais state, occurring in Cerrado biome, and were cited in one study. *L. brunioides* main threats are the deterioration of the habitat by mining, cattle raising, and fires (CNCFlora 2022). *L. canescens* was cited in a study in Santa Catarina coast (Merétika et al. 2010) and was probably cultivated in a homegarden. This species is reported in CNCFlora (2022) as being used and threatened by harvesting, but we found no evidence in literature about where this extraction occurs and by whom. The threat situation of *S. flexicaulis* is more related to the punctuality of its occurrence, associated with the expansion of agricultural and mining activities (CNCFlora 2022).

In the “Endangered” category we found 15 species, with emphasis on *Ocotea odorifera* (Vell.) Rohwer, cited in 9 studies; *Araucaria angustifolia* (Bertol.) Kuntze, cited in 5 studies; and *Virola bicuhyba* (Schott ex Spreng.) Warb. and *Annona dolabripetala* Raddi, cited in 2 studies. *O. odorifera* occurs in the northeast, south, and southeast of Brazil, being widely exploited for oil extraction and timber use, and the species has reproduction and dispersion characteristics that make its natural regeneration difficult and contribute to the current situation of threat (CNCFlora 2022). *A. angustifolia* is a wood species of great commercial value, which has suffered a very severe population reduction over the last century. It is

estimated that its forests (mixed ombrophilous forest, which is part of the Atlantic Forest biome) have had a reduction of more than 80% of its original cover. Currently, the species is still marketed as wood (although mostly illegal) and also as food, as its seeds are highly appreciated (CNCFlora 2022). *V. bicuhyba* is an endemic Brazilian species, occurring from the south to the northeast of Brazil. It is one of the most used woods for civil construction and its extracts have medicinal potential, which were widely used locally and also started to be explored by the pharmaceutical industry. The main threats are associated with selective extractivism and habitat conversion (CNCFlora 2022). *A. dolabripetala* is an endemic species of the municipality of Ubatuba (state of São Paulo); the main threats are related to its restricted distribution associated with the destruction of its habitats by urban expansion (CNCFlora 2022).

Eleven species are classified as vulnerable, with emphasis on *Cedrela fissilis* Vell. (cited in 13 studies), *Euterpe edulis* Mart. (6 studies), *Carapichea ipecacuanha* (Brot.) L.Andersson, *Cedrela odorata* L., *Melanoxylon brauna* Schott, and *Plinia edulis* (Vell.) Sobral (2 studies). *C. fissilis* is a species widely distributed throughout the country, but its population has been greatly reduced due to logging and habitat degradation (CNCFlora 2022). *E. edulis* is a palm widely distributed in the Atlantic Forest area and in part of the *Cerrado* and harvested since the colonial period. In preserved forests, it usually presents high abundance, but its intense exploitation for the extraction of palm heart (apical meristem) aiming at commercialization and the reduction of forest areas in the Atlantic Forest has led this species to the threat of extinction (CNCFlora 2022), also threatening the associated fauna (Pizo and Vieira 2004) and the dynamics of forest regeneration (Muler et al. 2014). However, it can be exploited with sustainability (Reis et al. 2000) both for palm heart and for *açaí* pulp. *C. ipecacuanha* is a species sought after by extractors of medicinal roots and has been commercially exploited since the eighteenth century, without incentives for cultivation, causing the decline of its populations. In addition, the fragmentation and degradation of their habitat are other threats. Thus, research on cultivation is recommended to meet the demands of commercial use and export for medicinal purposes (CNCFlora 2022). *C. odorata* is a widely distributed species that has been intensively exploited in the last 200 years due to the high commercial value of its wood, which has led to a decline in the populations of this species (CNCFlora 2022). *M. brauna* is an abundant species in some regions of the Atlantic Forest, but has habitat specificity. Illegal logging for logging is the main threat to this species (CNCFlora 2022). *P. edulis* is a species of edible fruit that is much appreciated and therefore widely cultivated, but which is rare in wild environments. It occurs in environments that have experienced great loss of habitat in recent years (CNCFlora 2022).

Among the “Near Threatened” species, we highlight *Handroanthus impetiginosus* (Mart. ex DC.) Mattos (nine studies), *Bowdichia virgilioides* Kunth (seven studies), *Smilax japicanga* Griseb. (three studies), *Amburana cearensis* (Allemão) A.C.Sm., *Lychnophora pinaster* Mart., *Ocotea puberula* (Rich.) Nees, and *Piper scutifolium* Yunck. (two studies). *H. impetiginosus* is a widely distributed species, but its timber potential has led its populations to decline. *B. virgilioides* is

also a species with a wide distribution, but due to its use for ornamentation and timber, it was considered almost threatened. *S. japicanga* is a species restricted to the Atlantic Forest biome, and the loss of habitat associated with its use for medicinal purposes can lead to a reduction in its population. *A. cearensis* is a widely distributed species, but it is threatened by selective logging because of its commercial timber value. *L. pinaster* is a species that occurs in the Cerrado, only in the state of Minas Gerais, suffering pressure from various human activities, such as mining, cattle raising, and urban expansion. *O. puberula* is a widely distributed species whose wood has commercial value, and the risk to populations is associated with habitat degradation, extraction of the species, and the existence of a fungus that attacks its fruits. *P. scutifolium* is a rare species, occurring in only three Brazilian states.

“Least Concern” is the status of most species threatened cited in the studies, especially *Mikania glomerata* Spreng. (34 studies), *Passiflora edulis* Sims (29 studies), *Pfaffia glomerata* (Spreng.) Pedersen (17 studies), *Genipa americana* L. (12 studies), *Jacaranda puberula* Cham. (12 studies), *Peperomia pellucida* (L.) Kunth (11 studies), and *Plantago australis* Lam. (10 studies). *M. glomerata* occurs in the northeast, south, and southeast regions. *P. edulis* is a species widely distributed in Brazilian biomes and also cultivated for food, medicinal, and ornamental purposes. *P. glomerata* is an endemic species from Brazil, widely distributed. *G. americana* is a species of wide distribution in the country, with economic food potential, since its fruits are used in the preparation of sweets. *J. puberula* is a widely distributed species that is used in construction, in cellulose production, for medicinal purposes, and in environmental restoration. *P. pellucida* and *P. australis* are also widely distributed species.

The threat analysis of the main species that are on the red list of the Brazilian flora shows that the risks and threats are not directly related to medicinal use, but mainly to factors of habitat destruction and selective logging for other commercial uses, such as timber and wood production. Only three species deserve more attention in relation to their extraction for medicinal purposes, and studies on sustainable extraction and possibilities for commercial cultivation are strongly recommended: *C. ipecacuanha*, *S. japicanga*, and *V. bicuhyba*. *C. ipecacuanha* was already known and used by the Brazilian Indigenous people before the arrival of the colonizers, being known as “vine that makes you vomit.” Due to its intense collection in the 1950s, its natural population was almost decimated, and therefore, it is at great risk of extinction. The main chemical constituent is emetine, which is already produced by chemical synthesis, but the species continues to be extracted today as a raw material for the pharmaceutical industry and the trade of medicinal plants (Lorenzi and Matos 2008). *S. japicanga* is also a plant used for centuries by the Indigenous peoples of the Americas. It is a plant widely used in the world for the treatment of rheumatism, syphilis, and other sexually transmitted infections (Lorenzi and Matos 2008). The medicinal use of the species *V. bicuhyba* is performed by extracting exudate from the stem, a substance popularly known as “sangue-de-bicuiba” (Reitz et al. 1978). Despite little information on the development of seedlings in experimental planting, it is one of the species considered to be of high conservation priority by Quilombola communities in southeastern Brazil, being the focus of preservation

in the management within the territories (Rodrigues et al. 2020). The threats to these species combine both habitat suppression and their medicinal use, and their conservation can be supported through studies that evaluate and encourage the sustainable use of this species by the IPLCs.

3.4.3 Exotic Species and the Threat of Invasive Species

Invasive alien species are one of the most important threats to biodiversity (Mollot et al. 2017). The use and favoring of these species by humans can bring risks to native ecosystems. Among the species recorded for medicinal use in the Atlantic Forest, 55 are identified as potentially invasive species by the Hórus Institute database (Base de Dados Nacional de Espécies Exóticas Invasoras 2022), although some species in this list are invasive in other biomes and not necessarily in the Brazilian Atlantic Forest.

Within this set of potentially invasive species used as medicinal plants, 13 species appeared in more than 5% of the studies: *Psidium guajava* (33% of studies), *Momordica charantia* L. (18%), *Ricinus communis* L. (14%), *Mangifera indica* L. (11%), *Morus nigra* L. (10%), *Sechium edule* Jacq. (10%), *Bryophyllum pinnatum* (Lam.) Oken (9%), *Euphorbia tirucalli* L. (9%), *Syzygium cumini* (L.) Skeels (9%), *Coix lacryma-jobi* L. (9%), *Eriobotrya japonica* (Thunb.) Lindl. (9%), *Morus alba* L. (9%), and *Cinnamomum verum* J.Presl (6%). Of these, eight are species from the Asian continent (*M. charantia*, *M. alba*, *M. indica*, *M. nigra*, *S. cumini*, *C. lacryma-jobi*, *C. verum*, *E. japonica*). With the exception of *C. lacryma-jobi*, which has medicinal uses and for making bio-jewels, the other six are widely cultivated for food and culinary purposes, in addition to other uses, such as ornamental and medicinal. Three have their origin in the African continent: *R. communis* is one of the main species planted in the world for the production of biodiesel; *E. tirucalli* is used for medicinal and ornamental purposes; and *B. pinnatum* is used as a medicinal and ornamental and propagated as a plant that brings good luck. Two have their natural distributions in America, but are not native to Brazil, *P. guajava* and *S. edule*, being widely cultivated for food purposes. It is worth to notice that although all these exotic species have an invasive potential, their degree of threat is variable: while some are well established as invasive (e.g., *R. communis*, *S. edulis*, Zenni and Ziller 2011), others cannot be behaving as invasive depending on the context (e.g., *S. cumini*, *P. guajava*, Londe et al. 2020).

As much as ethnobotanical studies have highlighted the importance of introduced species in local pharmacopeias as vital resources in the health care of communities (Bennett and Prance 2000) or for contributing to the resilience of local medical systems based on the diversification hypothesis (Medeiros et al. 2017), it is important to consider the invasive potential of some species and the threats and risks that use and cultivation can bring to ecosystems and native biodiversity. In addition, as discussed by Bennett and Prance (2000) many species were initially introduced due to their primary use as food and ornamentals, with medicinal use being only

secondary. Thus, the debate about the impact of these invasive species on native biodiversity and ecosystems, and about strategies to replace their use by native species (or introduced ones, without invasive potential), needs to be carried out in an integrated way not only restricted to the context of pharmacopeias.

Furthermore, it is important to consider that the process of European colonization and the diaspora of African peoples in Brazil mean that many of the exotic plants, and some with invasive potential, play a fundamental role in the constitution of the cosmovision and identity of the IPLCs. Ecological actions aimed at controlling these species must consider the efforts of IPLCs to maintain their cultural heritage, involving them in conservation and management strategies so that their knowledge and worldviews can be recognized and valued (Pagnocca et al. 2020).

3.4.4 Protected Areas, Traditional Territories, and the Conservation of Medicinal Species in the Atlantic Forest

Regarding the communities studied, at least nine segments of Brazilian traditional peoples and communities were analyzed: family farmers (whose knowledge on medicinal plants was studied by 8% of the articles reviewed), Caiçaras (7%), Quilombolas/Maroons (7%), artisanal fishers (4%), Indigenous peoples (4%), herbalists (“Raizeiros” in Brazilian Portuguese) or healers (3%), descendants of European immigrants (3%), extractivists (1%), and people of Afro-Brazilian religions (“Povos de Terreiro” in Brazilian Portuguese) (1%) (Fig. 3.2). In addition, 5% of the studies investigated urban communities and more than a half (57%) did not identify the type of human social group/community studied. The fact that many studies do not identify the type of communities makes it difficult to understand the cultural diversity related to medicinal plant knowledge and to recognize the holders of this traditional knowledge. Currently, the Brazilian law on access to traditional knowledge associated with genetic heritage (Brasil 2015; see also Zank et al. 2019b) requires that this identification of holders be carried out in scientific publications, and it is expected that this scenario will progressively change to ensure the proper recognition and benefits to the traditional knowledge holders.

In addition, 14% of the studies were carried out in (or adjacent to) protected areas created for biodiversity conservation, of which 9% were associated with categories of full protection and 5% with categories that allow the sustainable use of resources (Fig. 3.2). Only 2% of the studies included the information that the study was carried out in Indigenous or Quilombola land, in legally protected territories. This small percentage for Quilombola territories may be due to the legal land tenure process for recognizing Quilombola territories: although more than 3500 Quilombola communities are recognized in Brazil (Brasil 1988, 2007; Fundação Palmares 2022), few of them (only 198) have their territories demarcated with the legal ownership of their communal lands (CPISP 2022). These data may reflect both the lack of information

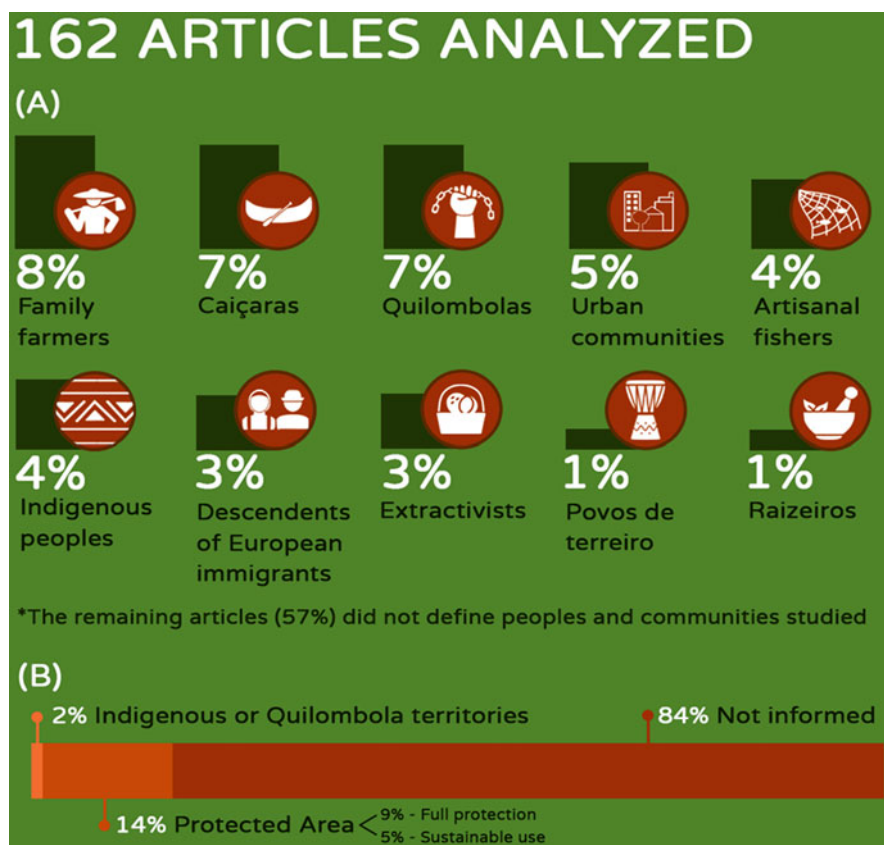


Fig. 3.2 Profile of the 162 articles analyzed about ethnobotany and medicinal plants in the Brazilian Atlantic Forest regarding the communities studied and the presence of protected areas and traditional territories. (a) Peoples and communities. (b) Protected areas and traditional territories

in the studies about the presence of protected areas and traditional territories, but also the low percentage of protected areas in the Atlantic Forest region, where they comprise 9% of the original territory of this biome (2% in the categories of full or integral protection) (Fig. 3.3).

The spatial distribution of the analyzed studies in relation to the presence of environmental protected areas shows that the wealth of plants known and used by IPLCs and other human groups in the Atlantic Forest is not directly related to legally protected areas. The exception is the southeastern region, mainly on the coast of the states of São Paulo and Paraná, where the largest preserved fragments of the Atlantic Forest and protected areas are found. In relation to traditional territories, these are few, small, and scattered in the Atlantic Forest and often close to urbanized areas. According to Lyver et al. (2019), the greatest vulnerability risk for local systems,

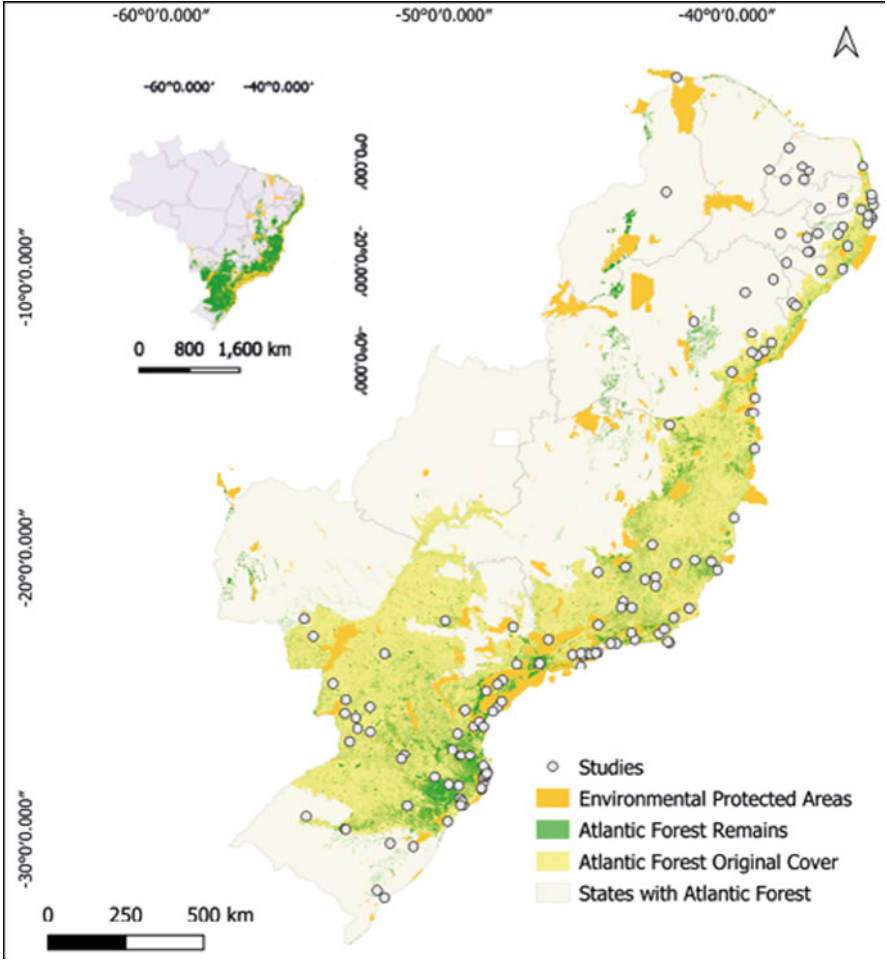


Fig. 3.3 Map of the Atlantic Forest domain with the location of all analyzed studies and environmental protected areas

which includes traditional health practices, is the lack of guaranteed access to territories where local ecological knowledge is generated, maintained, and innovated.

The establishments of protected areas that allow the sustainable use of resources as well as the legal recognition of traditional territories are unique opportunities to support the conservation of plant diversity. As important as the conservation of biodiversity is the need to guarantee the health and well-being of IPLCs and traditional knowledge holders, as well as their associated knowledge and practices.

3.5 Conclusion

The Brazilian Atlantic Forest harbors a great wealth of medicinal plants known and used by IPLCs, including both native and exotic species. About 10% of native plants are in some category of threat of extinction at the national level, which is mainly a reflection of habitat changes, including the expansion of large-scale agriculture and urbanization. The use of some exotic species with invasive potential also lights an alert to consider them in conservation and management plans, within the context of the use of these species. In order to face the threats both for medicinal plant biodiversity and especially for their knowledge holders, it is essential to recognize and expand the areas of traditional territories and conservation units for sustainable use in the Atlantic Forest region, in order to protect the knowledge and practices of the IPLCs.

In addition, we emphasize the need of researchers and scientists to properly identify the communities studied and the legitimate holders of ethnobotanical knowledge on medicinal plants, as well as the presence of protected areas (conservation units and traditional territories) in their publications. Without this information, the outline of strategies and priorities for the conservation and development tends to be fragile.

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Chapter 4

Climate Change Impact on Medicinal Plants: An Insight from the IUCN Red List of Threatened Species



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Abstract Medicinal plants are important for human life and wellbeing. Worryingly, climate change and severe weather have become serious threats to the diversity and sustainable use of medicinal plants. The aim of this work is to present the most current information on the impact of climate change on the world's medicinal plant species, with special emphasis paid on data provided by the IUCN Red List of Threatened Species. Using “Taxonomy: Plantae,” “Use and Trade: Medicine—human & veterinary,” and “Threats: Climate change & severe weather” filters on the advanced search of the IUCN Red List website, this work provides information on the diversity of medicinal plants (family, species, and growth form diversity) impacted by climate change, their use (diseases cured, plant parts used, active compound or secondary metabolites, and side effects), habitat ranges, geographical distribution, and conservation status according to the IUCN Red List Category and Criteria. We also inform conservation actions and research activities needed to ensure the future of medicinal plants and their sustainable uses.

Keywords Conservation status · Drought · Habitat shifting · IUCN Red List category and criteria · Temperature extreme

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4.1 Introduction

Medicinal plants are important for human life and wellbeing. The healthcare of about three-quarters of the world's population in developing countries depends on medicinal plants (World Health Organization 2002; He et al. 2018). Furthermore, medicinal plants provide a significant contribution to national and global economic development. At the national level, the trade on medicinal plants is an important source of income for rural people in some Asian countries, including India, Nepal, and China (Chen et al. 2016; Ghimire et al. 2016; He et al. 2018). In Nepal, the value of the trade is in the range of USD 11–48 million/year (Caporale et al. 2020). On the global scale, medicinal plant trades between countries can support regional and global economic development (Asl Roosta et al. 2017; He et al. 2018). An average of 400,000 tons/year of medicinal plant materials is traded globally, equal to the value of USD 1.2 billion (Lange 2001). The main exporter countries are Chile and Peru from South America; Egypt and Morocco from Africa; Poland, Bulgaria, and Albania from Europe; and China and India from Asia (Asl Roosta et al. 2017), while the major consumers are Japan, the USA, and Europe (Vasisht et al. 2016).

Climate change and severe weather have become serious threats to the diversity and sustainable use of medicinal plants (Applequist et al. 2020; Rana et al. 2020). The effect will be more severe if the threats are combined with habitat loss and over-harvesting. Several studies (e.g., Vasisht and Kumar 2002; FAO 2005, and UN Comtrade 2016) reported that about 80–90% of medicinal plants circulated in the global market are harvested directly from wild populations. All these threats will put the medicinal plant species on the brink of extinction.

The aim of this work is to present the most current information on the impact of climate change on the world's medicinal plant species, with special emphasis paid on data provided by the IUCN Red List of Threatened Species (IUCN 2022, <https://www.iucnredlist.org/>). The IUCN Red List has become the most widely used tool to measure the extinction risk of a species (Rodrigues et al. 2006; Marsh et al. 2021; Cazalis et al. 2022). Using “Taxonomy: Plantae,” “Use and Trade: Medicine - human & veterinary,” and “Threats: Climate change & severe weather” filters on the advanced search of the IUCN Red List website, this work provides information on the diversity of medicinal plants (family, species, and growth form diversity) impacted by climate change, their use (diseases cured, plant parts used, active compound or secondary metabolites, and side effects), habitat ranges, geographical distribution, and conservation status according to the IUCN Red List Category and Criteria (IUCN 2012). We also inform conservation actions and research activities needed to ensure the future of medicinal plants and their sustainable uses.

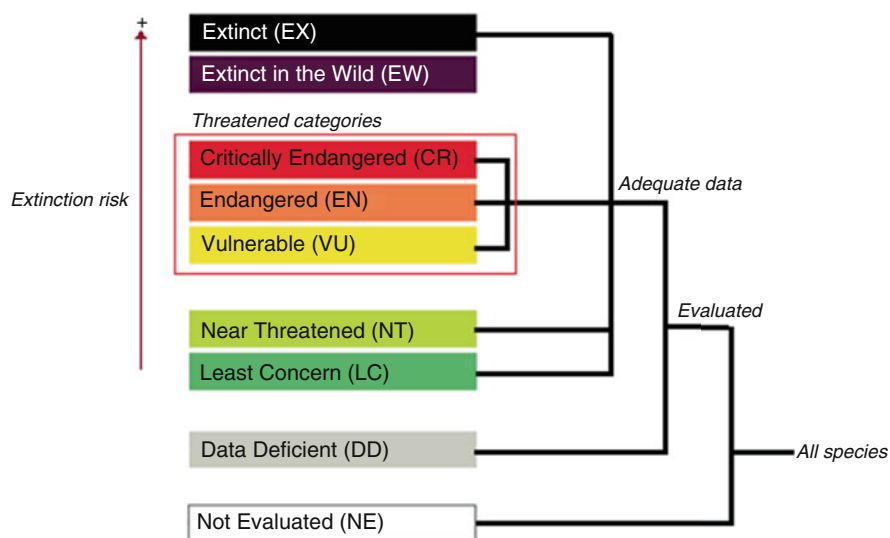


Fig. 4.1 Structure of the IUCN red list categories. (Modified from IUCN 2012)

4.2 The Nature of the IUCN Red List Category and Criteria

In the IUCN Red List, species can be classified into nine categories (Fig. 4.1). The category of extinct and extinct in the wild is self-explanatory. A taxon is declared to be extinct when the last individual has died, while extinct in the wild means that the taxon is extinct in its natural habitat. The next three categories (critically endangered, endangered, and vulnerable) are referred as ‘threatened’ and are assigned to a taxon based on several quantitative criteria. The category near threatened is assigned to a taxon that is not qualified as threatened now, but may be close to qualifying as threatened. When a taxon does not qualify as threatened or near threatened, the category least concern can be applied. The category data deficient is applied to a taxon with insufficient information to conduct a sound status assessment. Finally, the taxa that have not been evaluated against the Red List Criteria are assigned as not evaluated.

To assess whether a taxon is threatened or not, there are five criteria that can be used. The criteria are (IUCN 2012) (1) reduction of population size (A), (2) geographic range (B), (3) small population size and decline (C), (4) very small or restricted population (D), and (5) quantitative analysis (E). A taxon needs to meet only one of the criteria to be listed in any of the categories of threat.

4.3 Impacts of Climate Change on Medicinal Plants

There are currently 58,343 plant species included in the IUCN Red List assessment, of which 3358 species (5.8%) have medicinal uses (<https://www.iucnredlist.org/>, as per 20 February 2022). From this number, climate change and severe weather have impacted a total of 239 medicinal plant species from 177 genus and 81 families. Figure 4.2 shows that the highest number of species comes from the family of Fabaceae (19 species), followed by Orchidaceae (14 species), Lamiaceae (13 species), Fagaceae (10 species), and Asteraceae (9 Species).

According to the Plant Growth Forms Classification Scheme (IUCN 2013), plant species are categorized into 24 growth forms. Out of these, threatened medicinal plants are composed of 12 life forms: climber, herb, large tree, medium tree, small tree, palm, shrub, shrub-climbing, succulent shrub, succulent small, succulent tree, and vine. Herbs (32.6%), followed by small tree (25.9%), large tree (19.7%), and shrub (10.9%), are the most common growth form of the threatened medicinal plants (Fig. 4.2).

The most common impact is habitat shifting and alteration, which is observed in 126 species or more than 52.7%, followed by drought (104 species), storms and flooding (39 species), temperature extremes (33 species), and other impacts (10 species) (Fig. 4.3). Climate change has forced plants to shift into new ranges (Patni et al. 2021). Among medicinal plants affected by habitat shifting and alteration are *Melicope reflexa* (H.St.John) T.G.Hartley & B.C. Stone (Weisenberger et al. 2015), *M. adscendens* (H. St. John & E. P. Hume) T.G. Hartley & B.C. Stone (Weisenberger et al. 2020), and *M. lydgatei* (Hillebr.) T.G. Hartley & B.C. Stone (Keir et al. 2015). These species belong to the family Rutaceae and are endemic to

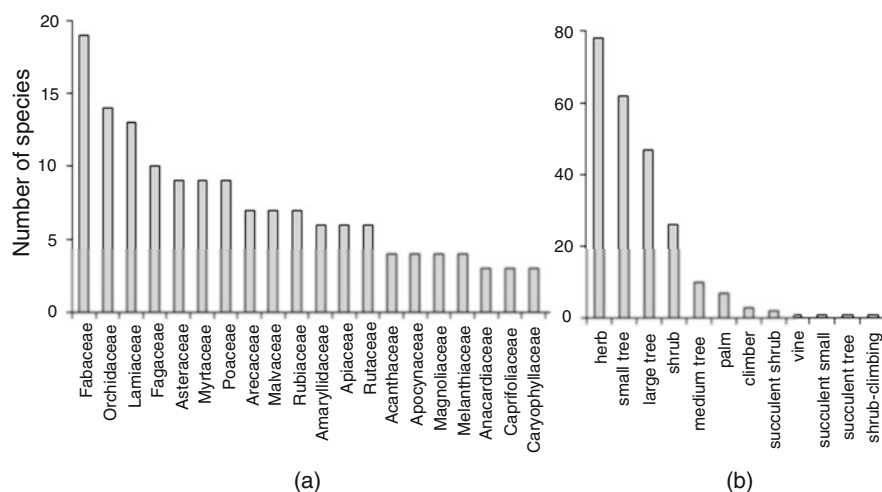


Fig. 4.2 The family (a) and growth form (b) diversity of medicinal plant impacted by climate change and severe weather

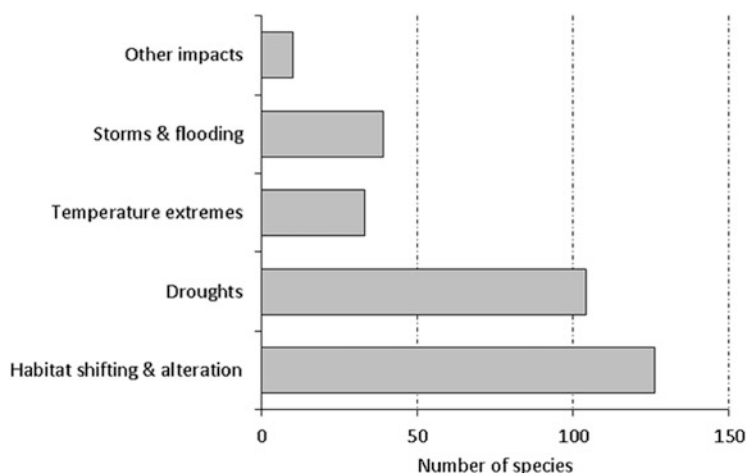


Fig. 4.3 Impacts of climate change on medicinal plants based on data of the IUCN red list

the Hawaiian Islands. Local people use the leaves and barks of these species for medicinal purposes. All the species are assessed as critically endangered and are extremely vulnerable to habitat shifting and alteration due to climate change. According to Fortini et al. (2013), the climate change vulnerability index of *M. reflexa*, *M. adscendens*, and *M. lydgatei* is 0.679, 0.88, and 0.873, respectively.

Climate change that raises the temperature and reduced precipitation affect the phenology of plant species by shortening their flowering season (Nam and Kim 2020). A historical pollen-data series from 1992 to 2011 of *Artemisia granatensis*, an endangered medicinal plant species from Sierra Nevada (Spain), indicated that the species has a shorter flowering season and appeared to have delayed the start of flowering and brought forward the end of flowering (Cariñanos et al. 2013). Climate change may also affect their potential distribution in the future. For instance, *Nardostachys jatamansi*, a highly exploited medicinal plant in the Himalaya region, was predicted to have expanding distribution areas by 2050 and 2070 as more suitable areas will be available under some greenhouse gas emission scenario (Li et al. 2019; Rana et al. 2020).

Drought will be more severe, persistent, and widespread due to climate change (Dai 2013; Huang et al. 2016). The impact will be more significant in semi-arid regions that are currently experiencing substantial water stress (Seager et al. 2013, 2014). St. Catherine Protected Area in southern Sinai, Egypt, is an example of area that has been subjected to extreme drought (Mosallam 2007; Khafagi et al. 2012). Due to the extreme drought condition of the protected area, the endemic medicinal plants *Ballota kaiseri* Täckh. (Lamiaceae), *Silene oreosinaica* Chowdhuri (Caryophyllaceae), and *Rosa Arabica* Crep. (Rosaceae) have been assigned the category of critically endangered and their population trends are decreasing (Omar 2017; Omar and Elgamal 2021a, b).

4.4 The Use of Medicinal Plants Impacted by Climate Change

Throughout human history, knowledge of the use of medicinal plants has been applied and preserved for generations to cure various diseases for both humans and livestock (Khatib et al. 2021). Based on the IUCN Red List data, 61.09% of the affected medicinal plants are known for curing particular diseases (approximately 245 types of diseases). The most common diseases include diarrhea (3.35%), stomach (infections and ulcers) (2.99%), asthma (2.46%), coughs (2.46%), fever (2.11%), headache (2.11%), and malaria (1.94%) (Fig. 4.4). Meanwhile, despite the relatively low percentage or unsuccessful attempt, some diseases, such as warts, can also be treated with such plants.

Globally, diarrhea is the third leading cause of childhood mortality after pneumonia and preterm birth. It is responsible for substantial mortality of young children under 5 years: every third child died due to this disease (Dadonaite et al. 2018). It is also prevalent throughout the year both in the rainy and dry seasons and is associated with climate change and modern lifestyles (Mellor et al. 2016). Similar to diarrhea, cough, fever, and headache are among common diseases. Therefore, the use of herbal medicines to treat such diseases has increased over the past decades. Plant families widely used to treat diarrheal diseases are Fabaceae (*Pterocarpus erinaceus*, *Ceratonia siliqua*, *Sophora tomentosa*) and Anacardiaceae (*Pistacia atlantica*, *Amphipterygium adstringens*, *Amphipterygium glaucum*).

The use of medicinal properties may be derived from various parts of the plant including roots, rhizomes, bark, stem, branches, buds, leaves, flowers, fruits, seeds, oil, and resin. This knowledge is based on the experience and culture of the local community, while the processing is carried out in a simple manner with simple equipment (Khatib et al. 2021). Ethnobotanical use categories in this research showed that barks were the commonly used parts (18.50%), followed by leaves

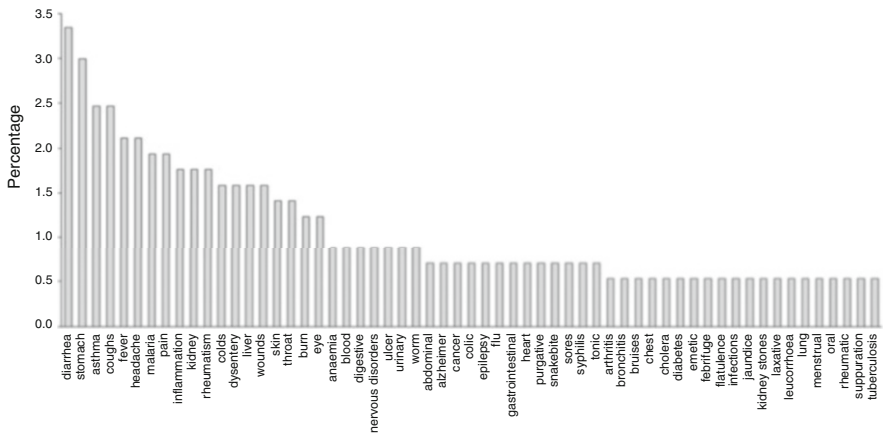


Fig. 4.4 Type of diseases cured by medicinal plants impacted by climate change

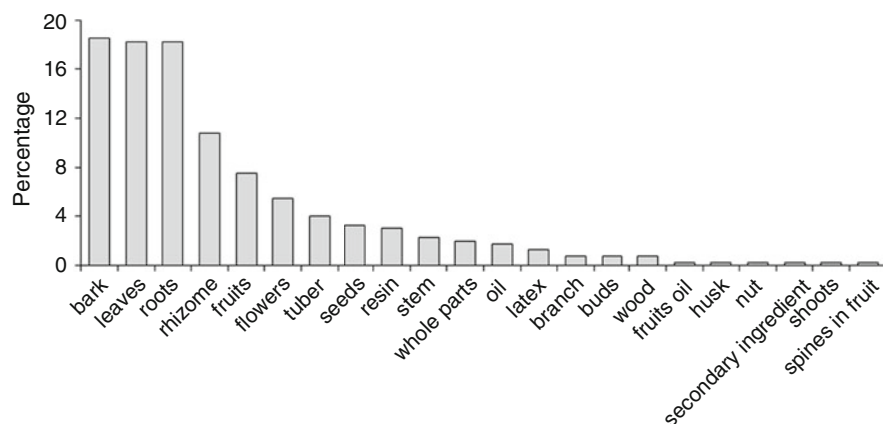


Fig. 4.5 Parts of climate change-impacted medical plants used to cure diseases

(18.25%), roots (18.50%), rhizome (10.75%), fruits (7.50%), and flower (5.50%) (Fig. 4.5). Meanwhile, the other parts of the plant are rarely used. Barks and leaves are widely used as they are relatively available and simple with high anti-inflammatory active compounds that can inhibit inflammation by at least 50% (Suirta et al. 2017). However, the removal of barks as medicinal ingredients will harm the plants. It must be a consideration on the sustainable harvesting, particularly if the plant is naturally rare, endangered/critically endangered, or in the wild and has not been cultivated.

Active compounds or secondary metabolites contained in medicinal plants are varied in type and function. Approximately 129 medicinal properties of plants have been identified and included in the IUCN Red List. The identified bioactive compounds have the roles of anti-inflammatory (4.62%), antioxidant (4.62%), antimicrobial (4.10%), and antibacterial (4.10%) and contain flavonoids (3.59%). Antioxidants are scientifically interesting compounds due to their various benefits, including anti-inflammatory used in pharmacy, pharmaceutical, and cosmetic industries (Zehiroglu and Ozturk Sarikaya 2019). Most of the flavonoids contain quercetin, which has an antioxidant and anti-inflammatory activity and can be found in vegetables, fruits, and medicinal herbs. The anti-inflammatory activity contained in the Fabaceae family contributes to the NO production inhibitory activity by suppressing nitric oxide production and scavenging free radicals (Bakasatae et al. 2018). Other beneficial components in medicinal plants are also identified despite their relatively low content.

In addition to the medicinal properties, plants have many significant benefits for both humans and animals, in the context of economics and ecology. Based on the data of IUCN (2022), the most common uses of plants other than medicinal purposes are food (both fruit and all parts of the plant) (11.52%), firewood (7.13%), construction (6.76%), timber (6.03%), furniture (5.30%), ornamental (6.58%), fodder (3.11%), and other various uses by community. The utilization of plants, however,

must highlight the aspects of economic and ecological sustainability, particularly for naturally rare, in danger of extinction, and nonconserved plants and specifically for timber extraction that usually accelerates the occurrence of damage, scarcity, and extinction due to tree felling. Excessive and illegal logging is a great threat to the sustainability and conservation aspects of a species.

4.5 Habitat and Distribution of Medicinal Plants Impacted by Climate Change

A total of 232 or more than 97% medicinal plants impacted by climate change are found on terrestrial system. Forests are habitats where most medicinal plants (67.7%) grow, followed by shrublands (28%) and grasslands (18.8%) (Fig. 4.6). Due to their significant contribution to affordable healthcare and livelihood security, medicinal plants are one of the most valuable non-timber forest products (Astutik et al. 2019).

Pattern of terrestrial biodiversity including plants and animals in the largest scale (continent or subcontinent) is described in the biogeographical realm (Dasmann 1972; Udvardy 1975; Olson et al. 2001; Boucher et al. 2013). There are seven biogeographical realms that are currently recognized: Neotropic, Afrotropic, Nearctic, Australasia, Palearctic, Indo-Malay, and Oceania (Udvardy 1975). Neotropic covers Central and South America. Afrotropic includes sub-Saharan Africa. Nearctic extends from north Mexico of Tehuantepec, USA to Canada. Australasia includes Australia, New Zealand, and also New Guinea. Palearctic includes Northern Africa and Eurasia. Indo-Malaya extends from South to Southeast Asia and Oceania covers all across Pacific Islands (Boucher et al. 2013).

Medicinal plants that are currently listed under the IUCN Red List of Threatened Species are found in all seven biogeographical realms. Figure 4.7 shows that the

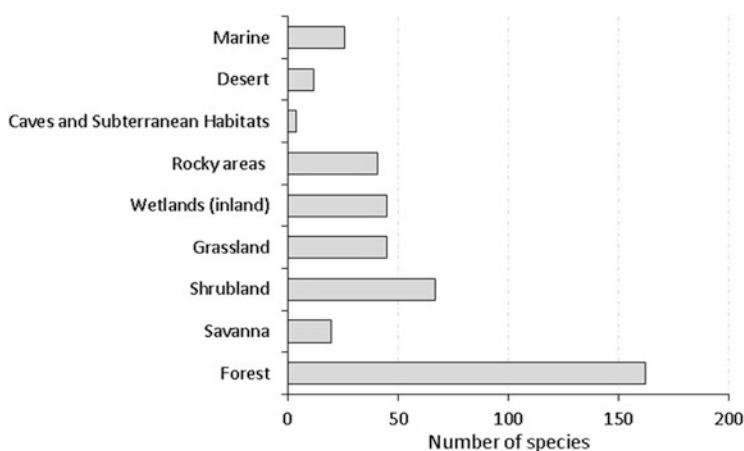


Fig. 4.6 Number of medicinal plants impacted by climate change based on habitats

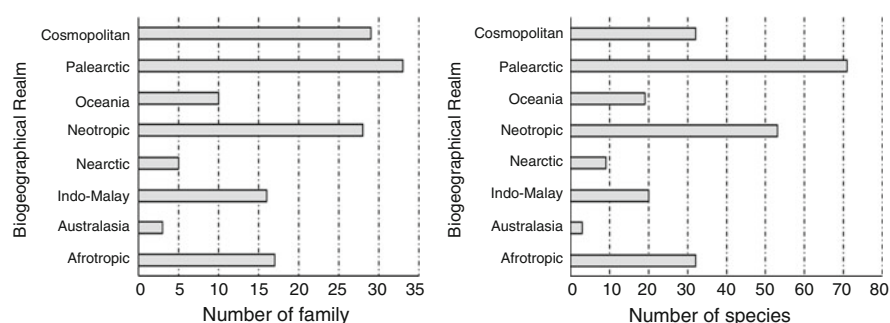


Fig. 4.7 Distribution of medicinal plants impacted by climate change and severe weather in biogeographical realms of the world

highest number of the family of threatened medicinal plants is found in Palearctic (33) followed by Neotropic (28), Afrotropic (17), Indo-Malay (16), Oceania (10), Nearctic (5), and Australia (3). However, significant numbers of threatened medicinal plants are also found in several biogeographical realms (Cosmopolitan, 29 families). The diversity pattern of the number of species and family of the threatened medicinal plants across biogeographical realm is similar. The highest species number occurs in Palearctic (71), followed by Neotropic (53), Afrotropic (32), Indo-Malay (20), Oceania (19), Nearctic (9), and Australia (3). The number of species that are found in more than one biogeographical realm is equal to Afrotropic. Palearctic particularly temperate broadleaf and mixed forests and temperate conifer forests was included as a highly vulnerable forest ecoregion (Wang et al. 2019). These forests and plant species that composed the communities are potentially threatened due to climate change in the future (Wang et al. 2019). Similarly, forests and species that are found in the islands including Indo-Malay, Australasia, and Oceania that are commonly near the equator are also vulnerable to climate change (Veron et al. 2019). According to recent climatic modeling analysis on medicinal plants in Indonesia, most species will have decreasing distribution area by 2050 and 2080 (Cahyaningsih et al. 2021). Many of them will also shift to new distribution area and more than half of the studied medicinal plant species will have a declining population size, mostly due to losing their distribution areas (Cahyaningsih et al. 2021; Elsen et al. 2022).

In terms of growth forms, Fig. 4.8 shows that the number of variations in growth form is largest in Neotropic (9) followed by Afrotropic and Palearctic (7), Oceania (6), Indo-Malay (4), Nearctic (3), and Australasia (2). Herbs are mainly dominant in the Palearctic and Indo-Malay, while small trees in Neotropic and Afrotropic.

Of our studied species, about 40 species are considered as introduced species or found outside their distribution region (Fig. 4.8). Half of these introduced medicinal plants were cosmopolitan, which were found in more than one biogeographical realm. About ten species were introduced in Palearctic, four in Nearctic, two in Neotropic, and one species in other biogeographical realms. Herbaceous introduced species are strong competitors to the native under climate change scenario (Haeuser et al. 2019). Thus, ongoing climate change will likely promote the naturalization of

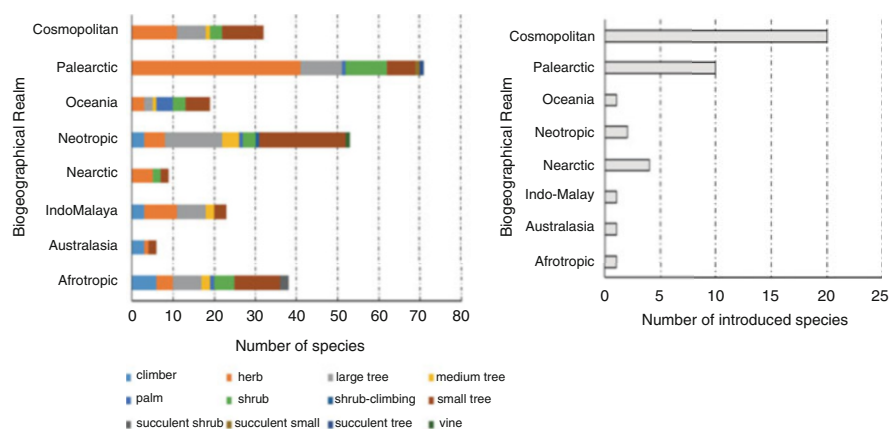


Fig. 4.8 Growth form variation and number of introduced medicinal plant species across biogeographical realms of the world

the introduced plant species. However, in some cases the situation goes to the opposite. Analysis on non-native trees and shrubs in South Africa under future climate change scenario showed that over half of them will experience a decrease in their distribution (Bezeng et al. 2017).

4.6 Conservation Status of Medicinal Plants Impacted by Climate Change

More than 43.5% of the species impacted by climate change are threatened with extinction, i.e., with the category CR (19 species), EN (51 species), and VU (34 species). Furthermore, there are 17 and 114 species with the category NT and LC, respectively. Four species are assessed as DD due to unclear exact distribution of the species. Distribution of the IUCN Red List categories of medicinal plants impacted by climate change can be seen in Fig. 4.9.

Most of the threatened medicinal plants are assessed under criterion B (50%), followed by criterion A (25.4%), criterion C (14.2%), and criterion D (10.4%) (Fig. 4.10). None of the assessments used criterion E to assign any of the threatened categories. Compared to other criteria, criterion B is the easiest to be completed in terms of data requirement (Robiansyah and Wardani 2020). The criterion is based on the occurrence data of a species, which can be obtained from databases, published scientific papers, and herbarium records. For criterion E, it requires high-quality data and therefore is rarely used in the assessment (Collen et al. 2016).

Figure 4.10 shows that more than half (54.4%) of the medicinal plants impacted by climate change has a decreasing trend in their population size. There are 37 species or around 15.5% have a stable population size. The medicinal herb *Chaya Silvestre* (*Cnidoscolus souzai* McVaugh) from the family Euphorbiaceae is the

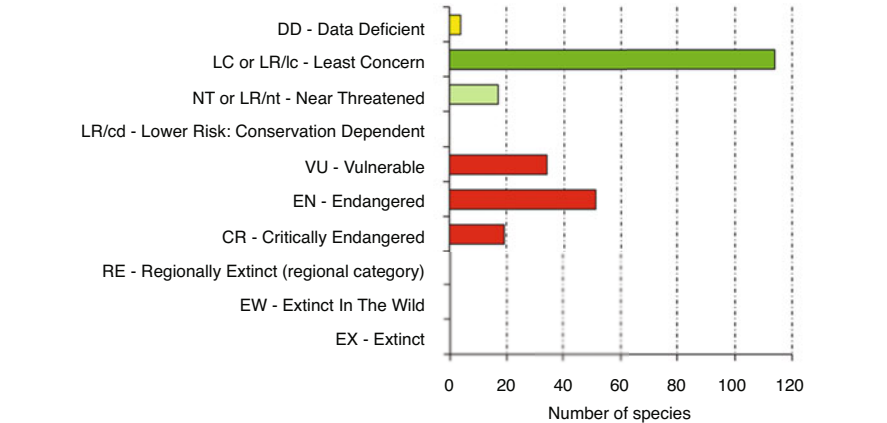


Fig. 4.9 Distribution of the IUCN red list categories of medicinal plants impacted by climate change

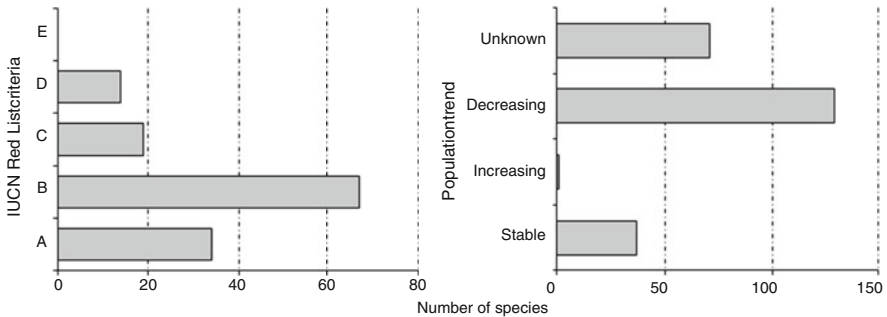


Fig. 4.10 Criteria of the IUC red list used in the assessment (left) and population trend of medicinal plants impacted by climate change (right)

only species with an increasing population trend (Fuentes and Samain 2019). The species, used traditionally to treat rheumatism, pain, and inflammation (Zapata-Estrella et al. 2014), is restricted to the Yucatan Peninsula and has adapted to disturbed sites. There are 71 species (29.7%) with unknown population trends. Therefore, further studies on population size and trend are required in order to monitor the population status and to conserve the species.

Conservation status assessment on medicinal plants affected by climate change was started in 2010 with 16 species. The number of assessment fluctuated after that and starts to increase in 2019 onwards (Fig. 4.11). There are 27 assessments that need updating and most of them are those published before 2016. Among medicinal plants needed updating are the endangered *Crinum thaianum* J. Schulze (Amaryllidaceae) and *Acridocarpus monodii* Arènes & P. Jaeger ex Birnbaum & J. Florence (Malpighiaceae) published in 2011, and *Aloe bella* G.D. Rowley (Asphodelaceae) published in 2013.

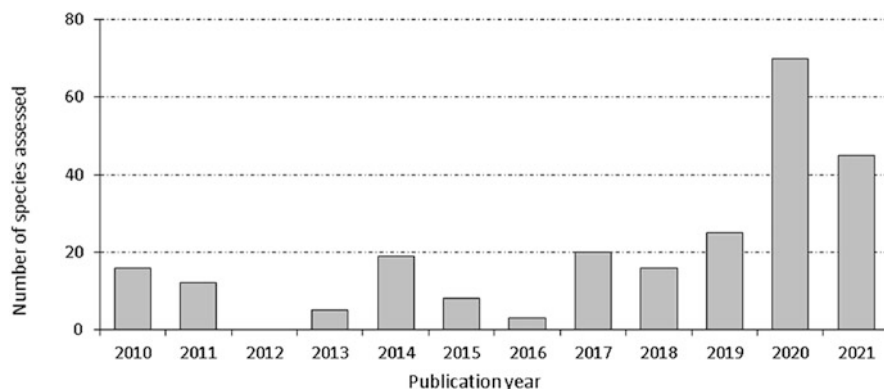


Fig. 4.11 Number of medicinal plants assessed according to its publication year

4.7 Conservation Actions

The conservation and research efforts for medicinal plants need to be done immediately to prevent the scarcity and the extinction of medicinal plant species. Special attention needs to be given to the medicinal plant species which are harvested in a huge number and harvested from wild populations. The IUCN data show the following six conservation actions needed for medicinal plant species: (1) habitat protection, (2) habitat management, (3) species management, (4) education and awareness, (5) law and policy, and (6) livelihood, economic, and other incentives.

Plant conservation has a major objective to preserve the viability of existing populations through in situ programs (Volis and Blecher 2010). In situ conservation does not require a complex adaptation or complex acclimatization process and allows the protection of targeted plant population together with other native plants, natural communities, and their intricate network of relationships (Gepts 2006). It can increase the amount of diversity that can be conserved (Forest et al. 2007) and strengthen the relationship between plant resource conservation and sustainable use (Long et al. 2003). The successful in situ conservation depends on the rule, regulation, and compliance of potential medicinal plants within growth habitats (Soule et al. 2005; Volis and Blecher 2010).

In situ conservation efforts worldwide have focused on establishing protected areas and taking an ecosystem-oriented rather than species-oriented approach (Ma et al. 2012). Habitat protection through protected areas plays a vital role in protecting plants in their natural habitat. Protected areas have significantly lower rate disturbance than nearby private forest (Zhang et al. 2010). For medicinal plants impacted by climate change, a total of 142 species (59.4%) are located within protected areas. The number, however, falls to only 69 species (28.9%) if it considered only the threatened species. There are seven critically endangered medicinal plants that are not recorded inside protected areas, and they are *Cryosophila williamsii* (Arecaceae), *Gentiana kurroo* (Gentianaceae), *Magnolia yajlachhi*

(Magnoliaceae), *Melicope lydgatei* (Rutaceae), *Melicope reflexa* (Rutaceae), *Pritchardia woodii* (Arecaceae), and *Sonchus erzincanicus* (Asteraceae). In addition to habitat protection, habitat management actions are also required by 92 medicinal plants impacted by climate change which include invasive species control and habitat restoration.

Under species management, the following actions are needed for the conservation of medicinal plants impacted by climate change: harvest management (required by 28 species), trade management (25 species), limiting population growth (6 species), species recovery (38 species), and species reintroduction (42 species). In addition, ex situ conservation is now increasingly being recognized as an important method of conservation. It complements in situ approaches and, through the living collections and seed banked materials, can support horticultural research, phenological observations, maintenance of the displays, and provision of material for seed exchange and for habitat restoration (Heywood 2017). Based on the data of BGCI (2022), a total of 180 (75.3%) species of medicinal plants impacted by climate change has been conserved at ex situ collections which include 74 species threatened with extinction. The number of medicinal plants represented at ex situ collections should be increased, especially for those with critically endangered status such as *Ballota kaiseri* (Lamiaceae), *Calatola mollis* (Metteniusaceae), *Hazomalania voyronii* (Hernandiaceae), *Magnolia yajlachhi* (Magnoliaceae), *Meconopsis bhutanica* (Papaveraceae), *Pritchardia woodii* (Arecaceae), *Silene oreosinaica* (Caryophyllaceae), and *Sonchus erzincanicus* (Asteraceae).

4.8 Conclusion and Opportunities for the Future

Climate change and severe weather have impacted a total of 239 medicinal plant species from 177 genus and 81 families. The highest number of species comes from the family of Fabaceae, whereas the most common growth form of the impacted medicinal plants is herbs. The most common impact caused by climate change is habitat shifting and alteration, which is observed in 126 species or more than 52.7%. More than 43.5% of the species impacted by climate change are threatened with extinction. There are 142 species (59.4%) that are located within protected areas and 180 (75.3%) species that have been conserved at ex situ collections.

To support the survival and sustainable use of medicinal plants impacted by climate change, further research on at least the following six areas are required: (1) population size, distribution, and trends, (2) threats, (3) life history and ecology, (4) harvest and use, (5) taxonomy, and (6) conservation actions. Monitoring on population, habitat, harvest, and trade trends is also important to inform the most current condition of the medicinal plant populations and their habitats. Furthermore, a specific conservation action plan needs to be developed to enhance the conservation of medicinal plants. This plan should include species and habitat as well as harvest and trade management programs.

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Chapter 5

Securing Conservation Status of *Paris polyphylla*, a Medicinally Important Plant of the Indian Himalayan Region



Mohd Tariq, Shyamal Kumar Nandi, and Indra Dutt Bhatt

Abstract The Himalayan region is a plethora of high-value and important medicinal and aromatic plants. Besides natural hazards, various anthropogenic activities like deforestation, over-exploitation, illegal removal and trading, trampling and grazing by animals and other factors have resulted in placing many of these plant species in decreased status. Conserving these plant species is thus a crucial exercise. One among such species is *Paris polyphylla* Smith (family Melanthiaceae) which has presently emerged as a key plant, with a wide range of therapeutic applications, and has been indigenously used in traditional medicines since early times. Phytochemical investigation on various varieties of *P. polyphylla* showed that apart from steroidal saponins, other constituents such as flavonoids, phenylpropanoids, ecdysteroids, phenolic glycosides, etc. are present. In view of its medicinal importance, the species is in high demand in the national and international market and is being collected uncontrolled from the wilds. Therefore, urgent steps need to be undertaken to bring this high-value species under cultivation and improved management practices. The chapter reviews various aspects on the species, particularly habitat, distribution, ethnobotany, pharmacological aspects, phytochemical contents, various propagation (including biotechnological) techniques and reintroduction and cultivation plans. Various research gap areas, opportunities and conservation strategies for sustainable utilization of this species are discussed.

Keywords *Paris polyphylla* · Ethnobotany · Pharmacology · Ecological niche modelling · Propagation

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5.1 Introduction

The Indian Himalayan Region (IHR) is over 5.3 lakh km² that is comprised of vast mountain ranges extending over 2500 km in length between Indus and Brahmaputra river system and rising from low-lying plains to over 8000 m and spreads over 12 states, namely, Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Sikkim, Arunachal Pradesh, Nagaland, Manipur, Mizoram, Tripura, Meghalaya, West Bengal hills (Darjeeling) and Assam (Karbi Anglong and Dima Hasao; Fig. 5.1). It represents a great deal of variations with respect to altitude, latitude, longitude, topography and geography and forms a wide variety of habitats and climates. This has resulted in the growth and development of a variety of forest types, flora, fauna and landscapes (Singh and Singh 1992).

The IHR, recognized as one among the 36 biodiversity ‘hot spots’ in the world, is known for its uniqueness, richness and representatives at all levels (gene, species and ecosystem). The rich plant diversity in the region is represented by over 8000 angiosperms, 44 gymnosperms, 600 pteridophytes, 1737 bryophytes, 1159 lichens and 6900 fungi (Samant et al. 1998). Also, the region accounts for about 50% of India’s total flowering plants, of which 30% of the flora is endemic to this region (Mehta et al. 2020). Many of these species are being used extensively as food/wild edible, fuel wood, fodder, fibre, agricultural tools, raw or processed high-value drugs, aromatics and nutritive additives and for religious and variety of purposes. Inhabitants of the region are traditionally well familiar with the healing properties of the available plants, and therefore, their dependency on medicinal and aromatic plants (MAPs) for primary healthcare forms an integral part of their life and basis for

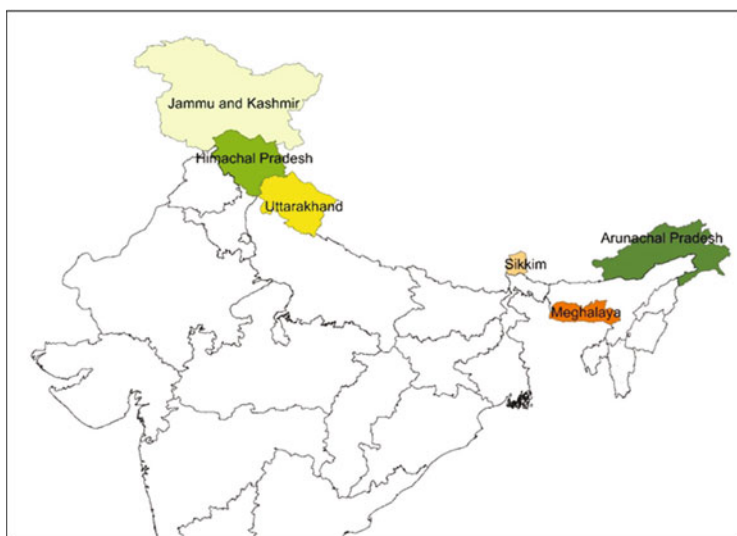


Fig. 5.1 Map showing the occurrence of *Paris polyphylla* Smith in the Indian Himalayan Region

their livelihood (Samant and Dhar 1997). The extracts of many of these plants have been used in traditional medicine since centuries and have also gained significant importance in the preparation of modern medicines. The Himalayan region thus serves as a major habitat of a large number of MAPs, and many compounds of plant origin have resulted in the formulation of modern medicines and, therefore, acclaimed as a rich source of therapeutic agents all over the world. In an estimate, about 80% of the developing nations inhabitants are directly or indirectly dependent on these MAPs for their primary healthcare needs (WHO 2011); of these, nearly 85% of people use plants or their extracts as active substance (Kala 2000).

5.2 Botanical Description of *Paris polyphylla*

The term '*Paris*' is derived from the word 'pars' means equal and which refers to the symmetry of plant and multiples of four in which its foliage, flowers and fruits grow. Flowers are bisexual, solitary, terminal and greenish at the apex, subtended by 4–9 lanceolate long-pointed leaf-like bracts. Fruit is berry in character, oval or round with 50–60 seeds, and becomes red on ripening. Seeds are enveloped by red, succulent aril. *Paris polyphylla* is a monoecious plant where male and female reproductive organs are present in the same flower. The life cycle is concluded in 2 or more years. It is a slow germinating plant which takes about 7 months to sprout from the seed. Key identifying characteristics are the whorled leaves usually 5–12 (5–16 cm long; 1.5–4 cm wide), with a long petiole (stalk), smooth margin and parallel venation.

5.3 Habitat and Distribution of *Paris polyphylla*

Uttarakhand, a well-known Indian state for its biotic wealth and diverse cultural mosaic, harbours maximum number of medicinal plants in comparison to other states of IHR (Kala et al. 2004). A total of 3500 higher plant species have been reported from this region and about 701 of them are used in different forms as medicine (Kala et al. 2004; Nautiyal et al. 1997). In contrast, the Trans-Himalayan region of India (e.g. Ladakh and Lahaul-Spiti) harbours about 337 species of medicinal plants (Kala 2002), which is low compared to other parts of the Himalaya. It has been reported that more than 208 species of medicinal plants are consumed as raw, roasted, boiled, fried and cooked or in the form of oil, spices, jams and pickles (Samant et al. 2001).

The target species *P. polyphylla* Smith var. *Polyphylla* (APG IV 2016) is a perennial herbaceous plant of Melanthiaceae family (earlier Trilliaceae and Liliaceae) with a wide distribution in the west Himalaya at an altitudinal range of 1800–3300 m asl (IUCN 2004; Tariq et al. 2016) and is commonly found in India, Nepal and Bhutan with nativity to southwest of China, Yunnan province (Li 1984).

In India, it is reported to be found growing in Jammu and Kashmir, Himachal Pradesh, Uttarakhand (earlier Uttaranchal), Sikkim, Meghalaya and Arunachal Pradesh (Fig. 5.1). It is a glabrous and perennial herb that grows on light sandy and moistened places and humus-rich soil with partial or full shade (Madhu et al. 2010).

5.4 Ethnobotany

P. polyphylla has emerged out as most vital medicinal plants of the Himalayas, often utilized in traditional medicines (Kunwar et al. 2013). It is used as antiphlogistic, antipyretic, anthelmintic, antispasmodic, digestive and expectorant and for the treatment of vermifuge problems, headache and intestinal worms (IUCN 2004; Madhu et al. 2010; Kunwar et al. 2013). Traditional importance of *P. polyphylla* is due to the occurrence of steroidal saponins, one of the most structurally diverse and extensively distributed secondary metabolites in plants. The species has a wide range of therapeutic applications and indigenously used in traditional medicines (TM) since ages (Li 1998) in snake bites, insect bites, wounds, fever and food poisoning (Madhu et al. 2010; Dutta 2007). The rhizome is fed to cattle to lessen diarrhoea and dysentery (Baral and Kurmi 2006). It is beneficial in the treatment of asthma, heart and muscular malady and bronchitis and for spermicidal enhancement and as sedative (Liu et al. 2009). Rhizomes are used for stomachaches, in ethnopediatrics for diarrhoea and as tonic by the local inhabitants (Nautiyal and Nautiyal 2004). A mixture of its rhizomes and roots can be used in treating poisonous snake bites, insect bites and boils (Tang 2003; Huang et al. 2005). The dried rhizome has been used to treat fractures, parotitis and abscess in folk medicine for a long time and has played a vital role in medicine development for anti-tumour, immunity adjustment and development, analgesic and inflammation (Wang and Liu 2010; Yang et al. 2011). It has been reported that steroidal saponins (polyphyllin D, dioscin, diosgenin and balanitin 7; Fig. 5.2) are the main and active components present in *P. polyphylla* (Deng et al. 2008; Cheung et al. 2005).

5.5 Pharmacological Activities

P. polyphylla has wide range of pharmacological activities including haemostatic, antitumor, antifertility effects. Steroidal saponins are the primary active constituents of genus *Paris* that have various pharmaceutical applications and possess a broad range of pharmacological (Fig. 5.3) and biological activities (Fig. 5.4). A large number of studies indicated detection and quantification of saponins, a bioactive compound from the genus *Paris* (Chen et al. 2016). They serve as a starter for the semi-synthesis of steroidal drugs by various cosmetic industries (Sparg et al. 2004). They are also believed to form the main composition of many plant drugs and folk

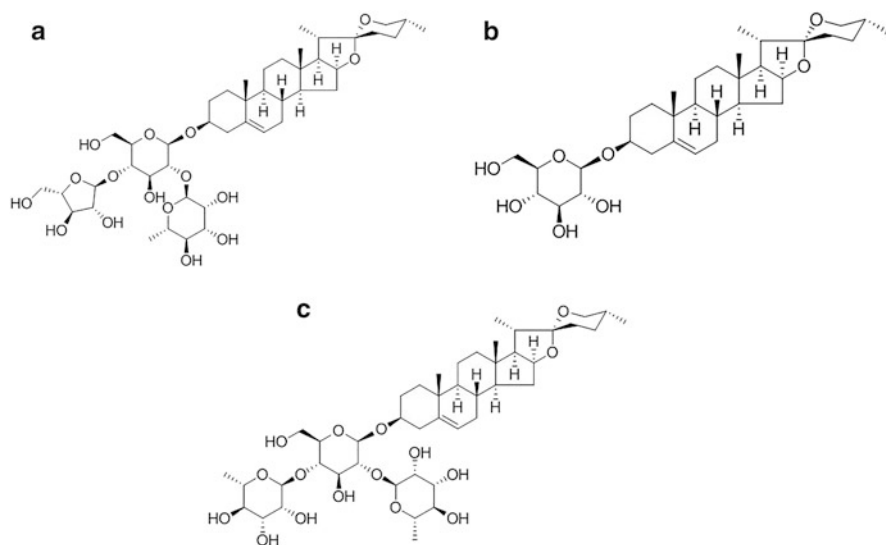


Fig. 5.2 Structures of some of the active constituents of *Paris polyphylla*: (a) polyphyllin D, (b) diosgenin and (c) dioscin

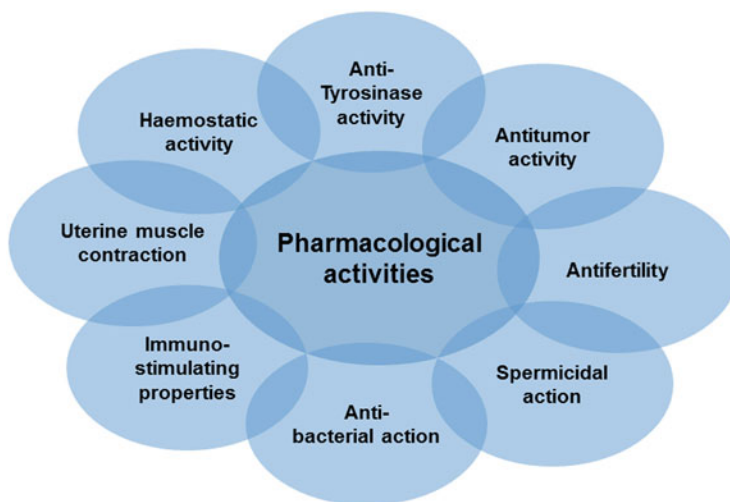


Fig. 5.3 Some of the reported pharmacological activities of *Paris polyphylla*

medicines and are thought to be responsible for various pharmacological activities (Estrada et al. 2000). A number of studies reported the extensive utilization of this species in various formulations by Chinese system of medicine (CSM) (Gairola et al. 2014; Chen et al. 2016). Phytochemical investigation on various varieties of



Fig. 5.4 Other biological activities of saponins

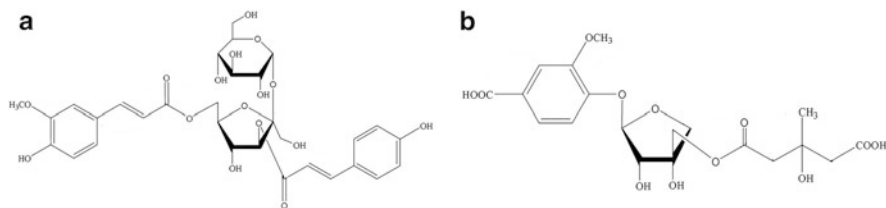


Fig. 5.5 Structures of (a) parispolyside F and (b) parispolyside G

P. polyphylla showed that apart from steroidal saponins, various other constituents such as flavonoids, phenylpropanoids, ecdysteroids, phenolic glycosides, etc. are present (Wang et al. 2006; Man et al. 2009; Wei et al. 2014). The phytochemical investigation of *Paris* has shown the presence of up to 126 compounds including 2 new compounds parispolysides F and G (Fig. 5.5). Predominantly, steroid saponins and flavonoids are the most frequent secondary metabolite constituents within the genus *Paris* (Zhang et al. 2007).

5.6 Conservation Initiatives

5.6.1 Ecological Niche Modelling (ENM) Studies

Conservation of high-value medicinal plants constitutes a broader part of medicinal plant research and traditional knowledge sustenance is associated with biodiversity conservation. Prediction and mapping of rare, endangered and threatened (RET) plant species is an important aspect of conservation, distribution and rehabilitation of many medicinal and aromatic plants that are of economic importance. Ecological niche modelling (ENM) or habitat distribution modelling (HDM) technique is now used for predicting and mapping of RET plant species. This technique is presently applied for monitoring, restoration and management and species conservation measures for various RET plant species in their natural habitat. It depicts the prior information about the distribution and ecological features of a particular plant species which may aid in decreasing over-exploitation and promoting sustainable harvesting (Bhattacharyya and Sarma 2010).

The ecological niche can be defined as the set of ecological conditions (abiotic factors) under which a species is able to persist and produces offspring without immigration (Grinnell 1917, 1924). It is a tool that uses computer-based algorithms to generate predictive distribution maps in a geographic space by correlating the point distributional data with a set of environmental raster data (Fig. 5.6). A substantial amount of work has been carried out globally using ENM for various applications (Peterson 2003; Ferrier 2002; Giriraj et al. 2008; Irfan-Ullah et al. 2007; Waltari et al. 2007; Eyre et al. 2022) which focuses on geographic distribution modelling, niche quantification, testing of ecological hypotheses, species recovery planning, setting up of artificial restoration sites, etc. In a recent report using ENM the habitat suitability of *Dactylorhiza hatagirea*, a critically endangered and endemic medicinal orchid species of the Himalayan region, was identified (Singh et al. 2022). This study delineated the fundamental niche baseline map of *D. hatagirea* in the western Himalayas and highlighted regions/areas where conservation and management strategies should be intensified in the next 50 years. However, no such effort has been undertaken for conservation of *Paris polyphylla* by determining its potential distribution in IHR. In our study in the west Himalaya described here we will deal with the understanding of the ecology of *P. polyphylla* and estimating its geographic distribution using MaxEnt modelling approaches.

The study area, Pindar Valley (2000–4000 m asl; 30° 1'–30° 10' N; 78° 53'–80° 12' E), a representative of western Himalaya, falls in the buffer zone of Nanda Devi Biosphere Reserve (NDBR) and upper reaches of Kumaun Himalayas. Field surveys were conducted in ten different sites of Pindar valley. Site characteristics of different populations of target species and phytosociological attributes of selected populations were collected. In each site, a minimum of 20 quadrats of 1 m × 1 m were laid randomly (Bankoti 1990). Individuals of target species and their associates were recorded from all the quadrats. A total of 200 quadrats were laid in all the 10 sites. The pooled quadrat information was used to analyse the phytosociological

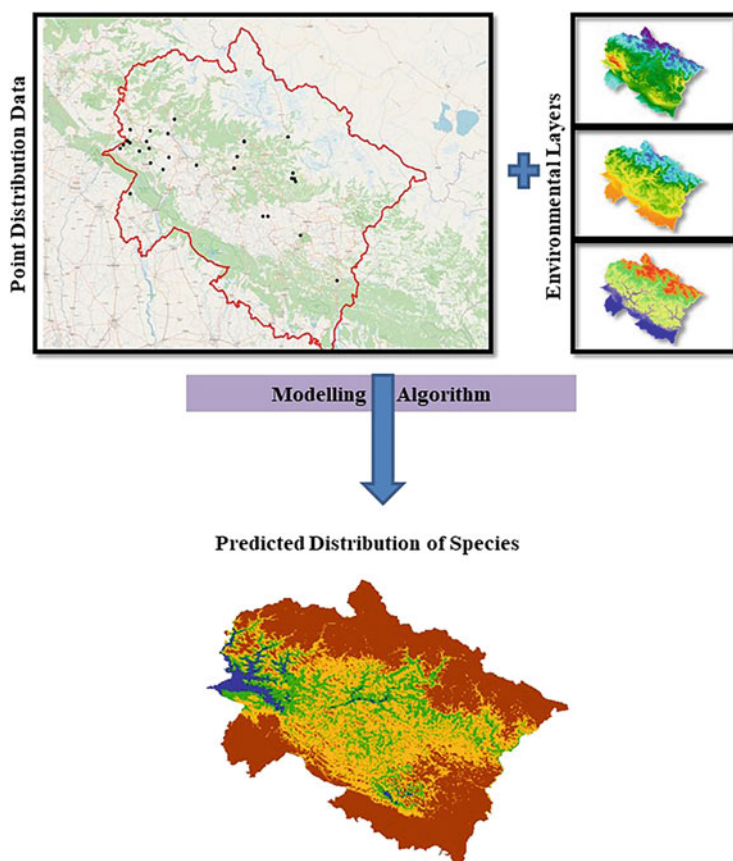


Fig. 5.6 Broad outline of ecological niche modelling procedure (Sharma et al. 2018)

parameters such as frequency, density, relative density and A/F ratio. Nineteen bioclimatic variables (Hijmans et al. 2005) having spatial resolution of 30 s (ca. 1 km) were downloaded from WorldClim dataset (www.worldclim.org). The study area was extracted from all the bioclimatic variable layers. Digital elevation model (DEM) from Shuttle Radar Topography Mission (SRTM; <https://earthexplorer.usgs.gov/>) with 90 m resolution was downloaded and used to generate layers for slope, aspect and elevation. *P. polyphylla* was found growing under the canopy of *Quercus semecarpifolia* and mixed deciduous forest mostly on south facing slopes. The frequency ranged from 25% to 95% and abundance/frequency ratio revealed random distribution of species. The species depicts localized distribution, which offers opportunities for large-scale cultivation. MaxEnt modelling was found to be successful in predicting Dwali site (ca. 2500 m asl) as a potential distribution area of *P. polyphylla* with a validation based on vegetation studies carried out in the field and it is concluded that this site could serve as a reintroduction site as well. This approach has been found to be a very promising tool in species

conservation planning and in predicting the potential distribution of other high-value and medicinal plants of IHR. The MaxEnt model's Jackknife test of variable importance showed that variables, i.e. precipitation of driest and wettest period, are the two most important predictors of *P. polyphylla* habitat distribution. The probability of the presence of *P. polyphylla* has correlation with elevation, precipitation, isothermality and temperature. The model output shows temperate forest characterized by broadleaf evergreen, deciduous broadleaf and evergreen coniferous species to be the preferred habitat for the occurrence of *P. polyphylla* with an area occupancy of 790.85 km², suggesting its habitat in moist and humus-rich soil with canopy from full to partial shade (Tariq et al. 2021).

5.6.2 Conventional Methods

Under natural conditions most medicinal herbs propagate via seeds and underground parts (tubers, bulbs, runners or rhizomes); these methods are well-recognized methods of multiplication and conservation of most species. Although multiplication via seed is a simpler and desirable option, due to harsh climatic conditions, slow growth, erratic flowering and fruiting, unsystematic harvesting and trampling of plants by humans and/or animals, several of the Himalayan herbs do not form viable seeds (Pandey et al. 2000). Furthermore, seeds of many alpine plants undergo dormancy, an adaptation strategy to overcome such conditions prevailing at higher altitudes for long periods and require favourable conditions to germinate. In view of these reasons such seeds need to be influenced by physical and/or chemical treatments for uniform germination (Pandey et al. 2000, 2001). Seeds of *P. polyphylla* possess prolonged dormancy, less viability and very poor/low germination rate along with slow growth and annual fluctuations in seed production (Li 1984, 1986). Further, seeds do not germinate under laboratory conditions following plant growth regulator (PGR) treatments. Therefore, large-scale propagation via seeds does not appear to be a viable and promising method for this species.

Propagation via vegetative method is another method of multiplication of several Himalayan herbs and has been established as an alternate, simple and less expensive means of reviving and reducing the risk of vulnerability and preserving the unique characteristics of a plant in the subsequent progeny. Propagation protocols are reported for a number of important plants having commercial and medicinal value and are being multiplied using their underground vegetative parts (rhizome, tubers, runners, roots, etc.) including ginger (Rahman et al. 2009), *Podophyllum hexandrum* (Nadeem et al. 2000; Nandi et al. 2016), *Picrorhiza kurroo* (Nautiyal et al. 2001; Chandra et al. 2006), *Aconitum atrox* (Rawat et al. 1992), *Nardostachys jatamansi* (Nautiyal and Nautiyal 2004), *Trillium govanianum* (Chauhan et al. 2020), stem cuttings in *Strychnos henningsii* (Kipkemoi et al. 2013), through sucker plantlets in *Musa* species (Baiyeri 2005) and many others. Vegetative propagation using rhizome fragmentation and splitting/cutting followed by PGR treatment has been reported for *P. polyphylla* by various workers (Jamir et al. 2015; Danu et al.

Table 5.1 Sprouting and rooting response (%) following treatments of rhizomes with different PGR concentration and combinations

Treatment (μM) [*]	% Sprouting ^{**}	% Rooting ^{**}
GA ₃ (10)	60.00 $\pm$ 1.15 ^{cd}	54.00 $\pm$ 2.31 ^{cde}
GA ₃ (100)	66.00 $\pm$ 2.08 ^{ef}	68.33 $\pm$ 1.67 ^f
GA ₃ (1000)	70.00 $\pm$ 1.15 ^f	49.33 $\pm$ 1.76 ^c
IBA (10)	42.67 $\pm$ 1.45 ^b	41.00 $\pm$ 2.08 ^b
IBA (100)	55.67 $\pm$ 1.20 ^c	50.33 $\pm$ 1.20 ^{cd}
IBA (1000)	64.00 $\pm$ 1.53 ^{de}	66.00 $\pm$ 1.00 ^f
GA ₃ + IBA (10 each)	56.33 $\pm$ 1.33 ^c	59.67 $\pm$ 1.45 ^c
GA ₃ + IBA (100 each)	69.00 $\pm$ 1.00 ^f	55.33 $\pm$ 1.45 ^{de}
GA ₃ + IBA (1000 each)	84.00 $\pm$ 1.00 ^g	75.00 $\pm$ 1.73 ^g
Control	31.67 $\pm$ 2.73 ^a	32.67 $\pm$ 2.91 ^a

Values are represented as means of triplicate $\pm$ standard error

Mean value followed by the same letter(s) in a column is not significantly different ($p < 0.05$) by DMRT

^{*}Each treatment consisted of five rhizomal buds

^{**}Scored after 100 days of planting

2015). The effect of PGRs in vegetative propagation along with the effect of soil texture on sprouting of rhizome segments was observed (Danu et al. 2015). These workers reported that a combination of GA₃+ IBA (100 mg L⁻¹ each) exhibited maximum influence over various morphological parameters; moreover, rhizomes showed better sprouting and rooting (76.6% and 73.3%) in soil texture having soil/loam/sand (3:2:1, v/v). Jamir et al. (2015) have reported the production of clonal planting material via rhizome cuttings/splitting and group rhizome fragmentation; rhizome fragmentation showed higher survival (75.0%) in comparison to that of rhizome cutting/splitting (49%). In a recent study, Puwein and Thomas (2019) developed an efficient propagation technique for fruiting and flowering, wherein 93.3% and 53.3% of sprouting and fruiting were observed, respectively. However, none of the abovementioned workers were able to mass propagate and reintroduce them in their natural habitat. In a recent investigation (present study) in our laboratory at GBPNiHE, Kosi-Katarmal, Almora, an attempt has been made to refine the abovementioned protocols for large-scale production of *P. polyphylla* plants and subsequently reintroduce them in their natural habitat. Rhizome fragmentation technique was found to be quite successful as far as success in sprouting and rooting was observed; the data recorded revealed a varied response of PGRs and most of them were effective (Table 5.1).

For group rhizome fragmentation, ‘aggregated rhizome masses’ or ‘rhizome clumps’ (Fig. 5.7a) were separated to produce more individual rhizomes (Fig. 5.7b) of *P. polyphylla*. Of all the rhizomes planted, 91% sprouted successfully (Fig. 5.7c) with increased biomass (Fig. 5.7d) and formed complete and mature plants (Fig. 5.7e–h) after 90 days of sowing, while the others failed to show any morphogenetic response. Using this technique, the mortality rate was observed to be very less and with more biomass. The plants obtained were also found to flower (Fig. 5.7g). Thus, propagation using rhizome fragmentation technique appeared to



Fig. 5.7 Different stages during vegetative propagation of *Paris polyphylla* through rhizome fragmentation: (a) aggregated rhizome masses showing multiple buds, (b) fragmented rhizomes ready for sowing, (c) regenerated plants in plastic pots (4–5 weeks), (d) plants with increased biomass and well-developed root system (10 weeks), (e) plants during vegetative phase, (f) plants in their reproductive stage with intact berry after treating with 100 μM of GA_3 + IBA, (g) a close view of a flower on a plant and (h) fruit containing ripened seeds

be very convenient and cost effective and can help in the conservation of this high-value medicinal plant.

5.6.3 Biotechnological Methods

Biotechnological method (in vitro propagation technique) holds tremendous potential and is an alternative and relatively more recent method of multiplication that has been widely applied for numerous plant species of IHR (Nandi et al. 2016; Nandi and Rawat 2019). This method is also being effectively utilized for rapid multiplication of RET species as well as elite clones of a species. The different biotechnological methods that are commonly applied for plant multiplication, genetic enhancement and secondary metabolite production include in vitro regeneration (micropropagation, genetic transformation and somatic embryogenesis), hairy root cultures and plant cell culture. Different parts of plants, e.g. leaves, stem, tubers, roots, rhizomes, runners or their parts, have been used as explant for several species including trees, forest species, horticultural and agricultural crops, vegetables and medicinal and aromatic plants. It would be important to mention that in vitro propagation of a number of medicinally important plant species of the IHR has been reported. Some examples include *Taxus* spp. (Chee 1996; Abbasin et al. 2010; Hussain et al. 2013), *Aconitum balfourii* (Pandey et al. 2004), *Aconitum heterophyllum* (Giri et al. 1993; Jabeen et al. 2006), *Aconitum ferox* (Singh et al. 2020), *P. hexandrum* (Nadeem et al. 2000; Chakraborty et al. 2010), *P. kurrooa* (Upadhyay et al. 1989; Chandra et al. 2004, 2006; Sood and Chauhan 2009; Patial et al. 2012), *Saussurea obvallata* (Joshi and Dhar 2003), *Valeriana jatamansi* (Purohit et al. 2015), *Habenaria edgeworthii* (Giri et al. 2012), *Amomum subulatum* (Purohit et al. 2017) and *Zanthoxylum armatum* (Purohit et al. 2016, 2019).

These workers have used elite planting material to establish cultures, induce multiple shoots, improve rooting and subsequently develop suitable methods for hardening before transferring to field. In some cases, genetic fidelity analysis, survival and growth of such in vitro raised have been monitored for their performance in the field. Therefore, in vitro methods would be a viable option for large-scale multiplication as reported for several plants. Moreover, this technique acts as a powerful tool not only to multiply difficult to propagate rare, endangered or useful species but also for commercial cultivation. While undertaking in vitro propagation work, use of nodal segments and/or attempting via somatic embryogenesis has proven to be a simple and reliable method for mass production of desired clones, as reported in many cases (Purohit et al. 2015; Sunandakumari et al. 2004; Nadeem et al. 2000). Somatic embryogenesis and subsequent germination are beneficial during in vitro propagation as it eliminates the additional step of root induction which is generally required for raising plants and has been reported particularly for *P. hexandrum* (Nadeem et al. 2000), *Pelargonium sidoides* (Kumar et al. 2015), *Lachenalia viridiflora* (Kumar et al. 2016) and others. In our Institute, the use of nodal explants for in vitro regeneration has been reported for many species,

including *Camellia sinensis* (Bag et al. 1997), *Thamnocalamus spathiflorus* (Bag et al. 2000), *P. kurroa* (Chandra et al. 2006), *Valeriana jatamansi* (Purohit et al. 2015), *A. subulatum* (Purohit et al. 2017) and *Z. armatum* (Purohit et al. 2016).

In view of the importance of *P. polyphylla*, it is important to apply such in vitro propagation protocol for this species. However, only a limited number of studies are available on this species. An efficient and reproducible high multiplication protocol for *P. polyphylla* var. *yunnanensis* has been reported (Li et al. 2008), but details are not available. In a subsequent study, effective propagation protocol of *P. polyphylla* via direct somatic embryogenesis using immature zygotic embryos and subsequent plant regeneration on different types of growth media, along with enhancement of active constituents, was reported (Raomai et al. 2014). These workers obtained highest frequency of somatic embryogenesis (32.6%) with maximum number (28.7 ± 1.7) of somatic embryos/explant on 1/2 strength Murashige and Skoog's (MS; Murashige and Skoog 1962) medium, directly without an intermediate callus formation. The high frequency of somatic embryo germination (94.0%) occurred on 1/2 strength MS medium containing 0.5 mg/L GA₃ and highest percentage of seedling to plantlet conversion was observed in medium supplemented with 0.05 mg/L BAP and 0.1 mg/L NAA. In another study by Raomai et al. (2015), an efficient regeneration protocol has been reported where preformed shoot was sliced into three equal portions, viz. basal, middle and apical. These portions were further sliced into small pieces (0.5 mm thickness) and used as transverse thin cell layer (tTCL) explants for plant regeneration. Mini rhizome (MR) was induced from these tTCL explants only from basal and middle portions. The highest response percentage (86.6%) of MR formation with maximum fresh weight (1.05 ± 0.08 g) was achieved from basal sections when cultured on 1/2 strength MS medium containing 0.5 mg/L BAP. In a recent study the use of 50% activated charcoal supplemented with varying concentrations of 6-benzylaminopurine (BAP; 0.5 µg/mL) either individually or in combination with auxin α-naphthalene acetic acid (NAA; 0.2 µg/mL) with 80% of shoot response was reported (Puwein and Thomas 2019).

Although the above studies have reported in vitro shoot multiplication in *P. polyphylla*, these do not mention the development of root system. Therefore, an efficient in vitro propagation protocol was developed in our laboratory using nodal explants taken from mother plant (Fig. 5.8a) using 1/2 strength MS medium along with PGR combinations of BAP, NAA and GA₃. Rhizomal buds were used as explant and all cultures were maintained in a culture room (25 ± 1 °C; 16 h light/ 8 h dark photoperiod) with irradiance of 40 µmol/m²/s by cool white fluorescent tubes (Philips TI 40 W/54, India). Among the various treatments used, 1/2 strength MS medium containing BAP (0.5 µM), NAA (0.1 µM) and GA₃ (0.1 µM) resulted in bud initiation within 2 weeks of inoculation (Fig. 5.8b) followed by induction of single leaf with root after 6 weeks (Fig. 5.8c). The explants cultured responded by forming multiple shoots and roots (8–10 weeks; Fig. 5.8d). After 12 weeks, the in vitro raised plantlets were taken out from the culture medium (Fig. 5.8e) and their basal portion washed thoroughly. These plantlets were then treated with Bavistin solution (0.5%, w/v; 10 min) to prevent fungal contamination and transferred to trays containing soil and farmyard manure (3:1, v/v; Fig. 5.8f). Further, hardening was



Fig. 5.8 Various stages during in vitro propagation of *Paris polyphylla*: (a) mother plant, (b) a rhizomal bud cultured on $\frac{1}{2}$ MS medium ($0.5 \mu\text{M}$ BAP; $0.1 \mu\text{M}$ NAA; $0.1 \mu\text{M}$ GA₃) and bud initiation in a 2-week-old culture, (c) induction of single leaf along with root formation (6 weeks), (d) plants with multiple shoots (8–10 weeks old), (e) plants with well-developed root system (10–12 weeks old), (f) plants growing in pots containing soil and farmyard manure (3:1, v/v) in mist chamber, (g) plants in polyhouse condition for hardening to natural condition and (h) plant reintroduced in natural conditions (near Khati Village, Pindar Valley; 2281 m) after 24 weeks of culture

carried out in a polyhouse with watering on alternate days. These plants were maintained under humid conditions in a greenhouse (95–100% relative humidity; initially for 3–4 weeks) followed by transfer to polyhouse for another 8–10 weeks (Fig. 5.8g). Field reintroduction of these plants (tissue culture raised) was eventually performed in their natural conditions (Fig. 5.8h) in Pindar Valley (District Bageshwar, Uttarakhand; 2281 m; 30°6'37.8"N, 79°55'71.5") after 24 weeks of culture.

5.7 Research Gaps, Opportunities and Future Perspective

The rich plant diversity comprising of a large number of MAPs growing in a wide range of geo-climatic zones with unique ecosystem features justifies Uttarakhand as an herbal state, along with some other regions in the IHR (Phondani et al. 2010; Jalal et al. 2008). These medicinal plants are remarkable sources of secondary metabolites and such desired molecules are used in modern drugs and nutraceuticals. In addition, a large number of plants of the IHR still remain unexplored and such plants can be tested for the discovery of new molecules for newer applications/remedies. With the ever-increasing demand of secondary metabolites and bioactive plant metabolites by pharmaceutical industries and the concept of 'plant naturals', various technologies need to be optimized for higher production. Therefore, the requirement of MAPs in future will be manifold.

Under natural conditions the plant propagates via seeds and rhizomes; these are well-recognized methods for multiplication and conservation of many important and/or endangered species. In addition, in vitro method can also be adopted for multiplication of this species as this technique has proven to be an alternate and effective means of rapid propagation that can facilitate the recovery of RET Himalayan herbs (Chandra et al. 2006; Nadeem et al. 2000; Nandi et al. 2002, 2016; Pandey et al. 2004; Purohit et al. 2015, 2016, 2017, 2019; Rawat et al. 2018; Deb et al. 2018). Recently, in vitro plant regeneration through direct somatic embryogenesis for this species as well as protocol using thin cell layer culture was developed (Raomai et al. 2014, 2015). Thus, conventional as well as in vitro methods can be employed for mass scale multiplication of quality propagules for systematic cultivation in newer locations, particularly in similar and/or closer to their natural habitats (in alpine and sub-alpine areas). For species reintroduction, prior ENM studies would be desirable.

There is a plethora of Himalayan medicinal plants that are being commercially utilized for their pharmaceutical values by the industries. Hence, on account of high demand of these plants, large-scale collection is still continuing from the wilds; thus, such species are under tremendous pressure in their natural habitat. Therefore, use of biotechnological methods particularly in vitro techniques as efficient and rapid means for mass multiplication and subsequent cultivation for secondary metabolite production would be highly desirable. Moreover, involving local wisdom (people with knowledge on medicinal plants and its use) and integrating expertise from other

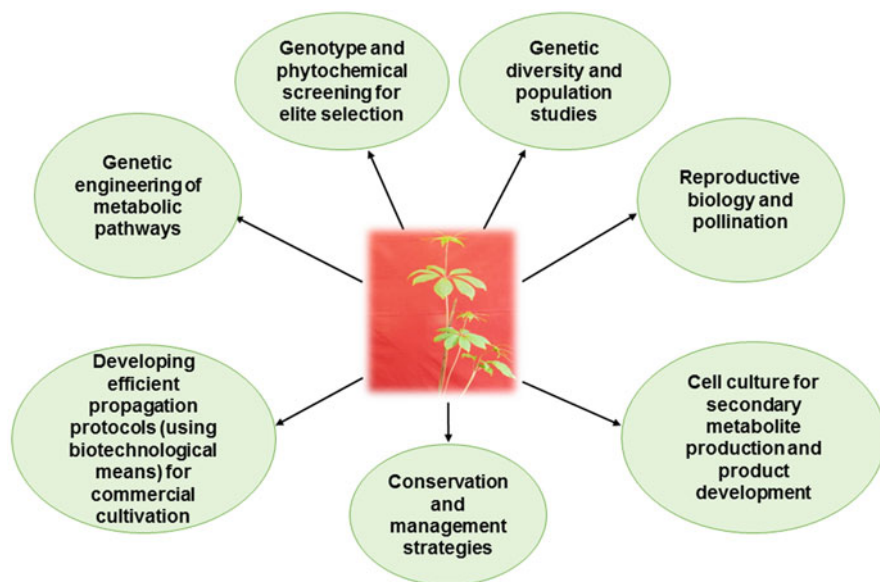


Fig. 5.9 Schematic representation depicting conservation implication of *Paris polyphylla* and research opportunities

disciplines (forestry, phytochemistry, conservation biology, biotechnology, etc.) can effectively improve the health status of various important and high-value MAPs of the IHR. In vitro methods can also provide the much-needed elite planting material and meet the overall goal of conservation.

Due to extensive collection from the wild and habitat loss, *P. polyphylla* is under tremendous pressure. Hence, it would be relevant to minimize the biodiversity loss and systematically improve the habitat and status of the species. Some of the major areas where little or very limited studies have been reported and require immediate focus (gap areas) include genetic diversity and population studies, genotype and biochemical/phytochemical screening for elite selection, reproductive biology and pollination, genetic engineering and understanding of metabolic pathways, development of reproducible and efficient propagation protocols for commercial cultivation and developing conservation and management strategies. These gap areas also form important R&D opportunities (Fig. 5.9), and exercising interdisciplinary and multipronged approach would result in better understanding of the species and subsequently pave the way for sustainable resource utilization and improved status.

P. polyphylla being a commercially important species of the IHR, a detailed study on the abovementioned aspects (see Fig. 5.9), will further strengthen the available information and aid in developing conservation strategies. The present study therefore deal with the integration of all abovementioned aspects like studies on understanding of ecology and formulation of various conservation strategies using application of ENM, identification of elite individual based on morphological and

phytochemical diversity, optimization of active constituents (polyphenolics) and development of propagation protocols of *P. polyphylla* for conservation and sustainable utilization. Biotechnological and ecological approaches are extremely important and essential for setting up of conservation and management step or plan for plant species that are mainly rare and endangered. *P. polyphylla* is categorized as endangered (CAMP) and thus needs such approaches for its long-term survival and sustainable utilization. Considering the ecological features and population status of this species, proper designing and implementation of effective conservation programme need to be undertaken using ENM techniques. The use of biotechnological and conventional methods of multiplication and subsequent reintroduction of the hardened plants in natural habitats and niche areas will be a significant initiative for its conservation. Such steps have been already undertaken in several areas of the IHR with satisfactory results. Since the genus is important and has potential commercial application, immediate attention should be focused on its management, conservation, sustainable utilization and most importantly its cultivation promoted at a broader level.

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Chapter 6

Endophytic Fungal Diversity in Solanaceous Medicinal Plants and Their Beneficial Impact



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Abstract Plants are not individual entities in the natural environment and are always associated with versatile groups of microflora including bacteria, fungi, actinomycetes, etc. Among those microorganisms, some of them reside symbiotically within plants, termed endophytes. Nowadays, endophytism is an emerging field in research, dealing with the study of a particular plant-endophyte relationship and providing opportunities for utilization of several beneficiary applications. Cross-association between microbes and plants always results in an enormous amount of bioactive secondary compound production, along with enhancement and modulation of plant growth, through the production of different growth substances, including phytohormones. Fungal endophytes help adapt the plant to the new eco-geological region by increasing biotic and abiotic stress tolerance. In recent years, the actual interrelationship between endophytic fungi and their host plants was demonstrated, which correspondingly helps to explore themselves for biotechnological purposes. The family Solanaceae is well known as the vegetable family. However, numerous medicinal plants, characterized by their ethnomedicinal values, are also included, which act as a good bioresource for different pharmaceutically important phytocomponents. The present chapter mainly focuses on exploring the diversity of fungal endophytes associated with medicinally important plant species of Solanaceae and their beneficial impacts. The advantageous properties of fungal endophytes studied in this chapter include enhanced production of plant growth

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regulatory and medicinal compounds, biocontrol activity, bioremediation ability, and pharmacological activities.

Keywords Biocontrol · Bioremediation · Endophytic fungi · Medicinal plants · Solanaceae

6.1 Introduction

Endophytism is the unique relationship between a plant and an endo-symbiotic group of microorganisms, which does not impose any harmful effects on the plant. The plant system was believed to be sterile until 1887, when Galippe discovered bacteria commonly occurring inside plant tissues (Galippe 1887). In 1809, the term endophyte was first coined and supposed to be the parasitic fungi associated with plants. According to Marella (2014), endophytes are classified into two distinct groups, i.e. obligate and facultative endophytes. The first ones depend on the host plant and pass their entire life cycle, whereas the facultative ones pass a portion of their life cycle within the host and the other part outside the host plant. Although most of the associations between microbes and plants are symbiotic, some antagonistic relationships can also be found. Endophytic groups of microorganisms, including bacteria, fungi, and actinomycetes, were reported to cause beneficial impacts on plants. The association of microorganisms with the plant can cause the enhancement of bioactive component production in plants by synthesizing and accumulating different bioactive compounds within the host plant (Vishwakarma et al. 2020). The endophytes also promote plant growth by providing nutrients, along with different groups of phytohormones (Poveda et al. 2021). Endophytes are ubiquitous and can reduce abiotic stresses like salinity and droughts (Abo Nouh et al. 2021). Biotic stress is also relieved by some endophytes, which cordially function to protect the plants from phytopathogens (Oukala et al. 2021). The utilization of microbes in sustainable agriculture provides an enormous scope for increasing productivity. Mechanism of endophytic interaction and enhancement of desired products are now deliberated properly and significantly (Kemen et al. 2015; Müller et al. 2016).

The commercially important plant family Solanaceae, commonly known as Nightshade, is a moderately large and diverse family of trees, shrubs, and herbs, which includes 98 genera and approximately 2800 species occurring around the world (Dupin et al. 2017). According to Hunziker (1979), the family Solanaceae is originated in South America and then distributed to other parts of the world. It occurs in warm tropical climatic zones of all continents except Antarctica, from sea level to the altitudes of 5000 m, and grows in different heterogeneous environments (Barboza 2013). The family is enriched with different important plant species and used majorly as food medicine and some of them being ornamental plants. Genera like *Solanum*, *Capsicum*, *Physalis*, and *Lycium* are widely cultivated species and used as food in different countries worldwide. The ornamental plants include *Browallia* sp., *Brugmansia* sp., *Petunia* sp., *Salpiglossis* sp., *Schizanthus* sp., *Solanum aviculare*, *S. capsicastrum*, *S. laciniatum*, *S. rantonnetii*, *S. seaforthianum*, and *S. wendlandii* (Kaunda and Zhang 2019; Palchetti et al. 2020).

The endophytes associated with Solanaceae play a pivotal role in plant growth, fitness, better productivity, disease resistance, etc. Diverse groups of bioactive secondary metabolites are also often produced by them (Naik 2017; Kushwaha et al. 2019a, b, c; Long et al. 2019; Elkhoully et al. 2021; Pratiwi et al. 2021). Production of this type of bioactive compounds by endophytes and accumulation by plants cause a healthy mutual relationship between them. The beneficial impact of the endophytes on the species of Solanaceae is the main focus of interest of this present chapter. We also aim to understand the potentiality of application of endophytes in human welfare as well as in the agriculture industry by using them as a natural resource.

6.2 Impact of Endophytes on Medicinal Plants

Medicinal plants are traditionally used worldwide for the preparation of different remedies against various diseases. The productivity of bioactive compounds and bioactivity in medicinal plants depends on different parameters like temperature, moisture, soil condition, and the presence of soil microbes (Haque et al. 2017; Porwal et al. 2020; Amare and Desta 2021; Chakraborty et al. 2022). Soil microbes, including bacteria, fungi, and actinomycetes, play an essential role in plants' growth regulation by producing different phytohormones, antimicrobial substances, bioactive secondary metabolites, and essential enzymes (Fig. 6.1). In addition, they also help in proper plant growth through the maintenance of soil quality by improving the solubilization of nutrients and essential ions to the plant (Chakraborty et al. 2019). The presence of different endophytic microbes can cause the production of

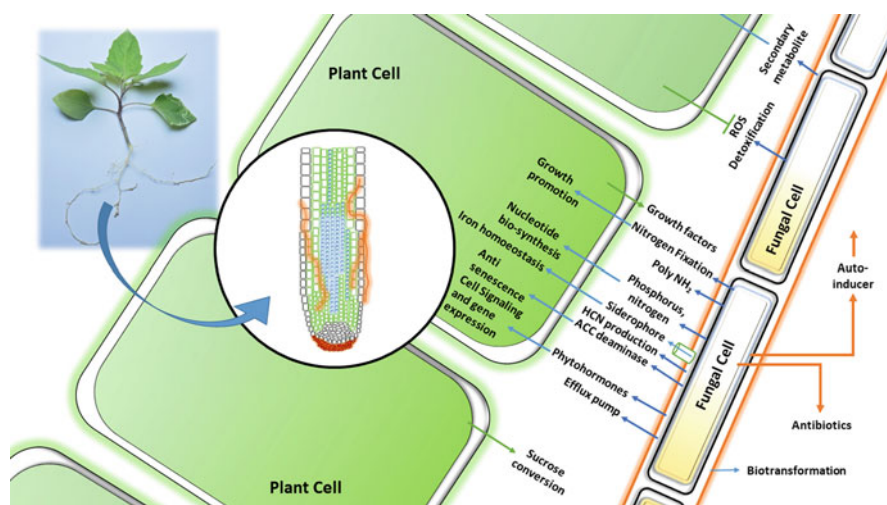


Fig. 6.1 Schematic representation of the beneficial impacts of endophytic fungi on plant

phytohormones like indole-3-acetic acid (IAA) and gibberellic acid (GA), which ultimately leads to plant growth promotion (Eid et al. 2019). The presence of pathogenic bacteria in association with plants can cause problems like slow growth and disease of plants, which can be eradicated by beneficial endophytes through the production of antimicrobial substances (Gao et al. 2010). Different types of detoxification enzymes such as protease, lipase, amylase, pectinase, etc., can also be produced by endophytes during mutualistic association with plants, which alternatively suppress plant-pathogen interactions (Ghosh et al. 2021). Some fatal human disease-causing microorganisms also can be treated by endophytes due to the production of potent antimicrobial compounds in them (Pimentel et al. 2011). Bioactive compounds such as alkaloids, flavonoids, terpenoids, phenolics, and steroids are also produced by endophytes while associated with plants that can be used for different pharmacological, industrial, and biotechnological development purposes (Eid et al. 2021). The presence of different types of chemical contaminants, pollutants, and toxins in the soil can harm the growth of both plants and endophytes. Removal of these materials through the process of bioremediation by endophytes also takes place in the soil environment (Sharma and Kumar 2021). Endophytes can also protect plants to survive in a different type of biotic stressed condition, through biocontrol by producing anti-insecticidal compounds (Morales-Cedeno et al. 2021). Microbes associated with plant roots take part in biogeochemical cycles by decomposing dead matter and solubilizing nutrients and essential ions into the soil that can further help the plant accumulate those substances. Microorganisms can also help improve the pharmacological industries by providing different metabolic by-products. This type of mutualistic endophytic relationship is also helpful for sustainable agricultural practices, drug development, industrial enzyme production, and biofertilizer production, due to versatile activities with eco-friendly practices (Moharram et al. 2016; Naik 2017; Kuźniar et al. 2019).

6.3 Applications of Endophytes in Biotechnology

With the growing world population and increasing demands, endophytes can prove to be new sources for biotechnological interventions and commercial exploitation. Endophyte biotechnology is currently a topic of interest as endophytes are ubiquitous and provide several functions that can be biotechnologically implemented for the betterment of humanity. Endophytes are essential sources for the production of pharmaceutically important bioactive metabolites and enzymes, and they can serve as biofertilizers, and they have applications in bioremediation and biotransformation processes (Kuźniar et al. 2019).

Plants can synthesize different secondary metabolites of therapeutic use. Endophytes residing within plants can mimic the synthesis of those plant secondary metabolites. Endophytic fungi can synthesize bioactive compounds with diverse activities like antimicrobial, antioxidant, anti-inflammatory, antidiabetic, and anticancerous properties (Ruma et al. 2013). The endophytic fungus *Stachybotrys*

chartarum can produce chartarlactams A–P and phenyl-spirodrimanones, which have shown potential antihyperlipidemic activity (Li et al. 2014). Several studies have reported that the endophytic fungi *Alternaria alternata*, *Alternaria* sp., and *Aspergillus niger* in different hosts can produce vinbrastine, lapachol, and lovastatin (Guo et al. 1998; Sadananda et al. 2011; Raghunath et al. 2012; Rana et al. 2020). Thus, there is a scope for industrial-level production of these bioactive compounds from endophytes, using different approaches. For the commercial-scale production of taxol from endophytic fungi, improved fermentation techniques have been adopted (Li et al. 1996; Wang et al. 2000; Sun et al. 2008; Zhou et al. 2010; Naik et al. 2018).

The biotransformation ability of endophytes is advantageous over chemical synthesis as it produces novel compounds, increases compound productivity, and can bypass problems of the chemical analysis process, which ultimately leads to the basic information required for the development of particular biosynthetic pathways (Suresh et al. 2006). For example, terpene biotransformation can produce industrially important enantiomerically pure flavours and fragrances (Krings and Berger 1998; Molina et al. 2012). In a study by Molina et al. (2012) three fungal strains have biotransformed α -pinene into industrially important verbenol.

Currently, in agricultural industries, there is a need to develop biofertilizers, bio-inoculants, and bio-preparations to substitute chemical fertilizers for the betterment of the growth and yield of crop plants. Biofertilizers are living organisms that, when applied to the plants and soil, can promote plant growth by providing nutrients to the host plant by colonizing the rhizosphere and plant interior (Kuźniar et al. 2019). As endophytes often produce IAA and siderophores (iron ion-chelating compounds), can fix nitrogen, and solubilize inorganic phosphate for better plant uptake, they are good candidates to be considered as biofertilizers. Endophytes in associated conditions with plants can also reduce hazardous contaminants or lower their bioavailability in the soil through the process of phytoremediation, bioaugmentation, and biostimulation, which are environment-friendly and low-cost methods compared to physicochemical approaches (Kuźniar et al. 2019).

6.4 Importances of Medicinal Plants of Solanaceae

The Solanaceae is an important and valuable family for humanity, as it provides a significant portion of vegetables consumed by people, such as potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and pepper (*Capsicum annuum*, *C. frutescens*). Plants like potato, tomato, pepper, and tobacco (*Nicotiana tabacum*) are used as vegetables in several parts of the world with different climates (Olmstead et al. 2008). Several other species like *Physalis ixocarpa* (tomatillo), *Physalis peruviana* (Cape gooseberry), *Solanum aethiopicum* (mock tomato), and *S. muricatum* (pepino) and *S. torvum* (turkey berry) are also cultivated for food (Rubatzky and Yamaguchi 1997).

Besides being a major vegetable family, Solanaceae is also a resource of a vast number of plants with medicinal potency. Solanaceous species contain alkaloids and

are known for their medicinal, psychotropic, and poisonous properties. Some of the well-known medicinal plants are belladonna (*Atropa belladonna*), mandrake (*Man-dragora officinarum*), henbane (*Hyoscyamus niger*), thorn apple (*Datura stramonium*), ashwagandha (*Withania somnifera*), etc. (Palchetti et al. 2020). The plant genera including *Atropa*, *Datura*, and *Hyoscyamus* contain various species with different important bioactive compounds that make the plant species medicinally active (Shah et al. 2013). Many plant species of the Solanaceae family were used traditionally from the ancient past. *Brugmansia candida* is a well-known species reported as a medication to treat dermatitis, orchitis, arthritis, headaches, and infections (Feo 2004). Another plant species of this family, *Capsicum annuum* was used as remedies for sinus infection, sore throat, cancer, asthma, and cough (Duke 1993; Khare 2004). *Datura* is reported to be an essential medicinal genus, at different intervals of time by many researchers. *D. stramonium* is an important species under the respective genus and reported for its use as anthelmintic and anti-inflammatory and for worm infestation, toothache, and fever and as insect repellent (Guarrera 1999; Das et al. 2012; Soni et al. 2012). According to Duke (2008), *Solanum lycopersicum* is also an effective treatment for burns, scalds, sunburn, and toothache. *W. somnifera* is an important species with diverse properties in treating chronic fatigue, weakness, dehydration, bones and loose teeth, debility, and muscle tension (Mir et al. 2012). Antimicrobial resistance is a major issue nowadays, chiefly caused by misuse or overuse of antibiotics. The discovery of new effective drugs is declining, a serious global concern. From ancient times, medicinal plants are used to treat different bacterial and fungal diseases because of their unique and immense chemical diversity. Some of the plant species under the Solanaceae family are also found very active against pathogens causing human disease. *Capsicum baccatum*, *Datura innoxia*, *Solanum aethiopicum*, and *Nicotiana glauca* were reported vastly for their antibacterial and antifungal activity (Bard et al. 2014; Dracatos et al. 2014; Singh and Suresh 2016; Chen et al. 2018).

6.5 Diversity of Fungal Endophytes Associated with the Family Solanaceae

Endophytism is the relationship between plants and microbes without harming themselves. Endophytes are associated with plants in various forms, such as fungi, actinomycetes, mycoplasma, and bacteria. Endophytes are isolated from the Solanaceae plants from different parts such as the leaf, petioles, root tissues, seeds, and fruits. In this study, we are mainly focused on the fungal endophytes associated with the plants of the Solanaceae family. Although several pathogenic fungi are harmful to plants, endophytic fungi are mainly heterotrophic organisms associated with different plants symbiotically, without causing disease in them. The endophytic fungi associated with the plant Solanaceae are mainly ascomycetes and basidiomycetes. The endophytes are isolated by crushing or cutting the tissue and culturing it in

suitable media for their growth. Primary identification of the endophytes was done by testing them morphologically and biochemically. The molecular identification of the strains is now done based on ribosomal DNA or internal transcribed spacer (ITS) sequence analysis. Based on this type of modern molecular approach, phylogeny analysis can also be done to identify the organism and its ancestral. Approximately a total of 30 plant species under the family Solanaceae were reported to be associated with fungal endophytic relationships to date (Table 6.1). Twenty-two fungal endophytes including *Aspergillus aculeatus*, *Colletotrichum gloeosporioides*, and *Glomerellales* sp. were reported from the species of *Brugmansia aurea* Lagerh. (Singh et al. 2017). Another study on *Brunfelsia uniflora* (Pohl) D. Don reported an association of four endophytic fungi, namely, *Nigrospora oryzae*, *Nodulisporium* sp., *Phomopsis archeri*, and *Penicillium* sp. (Marsola et al. 2021). The plants including *Lycium barbarum* L., *Lycium chinense* Mill., *Physalis alkekengi* L., etc. are also reported various times for their association with fungal endophytes.

6.6 Beneficial Impacts of Fungal Endophytes from Solanaceous Medicinal Plants

The diverse range of endophytic fungal species associated with the family Solanaceae (Fig. 6.2) imparts different beneficial features, including plant growth promotion, biocontrol of plant pathogens, different bioactive compound production, pharmacological activities, and heavy metal stress amelioration and bioremediation ability.

6.6.1 Plant Growth Promotion

The microbiome associated with plants plays an important role in positively regulating host plant growth along with improving soil quality. Endophytic fungi colonize the internal tissues of plants without causing any detrimental effect to the plants and developing disease symptoms. Instead, they form mutualistic relationships with the plant and prove themselves beneficial to the host plant by various direct and indirect mechanisms.

Direct mechanisms include phytohormone synthesis. Phytohormones are considered as plant growth regulators that are biochemically synthesized by plants and which can mediate growth and developmental processes in normal or stressful conditions, by acting locally or in different sites of the plant after transportation. Phytohormones control other cellular processes in targeted cells and regulate the formation of plant organ systems. Some of the important phytohormones are auxins, cytokinins, gibberellins, abscisic acid, ethylene, and brassinosteroids, which control many physiological and biochemical processes (Peleg and Blumwald 2011; Iqbal

Table 6.1 Endophytes associated with the medicinal plant species of Solanaceae

Sl. No	Plant species with endophyte association	IUCN status	Fungal strains name	Endophyte association (references)
1.	<i>Atropa bella donna</i> L.		<i>Mycelia sterilia</i>	Krohn et al. (2001)
			<i>Piriformospora indica</i>	Noora et al. (2017)
				Noora and Shahabivand (2018)
2.	<i>Brugmansia aurea</i> Lagerh	Extinct in the wild	<i>Acremonium</i> sp., <i>Alternaria porri</i> , <i>Aspergillus aculeatus</i> , <i>Aspergillus brasiliensis</i> , <i>Aspergillus niger</i> , <i>Aspergillus</i> sp., <i>Aspergillus stellifer</i> , <i>Colletotrichum gloeosporioides</i> , <i>Colletotrichum queenslandicum</i> , <i>Colletotrichum</i> sp., <i>Diaporthe longicolla</i> , <i>Fusarium nematophilum</i> , <i>Glomerellales</i> sp., <i>Macrophomina phaseolina</i> , <i>Macrophomina</i> sp., <i>Neoscytalidium hyalinum</i> , <i>Penicillium oxalicum</i> , <i>Periconia</i> sp., <i>Phoma gardenia</i> , <i>Talaromyces</i> sp., <i>Talaromyces trachyspermum</i>	Singh et al. (2017)
			<i>Macrophomina phaseolina</i>	Singh et al. (2021)
3.	<i>Brunfelsia uniflora</i> (Pohl) D.Don	Least concern	<i>Nigrospora oryzae</i> , <i>Nodulisporium</i> sp., <i>Phomopsis archeri</i> , <i>Penicillium</i> sp.	Marsola et al. (2021)
4.	<i>Datura innoxia</i> Mill.		<i>Diaporthe</i> sp.	Sharma et al. (2018)
			<i>Aspergillus carneus</i> , <i>A. flavus</i> , <i>A. fumigatus</i> , <i>A. niger</i> , <i>A. ochraceus</i> , <i>A. terreus</i> var. <i>africanus</i> , <i>A. ustus</i> , <i>A. versicolor</i> , <i>Chaetomium atrobrunneum</i> , <i>C. hexagonosporum</i> , <i>Cladosporium cladosporioides</i> , <i>C. cucumerinum</i> , <i>C. oxysporum</i> , <i>C. spongiosum</i> , <i>C. uredinicola</i> , <i>Cochliobolus spicifer</i> , <i>Emericella nidulans</i> var. <i>lata</i> , <i>Eurotium chevalieri</i> , <i>Mucor hiemalis</i> , <i>Penicillium aurantiogriseum</i> , <i>P. chrysogenum</i> , <i>P. spinulosum</i> , <i>P. steckii</i> , <i>P. waksmanii</i> , <i>Periconia atropurpurea</i> , <i>Phialophora fastigiata</i> , <i>Scopulariopsis brevicaulis</i> , <i>Stemphylium botryosum</i> , sterile mycelia, <i>Ulocladium alternariae</i>	El-Said et al. (2016)
5.	<i>Datura metel</i> L.		<i>Fusarium solani</i>	Kuriakose et al. (2014)
			<i>Bipolaris</i> sp.	

(continued)

Table 6.1 (continued)

Sl. No	Plant species with endophyte association	IUCN status	Fungal strains name	Endophyte association (references)
				Maehara et al. (2020)
			<i>Colletotrichum gloeosporioides</i>	Anantha Krishna (2018)
			<i>Colletotrichum boninense</i> , <i>Colletotrichum incarnatum</i> , <i>Colletotrichum siamense</i> , <i>Colletotrichum gloeosporioides</i> , <i>Phomopsis</i> sp., <i>Fusarium solani</i>	Naik 2017
6.	<i>Datura stramonium</i> L.		<i>Alternaria</i> sp.	Sun et al. (2012)
			<i>Fusarium oxysporum</i>	Chen et al. (2013)
			<i>Gyroporus castaneus</i> , <i>Alternaria tenuissima</i> , <i>A. alternata</i> , <i>Colletotrichum</i> sp., <i>Talaromyces</i> sp., <i>Alternaria</i> sp., <i>Sporothrix schenckii</i> , <i>Trichoderma longibrachiatum</i> , <i>Pilobolus crystallinus</i> , <i>Rhodotorula mucilaginosa</i> , <i>Bipolaris setariae</i>	Tapfuma et al. (2019)
7.	<i>Hyoscyamus muticus</i> L.		<i>Acremonium strictum</i> , <i>Alternaria alternata</i> , <i>Aspergillus candidus</i> , <i>A. flavus</i> , <i>A. fumigatus</i> , <i>A. nidulans</i> , <i>A. niger</i> , <i>A. ochraceus</i> , <i>A. oryzae</i> , <i>A. sydowii</i> , <i>A. terreus</i> , <i>A. ustus</i> , <i>A. versicolor</i> , <i>Chaetomium globosum</i> , <i>Cladosporium cladosporioides</i> , <i>C. herbarum</i> , <i>C. oxysporum</i> , <i>Curvularia clavata</i> , <i>C. lunata</i> , <i>Drechslera hawaiiensis</i> , <i>Fusarium camptoceras</i> , <i>F. aquaeductum</i> , <i>F. solani</i> , <i>F. subglutinans</i> , <i>F. tricinctum</i> , <i>Gliocladium roseum</i> , <i>Humicola grisea</i> , <i>Mucor hiemalis</i> , <i>M. racemosus</i> , <i>Myrothecium roridum</i> , <i>Paecilomyces lilacinus</i> , <i>Penicillium brevicompactum</i> , <i>P. citrinum</i> , <i>P. chrysogenum</i> , <i>P. funiculosum</i> , <i>P. waksmanii</i> , <i>Rhizopus stolonifer</i> , <i>Rhizoctonia solani</i> , <i>Thyrostromella myriana</i> , <i>Neoscytalidium dimidiatum</i> , <i>Trichurus spiralis</i> , <i>Ulocladium atrum</i> , <i>U. chartarum</i> , <i>Verticillium nubilum</i>	Abdel-Motaal et al. (2010)
			<i>Pseudozyma japonica</i> , <i>P. hebania</i> , <i>P. egyptica</i> , <i>P. mutica</i>	Abdel-Motaal et al. (2009)
			<i>Alternaria alternata</i> , <i>Aspergillus flavus</i> , <i>A. fumigatus</i> , <i>A. niger</i> , <i>A. terreus</i> , <i>A. versicolor</i> , <i>Chaetomium globosporum</i> , <i>C. globosum</i> , <i>Cladosporium chlorocephalum</i> ,	El-Said et al. (2016)

(continued)

Table 6.1 (continued)

Sl. No	Plant species with endophyte association	IUCN status	Fungal strains name	Endophyte association (references)
			<i>C. cucumerinum</i> , <i>C. oxysporum</i> , <i>C. sphaerospermum</i> , <i>C. spongiosum</i> , <i>C. uredinicola</i> , <i>Cochliobolus spicifer</i> , <i>Cordella clarkia</i> , <i>Eurotium chevalieri</i> , <i>Penicillium aurantiogriseum</i> , <i>P. chrysogenum</i> , <i>P. duclauxii</i> , <i>P. funiculosum</i> , <i>P. rubrum</i> , <i>Periconia atropurpurea</i>	
			<i>Aspergillus flavus</i> , <i>A. fumigatus</i> , <i>A. oryzae</i> , <i>A. terreus</i> , <i>A. versicolor</i> , <i>Myrothecium roridum</i> , <i>Rhizopus stolonifer</i> , <i>Aspergillus cristatus</i> , <i>A. flavipes</i> , <i>A. sulphureus</i> , <i>A. sydowii</i> , <i>Cordana musae</i> , <i>Cunninghamella echinulata</i> , <i>C. elegans</i> , <i>Emericellopsis minima</i> , <i>Emericellopsis</i> sp., <i>Fusarium dimerum</i> , <i>F. nivale</i> , <i>F. solani</i> , <i>Microascus albonigrescens</i> , <i>M. niger</i> , <i>Myrothecium verrucaria</i> , <i>Penicillium canescens</i> , <i>P. duclauxii</i> , <i>P. janthinellum</i> , <i>P. oxalicum</i> , <i>P. roqueforti</i> , <i>P. verruculosum</i> , <i>P. waksmanii</i> , <i>Stachybotrys bisbyi</i> , <i>S. chartarum</i> , <i>S. elegans</i> , <i>Thyrostromella myriana</i> , <i>Torula graminis</i> , <i>Ulocladium botrytis</i> , <i>Aspergillus flavus</i> var., <i>A. terreus</i> var. <i>aureus</i> , <i>A. thomii</i> , <i>Chaetomium cochlioides</i> , <i>Cladorrhinum foecundissimum</i> , <i>Humicola grisea</i> , <i>Mucor circinelloides</i> , <i>M. racemosus</i> , <i>Phoma</i> sp., <i>Absidia corymbifera</i> , <i>Aspergillus alutaceus</i> , <i>Cladosporium herbarum</i> , <i>Eurotium repens</i> , <i>Gibberella gordonii</i> , <i>Monilia pruinosa</i> , <i>Penicillium purpurogenum</i> , <i>Rhizopus arrhizus</i> , <i>Thanatephorus cucumeris</i> , <i>Trichoderma viride</i> , <i>Ulocladium alternariae</i>	El-Zayat et al. (2008)
			<i>Aspergillus tubingensis</i>	Elkhouly et al. (2021)
8.	<i>Lycium barbarum</i> L.		Arbuscular mycorrhizal fungi and dark septate endophytic fungi	Zhang et al. (2010)
			<i>Bipolaris</i> sp.	Long et al. (2019)
			Dark septate endophytic fungi (<i>Paraphoma chrysanthemicola</i>)	Zhang et al. (2012)
			<i>Aspergillus</i> sp., <i>Penicillium</i> sp., <i>Emericella</i> sp.	Dai and Du (2017)
				Xu et al. (2017)

(continued)

Table 6.1 (continued)

Sl. No	Plant species with endophyte association	IUCN status	Fungal strains name	Endophyte association (references)
			<i>Alternaria</i> sp., <i>Aspergillus</i> sp., <i>Bipolaris</i> sp., <i>Chaetomium</i> sp., <i>Cladosporium</i> sp., <i>Fusarium</i> sp., <i>Penicillium</i> sp., <i>Preussia</i> sp., <i>Schizothecium</i> sp., <i>Sordaria</i> sp., <i>Taifanglania</i> sp., <i>Thielavia</i> sp., <i>Tricharina</i> sp., <i>Xylaria</i> sp.	
			<i>Fusarium nematophilum</i>	Siyuan et al. (2020)
			Arbuscular mycorrhizal fungi, dark septate endophytes	Sijie et al. (2021)
9.	<i>Lycium chinense</i> Mill.		<i>Colletotrichum brevisporum</i> , <i>Colletotrichum fruticola</i> , <i>Colletotrichum truncatum</i> , <i>Glomerella magna</i> , <i>Acremonium</i> sp., <i>Acremonium strictum</i> , <i>Cochliobolus lunatus</i> , <i>Cochliobolus</i> sp., <i>Fusarium</i> cf. <i>equiseti</i> , <i>Fusarium equiseti</i> , <i>Hypocrea citrina</i> , <i>Nemania</i> sp., <i>Nemania serpens</i> , <i>Nemania diffusa</i>	Paul et al. (2014)
			<i>Micromonospora lycii</i>	Zhao et al. (2016)
			<i>Phomopsis</i> sp., <i>Alternaria</i> sp., <i>Fusarium</i> sp., <i>Colletotrichum</i> sp., <i>Myrothecium</i> sp., <i>Cladosporium</i> sp., <i>Helminthosporium</i> sp., <i>Phomopsis</i> sp., <i>Fusicoccum</i> sp., <i>Chaetomium</i> sp., <i>Trichoderma</i> sp.	Lu et al. (2015)
10.	<i>Lycium europaeum</i> L.		<i>Paraglomus majewskii</i> , <i>Entrophospora schenckii</i> , <i>Scutellospora nigra</i> , <i>Glomus viscosum</i> , <i>G. versiforme</i> , <i>G. mosseae</i> , <i>G. clarum</i> , <i>G. claroideum</i> , <i>G. intraradices</i> , <i>G. glomerulatum</i> , <i>G. etunicatum</i> , <i>G. ambisporum</i> , <i>Diversispora spurca</i> , <i>Gigaspora pellucida</i> , <i>G. margarita</i>	Touati et al. (2013)
11.	<i>Lycium ruthenicum</i> Murray		<i>Aspergillus tubingensis</i>	Ma et al. (2016)
			<i>Alternaria chlamydospora</i> , <i>Alternaria</i> sp., <i>Preussia terricola</i> , <i>Laburnicola</i> sp., <i>Alternaria tellustris</i> , <i>Apiosordaria backusii</i> , <i>Fusarium petersiae</i> , <i>Microascus alveolaris</i> , <i>Herpotrichia striatispora</i> , <i>Fusarium</i> sp., <i>Emericellopsis maritime</i> , <i>Alternaria chartarum</i> , <i>Exserohilum pedicellatum</i> , <i>Cyphellophora olivacea</i> , <i>Enterocarpus grenotii</i> , <i>Thermomyces verrucosus</i> , <i>Slopeiomyces cylindrosporus</i>	Han et al. (2021)

(continued)

Table 6.1 (continued)

Sl. No	Plant species with endophyte association	IUCN status	Fungal strains name	Endophyte association (references)
			<i>Aspergillus oryzae</i>	Xiu-fang et al. (2015)
			<i>Irpex lacteus</i>	Wang et al. (2013)
			<i>Coprinellus radians</i>	Xiao-xin et al. (2018)
12.	<i>Nicotiana benthamiana</i> Domin		<i>Cladosporium cladosporioides</i>	Dastogeer et al. (2017a)
			<i>Cladosporium cladosporioides</i>	Dastogeer et al. (2018)
			<i>Penicillium oxalicum</i> , <i>Lecythophora</i> sp., <i>Cladosporium</i> sp., <i>Sarocladium</i> sp., <i>Hongkongmyces</i> sp., <i>Aspergillus ochraceus</i> , <i>Cladosporium cladosporioides</i> , <i>Rasamsonia piperina</i> , <i>Penicillium simplicissimum</i> , <i>Ascomycota</i> sp., <i>Botrytis cinerea</i> , <i>Ascomycota</i> sp., <i>Thozetella</i> sp., <i>Fusarium</i> sp.	Dastogeer et al. (2017b)
13.	<i>Nicotiana tabacum</i> L.		<i>Microidium</i> sp., <i>Cladosporium</i> sp., <i>Pseudopithomyces</i> sp., <i>Irpex</i> sp., <i>Lysurus</i> sp.	Yuan et al. (2018)
			<i>Arthrinium arundinis</i>	Zhang et al. (2018)
			<i>Paecilomyces</i> sp.	Xu et al. (2020)
			<i>Fusarium graminearum</i> , <i>Alternaria botrytis</i>	Vianna et al. (2020)
			<i>Streptomyces</i> sp., <i>Nocardia</i> sp.	Subhashini and Singh (2014)
			<i>Piriformospora indica</i>	Hui et al. (2015)
			<i>Acrocalymma vagum</i>	Jin et al. (2018)
			<i>Aspergillus flavus</i> , <i>Aspergillus niger</i> , <i>Aspergillus terreus</i> , <i>Aspergillus</i> sp., <i>Penicillium sublateralitium</i> , <i>Penicillium</i> sp.	Subhashini (2018)
			Sordariomycetes, Dothideomycetes, Eurotiomycetes, Agaricomycetes, Leotiomycetes, Hypocreales, Capnodiales, Eurotiales, Nectriaceae, Davidiellaceae, Trichocomaceae	Zhou et al. (2019)
			<i>Fusarium</i> sp., <i>Pyrenochaeta</i> sp.	Jin et al. (2017)
			Pleosporales sp., <i>Phoma</i> sp., <i>Alternaria</i> sp., <i>Fusarium</i> sp.	Liu et al. (2015)
			<i>Piriformospora indica</i>	Sherameti et al. (2005)

(continued)

Table 6.1 (continued)

Sl. No	Plant species with endophyte association	IUCN status	Fungal strains name	Endophyte association (references)
			<i>Curvularia trifolii</i> , <i>Leptosphaerulina chartarum</i>	Yuan et al. (2020)
			<i>Leptosphaerulina chartarum</i> , <i>Curvularia trifolii</i>	Yuan et al. (2019)
			<i>Piriformospora indica</i>	Su et al. (2021)
14.	<i>Physalis alkekengi</i> L.		<i>Thielavia</i> sp.	Wijeratne et al. (2014)
			<i>Serendipita indica</i>	Heidari (2021)
15.	<i>Physalis angulata</i> L.	Least concern	Hyphomycetes fungus, <i>Fusarium</i> sp., <i>Colletotrichum</i> sp.	Palupi et al. (2021)
			<i>Penicillium verrucosum</i> , <i>Colletotrichum alienum</i> , <i>Fusarium subglutinans</i> , <i>Aspergillus nidulans</i> , <i>Mycelia sterilia</i> , <i>Rhizoctonia</i> sp.	Hastuti et al. (2021)
			<i>Penicillium verrucosum</i> , <i>Colletotrichum alienum</i> , <i>Fusarium subglutinans</i> , <i>Aspergillus nidulans</i> , <i>Mycelia sterilia</i> , <i>Rhizoctonia</i> sp.	Hastuti et al. (2020)
			<i>Trichoderma harzianum</i>	Tang et al. (2019)
			<i>Curvularia</i> sp., <i>Penicillium</i> sp., <i>Aspergillus</i> sp.	Mahardhika et al. (2021)
16.	<i>Physalis peruviana</i> L.	Least concern	<i>Colletotrichum phyllanthi</i>	Pratiwi et al. (2021)
17.	<i>Przewalskia tangutica</i> Maxim.		<i>Lachnum palmae</i>	Zhao et al. (2018)
18.	<i>Solanum betaceum</i> Cav.	Data deficient	<i>Acaulospora spinosa</i> , <i>Funneliformis geosporus</i> , <i>Glomus magnicaule</i> , <i>Glomus multicaule</i> , <i>Rhizophagus aggregatus</i> , <i>Sclerocystis rubiformis</i>	Surendirakumar et al. (2021)
19.	<i>Solanum cernuum</i> Vell.	Least concern	<i>Arthrobotrys foliicola</i> , <i>Bipolaris</i> sp., <i>Candida carpophila</i> , <i>Candida conglobata</i> , <i>Candida diddensiae</i> , <i>Colletotrichum gloeosporioides</i> , <i>Cryptococcus flavescens</i> , <i>Cryptococcus laurentii</i> , <i>Cryptococcus rajasthanensis</i> , <i>Diatrypella frostii</i> , <i>Glomerella acutata</i> , <i>Petriella setifera</i> , <i>Meyerozyma caribbica</i> , <i>Meyerozyma guilliermondii</i> , <i>Basidiomycota</i> sp., <i>Flavodon flavus</i> , <i>Hohenbuehelia grisea</i> , <i>Kwonilella mangroviensi</i> , <i>Oudemansiella canarii</i> , <i>Phanerochaete sordida</i> , <i>Phlebia subserialis</i> , <i>Phlebiopsis gigantea</i> , <i>Schizophyllum umbrinum</i> , <i>Mucor</i>	Vieira et al. (2012)

(continued)

Table 6.1 (continued)

Sl. No	Plant species with endophyte association	IUCN status	Fungal strains name	Endophyte association (references)
			<i>circinelloides</i> , <i>Arthrobotrys foliicola</i> , <i>Bipolaris setariae</i> , <i>Botryosphaeria dothidea</i> , <i>Colletotrichum gloeosporioides</i> , <i>Candida carpophila</i> , <i>Curvularia</i> sp., <i>Dothideomycetes</i> sp., <i>Edenia gomezpompae</i> , <i>Eutypella scoparia</i> , <i>Leptosphaeria</i> sp., <i>Fusarium equiseti</i> , <i>F. oxysporum</i> , <i>Petriella</i> sp., <i>Phoma moricola</i> , <i>Phoma</i> sp., <i>Meyerozyma guilliermondii</i> , <i>Basidiomycete</i> sp., <i>Cercospora viticola</i> , <i>Coprinellus radians</i> , <i>Flavodon flavus</i> , <i>Hapalopilus salmonicolor</i> , <i>Oudemansiella canarii</i> , <i>Phanerochaete sordida</i> , <i>Phlebia subserialis</i>	
20.	<i>Solanum chilense</i> (Dunal) Reiche	Least concern	<i>Funneliformis</i> sp., <i>Spizellomyces</i> sp., <i>Scutellinia</i> sp., <i>Paraphoma</i> sp., <i>Diversispora</i> sp., <i>Tricharina</i> sp., <i>Cadophora</i> sp., <i>Monosporascus</i> sp., <i>Penicillium</i> sp., <i>Oidiodendron</i> sp., <i>Talaromyces</i> sp.	Fuentes et al. (2020)
21.	<i>Solanum elaeagnifolium</i> Cav.		Dark septate fungi and arbuscular mycorrhiza	Osuna Ávila et al. (2021)
22.	<i>Solanum mauritanium</i> Scop.		<i>Aureobasidium pullulans</i> , <i>Hyalodendriella</i> sp., <i>Cladosporium</i> sp., <i>Fusarium</i> sp., <i>Penicillium chrysogenum</i> , <i>Talaromyces</i> sp., <i>Colletotrichum boninense</i> , <i>Paracamarosporium leucadendri</i>	Pelo et al. (2020)
23.	<i>Solanum melongena</i> L.		<i>Geotrichum candidum</i>	Mookherjee et al. (2018)
			<i>Cladosporium</i> spp., <i>Fusarium</i> spp., <i>Heteroconium chaetospira</i> , <i>Mycelium radialis atrovirens</i> , <i>Paecilomyces</i> spp., <i>Penicillium</i> spp., sterile white mycelium, <i>Phialocephala fortinii</i> , <i>Trichoderma</i> spp.	Narisawa et al. (2002)
24.	<i>Solanum nigrum</i> L.		<i>Alternaria</i> sp., <i>Aspergillus awamori</i> , <i>Aspergillus flavipes</i> , <i>Aspergillus flavus</i> , <i>Aspergillus niger</i> , <i>Cladosporium herbarum</i> , <i>Curvularia lunata</i> , <i>Curvularia</i> sp., <i>Monascus</i> sp., <i>Penicillium citrinum</i> , <i>Penicillium chrysogenum</i> , <i>Penicillium digitatum</i> , <i>Torula herbarum</i> , <i>Trichoderma harzianum</i>	Nayak (2015)

(continued)

Table 6.1 (continued)

Sl. No	Plant species with endophyte association	IUCN status	Fungal strains name	Endophyte association (references)
			<i>Aspergillus oryzae</i> , <i>Penicillium solitum</i> , <i>Penicillium brevicompactum</i> , <i>Hortaea werneckii</i> , <i>Petromyces alliaceus</i> , <i>Cladosporium herbarum</i>	Attia et al. (2021)
			<i>Microsphaeropsis</i> sp.	Xiao et al. (2010)
			<i>Aspergillus</i> sp., <i>Lasiodiplodia theobromae</i> , <i>Aspergillus oryzae</i>	El-Hawary et al. (2017)
25.	<i>Solanum quitoense</i> Lam.		<i>Diaporthe citri</i> , <i>Diaporthe melonis</i> , <i>Diaporthe phaseolorum</i> , <i>Diaporthe schini</i> , <i>Phomopsis asparagi</i> , <i>Phomopsis</i> sp., <i>Colletotrichum</i> sp., <i>Neurospora tetrasperma</i>	Dávila et al. (2018)
26.	<i>Solanum rostratum</i> Dunal		<i>Purpureocillium</i> sp.	Kuchkarova et al. (2020)
27.	<i>Solanum sisymbriifolium</i> Lam.		<i>Acaulospora delitata</i> , <i>Acaulospora denticulate</i> , <i>Acaulospora lacunose</i> , <i>Acaulospora morrowiae</i> , <i>Acaulospora rehmi</i> , <i>Acaulospora spinosa</i> , <i>Acaulospora</i> sp., <i>Glomus rubiforme</i> , <i>Glomus lamellosum</i> , <i>Glomus intraradices</i> , <i>Glomus verruculosum</i>	Songachan and Kayang (2012)
28.	<i>Solanum torvum</i> Sw.		<i>Acaulospora delitata</i> , <i>Acaulospora scrobiculata</i> , <i>Acaulospora</i> sp., <i>Acaulospora tuberculata</i> , <i>Glomus badium</i> sp., <i>Glomus coronatum</i> , <i>Glomus fistulosum</i> , <i>Glomus intraradices</i> , <i>Glomus luteum</i> , <i>Glomus verruculosum</i> , <i>Scutellospora heterogama</i>	Songachan and Kayang (2012)
29.	<i>Solanum trilobatum</i> L.		<i>Curvularia lunata</i>	Kannan et al. (2016a)
			<i>Colletotrichum</i> sp., <i>Phoma</i> sp., <i>Aspergillus niger</i> , <i>Fusarium oxysporum</i> , <i>Cladosporium cladosporioides</i> , <i>Drechslera biseptata</i> , <i>Curvularia lunata</i> , <i>Rhizopus stolonifer</i>	Kannan et al. (2016b)
			<i>Colletotrichum lunata</i> , <i>Curvularia lunata</i> , <i>Phoma</i> sp., <i>Fusarium oxysporum</i> , <i>Cladosporium cladosporioides</i> , <i>Rhizopus stolonifer</i> , <i>Aspergillus niger</i> , <i>Drechslera biseptata</i>	Kannan et al. (2016c)
	<i>Withania somnifera</i> (L.) Dunal		<i>Chaetomium bostrycodes</i> , <i>Eurotium rubrum</i> , <i>Melanospora fusispora</i> , <i>Aspergillus awamori</i> , <i>Aspergillus auricomus</i> , <i>Aspergillus flavus</i> , <i>Aspergillus niger</i> , <i>Aspergillus pulvinus</i> , <i>Aspergillus</i>	Khan et al. (2010)

(continued)

Table 6.1 (continued)

Sl. No	Plant species with endophyte association	IUCN status	Fungal strains name	Endophyte association (references)
			<i>terreus</i> , <i>Aspergillus terreus</i> var. <i>aureus</i> , <i>Aspergillus terricola</i> , <i>Aspergillus thomii</i> , <i>Alternaria alternata</i> , <i>Cladosporium cladosporioides</i> , <i>Curvularia oryzae</i> , <i>Drechslera australiensis</i> , <i>Fusarium moniliforme</i> , <i>Fusarium semitectum</i> , <i>Myrothecium roridum</i> , <i>Penicillium corylophilum</i> , <i>Penicillium</i> sp., <i>Phoma</i> sp.	
			<i>Talaromyces pinophilus</i>	Sathiyabama and Parthasarathy (2018)
			<i>Fusarium</i> sp.	Singh et al. (2015a)
			<i>Fusarium</i> sp., <i>Alternaria</i> sp., <i>Penicillium</i> sp., <i>Aspergillus</i> sp., <i>Trichoderma</i> sp.	Nefzi et al. (2019)
			<i>Chaetomium globosum</i>	Kumar et al. (2013)
			<i>Fusarium oxysporum</i>	Vijayan et al. (2016a)
			<i>Alternaria</i> sp., <i>Cladosporium</i> sp., <i>Colletotrichum</i> sp., <i>Fusarium</i> sp., <i>Phomopsis</i> sp., <i>Xylaria</i> sp.	Atri et al. (2020)
			<i>Fusarium solani</i>	Vijayan et al. (2016b)
			<i>Aspergillus terreus</i> , <i>Penicillium oxalicum</i> , <i>Sarocladium kiliense</i>	Kushwaha et al. (2019b)
			<i>Aspergillus</i> sp., <i>A. niger</i> , <i>A. restrictus</i> , <i>Fusarium acutatum</i> , <i>F. armeniacum</i> , <i>F. avenaceum</i> , <i>F. begonia</i> , <i>F. dimerum</i> , <i>F. oxysporum</i> , <i>F. redolens</i> , <i>F. sambucinum</i> , <i>F. semitectum</i> , <i>F. solani</i> , <i>F. udum</i> , <i>F. verticillioides</i> , <i>Mucor</i> sp.	Salini et al. (2014)
			<i>Alternaria</i> sp., <i>Alternaria alternata</i> , <i>Alternaria pluriseptata</i> , <i>Aspergillus</i> sp., <i>Colletotrichum gloeosporioides</i> , <i>Fusarium</i> sp., <i>Fusarium moniliforme</i> , <i>Hyaline vegetativea</i> , <i>Nigrospora</i> sp., <i>Nigrospora oryzae</i> , <i>Talaromyces trachyspermum</i>	Sahu et al. (2019)

(continued)

Table 6.1 (continued)

Sl. No	Plant species with endophyte association	IUCN status	Fungal strains name	Endophyte association (references)
30.			<i>Alternaria alternata</i> , <i>Aspergillus aculeatus</i> , <i>Chaetomium globosum</i> , <i>Cladosporium cladosporioides</i> , <i>Eurotium rubrum</i> , <i>Petromyces flavus</i> , <i>Pleospora allii</i>	Moharram et al. (2016)
			<i>Alternaria alternata</i> , <i>Aspergillus flavus</i> , <i>Aspergillus niger</i> , <i>Cladosporium cladosporioides</i> , <i>Fusarium moniliforme</i> , <i>Penicillium</i> sp.	Tenguria and Khan (2010)
			<i>Aspergillus terreus</i> , <i>Penicillium oxalicum</i> , <i>Sarocladium kiliense</i>	Kushwaha et al. (2019c)
			<i>Aspergillus terreus</i>	Kushwaha et al. (2019a)
			<i>Fusarium semitectum</i>	Singh et al. (2016)
			<i>Fusarium solani</i> , <i>Corynespora cassicola</i> , <i>Penicillium chrysogenum</i>	Aswani et al. (2017)
			<i>Dothideomycetes</i> sp., <i>Alternaria tenuissima</i> , <i>Chaetomium globosum</i>	Susheel and Nutan (2013)
			<i>Acremonium butyri</i> , <i>A. cerealis</i> , <i>A. furcatum</i> , <i>A. kiliense</i> , <i>A. utilum</i> , <i>Alternaria alternata</i> , <i>A. brassicicola</i> , <i>A. citri</i> , <i>A. dianthi</i> , <i>A. raphani</i> , <i>Aspergillus flavo-furcatis</i> , <i>A. niger</i> , <i>A. ochraceus</i> , <i>A. terreus</i> var. <i>aureus</i> , <i>Cadophora meleini</i> , <i>Chaetomium globosum</i> , <i>Cladosporium cladosporioides</i> , <i>C. cucumerinum</i> , <i>C. sphaerospermum</i> , <i>C. spongiosum</i> , <i>C. uredinicola</i> , <i>Cochliobolus bicolor</i> , <i>C. spicifer</i> , <i>Curvularia ovoidea</i> , <i>Emericella nidulans</i> var. <i>lata</i> , <i>Epicoccum purpurascens</i> , <i>Memmoniella levispora</i> , <i>Mucor hiemalis</i> , <i>Myrothecium</i> , <i>Nigrospora sphaerica</i> , <i>Penicillium duclauxii</i> , <i>P. Erythromellis</i> , <i>P. funiculosum</i> , <i>Phoma leveillei</i> , <i>Quambalaria cyanobstans</i> , <i>Scopulariopsis brevicaulis</i> , <i>Setosphaeria rostrata</i> , <i>Stemphylium sarciniforme</i> , <i>S. vesicarium</i> , sterile mycelia, sterile mycelium black, <i>Ulocladium botrytis</i> , <i>U. tuberculatum</i>	El-Said et al. (2020)

Endophytic fungal classification : Divisions

- Ascomycota
- Glomeromycota
- Mucoromycota
- Basidiomycota

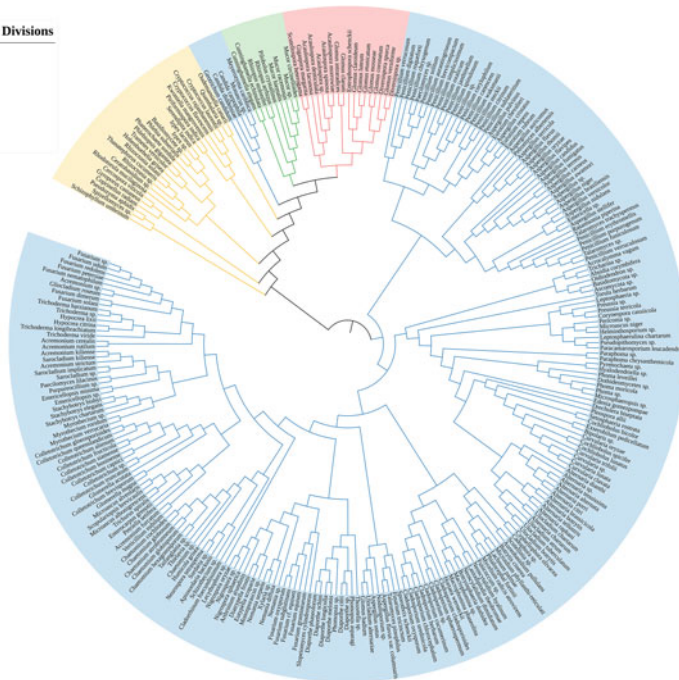


Fig. 6.2 Phylogenetic tree showing the relationship between fungal endophytes from Solanaceae, developed using available ITS1, ITS2, 5.8S rRNA, and 18S rRNA sequence data. On the basis of sequence homologies and following maximum parsimony approach, endophytic fungi were clustered into five clads denoted with different colours to represent the fungal divisions Ascomycota, Basidiomycota, Glomeromycota, and Mucoromycota

et al. 2014; Harris 2017). Auxins are important for plant growth promotion as they are chiefly responsible for root formation in plants. They increase the number of lateral root primordia, length of root hairs, and primary root length (Overvoorde et al. 2010; Harris 2017). IAA is an important plant growth-promoting auxin responsible for the induction of root growth in the plant (Chakraborty et al. 2019). IAA can be synthesized by the plants and also by their associated microorganisms. IAA production by the endophytic fungi *Alternaria* sp., *Alternaria alternata*, *Alternaria pluriseptata*, *Aspergillus* sp., *Colletotrichum gloeosporioides*, *Fusarium* sp., *Fusarium moniliforme*, *Hyaline vegetativea*, *Nigrospora* sp., *Nigrospora oryzae*, and *Talaromyces trachyspermum* in the medicinal plant *W. somnifera* has been observed in a study by Sahu et al. (2019). Pandey et al. (2018) have also reported similar findings in *W. somnifera* by the associated fungi *Penicillium* sp., *Aspergillus terreus*, *Trametes versicolor*, *Penicillium oxalicum*, *Colletotrichum capsici*, *Ceratobasidium* sp., *Penicillium* sp., *Aspergillus brasiliensis*, *Colletotrichum truncatum*, and *Hypocrea lixii* (Table 6.2).

Endophytic fungi can also promote plant growth indirectly by assisting plant nutrient uptake through nitrogen fixation, phosphate solubilization, and siderophore

Table 6.2 Plant growth promotional activities shown by endophytes associated with Solanaceae family

Sl. No.	Plant name	Associated endophytes	Plant growth-promoting activity	References
1.	<i>Brunfelsia uniflora</i> (Pohl) D.Don	<i>Nigrospora oryzae</i> , <i>Nodulisporium</i> sp., <i>Phomopsis archeri</i> , <i>Penicillium</i> sp.	Cellulase activity, lactase activity	Marsola et al. (2021)
2.	<i>Nicotiana benthamiana</i> Domin	<i>Cladosporium cladosporioides</i> , <i>Fusarium equiseti</i> , and <i>Thozetella</i> sp.	Promoted seedling growth by increasing shoot length and biomass and significantly enhanced moisture deprivation tolerance	Dastogeer et al. (2017b)
3.	<i>Nicotiana tabacum</i> L.	<i>Piriformospora indica</i>	Stimulation of nitrate assimilation	Sherameti et al. (2005)
4.	<i>Withania somnifera</i> (L.) Dunal	<i>Alternaria</i> sp., <i>Alternaria alternata</i> , <i>Alternaria pluriseptata</i> , <i>Aspergillus</i> sp., <i>Colletotrichum gloeosporioides</i> , <i>Fusarium</i> sp., <i>Fusarium moniliforme</i> , <i>Hyaline vegetativea</i> , <i>Nigrospora</i> sp., <i>Nigrospora oryzae</i> , <i>Talaromyces trachyspermum</i>	IAA production, siderophore production, phosphate solubilization, protease, lipase, chitinase, pectinase amylase, and cellulase activity	Sahu et al. (2019)
		<i>Fusarium</i> sp.	Chitinase, protease, and amylase activities	Nefzi et al. (2019)
		<i>Penicillium</i> sp., <i>Aspergillus terreus</i> , <i>Trametes versicolor</i> , <i>Penicillium oxalicum</i> , <i>Colletotrichum capsici</i> , <i>Ceratobasidium</i> sp., <i>Penicillium</i> sp., <i>Aspergillus brasiliensis</i> , <i>Colletotrichum truncatum</i> , <i>Hypocrea lixii</i>	IAA production, phosphate solubilization, nitrate reduction activity	Pandey et al. (2018)

production (Ullah et al. 2017; Nefzi et al. 2019). Nitrogen acts as an important limiting nutrient for normal plant growth and development. Plants can uptake nitrogen either as nitrate or ammonium ions or, in some cases, by nitrogen fixation with the help of nodule-forming rhizobacteria. Endophytic fungi can also deliver nitrate or ammonium ions to the root cells of plants. The association between the endophytic fungus *Piriformospora indica* and plant roots results in an enormous requisition of nitrogen from the environment to the plants. In *Nicotiana tabacum*, *Piriformospora indica* has been reported to stimulate nitrate assimilation (Sherameti et al. 2005) (Table 6.2). Plants are unable to uptake insoluble inorganic phosphate present in the soil, although the soluble form is a very important compound for the

proper growth of the plants. Root-associated fungal endophytes can solubilize inorganic phosphate so that the soluble form gets uptaken by the plants (Chakraborty et al. 2019). Pandey et al. (2018) and Sahu et al. (2019) have also reported phosphate solubilization by fungal endophytes in the medicinal plant *W. somnifera* (Table 6.2).

Iron is an essential mineral for the growth of all living organisms because of its role in different physiological processes such as electron transfer, oxygen metabolism, and several enzymatic reactions (Aguado-Santacruz et al. 2012). Microorganisms synthesize a low-molecular-weight protein siderophore for iron uptake under low bioavailability conditions and can assist plants in sequestering iron from the soil. Siderophores are extracellular chelating compounds, which can be divided into four types, rhodotorulic acid, coprogens, fusarinines, and ferrichrome. It primarily acts as a converter of insoluble soil iron into a soluble form under iron starvation, although it can also form complexes with other minerals (Bellenger et al. 2008; Braud et al. 2009; Chakraborty et al. 2019). In their study, Sahu et al. (2019) have shown that endophytic fungi *A. alternata*, *Alternaria pluriseptata*, *Aspergillus* sp., *Colletotrichum gloeosporioides*, *Fusarium* sp., *Fusarium moniliforme*, *Hyaline vegetativea*, *Nigrospora* sp., *Nigrospora oryzae*, and *Talaromyces trachyspermum* can produce siderophores in *W. somnifera*.

Endophytic fungal organisms also benefit plant growth by producing different enzymes, which may suppress pathogen growth inside plants through chitinolytic or proteolytic activity or may help plants utilize nutrients (Long et al. 2010). Nefzi et al. (2019) have reported that the endophytic fungus *Fusarium* sp. can produce the enzymes chitinase, protease, and amylase, when in association with the medicinal plant *W. somnifera*. Similar findings have been reported by Marsola et al. (2021) in the plant *Brunfelsia uniflora*, where the associated endophytic fungi *Nigrospora oryzae*, *Nodulisporium* sp., *Phomopsis archeri*, and *Penicillium* sp. showed cellulase and lactase activity. Sahu et al. (2019) have also observed protease, lipase, chitinase, pectinase, amylase, and cellulase activity by the endophytic fungi associated with the medicinal plant *W. somnifera*.

6.6.2 Biocontrol Activity

Plants are sessile entities and thus suffer from several pathogenic microorganisms' attacks. Although plants have defence systems that can counter those attacks, pathogens are often being able to oppose and overwhelm those protective mechanisms. Diseased plants from pathogenic attacks either lose their productivity or eventually succumb to the disease and fail to survive, depending on their intrinsic tolerance capacities. This proves to be costly, especially for commercial plant species. Solanaceous commercial vegetable species like *Solanum lycopersicum* and *S. melongena* suffer from diseases like 'Fusarium wilt of tomato' and 'Verticillium wilt of eggplant', caused by fungal pathogens *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and *Verticillium dahliae* subsequently. These soil-borne diseases are serious problems in growing regions worldwide and result in devastating losses in crop productivity. FOL causes major crop losses in protected

Table 6.3 Biocontrol activity of endophytes associated with Solanaceae family

Sl. No.	Plant name	Associated endophytes	Biocontrol activity	References
1.	<i>Hyoscyamus muticus</i> L.	<i>Alternaria alternata</i> , <i>Aspergillus fumigatus</i> , <i>Drechslera hawaiiensis</i> , <i>Fusarium solani</i> , <i>Penicillium citrinum</i> , <i>Neoscytalidium dimidiatum</i> , <i>Thyrostromella myriana</i> , <i>Ulocladium chartarum</i>	Antagonistic activity against plant pathogenic fungi <i>Gibberella zeae</i> and <i>Thanatephorus cucumeris</i>	Abdel-Motaal et al. (2010)
2.	<i>Solanum melongena</i> L.	<i>Cladosporium</i> spp., <i>Fusarium</i> spp., <i>Heteroconium chaetospora</i> , <i>Mycelium radicans atrovirens</i> , <i>Paecilomyces</i> spp., <i>Penicillium</i> sp., sterile white mycelium, <i>Phialocephala fortinii</i> , <i>Trichoderma</i> spp.	Antagonistic activity against eggplant <i>Verticillium</i> wilt disease-causing pathogen <i>Verticillium dahliae</i>	Narisawa et al. (2002)
3.	<i>Withania somnifera</i> (L.) Dunal	<i>Fusarium</i> sp.	Biocontrol of <i>Fusarium</i> crown and root rot, caused by <i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i>	Nefzi et al. (2019)

and open-field tomato cultivations (Bletsos et al. 1999; Narisawa et al. 2002; Cai et al. 2003; Lin et al. 2009; Abdallah et al. 2016). Chemicals and particularly fungicides have been used as a general solution to this situation. However, they lack the selectivity to act against pathogens and beneficial microorganisms. They also are not environment-friendly and often are overused or used unscientifically, thus causing undesirable pollution. Unscientific application of fungicides may also lead to the development of fungicide-resistant strains of pathogens, which nullifies the efficiency of the method (Aydi-Ben Abdallah et al. 2017, 2020). Again, disease resistance of new cultivars developed through genetic engineering has been overcome by the generation of new races of the pathogen (Abdallah et al. 2016).

In this scenario, endophytes as biological control agents for the diseases have become promising, environment-friendly, and sustainable alternatives. Endophytic fungal organisms reside within plant tissues, especially vascular tissues, without developing symptoms and causing harm to the host. As pathogens attack plant vascular tissues and move through them, endophytes can inhibit pathogen growth and limit disease severity more efficiently, by either direct antagonistic effect on the pathogens or via triggering host-induced resistance (de Lamo et al. 2018; Constantin et al. 2019; Passari et al. 2019; Aydi-Ben-Abdallah et al. 2020). Endophytic fungi isolated from some of the Solanaceae plants have showcased their promising biocontrol activities (Table 6.3). Endophytic fungal organisms *A. alternata*, *Aspergillus fumigatus*, *Drechslera hawaiiensis*, *Fusarium solani*, *Penicillium citrinum*, *Neoscytalidium dimidiatum*, *Thyrostromella myriana*, and *Ulocladium chartarum* isolated from the important medicinal plant *Hyoscyamus muticus* provided resistance against potent plant pathogenic fungi *Gibberella zeae* and *Thanatephorus*

cucumeris by antagonistic activity (Abdel-Motaal et al. 2010). In another study conducted by Narisawa et al. (2002), endophytic fungi isolated from eggplant *Solanum melongena* such as *Cladosporium* spp., *Fusarium* spp., *Heteroconium chaetospira*, *Paecilomyces* spp., *Penicillium* spp., *Phialocephala fortinii*, *Trichoderma* spp., *Mycelium radices atrovirens*, and sterile white mycelium inhibited the devastating eggplant *Verticillium* wilt disease-causing pathogen *Verticillium dahliae* through antagonistic activity. Nefzi et al. (2019) also showed that the endophyte *Fusarium* sp. isolated from the important medicinal plant *W. somnifera* could be a promising biocontrol agent as it successfully decreased *Fusarium* crown and root rot disease severity and suppressed the growth of the causal organism *Fusarium oxysporum* f. sp. *radices-lycopersici*.

6.6.3 Bioactive Compound Production

A natural bioactive compound means a group of compounds synthesized from plants, microorganisms, animals, and other living organisms with a high effectivity on different fields of study. Endophytic microorganisms can produce different types of bioactive compounds categorized as tannins, steroids, phenols, alkaloids, flavonoids, and polysaccharides, solely or with the association of plants. Fungus is a major resource of this type of bio-compounds with high therapeutic potential. Many compounds are reported as antibiotics, anticancer drugs, hypocholesterolemic agents, and immunosuppressants (Debbab et al. 2010). The compounds synthesized by fungi with the plant's association also help the plant resist biotic and abiotic stress, increasing resistance against harmful plant pathogens and insects (Cui et al. 2018; Liao et al. 2018). About 22,500 bioactive compounds were reported earlier from microorganisms, of which approximately 9000 of them are reported from fungi (Berdy 2005; Brakhage and Schroeckh 2011). Therefore, fungus-mediated compounds can be used in pharmaceutical industries to develop many crucial drugs for therapy. There are several reports of bioactive compounds synthesis by different endophytic fungi in association with the plant species of Solanaceae (Table 6.4; Fig. 6.3). Preussomerin is a group of compounds including preussomerins G, H, I, J, K, and L produced by the endophytic fungus mycelia sterilia with versatile bioactivity in association with medicinal plant species *A. belladonna* L. (Krohn et al. 2001). Another report on the plant *D. innoxia* Mill. in association with endophytic fungi *Diaporthe* sp. reported many bioactive compounds like xylarolide, xylarolide A, xylarolide B, and diportharine A (Sharma et al. 2018). According to Naik (2017), two major compounds, viz. hyoscyamine and scopolamine, are synthesized by a group of endophytic fungi including *Colletotrichum boninense*, *C. incarnatum*, *C. siamense*, *C. gloeosporioides*, *Phomopsis* sp., and *Fusarium solani* in association with the plant *Datura metel* L. In another plant *W. somnifera* (L.) Dunal six fungal endophytes, *A. alternata*, *Aspergillus aculeatus*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Petromyces flavus*, and *Pleospora allii* were found to be responsible for the synthesis of L-asparaginase, which is a potent

Table 6.4 Bioactive compounds produced by endophytes associated with Solanaceae family

Sl. No.	Plant name	Associated endophytes	Bioactive compounds	References
1.	<i>Atropa bel-ladonna</i> L.	Mycelia sterilia	Preussomerins G–L	Krohn et al. (2001)
2.	<i>Datura innoxia</i> Mill.	<i>Diaporthe</i> sp.	Xylarolide A, diportharine A, xylarolide B, and xylarolide	Sharma et al. (2018)
3.	<i>Datura metel</i> L.	<i>Bipolaris</i> sp.	Ophiobolin A	Maehara et al. (2020)
		<i>Colletotrichum boninense</i> , <i>Colletotrichum incarnatum</i> , <i>Colletotrichum siamense</i> , <i>Colletotrichum gloeosporioides</i> , <i>Phomopsis</i> sp., <i>Fusarium solani</i>	Hyoscyamine and scopolamine	Naik (2017)
4.	<i>Datura stramonium</i> L.	<i>Fusarium oxysporum</i>	Fusaric acid derivatives, 5-butyl-6-oxo-1,6-dihydropyridine-2-carboxylic acid, and 5-(but-9-enyl)-6-oxo-1,6-dihydropyridine-2-carboxylic acid	Chen et al. (2013)
		<i>Alternaria</i> sp.	Alternariol, alternariol-5-O-methyl ether, 30-hydroxyalternariol-5-O-methyl ether, altenusin, tenuazonic acid, and altertoxin II	Sun et al. (2012)
		<i>Alternaria</i> sp.	1,8-Dihydroxynaphthalene, anserinone B, phelligradin B, metacytofilin, phomopsidin, and vermoxocin A	Tapfuma et al. (2019)
5.	<i>Hyoscyamus muticus</i> L.	<i>Aspergillus tubingensis</i>	Anofinic acid	Elkhouly et al. (2021)
6.	<i>Lycium barbarum</i> L.	<i>Bipolaris</i> sp.	Bipolahydroquinones, cochlioquinones, and isocochlioquinones	Long et al. (2019)
7.	<i>Lycium ruthenicum</i> Murray	<i>Aspergillus tubingensis</i>	6-Isovaleryl-4-methoxypyran-2-one, Rubrofusarin B, asperpyrone A, and campyrone A	Ma et al. (2016)
		<i>Aspergillus oryzae</i>	Ergosterol, 5-hydroxybenzofuran-2 (3H)-one,	Xiu-Fang et al. (2015)

(continued)

Table 6.4 (continued)

Sl. No.	Plant name	Associated endophytes	Bioactive compounds	References
			2,5-di-hydroxyphenyl acetate, 5-hydroxymethylfuran-3-carboxylic acid, pyranonigrin A, campyrone A, 4-acetamidobutanoic acid, rubrofusarin B, and asperpyrone A	
8.	<i>Nicotiana tabacum</i> L.	<i>Paecilomyces</i> sp.	Paecilacadinol A, paecilacadinol B, ustusol B, ustusol D and ustusol E, 12-hydroxyalbrassitriol, 2-hydroxyalbrassitriol, deoxyuvidin B, 3 β ,9 α ,11-trihydroxy-6-oxodrim-7-ene, and 2 α ,11-dihydroxy-6-oxodrim-7-ene	Xu et al. (2020)
		<i>Arthrinium arundinis</i>	Diorcinols M, N, and O	Zhang et al. (2018)
9.	<i>Physalis alkekengi</i> L.	<i>Thielavia</i> sp.	Nor-spiro-azaphilones, thielavialides A – D, bis-spiro-azaphilone, thielavialide E, and pestafolide A	Wijeratne et al. (2014)
10.	<i>Physalis peruviana</i> L.	<i>Colletotrichum phyllanthi</i>	Ergosterol, ergosterol peroxide, tyrosol, uridine, 1-chloronaphthalene-2-ol	Pratiwi et al. (2021)
11.	<i>Przewalskia tangutica</i> Maxim.	<i>Lachnum palmae</i>	Palmaerones A–G, (R)-5-chloro-6 hydroxymellein, (3R,4R)-5-chloro-4,6 dihydroxymellein, palmaerin A, palmaerin, palmaerin D, trans-4-hydroxymellein, cis-4-hydroxymellein, (R)-5-hydroxymellein, (R)-6-hydroxymellein, mellein, and (R)-6-methoxymellein	Zhao et al. (2018)
12.	<i>Solanum melongena</i> L.	<i>Geotrichum candidum</i>	3-Methyl-1-butanol, isobutyl acetate, ethyl butanoate, ethyl but-2-enoate, ethyl 3-methylbutanoate, 3-methyl butyl acetate, isopentyl acetate, ethyl	Mookherjee et al. (2018)

(continued)

Table 6.4 (continued)

Sl. No.	Plant name	Associated endophytes	Bioactive compounds	References
			pentanoate, isobutyl isobutyrate, ethyl 2-methyl-2-butenate, β -ocimene, isobutyl 3-methylbutanoate, 1,4-cineole, 2-ethyl-1-hexanol, 3-methylbutyl, 2-methylpropanoate, (–)-(S)-limonene, ethyl (E)-2-hexenoate, 1,2-diethylbenzene, 1,4-diethylbenzene, terpinolene, pentyl pentanoate, nonanal, isopentyl 3-methylbutanoate, isoamyl valerate, 2-phenylethanol, isobutyl hexanoate, β -citronellal, benzyl acetate, naphthalene, dodecane, decanal, 3-ethylacetophenone, tridecane, tetradecane, pentadecane, hexadecane	
13.	<i>Solanum nigrum</i> L.	<i>Aspergillus</i> sp., <i>Lasiodiplodia theobromae</i> , <i>Aspergillus oryzae</i>	Ergosterol, ergosterol peroxide, indole-3-carboxylic acid (ICA), indole-3-acetic acid (IAA), (S,S)-sapihofuranone B, penicillic acid, cyclo(leu-pro), cyclo(Phe-pro), and 3-indolaldehyde	El-Hawary et al. (2017)
14.	<i>Solanum rostratum</i> Dunal	<i>Purpureocillium</i> sp.	Cerevisterol, adenosine, thymine	Kuchkarova et al. (2020)
15.	<i>Solanum trilobatum</i> L.	<i>Curvularia lunata</i>	Glycine, N-(phenyl acetyl)-, ethyl ester; 4-(6-methoxypyridazinyl-3-amino)- 1,2-naphthoquinone; hexadecanoic acid, 14-methyl-, methyl ester; 6-octadecenoic acid, methyl ester; [3-methyl-2-(4-nitrophenyl)-4-oxo-1,2,3,4-tetrahydropthalazin-1-yl]-, acetic acid, methyl ester;	Kannan et al. (2016a)

(continued)

Table 6.4 (continued)

Sl. No.	Plant name	Associated endophytes	Bioactive compounds	References
			9,15-octadecadienoic acid, methyl ester, (ZZ)-; pentadecanoic acid, 14-methyl-, methyl ester-; octa-decanedioic acid, 3,16-dioxo-, dimethyl ester	
16.	<i>Withania somnifera</i> (L.) Dunal	<i>Fusarium solani</i>	Taraxasterol, acenaphthylene, 3,4-oxadiazole, benzenamine	Aswani et al. (2017)
		<i>Chaetomium globosum</i>	Antibiotic Scheme 210971	Kumar et al. (2013)
		<i>Aspergillus terreus</i>	Withanolide A	Kushwaha et al. (2019a)
		<i>Aspergillus terreus</i> , <i>Penicillium oxalicum</i> , <i>Sarocladium kiliense</i>	Withanolide A	Kushwaha et al. (2019b, c)
		<i>Alternaria alternata</i> , <i>Aspergillus aculeatus</i> , <i>Chaetomium globosum</i> , <i>Cladosporium cladosporioides</i> , <i>Petromyces flavus</i> , <i>Pleospora allii</i>	L-Asparaginase	Moharram et al. (2016)
		<i>Talaromyces pinophilus</i>	Withanolide	Sathiyabama and Parthasarathy (2018)
		<i>Penicillium</i> sp., <i>Aspergillus terreus</i> , <i>Trametes versicolor</i> , <i>Sarocladium implicatum</i> , <i>Penicillium oxalicum</i> , <i>Colletotrichum capsici</i> , <i>Ceratobasidium</i> sp., <i>Penicillium</i> sp., <i>Aspergillus brasiliensis</i> , <i>Colletotrichum truncatum</i> , <i>Hypocrea lixii</i>	Withaferin A, 12-deoxy-withstramonolide, and withanolide A	Pandey et al. (2018)

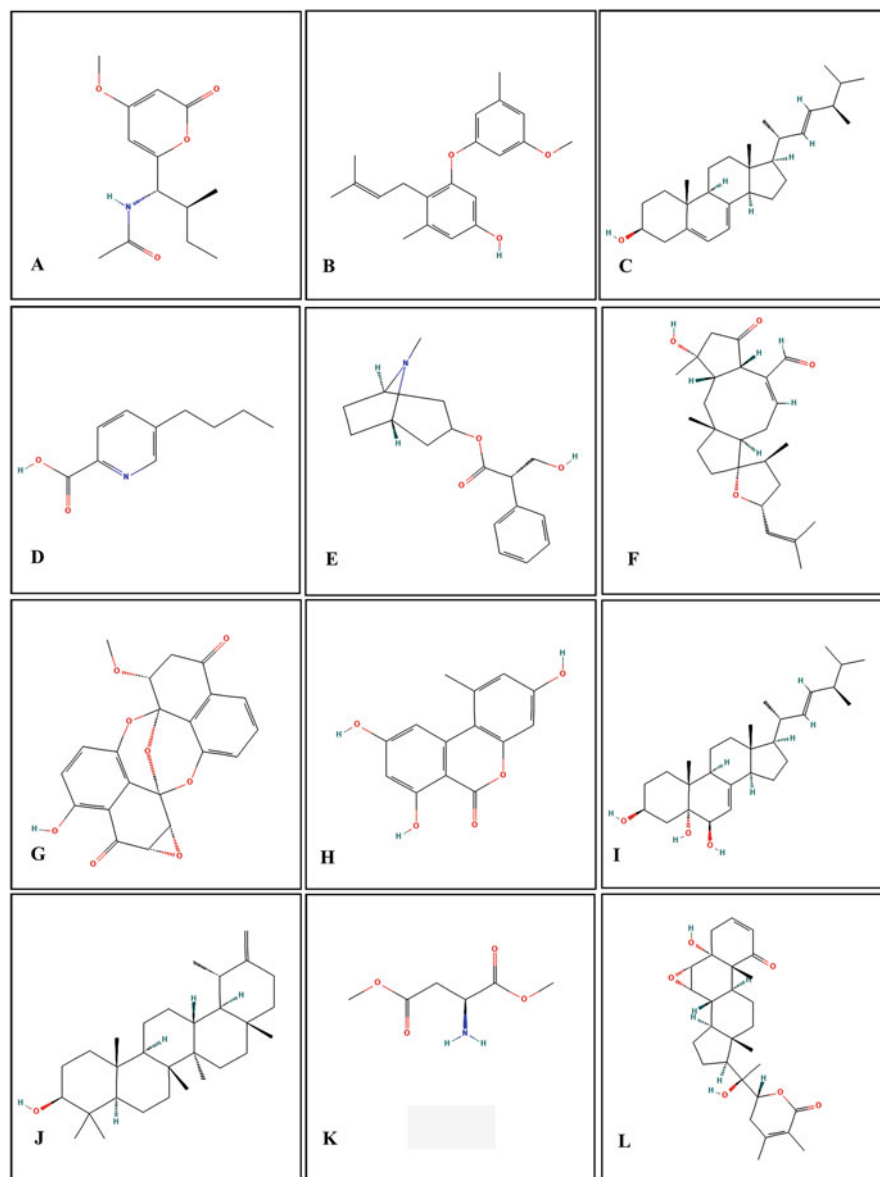


Fig. 6.3 Structure of some important bioactive compounds produced by fungal endophytes associated with solanaceous plant: (a) campyrone A, (b) diorcinol O, (c) ergosterol, (d) fusaric acid, (e) hyoscyamine, (f) ophiobolin A, (g) preussomerin I, (h) alternariol, (i) cerevisterol, (j) taraxasterol, (k) dimethyl L-aspartate hydrochloride, and (l) withanolide A

bioactive compound (Moharram et al. 2016). The endophytic species of *Aspergillus tubingensis* isolated from the medicinal plant *Hyoscyamus muticus* L. is reported to synthesize anofinic acid (Elkhouly et al. 2021). The endophytic fungi *Aspergillus tubingensis* and *Aspergillus oryzae* from the plant *Lycium ruthenicum* were explored for its active compounds like 6-isovaleryl-4-methoxy-pyran-2-one, rubrofusarin B, asperpyrone A, campyrene A, ergosterol, 5-hydroxy-benzofuran-2 (3H)-one, 2,5-dihydroxyphenyl acetate, 5-hydroxymethylfuran-3-carboxylic acid, pyranonigrin A, campyrene A, 4-acetamidobutanoic acid, rubrofusarin B, and asperpyrone A (Ma et al. 2016; Xiu-fang et al. 2015). *Nicotiana tabacum* L. is also a well-known medicinal plant species under the family Solanaceae and is reported for its association with different endophytic fungi like *Paecilomyces* sp. and *Arthrinium arundinis* with the production of a diverse group of bioactive compounds like paecilacadinol A, paecilacadinol B, ustusol B, ustusol D, and ustusol E, 12-hydroxyalbrassitriol, 2-hydroxyalbrassitriol, deoxyuvidin B, 3 β ,9 α ,11-trihydroxy-6-oxodrim-7-ene, 2 α ,11-dihydroxy-6-oxodrim-7-ene, and diorcinols M, N, and O (Zhang et al. 2018; Xu et al. 2020). Two plant species under the genus *Physalis*, namely, *P. alkekengi* and *P. peruviana*, reported for their association with fungal endophytes as well as for the production of different pharmacologically active compounds (Wijeratne et al. 2014; Pratiwi et al. 2021). *Solanum* is the type genus of the family Solanaceae with many medicinal plants, some of them are *S. melongena*, *S. nigrum*, *S. rostratum*, and *S. trilobatum*, which remain to be associated with fungal endophytes and contribute some of the important bioactive compounds (Mookherjee et al. 2018; El-Hawary et al. 2017; Kuchkarova et al. 2020; Kannan et al. 2016a). Palmaerones A–G are the bioactive halogenated dihydroisocoumarins with antimicrobial, anti-inflammatory, and cytotoxic activities and were reported from the symbiotic association of the plant *Przewalskia tangutica* and the fungus *Lachnum palmae* (Zhao et al. 2018). Another report also suggests the presence of nine unknown bioactive compounds from the fungal endophytes *Aspergillus oryzae* and *Lasiodiplodia theobromae* in association with *Solanum nigrum* L. (El-Hawary et al. 2017). According to Pratiwi et al. (2021), five bioactive secondary metabolites were isolated from the endophyte *Colletotrichum phyllanthi* associated with the solanaceous fruit Cape gooseberry (*Physalis peruviana*).

Several other compounds are yet to be discovered by means of exploring various other solanaceous plant-endophytic fungal associations. This scope was remained limited as many highly therapeutic plants were still underutilized. In the future modern analytical techniques, metabolic profiling, and revolutionary genomics can explore more endophytes, as well as many undiscovered bioactive principles in association with the plants of Solanaceae.

6.6.4 Pharmacological Activities

Endophytic fungi are the major resources of bioactive compounds discussed in the earlier section, which can show diverse pharmacological activities like anticancer, antioxidant, antimicrobial, and immunosuppressant (Bhagabaty and Joshi 2011).

Therefore, the fungal endophytes and the extracted substances from them can be exploited in pharma and other related industries. Residing these activities, exploration and utilization of microbial polysaccharides got high attention nowadays due to their application in the medical sector (Serrato et al. 2010). Several investigations reported that endophytic fungi are the primary resource for producing bio-polysaccharides (Yeh et al. 2014).

Endophytic fungi are isolated from different Solanaceae plant species and evaluated for their antibacterial, antifungal, and anticancer activities and for other activities several times (Table 6.5). Twenty-three fungal genera, including *Alternaria*, *Aspergillus*, *Cladosporium*, and *Setosphaeria*, were isolated from *W. somnifera* and extracted for application on bacterial strains to determine the antimicrobial potency. All the genera are found to be effective on the pathogenic bacterial strains (El-Said et al. 2020). Several other studies are also conducted for the antimicrobial activity of diverse endophytic fungal strains isolated from different solanaceous plants (El-Said et al. 2016; Ma et al. 2016; Hastuti et al. 2021). Plant-secreted volatile compounds are also found to be effective on fungal pathogens associated with roots. The fungal strain *Geotrichum candidum*, isolated from *Solanum melongena*, produces different volatile components having antifungal activity against pathogenic fungi *Rhizoctonia solani*, *Fusarium oxysporum*, *Pseudocercospora* sp., and *Cercospora* sp. (Mookherjee et al. 2018). The anticancer activity of fungal endophytes isolated from *Brugmansia aurea* was reported by Singh et al. (2017). According to Kuriakose et al. (2014), *Fusarium solani* associated with *D. metel* L. also showed the cytotoxic activity against human cancer cell lines, including HepG2, HeLa, MCF-7, OVCAR-3, and PC-3. Cytotoxic activity against HepG2, T-47D, HCT-11, HCT-6, and RAW 264.7 cell lines is also reported by endophytic fungi like *Cladosporium herbarum*, *Hortaea werneckii*, *Penicillium solitum*, and *Eurotium chevalieri*, which are associates of the plant species *Solanum nigrum* L. (Attia et al. 2021).

6.6.5 Heavy Metal Stress Amelioration and Bioremediation Ability

With the modernization of agricultural practices, increasing human activities, and industrialization, heavy metal pollution has become an apparent problem for the environment. Mining, the ever-growing use of fertilizers and pesticides, and irrigation with contaminated water from industrial emissions have resulted in contaminated soil. Plants can absorb and accumulate heavy metals from the soil; thus, it is developing significant threats for the crop quality and subsequently to the environment and public health because of the heavy metal toxicity, non-biodegradability, and bioaccumulation (Guo et al. 2010; Su et al. 2021). High concentrations of heavy metals such as cadmium and lead hamper plant growth by interfering with metabolic processes, nutrient homeostasis, and enzymatic activities (He et al. 2020). Heavy metals are also transferring through the food chain via crops, thus developing threats

Table 6.5 Biological activities shown by endophytes associated with the Solanaceae family

Sl. No.	Plant name	Associated endophytes	Biological activity	References
1.	<i>Atropa belladonna</i> L.	<i>Mycelia sterilia</i>	Antimicrobial activity of preussomerins against <i>Bacillus megaterium</i> , <i>Microbotryum violaceum</i> , <i>Mycotypha microspora</i> , <i>Eurotium repens</i> , <i>Fusarium oxysporum</i> , <i>Chlorella fusca</i>	Krohn et al. (2001)
2.	<i>Brugmansia aurea</i> Lagerh.	<i>Acremonium</i> sp., <i>Alternaria porri</i> , <i>Aspergillus aculeatus</i> , <i>Aspergillus brasiliensis</i> , <i>Aspergillus niger</i> , <i>Aspergillus</i> sp., <i>Aspergillus stellifer</i> , <i>Colletotrichum gloeosporioides</i> , <i>Colletotrichum queenslandicum</i> , <i>Colletotrichum</i> sp., <i>Diaporthe longicolla</i> , <i>Fusarium nematophilum</i> , <i>Glomerellales</i> sp., <i>Macrophomina phaseolina</i> , <i>Macrophomina</i> sp., <i>Neoscytalidium hyalinum</i> , <i>Penicillium oxalicum</i> , <i>Periconia</i> sp., <i>Phoma gardenia</i> , <i>Talaromyces</i> sp., <i>Talaromyces trachyspermum</i>	The anticancer activity was shown by endophytes against human cancer cell lines MCF-7, PC-3, A549, MIA-Pa-Ca Antimicrobial activity against <i>Aspergillus flavus</i> , <i>Fusarium solani</i> , <i>Sclerotinia</i> sp., <i>Fusarium oxysporum</i> , <i>Aspergillus fumigatus</i>	Singh et al. (2017)
3.	<i>Brunfelsia uniflora</i> (Pohl) D. Don	<i>Nigrospora oryzae</i> , <i>Nodulisporium</i> sp., <i>Phomopsis archeri</i> , <i>Penicillium</i> sp.	Antioxidant activity	Marsola et al. (2021)
4.	<i>Datura innoxia</i> Mill.	<i>Aspergillus flavus</i> , <i>A. fumigatus</i> , <i>A. niger</i> , <i>A. versicolor</i> , <i>A. ochraceus</i> , <i>A. ustus</i> , <i>Chaetomium atrobrunneum</i> , <i>C. hexagonosporum</i> , <i>Cladosporium spongiosum</i> , <i>C. uredinicola</i> , <i>Cochliobolus spicifer</i> , <i>Emericella nidulans</i> var. <i>lata</i> , <i>Mucor hiemalis</i> , <i>Penicillium aurantiogriseum</i> , <i>P. chrysogenum</i> , <i>P. steckii</i> , <i>P. waksmanii</i> , <i>Phialophora fastigiata</i> , <i>Stemphylium botryosum</i> , <i>Ulocladium alternariae</i>	Antibacterial activity against <i>Enterobacter aerogenes</i> , <i>Enterococcus faecalis</i> , <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Pseudomonas aeruginosa</i> , <i>Salmonella typhi</i> , <i>Salmonella typhimurium</i> , <i>Shigella flexneri</i> , <i>Staphylococcus aureus</i>	El-Said et al. (2016)

(continued)

Table 6.5 (continued)

Sl. No.	Plant name	Associated endophytes	Biological activity	References
		<i>Diaporthe</i> sp.	Cytotoxic activity against MIA-PA-CA-2, MCF-7, PC-3 cell lines, DPPH radical scavenging activity of isolated compounds	Sharma et al. (2018)
5.	<i>Datura metel</i> L.	<i>Fusarium solani</i>	Cytotoxic activity against human cancer cell lines HepG2, HeLa, MCF-7, OVCAR-3, and PC-3	Kuriakose et al. (2014)
6.	<i>Datura stramonium</i> L.	<i>Alternaria</i> sp.	Cytotoxic activity against UMG87 glioblastoma cells	Tapfuma et al. (2019)
7.	<i>Hyoscyamus muticus</i> L.	<i>Alternaria alternata</i> , <i>Aspergillus fumigatus</i> , <i>Drechslera hawaiiensis</i> , <i>Fusarium solani</i> , <i>Penicillium citrinum</i> , <i>Neoscytalidium dimidiatum</i> , <i>Thyrostromella myriana</i> , <i>Ulocladium chartarum</i>	Antifungal activity against <i>Alternaria alternata</i> , <i>Cladosporium cladosporioides</i> , <i>Cladorrhinum foecundissimum</i> , <i>Curvularia clavata</i> , <i>Gibberella zeae</i> , <i>Penicillium janthinellum</i> , <i>Thanatephorus cucumeris</i> , <i>Ulocladium chartarum</i>	Abdel-Motaal et al. (2010)
		<i>Alternaria alternata</i> , <i>Aspergillus flavus</i> , <i>A. niger</i> , <i>A. terreus</i> var. <i>Africanus</i> , <i>A. versicolor</i> , <i>Chaetomium globosporum</i> , <i>C. globosum</i> , <i>Cladosporium chlorocephalum</i> , <i>C. oxysporum</i> , <i>C. sphaerospermum</i> , <i>C. uredinicola</i> , <i>Cochliobolus spicifer</i> , <i>Cordella clarkii</i> , <i>Eurotium chevalieri</i> , <i>Penicillium aurantiogriseum</i> , <i>P. chrysogenum</i> , <i>P. funiculosum</i> , <i>P. rubrum</i>	Antibacterial activity against <i>Enterobacter aerogenes</i> , <i>Enterococcus faecalis</i> , <i>Escherichia coli</i> , <i>Klebsiella pneumonia</i> , <i>Pseudomonas aeruginosa</i> , <i>Salmonella typhi</i> , <i>Salmonella typhimurium</i> , <i>Shigella flexneri</i> , <i>Staphylococcus aureus</i>	El-Said et al. (2016)
		<i>Aspergillus tubingensis</i>	Antimicrobial activity against <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Candida albicans</i> , <i>Bacillus subtilis</i> , biofilm formation inhibitory activity, antioxidant activity Cytotoxic activity of anionic acid against carcinoma cell lines WI-35 HCT-116 Hep-G2 MCF-7	Elkhouly et al. (2021)

(continued)

Table 6.5 (continued)

Sl. No.	Plant name	Associated endophytes	Biological activity	References
8.	<i>Lycium barbarum</i> L.	<i>Bipolaris</i> sp.	Antimicrobial activity against <i>Bacillus subtilis</i> , <i>Clostridium perfringens</i> , <i>Pseudomonas viridiflava</i>	Long et al. (2019)
9.	<i>Lycium ruthenicum</i> Murray	<i>Aspergillus tubingensis</i>	Antibacterial activity of 6-isovaleryl-4-methoxypyran-2-one, rubrofusarin B, asperpyrone A, and campyrone A against <i>Escherichia coli</i> , <i>Staphylococcus aureus</i> , <i>Streptococcus lactis</i> , and <i>Pseudomonas aeruginosa</i>	Ma et al. (2016)
		<i>Aspergillus oryzae</i>	Antimicrobial activity against <i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> , <i>Bacillus subtilis</i> , <i>Fusarium oxysporum</i> , <i>G. saubinerii</i> , <i>C. mandshurica</i> , <i>B. cinerea</i>	Xiu-fang et al. (2015)
10.	<i>Nicotiana tabacum</i> L.	<i>Paecilomyces</i> sp.	Acetyl cholinesterase inhibitory activity of isolated compounds	Xu et al. (2020)
		<i>Arthrinium arundinis</i>	Antifungal activity of isolated compounds against <i>Alternaria alternata</i> and <i>Mucor hiemalis</i>	Zhang et al. (2018)
			Cytotoxic activity against THP-1 cell line	
11.	<i>Physalis angulata</i> L.	<i>Penicillium verrucosum</i> , <i>Colletotrichum alienum</i> , <i>Fusarium subglutinans</i> , <i>Aspergillus nidulans</i> , <i>Mycelia sterilia</i> , <i>Rhizoctonia</i> sp.	Antibacterial activity against <i>Bacillus subtilis</i> , <i>Escherichia coli</i> , and <i>Staphylococcus aureus</i>	Hastuti et al. (2021)
		<i>Curvularia</i> sp., <i>Penicillium</i> sp., <i>Aspergillus</i> sp.	Antibacterial effect against <i>Escherichia coli</i> and <i>Staphylococcus aureus</i>	Mahardhika et al. (2021)
12.	<i>Przewalskia tangutica</i> Maxim.	<i>Lachnum palmae</i>	Antimicrobial activity of isolated compounds against <i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i> , <i>Cryptococcus neoformans</i> , <i>Penicillium</i> sp., <i>Candida albicans</i>	Zhao et al. (2018)
			Inhibitory effects of isolated compounds on lipopolysaccharide (LPS)-stimulated NO production in raw 264.7 cells	

(continued)

Table 6.5 (continued)

Sl. No.	Plant name	Associated endophytes	Biological activity	References
			Cytotoxicity of the isolated compound against HepG2 cell line	
13.	<i>Solanum mauritianum</i> Scop.	<i>Aureobasidium pullulans</i> , <i>Hyalodendriella</i> sp., <i>Cladosporium</i> sp., <i>Fusarium</i> sp., <i>Penicillium chrysogenum</i> , <i>Talaromyces</i> sp., <i>Colletotrichum boninense</i> , <i>Paracamarosporium leucadendri</i>	Antibacterial activity against <i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella oxytoca</i> , <i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Mycobacterium bovis</i> , <i>Mycobacterium smegmatis</i>	Pelo et al. (2020)
14.	<i>Solanum melongena</i> L.	<i>Geotrichum candidum</i>	Antifungal activity of volatile compounds against <i>Rhizoctonia solani</i> , <i>Fusarium oxysporum</i> , <i>Pseudocercospora</i> sp., <i>Cercospora</i> sp.	Mookherjee et al. (2018)
15.	<i>Solanum nigrum</i> L.	<i>Cladosporium herbarum</i> , <i>Hortaea werneckii</i> , <i>Penicillium solitum</i> , <i>Eurotium chevalieri</i>	Cytotoxic activity against HepG2, T-47D, HCT-116, and RAW 264.7 cell lines	Attia et al. (2021)
16.	<i>Withania somnifera</i> (L.) Dunal	<i>Aspergillus</i> sp., <i>Fusarium solani</i> , <i>F. semitectum</i> , <i>F. avenaceum</i> , <i>F. oxysporum</i> , <i>F. redolens</i>	Antibacterial activity against <i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i> Showed immunomodulatory potential by increasing phagocytic activity	Salini et al. (2014)
		<i>Fusarium</i> sp.	Antibacterial activity of silver nanoparticles against <i>Escherichia coli</i> , <i>Salmonella typhi</i> , <i>Staphylococcus aureus</i>	
		<i>Fusarium solani</i> , <i>Corynespora cassiicola</i> , <i>Penicillium chrysogenum</i>	Antibacterial activity against, <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> , <i>Proteus</i> sp., <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> The relative percentage of reducing the power of about 90.62% (<i>Fusarium solani</i>), 57.47% (<i>Corynespora cassiicola</i>), and 52.37% (<i>Penicillium chrysogenum</i>) was observed in the ferric reducing power assay	Aswani et al. (2017)

(continued)

Table 6.5 (continued)

Sl. No.	Plant name	Associated endophytes	Biological activity	References
		<i>Fusarium solani</i>	Cytotoxic activity against HeLa cells	
		<i>Acremonium butyric</i> , <i>A. cereals</i> , <i>A. kiliense</i> , <i>A. rutilum</i> , <i>Alternaria alternata</i> , <i>A. citri</i> , <i>A. raphani</i> , <i>Aspergillus flavo-furcatis</i> , <i>A. niger</i> , <i>A. ochraceus</i> , <i>A. terreus</i> var. <i>aureus</i> , <i>Cadophora meleini</i> , <i>Chaetomium globosum</i> , <i>Cladosporium spongiosum</i> , <i>C. uredinicola</i> , <i>Cochliobolus bicolor</i> , <i>C. spicifer</i> , <i>Curvularia ovoidea</i> , <i>Emericella nidulans</i> var. <i>lata</i> , <i>Memmoniella levispora</i> , <i>Mucor hiemalis</i> , <i>Nectria bactridioides</i> , <i>Nigrospora sphaerica</i> , <i>Penicillium erythromellis</i> , <i>P. funiculosum</i> , <i>Quambalaria cyanbstens</i> , <i>Scopulariopsis brevicaulis</i> , <i>Setosphaeria rostrata</i> , <i>Stemphylium sarciniforme</i> , sterile mycelium black, <i>Ulocladium tuberculatum</i>	Antibacterial activity against <i>Enterobacter aerogenes</i> , <i>Enterococcus faecalis</i> , <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Pseudomonas aeruginosa</i> , <i>Salmonella typhi</i> , <i>Salmonella typhimurium</i> , <i>Shigella flexneri</i> , <i>Staphylococcus aureus</i>	El-Said et al. (2020)
		<i>Fusarium oxysporum</i>	Antibacterial activity of silver nanoparticles against <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Salmonella typhi</i> , <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i>	Vijayan et al. (2016a)
		<i>Chaetomium globosum</i>	Antifungal activity against <i>Sclerotinia sclerotiorum</i>	Kumar et al. (2013)

to human and animal health (Singh et al. 2015b; Khan et al. 2016). Heavy metals such as cadmium, arsenic, and lead can inhibit some human enzymes by binding with thiol groups (Jin et al. 2017). Cadmium is one of the most toxic, non-essential elements with no known biological functions; however, it can act as an agent of DNA damage and mutation even at minute concentrations. Thus, it is a potent carcinogenic for animals (Solis-Dominguez et al. 2007). Being highly mobile and water-soluble, cadmium gets easily absorbed and accumulated in plants, eventually entering the human body (Chirila and Carazeanu 2008; Khan et al. 2015).

Physicochemical methods were developed and used for heavy metal removal, including cadmium, from polluted water, and processes like filtration, evaporation recovery, chemical precipitation, oxidation/reduction, electrochemical treatment, ion exchange, membrane technology, and reverse osmosis, although these strategies are expensive, inefficient, and labour-intensive and lack selectivity (Chen et al. 2008; Tang et al. 2008; Xiao et al. 2010).

Compared to the earlier used physicochemical methods, the phytoremediation process has emerged as an environment-friendly, noninvasive, energy-efficient, and cost-effective technology (Tauqeer et al. 2016; Deng and Cao 2017; He et al. 2020). Heavy metal hyper-accumulating plant species show high tolerance to heavy metals and can absorb and accumulate much higher quantities of heavy metals than other normal plants. Hyperaccumulators such as *Thlaspi caerulescens*, *Arabidopsis halleri*, and *Solanum nigrum* are known for cadmium phytoremediation (Baker et al. 1994; Dahmani-Muller et al. 2000; Wei et al. 2005). However, phytoremediation efficiency is also measured by the plant biomass yields and not only by the metal accumulation ability of the plants (Xiao et al. 2010). A high level of heavy metals in soil and lower nutrient availability result in decreased plant growth and biomass yield, thus reducing the phytoremediation efficiency (Fang et al. 2017; Hussain et al. 2017; Khan et al. 2017; Wang et al. 2019; He et al. 2020). Other constraints, such as low bioavailability of heavy metals in the soil and lower root to shoot translocation rate of metals, also further reduce phytoremediation efficiency. These factors lead to the requirement of an extended period for soil clean-up. Synthetic compounds like EDTA, which improves heavy metal bioavailability and uptake, have been used for the betterment of the phytoextraction process. However, they possess disadvantages such as environmental persistence and leaching risks (Römken et al. 2002; Chen et al. 2010).

In natural ecosystems, mycorrhizal and endophytic fungi remain closely associated with the plants and form symbiotic relationships (Petrini 1986; Jin et al. 2017). Endophytic organisms can improve phytoremediation efficiency in two ways, by either increasing the bioavailability and bioabsorption of heavy metals or by improving plant tolerance to heavy metals or by increasing the plant biomass. In a study, Xiao et al. (2010) showed that the fungus *Microsphaeropsis* sp. isolated from the hyperaccumulator plant *Solanum nigrum* possesses a high biosorption capacity (247.5 mg/g) for cadmium compared to other known adsorbents, including biosorbents and activated carbon (Table 6.6). Plants affected with heavy metal stress develop an array of different mechanisms and repair systems that counter the heavy metal oxidative damage (Khalvati et al. 2010). Arbuscular mycorrhizal fungi and endophytic fungi like *Piriformospora indica* can help plants to further alleviate those stress symptoms and promote plant growth, resulting in a large increase in the biomass (Stein et al. 2008; Oelmüller et al. 2009; Talaat and Shawky 2011; Moradi and Salimi 2013; Unnikumar et al. 2013; Varma 2013). In their studies, Hui et al. (2015) and Su et al. (2021) showed that the endophytic fungus *Piriformospora indica* can alleviate cadmium stress tolerance in the host plant *Nicotiana tabacum* by upregulating expressions of antioxidant enzymes like peroxidase, glutathione synthase, and photosynthesis-related proteins, enhancing pigment and proline

Table 6.6 Heavy metal bioremediation and bioaccumulation abilities of endophytes associated with Solanaceae family

Sl. No.	Plant name	Associated endophytes	Bioremediation, bioaccumulation, and heavy metal stress amelioration abilities	References
1.	<i>Nicotiana tabacum</i> L.	<i>Piriformospora indica</i>	Induction of Cd stress tolerance by sequestering Cd in roots and by enhancing antioxidant enzyme, pigment, and proline contents, as well as by inducing higher expression of stress-related phytochelatin biosynthetic genes	Hui et al. (2015)
		<i>Acrocalymma vagum</i>	Heavy metal stresses amelioration by decreasing leaves' Cd, As, and Pb	Jin et al. (2018)
		<i>Piriformospora indica</i>	Alleviation of Cd stress tolerance by preferential accumulation of Cd in the roots and by upregulating expressions of peroxidase, glutathione synthase, and photosynthesis-related proteins	Su et al. (2021)
2.	<i>Solanum nigrum</i> L.	<i>Microsphaeropsis</i> sp.	High bioabsorption capabilities for Cd	Xiao et al. (2010)

contents, and by inducing higher expression of stress-related phytochelatin biosynthetic genes (Table 6.6). The tobacco plant or *Nicotiana tabacum* is one of the unique and valuable plants of Solanaceae as it possesses both phytoremediation ability and commercial value. In both the studies of Hui et al. (2015) and Su et al. (2021), *Piriformospora indica* also preferentially sequestered cadmium in the roots of *Nicotiana tabacum* and thus helped maintain the food safety value of the plant's edible parts, leaves. Jin et al. (2018) also showed that the fungus *Acrocalymma vagum* can reduce cadmium, arsenic, and lead content in the leaves of *Nicotiana tabacum*. Thus, the endophytic fungi have proved to be a promising agent for the process of phytoremediation and can be utilized to obtain better results.

6.7 Conclusion

Endophytism dealing with the study of a particular plant-endophyte relationship is a rapidly emerging and developing field in research, as endophytes can provide several beneficiary applications in both agricultural and pharmacological industries. They have proved to be better bio-alternatives over physicochemical approaches for their cost-effective and environment-friendly attributes. They can encourage the growth of medicinal plants and elevate bioactive metabolite production, can be applied as potent biofertilizers and biocontrol agents, and can assist in phytoremediation. Solanaceae is a diverse family of different plant species with medicinal importance. The plants, in turn, are large reservoirs of a significant number of fungal endophytes with potential application in the fields of medicine, food, and agriculture. This

review has been intended to showcase the plant-endophyte relationships reported in solanaceous medicinal plants and has shown the diversity of endophytes and their respective prospects for various applications. Several medicinal plants of the Solanaceae family have remained underutilized and unexplored for the presence of endophytes in them. Novel discoveries of fresh endophytic fungal strains, identification of their potential for useful applications, and industrial-level utilization of those beneficial features can bring us new light in the quest for human welfare, keeping hand in hand with developing sustainable approaches.

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Chapter 7

Genetic Studies on Threatened Medicinal Plants of Brazil: Mind the Gap!



Liliana Essi

Abstract This chapter presents a brief review of genetic diversity studies on endangered Brazilian medicinal plant species. Initially, a background on the diversity of Brazilian plants and the number of species threatened with extinction is provided, and subsequently, several cases of Brazilian medicinal plant species threatened with extinction and the studies that have been conducted on their genetic diversity are presented. The six species used as an example come from different Brazilian regions and ecosystems and different plant families as well as have different popular and commercial uses. A summary of genetic diversity findings available in literature is presented for *Aniba rosaeodora* Ducke, *Carapichea ipecacuanha* (Brot.) L. Andersson, *Colletia paradoxa* (Spreng.) Escal., *Hesperozygis ringens* (Benth.) Epling., *Paubrasilia echinata* (Lam.) Gagnon, H. C. Lima & G. P. Lewis and *Pilocarpus microphyllus* Stapf ex Wardlew. This chapter aimed to alert researchers to the knowledge gaps in the genetic diversity of Brazilian medicinal plant species with some degree of extinction threat. As a final remark, medicinal Brazilian species are particularly threatened by habitat destruction, and the number of studies on genetic diversity is scarce for most threatened taxa. For successful biodiversity conservation, environmental education is essential in parallel.

Keywords Endangered species · Molecular markers · Brazil flora · Folk medicine · Red List

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7.1 Introduction

Brazil is a biologically megadiverse country and stands out for its large number of plant species. According to the official list of Brazilian flora (Flora e Funga do Brasil 2022), 38,757 plant species occur in Brazil (native, cultivated and naturalised, excluding algae), 19,747 of which are endemic (Table 7.1).

Among the components of biodiversity, in Brazil, plants are the main raw material for the manufacture of herbal and other medicines. Plants are also used in popular and traditional practices as home and community remedies, a practice known as traditional medicine (Brasil 2006). In addition to its genetic heritage, Brazil has a rich cultural and ethnic diversity that have resulted in a considerable accumulation of traditional knowledge and technologies, passed on from generation to generation, among which the vast collection of knowledge on the management and use of medicinal plants stands out (Brasil 2006).

Some species in medicinal use have been reported as threatened species in books and lists of rare or threatened species published in Brazil; however, few species appear in the most current official lists. Ribeiro-Silva et al. (2001), in a report on aspects of legislation and trade of medicinal plants in Brazil, presented a survey of medicinal species in the official lists of Brazilian states and the country. The authors found 107 species on national lists and 54 on state lists at the time—numbers that seem nowadays to underestimate the risk of extinction of medicinal plants, when we consider more recent Red Lists, which provide more detailed data than those available in the early twenty-first century.

One of the great difficulties in assessing the extinction risk to medicinal plant species is the fact that the popular trade often uses closely related species (e.g. belonging to the same genus) for the same purpose. For example, in the southern region of Brazil, several different species of the genus *Achyrocline* (Less.) DC., known as *marcelas*, are used as tea and collected indiscriminately. It is known that this genus has common species, such as *Achyrocline flaccida* (Weinm.) DC. and *Achyrocline vauthieriana* DC., but also microendemic species, such as *Achyrocline anabelae* Deble—all used interchangeably by the population.

Since 2001, many advances in terms of trade legislation and herbal use have been made, as well as advances in the assessment of plant species’ extinction risk. However, the real number of endangered medicinal plant species in Brazil is still an open question. In addition to environmental depletion, there has been a notorious depletion of popular knowledge about the use of native plants in Brazil (Shanley and Rosa 2005; Cámara-Leret and Bascompte 2021). The extractivism of endangered

Table 7.1 Plant diversity in Brazil (excluding algae groups), according to the data from ‘Flora e Funga do Brasil’ (2022)

Plant group	Total species	Native	Endemic
Angiosperms	35,623	34,038	18,867
Bryophytes (s.l.)	1610	1584	354
Gymnosperms	116	26	3
Ferns and lycophytes	1408	1382	523
	38,757	37,030	19,747

species is undoubtedly a concern in terms of environmental conservation; however, recent studies (Brummitt et al. 2015) show that globally, the main human activity that leads to the risk of extinction is still the destruction of habitats.

A factor that has been causing a retraction in studies on medicinal plants in Brazil is the legislation on access to genetic heritage, protection and access to associated traditional knowledge and benefit sharing (*Lei n° 13.123, 2015*, and *Decreto n° 8.772, 2016*). The Brazilian Law 13.123 requires the registration of all activities related to access to associated traditional knowledge in a national system. Although the purpose of this legislation is to protect national traditional knowledge and guarantee the sharing of possible benefits to the population, the lack of clarity and multiple interpretations of the legislation as well as heavy fines for not registering research (or registering inadequately or incompletely) have generated a situation of insecurity among Brazilian researchers and institutions, discouraging them from research on native plants.

Several national scientific societies have spoken out against the excessive bureaucratisation of research on native species in Brazil, and working groups have tried to propose improvements to the system to make the registration smoother, as well as promoting lectures and informational meetings. The reality, however, is that the delay in simplifying registration activities has consequently delayed studies, which is extremely concerning for species that are or may be at risk of extinction.

Global data presented by Antonelli et al. (2020) showed that 13% (723) of plant species documented to be of medicinal use worldwide are threatened with extinction, of 5411 medicinal plant species assessed under the International Union for Conservation of Nature (IUCN) Red List. This high number of threatened species signifies the global challenge we have for the conservation of medicinal plants, as well as the conservation of biodiversity as a whole.

The most recent official list of threatened plant species in Brazil (Red List of Plant Species, CNCFlora 2022a) evaluated 6046 species and categorised 2953 of these as threatened with extinction (6.38–8.02%). This list contains many species with commercial importance, including species considered medicinal. A noteworthy factor in this list is the high number of species about 600 with data deficiency (DD).

The CNC Flora list does not categorise species in terms of their potential uses, but indicates threats, and in line with the findings of Brummitt et al. (2015) globally, habitat degradation is the main threat in most cases in Brazil.

Currently, the Red List of Plant Species in Brazil (CNCFlora 2022a) is under review and is expected to obtain a more accurate picture of the conservation status of these species. The Brazilian Red List follows the criteria of the IUCN. According to the IUCN Standards and Petitions Committee (2022), the threat categories are Vulnerable (VU), Endangered (EN) and Critically Endangered (CR) (Fig. 7.1).

According to the IUCN Standards and Petitions Committee (2022), several types of factors that can lead to extinction should be evaluated, for instance, environmental fluctuations (in the form of random changes in one or more model parameters), demographic stochasticity, expected future trends in the average values of model parameters (e.g. as a result of deteriorating habitat), genetic stochasticity, random

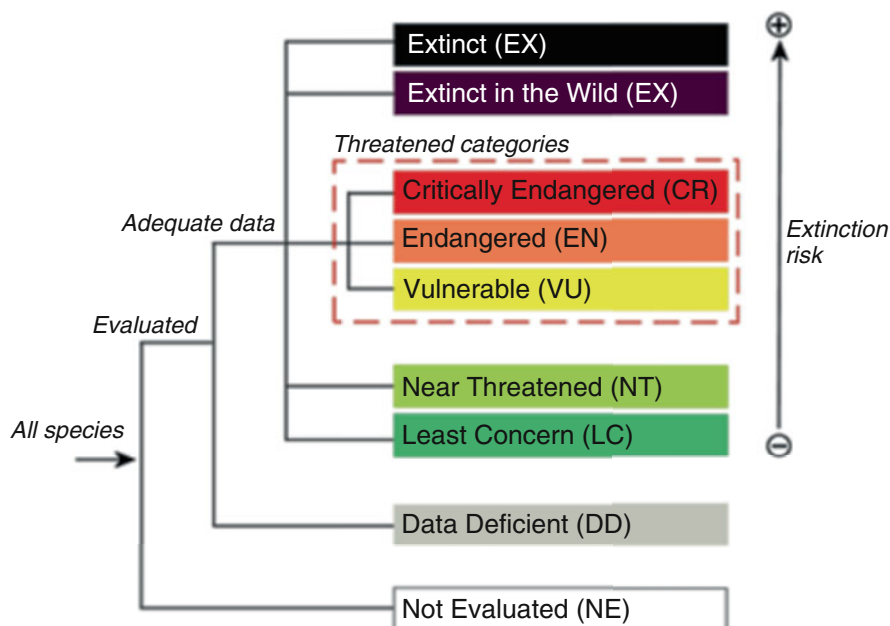


Fig. 7.1 Structure of IUCN categories for threatened species. Vulnerable (VU), Endangered (EN) and Critically Endangered (CR) are the categories for species considered under the threat of extinction. Data Deficient is the category for species with insufficient data to evaluate conservation status. (Source: IUCN Standards and Petitions Committee 2022)

changes in the sex ratio and low-probability, high-impact events (disturbances or catastrophes).

For Brazilian plants in general, there have been many advances in recent decades in the knowledge of the geographic distribution of species and monitoring of habitat degradation, thanks to a series of working groups, such as those for inventorying the flora in general (REFLORA project) and from the Brazilian National Flora Conservation Centre (CNC Flora). However, in recent years, due to government policies unfavourable to environmental protection, there have been several setbacks and a slowdown in the advancement of this knowledge and the protection of biodiversity; given the many existing gaps in the knowledge, this is concerning.

When we look at deeper knowledge about the 'genetic health' of natural populations of threatened species (evaluation of genetic diversity, population structure, gene flow, etc.), the situation is alarming. There is a great lack of studies on the genetic data of plant species under risk of extinction, as we will see below.

7.2 Genetic Studies of Threatened Species in Brazil: Identifying Gaps

Although genetic data are recognised as highly informative in improving conservation strategies of threatened plant species, and the use of molecular markers is a practice for the study of such species worldwide, in Brazil, there is a huge gap in such studies.

A bibliographic revision conducted by our research group (Essi et al. 2020), considering the last Red List of Plant Species in Brazil published in 2012 (CNCFlora 2022a), revealed a disturbing scenario: only 0.08% of the species officially listed as threatened (VU, EN, CR) or data deficient (DD) in Brazil had been featured in one or more studies focusing on their genetic characterisation (phylogenies excluded). The search recovered studies that focused on a total of only 145 species—mostly those with commercial interest as wood or food. The four species with the highest number of genetic studies were *Bertholletia excelsa* Bonpl. (*castanha-do-Pará*, Lecythidaceae), *Cedrela odorata* L. (*cedro-vermelho*, *cedro-rosa*, Meliaceae), *Euterpe edulis* Mart. (*palmiteiro*, Arecaceae) and *Swietenia macrophylla* King (*mogno-brasileiro*, Meliaceae) (Essi et al. 2020). *Araucaria angustifolia* (Bertol.) Kuntze (*pinheiro-do-Paraná*, Araucariaceae) has also received attention concerning its genetic diversity recently. It is noteworthy that researchers' focus is usually on trees or palms with very high economic use; threatened shrubs and herbs are still poorly studied.

Analysing the genetic diversity study data in the context of plant families and IUCN threat categories and species with deficiency of data, the negative highlights for Critically Endangered species are Orchidaceae (52 CR species, only 3 recovered studies) and Asteraceae (49 CR species, no study recovered). For the Data Deficient category, the negative highlights were Eriocaulaceae (42 DD species, no study recovered) and again Asteraceae (40 DD species, only 1 study recovered).

For medicinal species, only a few studies were recovered. *Swietenia macrophylla* King is a positive example, with 18 genetic studies recovered. It is a species used for both wood and medicinal purposes (Moghadamtousi et al. 2013; Telrandhe et al. 2022). Conversely, medicinal species like *Anemopaegma arvense* (Vell.) Stellfeld ex de Souza, *Dorstenia sucrei* Carauta and *Lippia alnifolia* Mart. & Schauer have not yet been studied concerning the genetic diversity of their Brazilian populations (Table 7.2).

Of the medicinal plants for which we recovered genetic studies, we selected six threatened species, two of which were studied in terms of genetic diversity by our research group (*Colletia paradoxa* (Spreng.) Escal. and *Hesperozygis ringens* (Benth.) Epling). Information about their medicinal uses is based on ethnobotanical or scientific essays or published articles or reports.

Table 7.2 Three examples of threatened medicinal species without genetic studies found for Brazil natural populations. Species name, family name, conservation status according to IUCN categories, vernacular names and distribution are based on a public database (Flora e Funga do Brasil, CNC Flora). Medicinal plant data are compiled from the literature cited

Species	Family	Conservation status	Vernacular name (Portuguese)	Distribution in Brazil	Medicinal uses
<i>Anemopaegma arvense</i> (Vell.) Stellfeld ex de Souza	Bignoniaceae	EN	Catuaba	Brazilian Cerrado	Used as an aphrodisiac and a tonic for nervous debility and memory loss (Mendes and Marques 2018) ^a
<i>Dorstenia sucreei</i> Carauta	Moraceae	DD	Carapiá	Atlantic Forest, endemic from Espírito Santo State	Used as eupeptic and digestive tonic; for ailments of the skin; as antispasmodic, emmenagogue, febrifuge, antidiarrheal and diuretic; and for fracture healing (Botsaris and Machado 1999) ^b
<i>Lippia alnifolia</i> Mart. & Schauer	Verbenaceae	VU	Alecrim-do-mato	Catinga and Cerrado, endemic from Bahia State	Used as an antiseptic and to treat diseases that affect the respiratory system, like bronchitis and asthma (Vilela et al. 2020)

^a Mendes FR, Marques LC (2018) *Anemopaegma arvense* (Vell.) Stellfeld ex De Souza. In: Albuquerque U, Pail U, Máthé Á (eds) Medicinal and Aromatic Plants of South America. Medicinal and Aromatic Plants of the World, vol. 5. Springer, Dordrecht. https://doi.org/10.1007/978-94-024-1552-0_8

^b Botsaris AS, Machado PV (1999) Memento terapêutico. Laboratório Flora Medicinal. J. Monteiro da Silva, Rio de Janeiro. 91p. 3. Vilela DAD, Silva BAO, Brito BMC et al. (2020) *Lippia alnifolia* essential oil induces relaxation on Guinea-pig trachea by multiple pathways, J. Ethnopharmacol. 246: 112162. <https://doi.org/10.1016/j.jep.2019.112162>

7.3 Threatened Medicinal Plants from Brazil: Examples of Studies

As mentioned above, the precise number of medicinal plants threatened with extinction in Brazil is hard to define. There are gaps in the knowledge about the species used in this continental country, and new studies are decelerating due to the increase of bureaucracy and reduction of proper financial support for research. The official lists of threatened species give us a clue; however, a relatively small number of taxa have been evaluated, in comparison with the incredible plant diversity of the country. I selected six species with occurrence in different regions of Brazil to illustrate the genetic studies being published. It is just a glimpse of the likely hundreds of medicinal plants from Brazil which are under threat. The category of threat is according to the last Brazilian Red List (CNCFlora 2022a), which follows the IUCN criteria. It is important to emphasise that the Brazilian Red List is undergoing updates in the current year, so some species' threatened status may change.

7.3.1 *Aniba rosaeodora* Ducke (Lauraceae)

Known as rosewood (*pau-rosa* in Portuguese), *Aniba rosaeodora* is considered Endangered (EN). This Amazonian tree is used popularly to control epileptic seizures (Teles et al. 2021) and considered to be antibacterial and antifungal (Pattnaik et al. 1997; Herman et al. 2016; Teles et al. 2021), antioxidant (Seol et al. 2016) and antiprotozoan (Bero et al. 2014; Teles et al. 2021). The species is known globally for its use as a component of fragrances and as an ornamental plant (Teles et al. 2021). For the production of fragrances, the entire individual is extracted, and its overexploitation for commercial purposes is a very important threat to the species. In Brazil, several preliminary studies on its genetic diversity were performed, resulting in monographies and reports, but very few articles have been published. Contim et al. (2005) characterised the nuclear DNA content and karyotype of the species, and Santos et al. (2007) worked on the establishment of random amplified polymorphic DNA (RAPD) molecular marker protocols. Angrizani et al. (2013) reported the isolation and characterisation of 11 highly variable microsatellite loci (simple sequence repeats, SSR) for *A. rosaeodora*, aiming to characterise the population genetic diversity and structure, gene flow and mating system of this threatened species. Population genetic studies for Brazilian rosewood material are still scarce (Santos et al. 2008a, b). Guizado et al. (2020) published an approach for the Peruvian population of the species with inter-simple sequence repeat (ISSR) markers and found that genetic diversity, including within-population diversity, was higher than that reported for rosewood populations in the central Brazilian Amazon, consistent with a pattern of fragmentation resulting from overexploitation.

The process of fragmentation is difficult to identify using the most common molecular markers, but is of utmost importance in conservation genetics. Fragmentation may result in an erosion of genetic variation and increased inter-population genetic divergence due to increased random genetic drift, elevated inbreeding and reduced gene flow. Not all fragmentation events lead to genetic losses and different types of genetic variation, but they are an important reminder that the monitoring of a threatened species must be maintained.

7.3.2 *Carapichea ipecacuanha* (Brot.) L. Andersson (*Rubiaceae*)

Considered Vulnerable (VU) by the IUCN, this shrub is popularly known as *poiaia*, *ipeca* or *ipecacuanha*. According to Teixeira et al. (2012), the species has high pharmacological value due to its levels of emetine and cephalin. In previous works, it has been cited under the synonym *Psychotria ipecacuanha* (Brot.) Stokes. Dependent on shady forest habitat and cross-pollination, it is sought after by medicinal root extractors and has been commercially exploited since the eighteenth century, with no incentives for cultivation (CNCFlora 2022b). There has been a population decline, with the extinction of subpopulations and a continuous decline in the next generations predicted. Its extraction, trade and export have not ceased; however, as its export volume has decreased due to the scarcity of subpopulations, a reduction of more than 30% of the population in three generations is predicted (CNCFlora 2022b).

Oliveira et al. (2010) screened 50 wild clusters of the species with 89 ISSR markers. They found that 291 aerial stems were grouped into 42 putative clones. The clonal groups generally consisted of aerial stems from the same cluster, and there was little or no genetic differentiation among aerial stems at the cluster level. These findings suggest that strategies designed to conserve the species in situ should not rely upon census data, which are based on the number of aerial stems per cluster and the number of clusters per population, because such data greatly underestimate the species' effective population size and genetic diversity. The study recommended federal protection for the species. Furthermore, de Sousa Queiroz et al. (2011) conducted a study on two nuclear DNA fragments of *C. ipecacuanha* in a phylogeographic context and identified a genetic depauperation in the Amazonian and Central American ranges of the species. Although the species is conserved in some conservation units in Espírito Santo and Minas Gerais states, the species is still very susceptible to extinction (CNCFlora 2022b).

7.3.3 *Colletia paradoxa* (Spreng.) Escal. (Rhamnaceae)

Known as *quina*, *C. paradoxa* is a thorny shrub from the grasslands of Southern Brazil that is threatened under the Critically Endangered (CR) category (Fig. 7.2). The roots and bark of the branches are used in folk medicine to reduce fever (Tortosa 1989). Bastos-Záchia et al. (2021a) demonstrated that the species has potential as a natural antioxidant, as well as exhibits moderate to significant antimycobacterial activity.

Our research group evaluated the genetic diversity and population structure of natural populations of *C. paradoxa* from Rio Grande do Sul and Santa Catarina states in Southern Brazil (Bastos-Záchia et al. 2021b) using the molecular technique ISSR. We found four genetic groups with admixture and a number of migrants close to one for generation ($N_m = 1.0465$). The number of polymorphic loci was relatively high, and there were a moderate to high Shannon index and Nei's genetic diversity. In the field, populations are highly fragmented and threatened by habitat degradation. Although the genetic data are somehow encouraging, *in loco* populations are clearly depauperate and rare. It is likely that the species will be maintained as threatened, perhaps with a change to a less severe category. Further

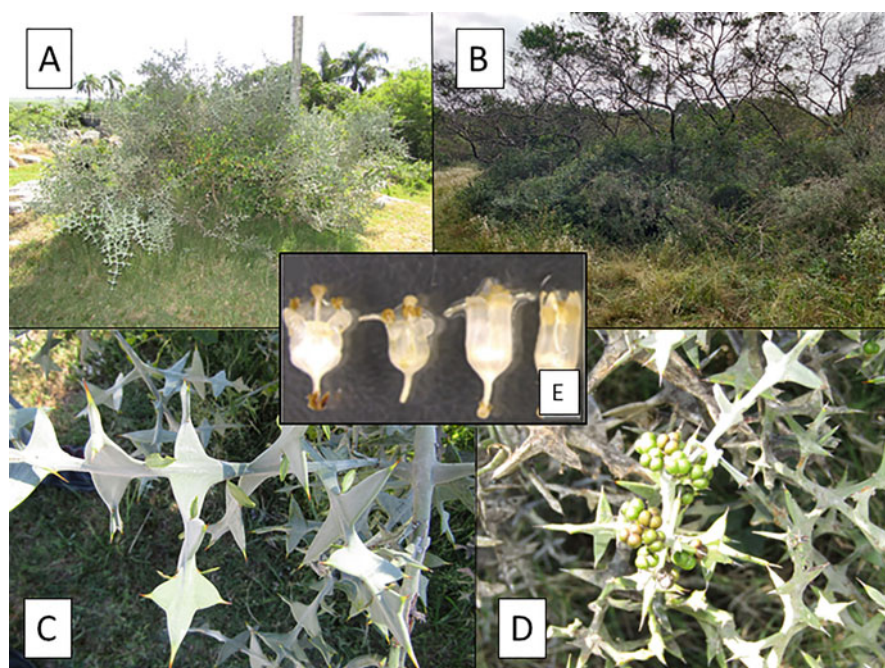


Fig. 7.2 *Colletia paradoxa* (Spreng.) Escal. (a) Habit. (b) Natural environment of the species in Mostardas municipality, RS State. (c) Detail of the branches with leaves. (d) Detail of immature fruits. (e) Detail of flowers showing the small calix and corolla tube. (Photos a, c and d: Liliana Essi. Photos b, e: Nelci Rolim Bastos Záchia)

studies are important to identify whether the diversity found is a result of fragmentation or if it is a favourable sign of population recovery potential.

7.3.4 *Hesperozygis ringens* (Benth.) Epling (Lamiaceae)

Known as *espanta-pulga*, *H. ringens* is a native and endemic species from Rio Grande do Sul state, Southern Brazil, Pampa biome (Fig. 7.3). Considered threatened, this aromatic shrub has several potential uses. Studies have shown antiparasitic (Ribeiro et al. 2010) and allelopathic action on some species of pests, parasites and weeds (Pinheiro et al. 2017; Lima et al. 2020), which favours it as an alternative in the production of antiparasitics and natural herbicides. Its other notable properties include anaesthetic (Silva et al. 2013) and bactericidal activity for the control of fish parasitic microorganisms, aspects of great interest in fish farming (Rosa et al. 2019).

Our research group evaluated the genetic diversity and population structure of natural populations of *H. ringens* (Leite et al. 2023), 16 years after the first molecular study on the species (Fracaro and Echeverrigaray 2006), using ISSR molecular markers. Low genetic diversity indices were found, with low averages for the Shannon index (I) = 0.267 and diversity (h) = 0.170. The percentage of polymorphic loci (P) was also below the usual for this molecular marker, and the population of Santa Maria municipality seems to be the least polymorphic, with P = 55.47% (Leite et al. 2023). Field observations detected a lack of seeds in this population, while all other populations seem to be producing healthy seeds. We suspect that the decline in genetic diversity for this population may be the explanation for this absence or reduction of seed production, but further studies need to be performed.

Fortunately, *H. ringens* seems to be well propagated by cuttings (Siqueira et al. 2020), as well as by seeds. The real threat to the species is habitat degradation: several natural Southern Brazilian grasslands are under disturbance and degradation, mainly because of the expansion of soybean monoculture (*Glycine max* (L.) Merr.) and pine forestation (*Pinus* spp.). *Pinus* L. species are considered very invasive in Brazilian Pampa, and this is a matter of concern to several plant species, especially endemic ones like *H. ringens*.

7.3.5 *Paubrasilia echinata* (Lam.) Gagnon, H.C. Lima & G.P. Lewis (Fabaceae)

Known in Brazil as *pau-brasil* and abroad as brazilwood, *P. echinata* is considered Endangered (EN). This tree, native to the Atlantic Forest, is used as a raw material for high-quality violin bows, is used in folk medicine for dysentery and diarrhoea and has been reported to strengthen the gums (da Silva Gomes et al. 2014) and act as an astringent and tonic (Cota et al. 2011). The species was part of the first economic

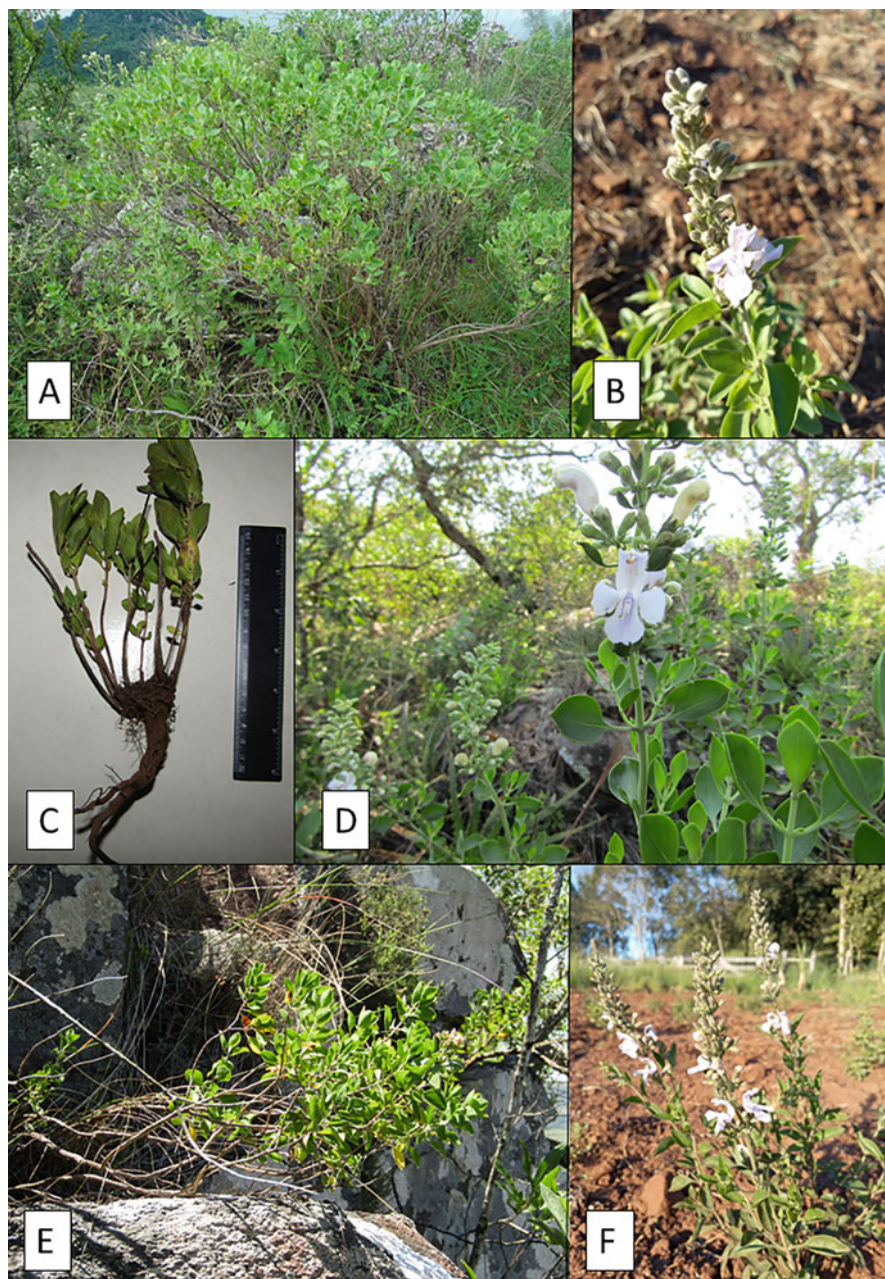


Fig. 7.3 *Hesperozygis ringens* (Benth.) Epling. (a) Habit. (b, f) Flowering branches of cultivated individuals. (c) Detail of underground root system. (d, f) Individuals in natural environments. (Photos: Liliana Essi)

cycle of the Brazilian colonial period and was overexploited due to the use of the timber to produce red pigment. Commonly cited in the literature by its synonym *Caesalpinia echinata* Lam., the species is now recognised within the phylogenetically isolated genus *Paubrasilia* (Lam.) E. Gagnon, H. C. Lima & G. P. Lewis (Gagnon et al. 2016).

Several molecular studies on *P. echinata* have been conducted, with different molecular markers, like RAPD (Cardoso et al. 1998), amplified fragment length polymorphism (AFLP) (Cardoso et al. 2005), SSR (Melo et al. 2007) and more recently plastome analysis (Aecyo et al. 2021). Fragmentation of the population is genetically an important concern, especially due to deforestation. According to CNCFlora (2022c), the wood of the species is still used for export due to its unique properties. An anatomical evaluation of morphotypes found that *P. echinata* presents variation in wood anatomy along a latitudinal range (Macedo et al. 2019), although wood anatomy does not support its categorisation into subspecies or varieties (Macedo et al. 2019).

In spite of the efforts for conservation of *P. echinata*, which is a flagship species with historical, cultural and economic importance, Bastos et al. (2022) identified important weaknesses of the current management models of the species in protected areas (PA). The authors verified socio-ecological dissociation both inside and outside the PAs. This indicates a possible risk of losing the local knowledge network and the native populations of brazilwood in these areas. This tree, which is a symbol for Brazil, is also a model case of the challenges for biodiversity conservation in developing countries. Unfortunately, the species will probably be maintained as threatened in the next revisions of the Brazil Red List, since the studies show that the species is still under extinction risk.

7.3.6 *Pilocarpus microphyllus* Stapf ex Wardlew. (Rutaceae)

Popularly known as *jaborandi*, *P. microphyllus* is considered Endangered (EN). The species is native to the north and northeast regions of Brazil and, like other species of *Pilocarpus* Vahl., is used medicinally. In folk medicine, *P. microphyllus* is used as a treatment for earache, evil eye, postpartum convalescence, colic bowel and sinusitis (Santos et al. 2014). *Jaborandi* harvesting in the wild is one of the most important income sources for many families, with pilocarpine being one of Brazil's most highly exported pharmaceutical substances, according to Abiquif (2020).

Monteiro et al. (2022) conducted a study of genetic diversity with 277 individuals from Carajás National Forest (CNF) using restriction site-associated DNA (RAD) sequencing and estimated the minimum number of individuals necessary to establish a germplasm. They indicated that at least 40 matrices sampled randomly from each population would be required to conserve genetic diversity in the long term. Fortunately, they found that genetic diversity has been maintained despite the continuous harvesting of raw leaf material in the area over recent decades. Their results may be

good guidelines for the creation of germplasm banks and for strategies for conservation and sustainable use of the species.

7.4 Conclusions

Firstly, the low representation of threatened species with some characterisation of genetic diversity is evident and worrying, including for plants of medicinal use. Secondly, it has been observed that few studies have continuity. I selected some cases with important studies being conducted and some cases where much investment is still needed to stop the risk of extinction. Although a small sample of a much larger and more complex universe, it is noteworthy in the literature that the most common cause of extinction is the deterioration of habitats, which puts hundreds of species at risk at the same time. Genetic diversity studies represent strategic tools to speed up the understanding of the evolutionary processes that are affecting species. However, the human factor is also decisive. The *P. echinata* case of socio-ecological dissociation identified by Bastos et al. (2022) illustrates very well the need for intense environmental education work and long-term follow-up. If human activity has been primarily responsible for the decline of many species, well-targeted human action can also prevent the disappearance of many species. Action, government policies that take science and people into account and a broader understanding of the importance of biodiversity conservation are needed. I do not like to think of plants as mere producers of food, drugs or products. However, the clarification of its more direct importance for the population can be a path towards a greater understanding of the importance of biodiversity per se and respect for the planet itself, our home.

Final Recommendations

- Investments in monitoring natural medicinal populations in Brazil, including genetic studies, even if preliminary, inside and outside of protected areas.
- Investment in environmental education, including of the adult population. Environmental education cannot wait for the new generations, especially because the new generations learn much more by example than by speech.
- Investment in the rescue of traditional knowledge and its re-signification, so that it is possible to stop not only genetic erosion but also cultural erosion.

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Chapter 8

Medicinal Plants of North-East India: Biodiversity and Their Ethnomedicinal Values



Judith Mary Lamo , Linu John, and Satyawada Rama Rao

Abstract North-East India being at the confluence of the Indian Malayan, Indo-China and Indian realms is known to be one of the ‘biodiversity hotspots’ of the world. The region is the adobe of diverse plant species with a high level of endemism. The region comprises of more than 200 angiosperm family, and many of the genera are economically and medicinally important. North-East India comprises of eight states which are inhabited by different tribes/communities of diverse ethnicities. These tribes/communities are dependent on local plants for their food, shelter and health care. The diversity of medicinal plants in North-East India is enormous and is reflected in the number of species and their ethnobotanical uses. The people of North-East India rely much on indigenous traditional knowledge system and use various parts of the plants for the treatment of various ailments. Knowledge about the diversity of medicinal plants is crucial for translational research, sustainable utilization and conservation.

Keywords North-East India · Angiosperm · Medicinal plants · Biodiversity

8.1 Introduction

Plants have been widely used in traditional medicine and ethnopharmacology. The traditional knowledge of utilizing plants as food, clothing, shelter and primary health care depends on the ethnic group they belong (Mao and Hynniewta 2000; Díaz et al. 2018; Ramawat and Arora 2021). India is reported to have the highest numbers of medicinal plants with about 7500 species (Shiva 1996; Chen et al. 2016). India is also known for its diversity in various forest products as well as in their traditional

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knowledge of medicine (Kala et al. 2006). These medicinal plants not only are used in health care but are also a source of livelihood for many Indian communities (Kumar et al. 2015).

North-East India which lies between 21°34' N to 29°50' N and 87°32' E to 97° 52' E is part of the Himalaya as well as Indo-Burma Biodiversity Hotspot (Mao et al. 2009). The region is considered to be the Vavilovian centres of origin and diversity of many plant species (Vavilov 1951; Chakravarty et al. 2012). Due to its unique geographical location, climatic conditions and edaphic and altitudinal variations, North-East India houses rich plant diversity such as orchids, bamboos, magnolias and medicinal plants, many of which are rare and endemic to the region. North-East India being one of the treasure houses for a variety of plant species (Meetei et al. 2012) contributes about 50% of India's biodiversity.

North-East India comprises eight states, viz. Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura. The region is inhabited by more than 225 tribes of different ethnicities (Chatterjee et al. 2006), and agriculture is the main source of livelihood. The different tribes of the region also have a rich indigenous traditional knowledge of the use of various plant species which has been passed down from generation to generation. The tribes/communities of North-East India strongly believed in their native folklore medicine for treating various ailments. The dependency of human in plants can be seen as 25% of the drug prescriptions which contain bioactive compounds derived from higher plants (Tiwari and Joshi 1990; Das and Choudhury 2012). The diversity of medicinal plants in North-East India is reflected by the number of species, the part of the plant use and their utilization by different tribes/communities. However, medicinal plants of North-East India are facing a constant threat due to lack of harvesting practices, overexploitation, changes in forest covers and other anthropogenic activities. The community setup in North-East India are known to have an active role in managing the forest ecosystem by initiating indigenous traditions of farming and harvesting and afforestation and conservation programmes and regulating the exploitation of medicinal plants. Though the depth of knowledge about indigenous traditional system and plant used is enormous, it is imperative to assess the diversity of medicinal plants and their ethnopharmacological uses and traditional knowledge. Such information is essential for translational research and provides a basis for designing conservation strategies and sustainable utilization.

8.2 Biodiversity of North-East India

North-East India, covering an area of about 262,179 sq.km, has the highest percentage of forest cover (64.66%) with respect to its geographical area (Table 8.1). It has a total forest cover of 169,521 sq.km and accounts for about 50% of India's biodiversity (Mao and Hynniewta 2000; Anonymous 2021). The state with the highest forest cover is Mizoram, followed by Arunachal Pradesh, Meghalaya and so on. The vegetation types of North-East India are classified into tropical moist deciduous

Table 8.1 Forest cover in the north-eastern states of India (Anonymous 2021)

State	Geographical area (sq.km)	Forest cover (sq.km)	Percentage forest cover (%)	Change in forest cover w.r.t. ISFR 2019
Arunachal Pradesh	83,743	66,431	79.33	−257
Assam	78,438	28,312	36.10	−15
Manipur	22,327	16,598	74.34	−249
Meghalaya	22,429	17,046	76.00	−73
Mizoram	21,081	17,820	84.53	−186
Nagaland	16,579	12,251	74.16	−235
Sikkim	7096	3341	47.08	−1
Tripura	10,486	7722	73.64	−4
Total	262,179	169,521	64.66	−1020

Table 8.2 Forest types in the north-eastern states of India

States	Forest types					
	Tropical moist deciduous forests	Tropical semi-evergreen forests	Tropical wet evergreen forests	Subtropical forests	Temperate forests	Alpine forests
Arunachal Pradesh	√	√	√	√	√	√
Assam	√	√	√	√	—	—
Manipur	√	√	√	√	√	√
Meghalaya	√	√	√	√	√	—
Mizoram	—	√	√	√	√	—
Nagaland	√	√	√	√	√	√
Sikkim	√	√	—	√	√	√
Tripura	√	√	√	—	—	—

forests, tropical semi-evergreen forests, tropical wet evergreen forests, subtropical forests, temperate forests and alpine forests. The type of forest found in the north-eastern states of India is summarized in Table 8.2.

A rich floral diversity with high level of endemism is found in North-East India which is attributed to its unique topographical variation, climatic condition and geographical location and is often related to the forest cover of the region. According to Takhtajan (1988), the region is the centre of origin of some angiosperms. About 8500 angiosperms are found in the region, of which 5000 species have been reported from Arunachal Pradesh, 3832 species from Assam, 2984 species from Manipur, 3272 species from Meghalaya, 2141 species from Mizoram, 2250 species from Nagaland, 5175 species from Sikkim and 1600 species from Tripura (Chatterjee et al. 2006; Sharma et al. 2008; Barooh and Ahmed 2014; Anonymous 2016; Kandel et al. 2019; Lasushe et al. 2022).

North-East India is a reservoir for a variety of fruits, vegetables, spices and ornamental, medicinal and aromatic plants (Asati and Yadav 2004). The region

exhibits a high diversity of species belonging to the families Orchidaceae, Fabaceae, Asteraceae, Poaceae and Rubiaceae. About 900 species of orchids are found in the north-eastern region, out of which 150 species are endemic to the region (De and Singh 2015). Arunachal Pradesh alone is reported to have the maximum number of orchids with about 577 species, followed by Sikkim (561 species), Meghalaya (380 species), Nagaland (387 species), Manipur (314 species), Mizoram (253 species), Assam (231 species) and Tripura (39 species) (Ninawe and Swapna 2017).

The region is popularly known as the treasure house of *Citrus* germplasm (Sharma et al. 2004) with about 23 species, 1 subspecies and 68 varieties being reported (Malik et al. 2012). North-East India is believed to be the centre of origin for *Citrus* with a wide diversity in both wild and cultivated species. The region also shows a wide diversity of both wild and cultivated banana with about 30 taxa, of which 19 are endemic to the region (Sabu et al. 2016).

A wide variety of species like *Curcuma*, *Zingiber* and *Hedychium* (Family: Zingiberaceae) are also found in this region. *Hedychium* is believed to have originated in the north-eastern region. The highest species diversity is found in Meghalaya with about 32 species followed by Arunachal Pradesh with 20 species (Ashokan and Gowda 2019).

There are about 94 species of Cucurbitaceae in India with the highest number of species being reported from the North-East and Peninsular India (Renner and Pandey 2013). Some of the important species are *Cucumis melo* (melon), *C. sativus* (cucumber), *Citrullus lanatus* (watermelon), *Momordica charantia* (bitter gourd), etc.

8.3 Diversity of Medicinal Plants and Their Uses

Plants have been used in traditional medicines, and this knowledge has been passed on from generations to generations. The different tribes of North-East India strongly believed in their native folklore medicine for treating various ailments. The practice of ethnomedicine by the indigenous people of North-East India is closely related to the local ecosystems as well as cultural landscapes (Gesler 1992; Anyinam 1995; Sangma and Sahoo 2017). The different tribes of North-East India use a variety of plant species as preventive medicines and for the treatment of various diseases. Local practitioners used different parts of the plant like leaves, roots, stem, bulbs, rhizomes, flowers, fruits and seeds in traditional medicine.

North-East India has a rich diversity of medicinal plants which are used in indigenous health practices as well as modern system of medicines (Gowthami et al. 2021). About 850 medicinal plants have been reported from the state of Meghalaya, 526 plants from Nagaland, 420 plants from Sikkim, 286 plants from Assam, 200 plants from Arunachal Pradesh, 194 plants from Tripura and 93 plant species from Mizoram (Mao et al. 2009; De 2019; Laldingliani et al. 2022). The diversity of medicinal plants in North-East India is reflected by the number of species, the part of the plant used and their utilization by different tribes/communities of North-East India (Table 8.3). The medicinal plants commonly used by the

Table 8.3 Diversity of medicinal plants of North-East India

Family	Species name	Tribes/communities	Part used	Uses	References
Acanthaceae	<i>Adhatoda vasica</i> Mill.	Assam, Sikkim	Flower, leaf and root	Fresh flowers' and leaves' decoction is used for nose bleeding; dysentery and blood vomiting; leaf paste is used in dysentery, cough, fever and gout	Jamir et al. (1999), Bhardwaj and Gakhar (2005), Sajem and Gosai (2006), Kongsai et al. (2011), Das and Choudhury (2012), Hussain and Hore (2015), Kichu et al. (2015), Debbarma et al. (2017), Sharma et al. (2001)
	<i>Adhatoda zeylanica</i> Medic.	Mizoram	Leaf	Leaf is applied to wound and cuts, and boiled water is administered to malaria patient	
	<i>Andrographis paniculata</i> Nees.	Arunachal Pradesh, Mizoram, Tripura	Whole plant	Used in malaria, jaundice, fever, stomach ailment and dog bite	
	<i>Barleria cristata</i> Alba	Assam	Aerial parts	Entire plant decoction is used against skin infections	
	<i>Phlogacanthus curviflorus</i> (Wall.) Nees.	Arunachal Pradesh	Leaf and root	Boiled leaf juice is used for cough and fever	
Aceraceae	<i>Phlogacanthus thyrsoiflorus</i> Nees.	Arunachal Pradesh, Nagaland, Tripura	Leaf and fruit	Used as expectorant and to treat asthma, stomach problems and fever	Sharma et al. (2001), Majumdar et al. (2006), Sajem and Gosai (2006)
	<i>Arum dioscoridis</i> Sibth. & Sm.	Assam, Tripura	Stem, root	Stem extract is applied directly to cure boils; root paste mixed with water is used for curing kidney and urinary disorders	
Amaranthaceae	<i>Acer laevigatum</i> Wall.	Mizoram	Leaf	Leaf decoction is used as an external application in sprains	Sharma et al. (2001), Sajem and Gosai (2006), Kongsai et al. (2011), Kichu et al. (2015)
	<i>Achyranthes aspera</i> L.	Assam	Leaf	Crushed leaves are used for the treatment of boils	
	<i>Achyranthes bidentata</i> Bl.	Sikkim	Leaf	Leaf decoction used as diuretic; leaf juice used externally in the treatment of leech bites	

(continued)

Table 8.3 (continued)

Family	Species name	Tribes/communities	Part used	Uses	References
Anacardiaceae	<i>Amaranthus gangeticus</i> L.	Nagaland	Leaf	Boiled leaves used in indigestion and as a laxative	Jamir et al. (1999), Kongsai et al. (2011), Debbarna et al. (2017)
	<i>Amaranthus spinosus</i> L.	Arunachal Pradesh	Roots	Root paste used against contagious skin infection	
	<i>Amaranthus viridis</i> L.	Mizoram	Leaf	Leaf juice is used in scorpion sting and in insect bites	
	<i>Mangifera indica</i> L.	Tripura	Bark and root	Used for toothache	
	<i>Spondias mangifera</i> Willd.	Nagaland	Leaves and bark	Fresh leaves are crushed and used topically for the treatment of foot and toe infection; grounded bark is applied as cooling agent in burns	
Apiaceae (Umbelliferae)	<i>Rhus javanica</i> L.	Manipur and Nagaland	Fruit	Fruit juice is used for diarrhoea and dysentery	Deb et al. (2009), Srivastava and Adi Community (2009), Sajem and Gosai (2006), Singh et al. (2011), Badola and Pradhan (2013), Nanda et al. (2013), Hussain and Hore (2015), Perme et al. (2015), Prakash (2021)
	<i>Centella asiatica</i> (L.) Urban	Arunachal Pradesh, Assam Manipur, Meghalaya, Sikkim, Tripura	Whole plant	Leaf decoction is used in conjunctivitis and eye irritation and as carminative and appetizer; crushed leaves are used for boils, tumours, gastritis and indigestion; used in leprosy, fever, asthma and bronchitis and for improving memory	
	<i>Eryngium foetidum</i> L.	Arunachal Pradesh, Manipur	Whole plant	Paste applied for intermittent headache	
	<i>Hydrocotyle asiatica</i> L.	Sikkim	Whole plant	Plant extract used in pneumonia, skin infection and toothache; plant juice used in sore throat, cough and fever; leaf paste used in dysentery	

Apocynaceae	<i>Alstonia scholaris</i> (L.) R.Br.	Assam, Tripura	Bark, root, shoot, latex	Fresh barks are used in asthma rheumatism, malaria and skin disease; root juice is used with milk in leprosy; shoot is used for treating piles; latex is used as vermifuge	Pandey et al. (1991), Sajem and Gosai (2006), Kichu et al. (2015), Singh et al. (2015), Debbarma et al. (2017)
	<i>Catharanthus roseus</i> (L.) G. Don	Assam, Nagaland	Leaf	Leaves are used for the treatment of gastritis, jaundice, diabetes, high blood pressure and nasal bleeding	
	<i>Holarrhena antidysenterica</i> (L.) Wall. ex G.Don.	Tripura	Leaf	Used in the treatment of dysentery or diarrhoea and as anthelmintic	
Araceae	<i>Acorus calamus</i> L.	Sikkim	Rhizome	Used in the treatment of epilepsy, fever, abdominal pain, asthma, cough and sore throat	Sharma et al. (2001), Deb et al. (2009), Singh et al. (2011), Nanda et al. (2013), Hussain and Hore (2015), Debbarma et al. (2017)
	<i>Amorphophallus campanulatus</i> (Roxb.) Bl.	Mizoram	Rhizome	Used in heart diseases	
	<i>Colocasia esculenta</i> (L.) Schott	Manipur, Nagaland	Rhizome, petiole and stem	Rhizome is used for diabetes; sap is used in bee stings and as a stimulant	
	<i>Homalomena aromatica</i> (Spreng.) Schott	Tripura	Stem	Cooked stem consumed as vegetable for cough	
	<i>Brassaiopsis mitis</i> C.B. Clarke	Sikkim	Root	Used in dysentery	
Araliaceae	<i>Panax assamicus</i> Banerjee	Arunachal Pradesh, Manipur, Meghalaya, Nagaland	Rhizome	Used as a tonic and vitalizer	Mao et al. (2009), Badola and Pradhan (2013), Hussain and Hore (2015)

(continued)

Table 8.3 (continued)

Family	Species name	Tribes/communities	Part used	Uses	References
Asclepiadaceae	<i>Panax pseudoginseng</i> Wall.	Sikkim	Rhizome	Used as stimulant and aphrodisiac and in headache, vomiting and weakness	
	<i>Asplenium phyllitidis</i> D. Don.	Arunachal Pradesh	Leaves, aerial parts	Known to have antioxidant and antimicrobial properties	Perme et al. (2015)
	<i>Calotropis gigantea</i> (L.) R.Br. ex Schult.	Tripura	Leaf, latex	Leaves used for rheumatism; latex used in snake bite	Debbarna et al. (2017)
Asteraceae	<i>Ageratum conyzoides</i> L.	Assam	Leaf	Crushed leaves are used directly on cuts and wounds	Sharma et al. (2001), Deb et al. (2009), Srivastava and Adi Community (2009), Das and Choudhury (2012), Badola and Pradhan (2013), Nanda et al. (2013), Hussain and Hore (2015), Perme et al. (2015), Myrchiang et al. (2018), Prakash (2021)
	<i>Artemisia indica</i> Willd.		Leaves, young seedlings	Used for treating skin allergy and breast cancer	
	<i>Artemisia nilagirica</i> Clarke	Arunachal Pradesh, Mizoram	Leaf	Used in malaria, stomachache and asthma and as anthelmintic	
	<i>Artemisia vulgaris</i> L.	Sikkim	Leaf	Used in treating nose bleed and asthma	
	<i>Bidens biternata</i> (Loureiro) Merrill & Sherff.	Manipur	Leaf	Used as diuretic and in liver infection	
	<i>Eupatorium adenophorum</i> Spreng.	Sikkim	Shoot, fruit	Shoot used as an antiseptic; seed paste used in mumps	
	<i>Eupatorium odoratum</i> L.	Meghalaya	Leaves	Used for wounds and stomach problem	
	<i>Mikania micrantha</i> Kunth	Manipur	Whole plant	Diuretic, anticancer, antidiarrhoeic, antidyenteric and antimalaria	
	<i>Spilanthes paniculata</i> Wall. ex DC	Meghalaya, Tripura	Leaf	Used for the treatment of stomach troubles, liver problems and dyspepsia	

Begoniaceae	<i>Begonia crenata</i> L.	Assam, Meghalaya	Whole plant	Entire plant decoction is used against skin infections and urination problems/disorders	Sharma et al. (2001), Sajem and Gosai (2006), Prakash (2021)
	<i>Begonia inflata</i> Clarke	Mizoram	Whole plant	Used in dysentery and malaria	
	<i>Begonia roxburghii</i> (Miq.) A.DC.	Assam	Rhizome/bulb	Applied on the body parts where the thorns are stuck to prevent further infection	
Berberidaceae	<i>Berberis asiatica</i> L.	Sikkim	Leaf, fruit and bark	Fruit and leaf juice used in diarrhoea and dysentery; bark and root decoction used in jaundice and fever	Badola and Pradhan (2013)
Betulaceae	<i>Alnus nepalensis</i> D. Don	Arunachal Pradesh	Bark, leaves	Used as diuretic, to reduce swelling and to prevent excessive sweating	Tag and Das (2004), Badola and Pradhan (2013)
	<i>Betula utilis</i> D. Don	Sikkim	Bark	Used as an antiseptic	
Bignoniaceae	<i>Oroxylum indicum</i> (L.) Kurz.	Arunachal Pradesh, Manipur, Nagaland, Sikkim, Tripura	Root, bark, seeds	Root used in the treatment of jaundice, diarrhoea, fever and ulcer; seeds used as anthelmintic; bark decoction used as antipyretic and bowel tonic	Wangjen et al. (2011), Das and Choudhury (2012), Badola and Pradhan (2013), Nanda et al. (2013), Kichu et al. (2015)
Bombacaceae	<i>Bombax ceiba</i> L.	Manipur, Sikkim	Bark, gum	Decoction is used to cure dysentery; gum is used for treating diarrhoea and dysentery	Badola and Pradhan (2013), Nanda et al. (2013)
Cannabaceae	<i>Cannabis sativa</i> L.	Arunachal Pradesh, Manipur, Nagaland, Sikkim	Leaf and seeds	Cooked leaves are eaten to cure dysentery; leaf decoction is used in small proportion during diarrhoea; powdered seeds are used in relieving body pain	Kongsai et al. (2011), Badola and Pradhan (2013), Nanda et al. (2013), Kichu et al. (2015)
Capparaceae	<i>Crataeva nurvala</i> Buch. Ham.	Tripura	Bark	Bark paste used for high fever and headache	Debbarma et al. (2017)

(continued)

Table 8.3 (continued)

Family	Species name	Tribes/communities	Part used	Uses	References
Caricaceae	<i>Carica papaya</i> L.	Arunachal Pradesh, Manipur, Sikkim, Tripura	Whole plant	lowers are boiled and taken to improve hearing; latex is used to remove skin freckles and blemishes; fruit decoction of green fruit with salt acts as diuretic; fruits are eaten in jaundice; seeds are used as anthelmintic; ripe fruit seeds are used as antidiysenteric; flower pedicel is used as antiemetic and eaten against liver enlargement	Srivastava and Adi Community (2009), Das and Choudhury (2012), Perme et al. (2015), Badola and Pradhan (2013), Nanda et al. (2013)
Caryophyllaceae	<i>Drymaria cordata</i> (L.) Willd. ex Roem. & Schult.	Sikkim	Leaf	Used for relieving headache, sinusitis and cold	Srivastava and Adi Community (2009), Badola and Pradhan (2013), Perme et al. (2015)
	<i>Drymaria diandra</i>	Arunachal Pradesh	Whole plant	Used for skin diseases; leaf juice for relief from sinusitis	
Chenopodiaceae	<i>Chenopodium album</i> L.	Sikkim		Reduces body pain	Badola and Pradhan (2013)
Combretaceae	<i>Terminalia myriocarpa</i> Muell.	Arunachal Pradesh	Fruit, leaves, bark	Bark extract is given in chest pain and as cardiac stimulant	Deb et al. (2009)
Crassulaceae	<i>Bryophyllum calycinum</i> Salisb.	Assam	Leaf	Crushed leaves are applied on burns and bruises	Sajem and Gosai (2006)
	<i>Kalanchoe pinnata</i> (Lam.) Pers.	Tripura	Leaf	Leaf paste used on wounds and burns; leaf juice taken during vomiting; leaf extract used in bloody dysentery	Debbarma et al. (2017)

Cucurbitaceae	<i>Benincasa hispida</i> (Thunb.) Cogn.	Manipur, Mizoram	Fruit, seeds	Fruit extract is used in dysentery, diarrhoea, cholera and diabetes; and along with tamarind and sugar, it is used for kidney stone; seeds are used as anthelmintic	Jamir et al. (1999), Sharma et al. (2001), Das and Choudhury (2012), Badola and Pradhan (2013), Nanda et al. (2013), Kichu et al. (2015), Debbarna et al. (2017)
	<i>Cucumis sativus</i> L.	Nagaland	Fruit and leaves	Fruit used in flatulence and hyperacidity; crushed fresh leaf used as mosquito and other insect repellent	
	<i>Cucurbita pepo</i> L.	Manipur, Sikkim	Seeds	Used as vermifuge	
	<i>Coccinia grandis</i> (L.) Voigt	Tripura	Leaf	Consumed against diabetes	
	<i>Luffa acutangula</i> L.	Nagaland	Flowers, fruits and leaves	Used as laxative and to aid in digestion	
Dilleniaceae	<i>Momordica charantia</i> L.	Sikkim, Tripura	Fruit, twigs	Fruit juice used as blood purifier and in controlling diabetes and as anthelmintic; twig extract used against dyspepsia	Deb et al. (2009), Majumdar et al. (2006)
	<i>Dillenia indica</i> L.	Arunachal Pradesh, Tripura	Fruit pulp, bark and leaves	Fruit decoction used for curing dandruff, bone fracture and diarrhoea; bark paste along with water used for blood cancer	
Dioscoreaceae	<i>Dioscorea floribunda</i> M.	Arunachal Pradesh, Nagaland	Root	Used in the treatment of intestine diverticulosis and gall bladder pain, for increasing energy and in the treatment of rheumatoid arthritis and back pain	Jamir et al. (1999), Srivastava and Adi Community (2009), Kongsai et al. (2011), Perme et al. (2015), Myrchiang et al. (2018)
	<i>Dioscorea bulbifera</i> L.	Meghalaya	Tuber	Used in dysentery and other stomach problem	

(continued)

Table 8.3 (continued)

Family	Species name	Tribes/communities	Part used	Uses	References
	<i>Tacca integrifolia</i> Ker-Gawl	Arunachal Pradesh	Rhizome, tuber	Used in the treatment of skin disease, leprosy, wound healing, stomach pain and dysentery	
Fabaceae	<i>Acacia concinna</i> (Willd.) DC	Tripura	Fruit, root, bark	Fruit used for treating diabetes; root decoction used for toothache and body pain	Sajem and Gosai (2006), Deb et al. (2009), Srivastava and Adi Community (2009), Nanda et al. (2013), Perme et al. (2015), Debbarma et al. (2017)
	<i>Bauhinia variegata</i> L.	Arunachal Pradesh	Flowers, leaves, root and buds	Used for the treatment of asthma, ulcer and digestive problem	
	<i>Cassia alata</i> L.	Arunachal Pradesh	Leaf	Leaf juice is applied in eczema and itching	
	<i>Lablab purpureus</i> L.	Manipur	Fruits	Used in common cold	
	<i>Mimosa pudica</i> L.	Assam, Manipur	Roots	Treatment of piles and removal of stones	
	<i>Parkia roxburghii</i> G. Don	Manipur	Leaf, bark, fruit	Antidiarrhoeic and antidyenteric	
Flacourtiaceae	<i>Gynocardia odorata</i> R. Br.	Sikkim	Seed	Seed extract used as massaging oil for infants	Badola and Pradhan (2013)
Fumariaceae	<i>Dicentra scandens</i> (D. Don) Walp.	Sikkim	Root	Powdered roots are administered during gastritis	Badola and Pradhan (2013)
Gentianaceae	<i>Swertia chirayita</i> (Roxb. ex Fleming) H. Karst.	Tripura	Whole plant	Decoction used for the treatment of fever and hepatitis B	Deb et al. (2009)
Geraniaceae	<i>Oxalis corniculata</i> L.	Sikkim	Aerial part	Used during cough, diarrhoea and dysentery and as appetizer	Badola and Pradhan (2013)
Juglandaceae	<i>Juglans regia</i> L.	Sikkim	Fruit	Powdered fruit used in reducing headache and blood pressure	Badola and Pradhan (2013)

Lamiaceae/ Labiatae	<i>Clerodendrum colebrookianum</i> Walp.	Manipur	Leaf	Leaves' infusion is anti-dandruff; boiled leaves are taken for diabe- tes and high blood pressure	Tag and Das (2004), Laloo et al. (2006), Sajem and Gosai (2006), Deb et al. (2009), Kongsai et al. (2011), Badola and Pradhan (2013), Nanda et al. (2013), Hussain and Hore (2015), Kichu et al. (2015), Prakash (2021)
	<i>Clerodendrum glandulosum</i> L.	Arunachal Pradesh, Assam	Leaf, fruit	Raw or cooked leaves are taken for diabetes and high blood pressure; fruit is used for the treatment of high blood pressure, bowel troubles and obesity	
	<i>Clerodendrum infortunatum</i> Auct non	Meghalaya	Root	Used as astringent	
	<i>Clerodendrum serratum</i> (L.) Moon.	Assam	Whole plant	Used to cure fever	
	<i>Clerodendrum serratum</i> L.	Arunachal Pradesh, Meghalaya	Whole plant	Treatment of fever and headache	
	<i>Clerodendrum viscosum</i> Vent.	Assam	Leaf	Used for diabetes, high blood pressure and asthma	
	<i>Elsholtzia communis</i> (Coll. & Hemsley) Diels	Manipur	Leaf and inflorescence	Used for tonsillitis	
	<i>Mentha arvensis</i> L.	Arunachal Pradesh, Manipur	Whole plant	Used for asthma and stomach problem	
	<i>Ocimum basilicum</i> L.	Sikkim	Leaf and seeds	Leaf juice used in cold and cough; seeds used in dysentery and gonorrhoea	
	<i>Ocimum sanctum</i> L.	Arunachal Pradesh, Sikkim	Leaf	Used in mouth ulcer and stomach problem and as an expectorant	
	<i>Cinnamomum indicum</i> L.	Sikkim	Flower	Used in stomachache	
	<i>Litsea cubeba</i> (Lours.) Pers.	Meghalaya	Bark, leaf	Used for food and mouth disease in cattle and as antiseptic	
Lauraceae					Mao et al. (2009), Badola and Pradhan (2013), Myrchiang et al. (2018)

(continued)

Table 8.3 (continued)

Family	Species name	Tribes/communities	Part used	Uses	References
Liliaceae	<i>Allium wallichii</i> Kunth.	Sikkim	Whole plant	Used as tonic, purgative and anthelmintic; leaf juice is applied on the head during high fever	Sharma et al. (2001), Sajem and Gosai (2006), Badola and Pradhan (2013), Hussain and Hore (2015)
	<i>Asparagus racemosus</i> Willd.	Assam, Mizoram, Sikkim	Root, leaf, fruits	Root paste is used orally in fever, cold and cough; dried leaves are powdered and taken orally to cure stomachache and urinary disorders; fruits are used to treat pimples	
	<i>Gloriosa superba</i> L.	Assam	Leaf	Anthelmintic	Rout et al. (2012)
Malvaceae	<i>Hibiscus rosa-sinensis</i> L.	Tripura	Flower bud	The paste is used for the treatment of excessive hair loss and dandruff problems	Majumdar et al. (2006)
Meliaceae	<i>Azadirachta indica</i> A. Juss. L.	Sikkim	Leaf	Used to control diabetes	Badola and Pradhan (2013), Prakash (2021)
Menispermaceae	<i>Toona ciliata</i> Roem.	Meghalaya	Bark	Used in joint pains	Myrchiang et al. (2018)
	<i>Tinospora cordifolia</i> (Willd.) Miers ex Hook. F. & Thoms.	Meghalaya	Whole plants	Leaf paste is applied in cuts and boils. Stem is applied externally and used in the treatment of bone fracture, body ache and diabetes	
Moraceae	<i>Artocarpus heterophyllus</i> Lamarck	Manipur, Mizoram, Nagaland, Sikkim	Latex	Applied to cure boils, bone fracture and skin diseases	Sharma et al. (2001), Laloo et al. (2006), Badola and Pradhan (2013), Nanda et al. (2013), Kiehu et al. (2015), Myrchiang et al. (2018), Prakash (2021)
	<i>Artocarpus lakoocha</i> Roxb.	Sikkim	Latex	Applied to cure boils and bone fracture	
	<i>Ficus elastica</i> Roxb. Ex Hornem.	Meghalaya	Young leaf	Ointment used for the treatment of body pain and paralysis	
	<i>Ficus glomerata</i> Roxb.	Manipur	Latex	Latex is applied over boils	

	<i>Ficus hispida</i> L.	Meghalaya	Bark, fruit and seeds	Used in anaemia, jaundice and fever	
	<i>Ficus palmata</i> L.	Meghalaya	Flower/fruit	Used for stomach pain	
	<i>Morus indica</i> L.	Sikkim	Leaf	Young leaf is used in inflammation	
Moringaceae	<i>Moringa oleifera</i> Lam.	Arunachal Pradesh	Seed pod, leaf	Used in the treatment of liver disorder	Deb et al. (2009)
Musaceae	<i>Musa paradisiaca</i> L.	Manipur	Stem	Used for liver infection, snake bite and diarrhoea	Nanda et al. (2013), Babina and Maibam (2021)
Myricaceae	<i>Myrica esculenta</i> Buch.-Ham. ex D. Don	Nagaland	Fruits and leaves	To treat cuts and wounds	Kichu et al. (2015)
Myrsinaceae	<i>Embellia ribes</i> Burm. F.	Arunachal Pradesh	Leaves and fruits	Used as anthelmintic and in diarrhoea	Nimachow et al. (2011)
Myrtaceae	<i>Eucalyptus globulus</i> Labill.	Nagaland	Leaf and fruit	Extract applied to treat scalp infection and dandruff	Jamir et al. (1999), Majumdar et al. (2006), Kongsai et al. (2011), Myrchiang et al. (2018)
	<i>Eugenia claviflora</i> Roxb.	Meghalaya	Leaf	Used in liver problem and abdominal pain	
	<i>Psidium guajava</i> L.	Nagaland, Tripura	Leaf	Used to treat tooth pain and decay; fresh leaves are boiled in water, and the extract is drunk in the treatment of severe diarrhoea	
	<i>Syzygium cumini</i>	Arunachal Pradesh	Fruit and bark	Used for stomach disorder, diarrhoea and dysentery	
Nepenthaceae	<i>Nepenthes khasiana</i> Hook.f.	Meghalaya	Roots	Root paste applied externally for treating corn and warts	Myrchiang et al. (2018)
Nyctaginaceae	<i>Mirabilis jalapa</i> L.	Assam	Leaf	Fresh leaf juice applied on itching skin	Rout et al. (2012)
Oleaceae	<i>Nyctanthes arborescens</i> L.	Sikkim	Leaf	Leaf juice used in fever	Badola and Pradhan (2013)

(continued)

Table 8.3 (continued)

Family	Species name	Tribes/communities	Part used	Uses	References
Orchidaceae	<i>Orchis latifolia</i> L.	Sikkim	Tuber and roots	Used as nerve tonic and aphrodisiac	Hussain and Hore (2015)
Passifloraceae	<i>Passiflora edulis</i> Sims.	Manipur	Fruits	Fruits are prescribed in liver enlargement	Nanda et al. (2013)
Phytolaccaceae	<i>Phytolacca acinosa</i> Roxb.	Sikkim	Root	Used for sinusitis	Badola and Pradhan (2013)
Piperaceae	<i>Piper betel</i> L.	Arunachal Pradesh, Nagaland	Leaf	Leaf rubbed with mustard oil is passed over flame and applied to the belly during stomachache	Jamir et al. (1999), Deb et al. (2009), Goswami et al. (2009), Badola and Pradhan (2013)
	<i>Piper longum</i> L.	Arunachal Pradesh, Sikkim	Root, leaf, stem and fruit	Powdered roots serve as vermifuge and relieve stomachache; fruits are taken during diarrhoea and dysentery; stem and fruit are used for gout, paralysis and arthritis and to improve the immune and digestive system	
Plantaginaceae	<i>Plantago erosa</i> Wall.	Arunachal Pradesh	Seeds, leaves	Used in constipation, to improve digestion and as astringent, diuretic, expectorant and anti-inflammatory agent	Srivastava and Adi Community (2009), Mishra et al. (2011), Perme et al. (2015), Myrchiang et al. (2018)
	<i>Plantago major</i> L.	Meghalaya	Whole plant	Minor cuts and wounds	
	<i>Scoparia dulcis</i> L.	Arunachal Pradesh	Whole plant	Used in the treatment of jaundice and diabetes and also as antioxi-dant, diuretic, analgesic and anti-inflammatory agent	
Polygonaceae	<i>Fagopyrum esculentum</i> Moench	Arunachal Pradesh, Sikkim	Whole plant	Leaf juice used in stomachache and gastritis; to control high blood pressure and for pain relief	Srivastava and Adi Community (2009), Mishra et al. (2011), Badola and Pradhan (2013)

Ranunculaceae	<i>Aconitum ferox</i> Wall. ex Ser.	Sikkim	Rhizome	Used in malaria, diarrhoea, indigestion, abdominal pain, cold and cough	Hussain and Hore (2015), Badola and Pradhan (2013), Perme et al. (2015)
	<i>Aconitum heterophyllum</i> Wall. ex Royle	Sikkim	Rhizome	Used in snake bite	
	<i>Coptis teeta</i> Wall.	Arunachal Pradesh	Roots	Fever, headache, gastric problem, ulcer, insomnia and vomiting	
Rosaceae	<i>Fragaria nubicola</i> Lindl. ex Lacaita	Sikkim	Roots	Used during cold and cough	Badola and Pradhan (2013)
Rubiaceae	<i>Hedyotis auricularia</i> L.	Tripura	Leaves	Used to cure dysentery and other abdominal disorder	Majumdar et al. (2006), Srivastava and Adi Community (2009), Nanda et al. (2013), Perme et al. (2015)
	<i>Mussaenda roxburghii</i> Hooker f.	Arunachal Pradesh, Manipur	Floral bracts	Applied to boils and used as antipyretic and diuretic and to treat blemishes	
	<i>Paederia foetida</i> L.	Manipur	Leaf, root, whole plant	Leaves and roots are used for toothache and gum problems; root paste with salt is applied over ringworm; stem is used in bone dislocation	
	<i>Rubia manjith</i> Roxb.	Arunachal Pradesh	Roots, fruits and leaf	Used to cure headache, cough and cold	
Rutaceae	<i>Aegle marmelos</i> (L.) Corr.	Assam	Bark, fruit	Used for the treatment of diarrhoea	Laloo et al. (2006), Theanphong et al. (2008), Srivastava and Adi Community (2009), Rout et al. (2012), Badola and Pradhan (2013), Nanda et al. (2013), Perme et al. (2015)
	<i>Citrus indica</i> Yu. Tanaka	Meghalaya	Leaves	As an antiseptic	
	<i>Citrus maxima</i> Burm.	Assam	Fruit	Ripe fruit juice used in reducing tremors	

(continued)

Table 8.3 (continued)

Family	Species name	Tribes/communities	Part used	Uses	References
Saxifragaceae	<i>Citrus medica</i> L.	Arunachal Pradesh, Manipur, Meghalaya, Sikkim	Fruits, root and rind	Roots are used as laxative, anthelmintic and diuretic; fruit is used as astringent and stimulant; preserved rind is used in dysentery	Sharma et al. (2001), Badola and Pradhan (2013), Hussain and Hore (2015)
	<i>Citrus sinensis</i> (L.) Osbeck	Manipur	Leaf, fruits	Leaf infusion given orally in sinus pain; fruit juice taken internally to cure bee stings and used to treat scurvy and intestinal ailments, as antidote and to treat weak eyesight, vomiting, skin diseases and haemorrhoids	
	<i>Citrus reticulata</i> Blanco	Sikkim	Fruit	Used as a skin cleanser	
	<i>Murray paniculata</i> (L.) Jack	Arunachal Pradesh	Whole plant	Analgesic, anti-diarrhoeal and anti-inflammatory agent	
	<i>Astilbe rivularis</i> Ham.	Sikkim	Root	Used in stomach problems, jaundice, toothache, cold and cough	
	<i>Bergenia ciliata</i> (Haw.) Stemb.	Sikkim	Rhizome and root	Dried rhizome is used as astringent and in ulcers, cough and spleen enlargement; roots are applied to cuts and burns	
	<i>Bergenia ligulata</i> (Wall.) Engl.	Mizoram	Root and leaf	Decoction of the root is given orally in diarrhoea and pulmonary infections; leaf juice is applied on boil and is also taken orally to dissolve kidney stones	

Scrophulariaceae	<i>Hemiphragma heterophyllum</i> Wallich.	Sikkim	Fruit	Fruit juice is used to relieve sore throat	Badola and Pradhan (2013), Hussain and Hore (2015)
	<i>Picrorhiza kurroa</i> Royle ex Benth.	Sikkim	Root	Used as a tonic and laxative and in fever, jaundice, anaemia, cold and cough	
Solanaceae	<i>Capsicum frutescens</i> L.	Assam	Fruit	Fruit paste applied on leech bite to stop bleeding	Jamir et al. (1999), Deb et al. (2009), Mao et al. (2009),
	<i>Datura fastuosa</i> L.	Sikkim	Leaf and seeds	Leaves are used to reduce pain and swelling; seeds are used in asthma	Srivastava and Adi Community (2009), Rout et al. (2012),
	<i>Solanum khasianum</i> C.B. Clarke	Arunachal Pradesh, Meghalaya, Sikkim	Root, fruit	Root decoction used in malaria and as anti-inflammatory and antifertility property; burnt fruit used in tooth decay	Badola and Pradhan (2013),
	<i>Solanum kurzii</i> Brace ex Prain	Manipur	Fruit	Fruit juice is used for allergy	Perme et al. (2015)
	<i>Solanum nigrum</i> L.	Arunachal Pradesh, Nagaland	Berries, leaves, shoots	Used to treat vomiting and diarrhoea, also to cure tuberculosis and cough and to reduce mild abdominal pain	
Symplocaceae	<i>Symplocos theifolia</i> D. Don	Sikkim	Fruit	Fruit juice used in diarrhoea	Badola and Pradhan (2013)
Thymelaeaceae	<i>Daphne camabina</i> Wall.	Sikkim	Root	Decoction used during poisoning	Badola and Pradhan (2013)
Urticaceae	<i>Elatostema platyphyllum</i> Wedd.	Sikkim	Seed	Seed paste used in cough	Badola and Pradhan (2013)
	<i>Girardinia palmata</i> (Forssk.) Gaudich	Sikkim	Root	Used in kidney problem, skin disease and toothache	

(continued)

Table 8.3 (continued)

Family	Species name	Tribes/communities	Part used	Uses	References
Verbenaceae	<i>Callicarpa arborea</i> Roxb.	Mizoram, Nagaland, Sikkim	Bark, fruit	Powdered bark is applied to cuts and wound; fruit juice is used orally during fever and toothache	Jamir et al. (1999), Bhardwaj and Gakhar (2005), Rout et al. (2012), Badola and Pradhan (2013)
	<i>Clerodendron colebrookianum</i> Walp.	Assam	Leaf	Decoction used in high blood pressure	
	<i>Clerodendron infortunatum</i> Gaertn.	Assam	Leaf	Leaf paste applied on bee sting-affected area	
	<i>Lantana camara</i> L.	Nagaland, Sikkim	Leaf	Used as an antiseptic and in the treatment of jaundice, cold and fever	
	<i>Verbena officinalis</i> L.	Nagaland	Whole plant	Used in high fever and malaria. Plant extract used as an appetizer	
Zingiberaceae	<i>Amomum dealbatum</i> Roxb.	Assam	Roots/rhizome	Rhizome or roots are applied to cure joint pains	Rao (1981), Sharma et al. (2001), Tag and Das (2004), Sajem and Gosai (2006), Das and Choudhury (2012), Badola and Pradhan (2013), Nanda et al. (2013), Hussain and Hore (2015), Singh et al. (2015), Myrchiang et al. (2018), Prakash (2021)
	<i>Amomum subulatum</i> Roxb.	Mizoram, Sikkim	Rhizome, fruit and seeds	Rhizome decoction is taken orally in diarrhoea, dysentery, food poisoning and jaundice; seed oil is used in stomach problem; decoction of fruit is used in teeth and gum problems and in combination with melon seeds is used as a diuretic	
	<i>Alpinia allughas</i> (Retz.) Roscoe	Meghalaya	Rhizome	Used for intestinal infections	
	<i>Alpinia nigra</i> (Gaertn.) Burt	Arunachal Pradesh	Rhizome, fruits and leaves	Used in the treatment of jaundice and gastric ulcer and as diuretic, appetizer and expectorant	

	<i>Costus speciosus</i> (Koenig. ex Retzius) Smith	Manipur, Meghalaya, Sikkim	Rhizome	Used in urinary problem; to treat body swelling and ear infection; as appetizer, carminative and anthelmintic; and to treat urinary disorder
	<i>Curcuma montana</i> Roxb.	Meghalaya	Rhizome	Treatment of body swelling and jaundice
	<i>Curcuma aeruginosa</i> Roxb.	Manipur	Rhizome	To cure liver enlargement; extract taken against dysentery
	<i>Curcuma angustifolia</i> Roxb.	Meghalaya	Rhizome	Ground rhizome is made into paste and used as gripe in chil- dren and also used for treating severe cough
	<i>Curcuma aromatica</i> Salisb.	Meghalaya, Sikkim	Rhizome	Used as diuretic, carminative, tonic and anthelmintic
	<i>Curcuma longa</i> L.	Assam, Manipur	Rhizome	Crushed rhizomes are used to counteract dyspepsia; paste is applied over wounds
	<i>Hedychium coccineum</i> Buch.-Ham. ex Wall.	Arunachal Pradesh	Whole plant	Cure asthma and indigestion
	<i>Hedychium spicatum</i> Sm.	Sikkim	Rhizome	Used in the treatment of liver disorders, vomiting and stomach problem
	<i>Kaempferia rotunda</i> L.	Sikkim, Tripura	Tuber	Used in the treatment of jaundice, swelling, fracture and mumps

different tribes of North-East India are discussed in the following sections. Prominent families which are used as medicinal plants in the region are Acanthaceae, Asteraceae, Cucurbitaceae, Fabaceae, Liliaceae, Musaceae, Rubiaceae, Rutaceae and Zingiberaceae (Mao et al. 2009).

8.3.1 *Banana (Family: Musaceae)*

Banana has many potential therapeutic uses besides its high nutritive value. Various plant parts such as fruit, pseudostem and inflorescence are used in ethnic dishes by the different communities of North-East India. *Musa paradisiaca* and *M. sapientum* are rich in starch. Bananas are also rich in carbohydrates; vitamins A, B6 and C; as well as minerals, such as potassium, magnesium, phosphorus and folate (Kumari et al. 2022). Bananas have antioxidant, anti-diabetic and anti-advanced glycation end-product properties and are used for the treatment of diarrhoea (Singh et al. 2013).

8.3.2 *Citrus (Family: Rutaceae)*

Citrus are well known for their medicinal and economic importance. They are also a good source of citric acid, flavonoids, phenolics, limonoid and ascorbic acids (Dugo and Di Giacomo 2002; Kumar et al. 2010). A few of the *Citrus* species of North-East India used for medicinal purposes are *Citrus indica*, *C. macroptera*, *C. limon*, *C. aurantiifolia*, *C. limetta*, *C. medica* and *C. sinensis*. The various plant parts used are fruit, fruit juice, peel of the fruit and seed paste for treating various ailments such as kidney, urinary and gastric problems, diarrhoea, cancer, scabies, jaundice, anaemia, fever, cold, ringworm, etc. (Leonardi et al. 2004; Hazarika 2012; Malik et al. 2012).

8.3.3 *Orchids (Family: Orchidaceae)*

Orchids are known for their beautiful flowers which have gained ornamental importance worldwide. Moreover, they are also highly valued for their medicinal properties. Orchid species such as *Malaxis muscifera*, *M. acuminata*, *Habenaria edgeworthii*, *H. intermedia* and *Eulophia nuda* have been widely used in Ayurvedic system of medicine (Ninawe and Swapna 2017). Some of the orchid species from North-East India used for medicinal purposes are *Cymbidium aloifolium*, *C. elegans*, *Dendrobium denudans*, *D. eriaflorum*, *D. transparens*, *D. devonianum*, *Vanda roxburghii*, *Rhynchostylis retusa*, etc. (Mao et al. 2009; Ninawe and Swapna 2017). Indigenous traditional knowledge of orchids from North-East India varies

among the ethnic groups. Leaves, roots, stem and bulbs are commonly used for treating various ailments.

8.3.4 *Hedychium* (Family: *Zingiberaceae*)

Hedychium are rhizomatous perennial herbs which are of ornamental, medicinal and horticultural values. Local practitioner/healers use various *Hedychium* species to treat various ailments. *Hedychium spicatum* has been mentioned in Ayurvedic system of medicine to treat cough, wound ulcer, fever and respiratory problems (Ghildiyal et al. 2012).

8.4 Over-collected and Threatened Medicinal Plants of North-East India

Medicinal plants have been used as a source of food, fodder and timber and for ethnobotanical purposes by the indigenous tribes of North-East India (Kala et al. 2006). With the increase in population, the demand on these medicinal plants is also increasing. In India, approximately US \$1 billion per year is estimated for medicinal plant-related trade (Joshi et al. 2004). The therapeutic importance of these medicinal plants has led to high market demand, leading to their over-collection. Over-collection of several medicinal plants, like *Dendrobium* spp., *Aconitum* spp., *Coptis teeta*, *Lycopodium pseudoclavatum*, *Panax assamicus*, *P. pseudoginseng*, *Paris polyphylla*, *Rubia manjith*, *Taxus wallichiana*, *Rhododendron*, *Zingiber* species, etc., has led to the depletion in their population (Mao et al. 2009; Ninawe and Swapna 2017). Many orchids like *Anoectochilus tetraplerus*, *Ascocentrum ampullaceum*, *A. miniatum*, *Dendrobium bensoniae*, *D. draconis*, *D. heterocarpum* and *D. wardianum* are facing a threat of survival (Hegde 2012). Other important medicinal plants which are in various threatened categories are listed in Table 8.4.

8.5 Threats and Conservation

Medicinal plants have been collected from wild, and their populations are decreasing drastically. These medicinal plants are facing a constant threat due to overexploitation, indiscriminate collection, changes in forest cover and anthropogenic and developmental activities. Major loss in forest cover is seen in the states of Arunachal Pradesh, Manipur, Nagaland, Mizoram and Meghalaya (Table 8.1).

Table 8.4 Some threatened medicinal plants of North-East India

Species	Family	Status ^a
<i>Acer oblongum</i>	Aceraceae	Endangered
<i>Acorus calamus</i>	Acoraceae	Endangered
<i>Rauvolfia serpentina</i>	Apocynaceae	Critically endangered
<i>Holarrhena antidysenterica</i>		Endangered
<i>Aralia chinensis</i>	Araliaceae	Vulnerable
<i>Panax assamicus</i>		Endangered
<i>Cinnamomum tamala</i>	Lauraceae	Threatened
<i>Paris polyphylla</i>	Liliaceae	Vulnerable
<i>Gloriosa superba</i>		Threatened
<i>Nepenthes khasiana</i>	Nepenthaceae	Endangered
<i>Boerhaavia diffusa</i> L.	Nyctaginaceae	Endangered
<i>Dendrobium nobile</i>	Orchidaceae	Endangered
<i>Malaxis muscifera</i>		Vulnerable
<i>Piper pedicellatum</i>	Piperaceae	Vulnerable
<i>Aconitum ferox</i>	Ranunculaceae	Endangered
<i>Aconitum heterophyllum</i>		
<i>Coptis teeta</i>	Ranunculaceae	Endangered
<i>Rubia manjith</i>	Rubiaceae	Vulnerable
<i>Hedyotis scandens</i>		
<i>Citrus medica</i>	Rutaceae	Threatened
<i>Bergenia ciliata</i>	Saxifragaceae	Vulnerable
<i>Smilax glabra</i>	Smilacaceae	Critically endangered
<i>Taxus wallichiana</i>	Taxaceae	Endangered
<i>Aquilaria khasiana</i>	Thymelaeaceae	Critically endangered
<i>Boesenbergia longiflora</i>	Zingiberaceae	
<i>Hedychium spicatum</i>		Vulnerable

Source: Mao et al. (2009), Gowthami et al. (2021), Lasushe et al. (2022), Ray and Saini (2022)

^a Status based on IUCN, CAMP or RDB

Decrease in the forest covers might be due to various biotic stresses, natural calamities, shifting cultivation, encroachment and other developmental activities.

Conservation of wild, endemic and rare medicinal plants of North-East India is of utmost importance to increase their populations in the wild. Conservation can be either in situ or ex situ. In situ conservation encompasses natural reserves and wild nurseries, while ex situ conservation comprises botanic gardens and seed banks (Chen et al. 2016). The North-east region has 5 biosphere reserves, 48 sanctuaries, 16 national parks and 2 world heritage sites (Yumnam 2008; Shukla 2012) which are in situ. Forests in North-East India are also regarded with respect as it is believed to be the abode of gods (Mao et al. 2009), and some consider trees and bamboos the symbols of gods and goddesses in their culture (Debbarma et al. 2017). Thereby, states like Assam, Arunachal Pradesh, Manipur, Meghalaya and Sikkim have well-preserved sacred grove where cuttings of trees and other plants are strictly

prohibited. Besides, there are also a number of reserved and protected forests in the region which are predominantly under the governance of the local community. Such places also serve as a refuge to many endemic, rare and threatened medicinal plants. The communities of North-East India thus have an intricate relationship and an active role in managing the forest ecosystem by initiating traditional knowledge of farming and harvesting and afforestation and conservation programmes and regulating the exploitation of medicinal plants.

Botanical gardens are important as they can sustain the ecosystems which enhance the survival of many rare and endangered plant species (Huang et al. 2002). Such gardens have been established for *Nepenthes* in Meghalaya and *Rhododendrons* in Sikkim. Botanical gardens also support a wide range of taxonomically and ecologically diverse plant species which can play further a role in conserving medicinal plants through the development of propagation and cultivation protocols (Maunder et al. 2001; Primack and Miller-Rushing 2009; Chen et al. 2016).

8.6 Conclusion

The tribes of North-East India are dependent on plants for food, shelter, clothing and providing affordable health care to the people. The knowledge of utilizing various plants for the treatment of different ailments have been passed down from generation to generation. The traditional knowledge about the diversity of medicinal plants and their medicinal values can significantly contribute to their sustainable utility and conservation. Moreover, this vast knowledge can be translated to modern medicine and therapeutics.

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Part II

Conservation of Medicinal Plants

Chapter 9

Strategies for Conservation and Sustainable Use of Medicinal Plants



**Monika Sharma, Lalit Saini, Prasann Kumar, Sonam Panigrahi,
and Padmanabh Dwivedi**

Abstract Plants have been used as medicines since prehistoric times, for the treatment of different diseases and to improve the health of man and animals. Medicinal plants play an important role in curing diseases and stimulating the well-being of the population. WHO (World Health Organization) claims that 80% population of the world depends on medicinal plants for primary well-being programs. Medicinal plants are used for the manufacturing of various herbal products, but these plants are decreasing day by day at a high speed. As the medicinal plants are decreasing, it can become a major issue in terms of the health of both man and animal in the coming times. The main reason for this is increasing population and industrialization, increased commercial exploitation, mostly unmanaged trading, and habitat destruction. In this chapter, we discuss worldwide changes, problems, strategies, and approaches for the conservation and sustainable use of medicinal plants and their resources. It can be a great start toward conserving the medicinal plants and their products for the betterment of the population. We have to look for both in situ and ex situ growing and conservation practices for use of medicinal plant resources for a long period. To improve the growing of medicinal plants, the use of biotechnology is the best option. Different tools of biotechnology are used for the selection, multiplication, and conservation of medicinal plants having critical genotypes. Due to the destruction of habitats and swamp areas, the biodiversity of medicinal plants is on decline. In the future, the adoption of pharmaceutical and clinical medicine must be supported by medicinal plants. These plants have an extraordinary wealth of chemical compounds that have a wide range of applications

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in the healthcare of humans. Therefore, there is a need to conserve and sustain the use of medicinal plants in the modern era.

Keywords Biotechnology · Diseases · Medicinal plants · Hunger · Poverty · Conservation · Sustainability

9.1 Introduction

Medicinal plants are an important source of novel drugs all around the world. In the Northern Hemisphere and a few parts of the Eastern Hemisphere, over 1200 medicinal plant species are utilized, out of that 85% species of medicinal plants are collected from the wild and natural resources. In the countries of the United States, about 117 out of 149 drug allocations are done on the basis of naturalistic sources (Nalawade et al. 2003). In those countries which are properly developed more than 24.5% of medications are formed by the species of wild plants, for the elementary healthcare up to 75% peoples of under developing countries are based on herbal or natural drugs. Many medicinal species are utilized by individuals who have been their principal custodians (Pérez-Escobar et al. 2020).

According to the data from the International Union for Conservation of Nature and the World Wildlife Fund for Nature, 45,000–80,500 flowering species of plants are used for curing and medicinal purpose globally. Over 1400 species of medicinal plants are threatened and destroyed by the loss of habitat and overexploitation, and with the rising population of human beings, about 21% of wild medicinal resources are depleted and consumed (Hamilton 2008). In the few decades, the species of medicinal plants are rapidly lost from the environment, and devastation of the habitat has been seen to raise the risk of disappearing medicinal plants, particularly in China, Kenya, Uganda, India, Tanzania, and Nepal (Heywood and Iriondo 2003; Zerabruk and Yirga 2012).

According to the World Health Organization, 20,000 species of medicinal plants were used for curing purposes in the 1980s. For medicinal drug formation, China alone uses more than 32,000 species of indigenous plants and accounts for 15.5% of the country's flora. In medicinal plants, thorough investigations are used for the long term and their conservation purpose. Different strategies of conservation are formed for the specific monitoring and inventorization status of medicinal plants with the help of combined measures of conservation, i.e., dependent on the ex situ and in situ strategies (Larsen and Olsen 2006). Also, another one is sustainable use which helps in the conservation of wild and natural resources of medicinal plants that are used in healthcare. In the present days, with the increasing population and the rising demands, the situation in the countries of South Africa and North Asian countries like China is serious (Upriety et al. 2012).

9.2 Distribution in India

In India, medicinal plants are present in a wide range of habitats and their surrounding environments. Up to 65% of medicinal plants are present in the tropical regions and the forests of that regions are spreading from Western to the Eastern Ghats, also the Vindhya, Aravalli Hills, Plateau of Chotanagpur and the Tarai areas of Himalayas Hills, and a few parts of northeast India comes under the wild resources of medicinal plants (Dhyani et al. 2021). A modest number of therapeutic plants are present in the mangroves and watery areas, and around 28% of medicinal plants are present in the temperate and alpine regions (Radha et al. 2021). In comparison to temperate and evergreen habitats, an immense number of medicinal herbs species are present in the vegetation of deciduous, i.e., moist and dry, regions. Most common species of medicinal herbs are present in a group of lichens, algae, gymnosperms, bryophytes, fungi, angiosperms, and pteridophytes (Shankar and Ved 2003).

9.3 Medicinal Plants at Threat

There are almost 25,906 species of medicinal plants which are documented for use. All species are analyzed which are not hybrid; still, 25,791 species are yet to be analyzed. Of those, 21% of species are marked on IUCN Red List, and 13% of species are set in the category of threatened species. The number of medicinal plants which are in the category of the IUCN Red List and are not reported to be used as medicines is significantly high. Some taxonomic groups are considered rich in medicinal plants as there is an increase in efforts to analyze the targeted species of medicinal plants, e.g., Cactaceae (Goettsch et al. 2015). Generally, it was found that the risk of extinction is more in non-medicinal plants. If a plant species is medicinal, the chances of it being categorized as threatened species on the IUCN Red List are five times lower as compared to other plant species. When all digitally accessible worldwide evaluations utilizing data from Threat Search (BGCI 2020) are included, the total number of medicinal species examined climbs to 10,673 (42%), although the main trends are largely comparable to those reported on the IUCN Red List.

Different risks included in the exploitation of medicinal plant species are described in Fig. 9.1. Given the well-documented conservation problems associated with the exploitation of specific medicinal species (Fig. 9.1), the comparatively small mean extinction risk for medicinal plants may surprise the reader. This low average extinction risk, on the other hand, is consistent with findings made over the last two decades that “weedy” and/or imported species are over-represented in traditional medicinal floras and that availability is a major component in explaining this (Stepp and Moerman 2001; Hart et al. 2017). The fact that weeds are over-represented among plants that are the sources of modern drugs is also consistent with the low mean extinction risk for medicinal plants, and a more recent report indicates that the likelihood of developing an alkaloid into a medicinal product is heavily influenced

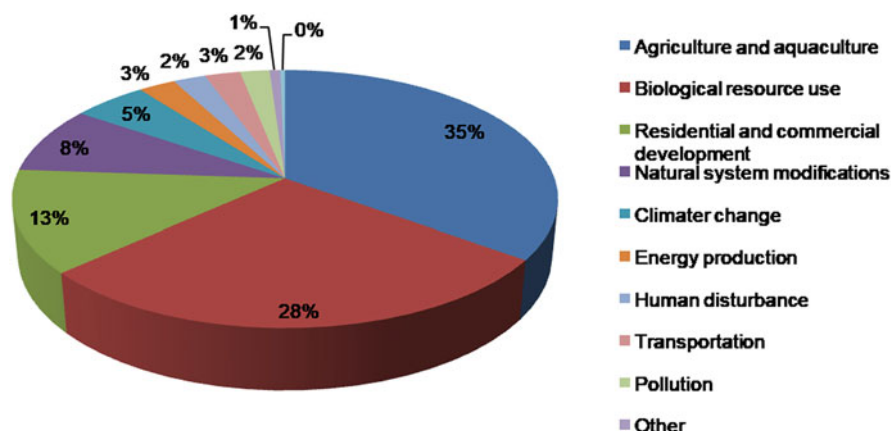


Fig. 9.1 Different threats reported for medicinal plant species. (Source: IUCN 2020)

by the abundance of the source species (Amirkia and Heinrich 2014). This latter finding also supports the notion that access and supply restrictions are a major impediment to the pharmaceutical industry's development of natural products (Harvey 2008). The importance of availability in deciding whether plants are recognized as beneficial as medicines extends beyond the issue of weedy or invasive species. The comparatively low mean extinction risk of medicinal plants is less surprising given that native range size is the biggest predictor of extinction risk in plants (Nic Lughadha et al. 2020). Another less quantifiable element is widespread human interest in medicinal plants (Petrovska 2012), particularly in areas where plants are used for basic healthcare. Increased attention frequently leads to exploitation, but it also has the potential to drive long-term stewardship of these natural resources.

9.4 Conservation Need

Conservation promotes long-term development by managing biological resources in ways that do not deplete the different varieties of medicinal plant species present in the world and do not devastate ecosystems. Collection, evaluation, propagation, identification of disease, removal, depositing, and distribution are all part of the process. Medicinal plants can be conserved with the help of their inherited resources by ex situ and in situ conservation methods. In the need for conservation, we can also include different traditional conservation methods (Engels and Ebert 2021). In rural areas, people are mostly dependent on their nearby forests for medicines and also for fuel wood. Due to the high prices of current medicine and fuel, they can't afford it. Implementing immediate action is necessary to protect against the degradation of the environment and losses occurring in the near surrounding environment. By discouraging the people to stop cutting the tree and encouraging them to plant new

varieties of indigenous species of trees that help them for their domestic purpose by conserving the medicinal plants (Opoku 2019).

9.5 Strategies to Conservation

Medicinal plant resources, especially from wild populations, are now being obtained in large numbers. The demand for natural resources in some nations like Europe, North America, and Asia has increased by 10–15% each year (Soulé et al. 2005; Semwal et al. 2007). There may be a point arising where the ability of the plant species to reproduce is decreased. Various sets of recommendations for medicinal plant conservation have been established, including both in situ and ex situ conservation. Botanic gardens and seed banks are good instances of ex situ conservation and future replanting, whereas natural reserves and wild nurseries are good examples of how to preserve plants’ medical efficacy in their natural habitats. The geographic distribution and biological features of medicinal plants must be determined to aid conservation efforts (Sheikh et al. 2002). All the conservation strategies of medicinal plants are represented in Fig. 9.2.

9.5.1 In Situ Conservation

Secondary metabolites play an important role in the curative functions of medicinal plants as they are synthesized and respond to stimuli in natural conditions and are not synthesized in culture conditions (Long et al. 2003). By in situ conservation, we can conserve more and more natural communities and national flora and can maintain their behavior and interactions (Ma et al. 2012). In situ conservation increases the amount of diversity that may be conserved while also establishing the link between resource conservation and long-term use. Around the world, in situ conservation efforts have focused on creating nature reserves and adopting an ecosystem-oriented

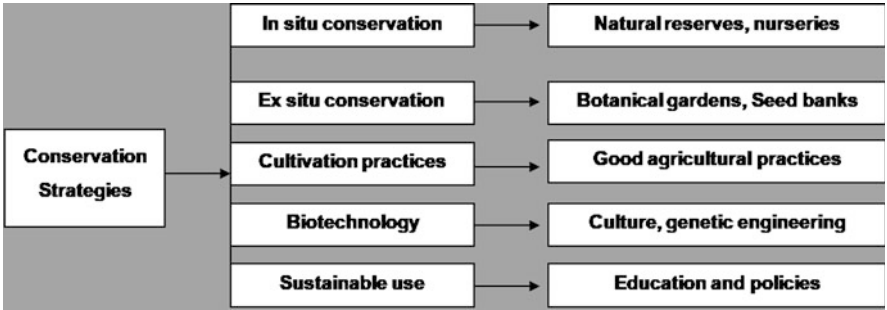


Fig. 9.2 Conservation strategies for medicinal plants

strategy rather than a species-oriented one. Rules, constraints, and the likelihood of medicinal plants' cooperation within growing habitats are all essential variables in in situ conservation success (Camm et al. 2002). The loss of medicinal plant resources is mostly due to habitat damage and degradation. Natural reserves are areas that have been set aside to protect and restore natural resources. On average, nearly 12,700 conservation areas are established around the world which covers almost 8–10% of the land surface (Rodríguez et al. 2007). To protect medicinal plants by preserving important natural habitats, specific habitat contributions and ecological processes must be examined. It is difficult to designate every natural wild plant habitat as a protected area due to budget restrictions and competing land uses (Chiarucci et al. 2001). In a protected area or a natural habitat or a place that is near the naturally grown plant species, a nursery can be built there to cultivate wild species, or species-wise cultivation can be established to domesticate the endangered species of medicinal plants (Huang et al. 2002).

9.5.2 *Ex Situ Conservation*

Ex situ conservation of medicinal plants is an important strategy, especially for plant species that are overexploited, are endangered, have low availability, and are sensitive to replanting diseases. Ex situ conservation is not much different from in situ conservation, but more focus is given here to conserving the medicinal plant species in their natural habitat (Yu et al. 2010). Ex situ conservation involves growing and naturalizing species to ensure their continued existence and, in some cases, to generate large quantities of planting material needed in the development of medications, and it is frequently a quick response to ensure medicinal plant resources are restored (Swarts and Dixon 2009). Botanical gardens are good examples of ex situ conservation of medicinal plants, and they play an essential role in the sustainability of the ecosystem due to which the rarely found and endangered species can survive (Pulliam 2000). In botanical gardens, many cultures and propagation techniques can be followed which can generate the breeds of plant species. They help in developing propagates of medicinal plant species and domesticate the variety of plant species. Seed banks, rather than botanic gardens, are recommended for preserving the biological and genetic diversity of wild plant species because they provide a superior strategy for preserving the genetic diversity of many medicinal herbs ex situ (Yuan et al. 2010). The most famous and known seed bank is the Millennium Seed Bank Project at the Royal Botanic Gardens which is established in the United Kingdom to conserve a variety of genetic wild and pure seeds (Maunder et al. 2001). Seed banks provide relatively quick and easy access to plant samples for property evaluation, which is useful information for protecting natural populations (Long et al. 2003).

Given the importance of plant medicine in people's healthcare, it is critical to preserve it for current and future generations. The following are some of the measures being taken to keep the situation under control:

9.5.2.1 Education

People should be educated about the relevance of plant medicine and the need of preserving the forests and woods that maintain this legacy. Medicinal plant collectors should not be the only ones who receive education; schoolchildren and youngsters should also be included. There are other indirect advantages gained from forests and woods, in addition to the direct benefits such as improvement of the local climate, watershed preservation, erosion prevention, etc. Protection of the medicinal plant species in sanctuaries, botanical gardens, national parks, and forest reserves should be emphasized.

9.5.2.2 Growing Medicinal Plant Species

Different exporters and growers should be encouraged to grow medicinal plants. The financial loan should be provided to the growers to cultivate more and more medicinal plants. There are some initiatives like the Darwin sponsoring cultivation of medicinal plant species in the botanical gardens of Ghana and the University of Ghana. Tamarack, a Tamale-based NGO, is also receiving help from the UNDP's Global Environment Facility for medicinal plant production. Commercial plant medicine makers, distributors, and dealers should invest some of their income in the establishment of the plantation of medicinal plants.

There are so many organizations and agencies which are involved in controlling and conserving the sustainable use of medicinal plants. Different activities are performed by the Species Survival Commission (SSC) of the International Union for Conservation of Nature (IUCN) to conserve medicinal plant species.

9.5.2.3 Policies

Government policies that acknowledge the value of medicinal plants to health and the economy, and encourage conservation and sustainable use of this resource, generate laws that preserve medicinal plants from extinction. WHO (World Health Organization), WWF (World Wildlife Fund for Nature), and TRAFFIC (the first public version of an international standard for sustainable wild collection of medicinal and aromatic plants) work on a global level to update the guidelines related to the conservation of medicinal plant species. Laws should be there to protect against the overexploitation of the medicinal plant species, and modern techniques should be adopted to conserve the plants. New policies should be adopted that can be updated to integrate new insights on how to effectively maintain wild populations by promoting the sustainable wild collection, the application of local knowledge and practices, and equitable benefit sharing.

9.5.2.4 Trade/Organizations

On average, about 60,000 plant species are used for medicinal, nutritional, and aromatic use, and more than 500,000 tons from these species are traded. Worldwide, near about half a million tons of medicinal and aromatic plants in dry form are traded each year. These are also traded substantially on national and local markets. The demand for medicinal plants and aromatic plant species is increasing internationally, and about 50% of plants are harvested from the wild type. Only a few nations dominate the pharmaceutical plant trade, with three major trading centers: Germany, the United States, and Hong Kong. About 80% of exports and imports of plants from the global market come from 12 different nations. The industrialized world's markets are the most important, whereas the majority of pharmaceutical plants are shipped from underdeveloped nations, unprocessed or just little processed, and of wild origin. Millions of families participate in collecting, with women typically playing a key role, and earn money from the trade, and it supplies industrial manufacturing of a wide range of medical and domestic items. Despite the dearth of precise statistics, existing evidence shows that commerce is growing. TRAFFIC and the Convention on International Trade collaborate with the IUCN Wildlife Trade Programme and the Endangered Species of Wild Fauna and Flora (CITES) to identify medicinal plant species with significant international trade and to assess whether and how countries that have signed the convention should monitor international trade. Therefore, different organizations should be encouraged to involve in the conservation of the species of medicinal plants. The organizations should be funded by the agencies or government for better performance and resource availability.

9.5.2.5 Conservation Status Assessment

We encourage and promote the evaluation of medicinal plant conservation status using the IUCN Red List categories and criteria, and we assist in training our members and partners to perform these assessments and others to use these approaches. We recently helped fund a Red List workshop in the Dominican Republic, which is part of the Caribbean island of Hispaniola, to teach personnel at the National Botanic Garden in Santo Domingo how to apply the IUCN Red List categories and criteria to prioritize medicinal plant species. These experts are now instructing other professionals throughout the Caribbean on how to apply these strategies to plant conservation.

9.5.2.6 Sustainable Use

There are agencies, like the German Federal Agency for Nature Conservation, WWF, and TRAFFIC (the first public version of an international standard for

sustainable wild collection of medicinal and aromatic plants), to analyze the sustainability of medicinal plants (ISSC-MAP). Legislation should prohibit the commercial overuse/misuse of wild plants, bark, roots, tubers, corms, and rhizomes of medicinal plant species. Defaulters should face significant fines as deterrence, in addition to the seizure of unlawfully obtained plant material. Long-term medicinal plant harvesting. Plants excavated for their roots, for example, are routinely covered with dirt, apparently to preserve the medication's efficacy, but it appears that the practice is, in fact, a conservation strategy.

Some of the traditional methods of protecting medicinal plants from destruction can be encouraged to conserve the species of medicinal plants like:

1. Medicinal plants should be surrounded by superstition, myths, and taboos to protect them from destruction.
2. To make people believe that some medicinal plants are sacred and should not be cut or used for fuel or another purpose.

9.5.2.7 Agricultural Practices

To conserve medicinal plant species, good agriculture practices have been developed to help manage to manufacture, assure good quality, and standardize herbal medications. Integrated application of good agricultural practices in the cultivation of medicinal plants ensures the growth of plant species that are of high quality, safe to use, and pollution-free (Chan et al. 2012). Good agricultural practices include various sites from propagation to conservation which includes environment, germ-plasm, culture techniques, insect-pest, and disease detection, macro, and microscopic inspection which greatly helps to cultivate and conserve the quality and variety of medicinal plants (Muchugi et al. 2008). The application of good agricultural practices (GAP) is actively promoted by several nations. Taking China as an example, they supported good agriculture practices continuously for the cultivation of medicinal plants in their natural habitat so that they can grow and preserve efficiently (Makunga et al. 2008). Organic farming is also a means to cultivate a sustainable agricultural system. It is a means of creating integrated and ecologically sustainable agricultural practices to ensure the good quality of medicinal herbs. Organic farming is characterized by not involving the use of chemical fertilizers, pesticides, insecticides, etc. in the cultivation purpose so that the quality of the herbs can be estimated. Organic fertilizers maintain soil nutrients and stability, which has a significant influence on medicinal plant development and the production of essential compounds (Rigby and Cáceres 2001). The biomass output of *Chrysanthemum balsamita* increased when organic fertilizer was utilized, and the essential oil content of the plant was higher than when there is no uptake of organic amendments.

9.6 Biosynthetic Approaches for Medicinal Molecules

Biotechnology is the finest choice for improving medicinal plant cultivation. For the selection, multiplication, and conservation of medicinal plants with important genotypes, many biotechnological methods are applied. The analysis of biosynthetic pathways in combination with the bioengineered fungal or bacterial cell produces new molecules which are bioactive and help in conserving the biodiversity of medicinal plant species. With the increase in tools and techniques available like genomes, transcriptomes, and heterologous hosts, the connection of biosynthetic genes with bioactive molecules is possible (e.g., *Aspergillus oryzae*) (Harvey et al. 2018; Skellam 2019). This technique is generally followed in research for achieving large-scale production of medicinal plant species at the industrial level (Steiniger et al. 2017). Modern genetic and biotechnological techniques make use of fungal fermentation for the synthesis of bioactive compounds in medicinal plants. This is an ideal approach to conserving the plant species which are at threat (Pyne et al. 2019). Plants have a very long and branched biosynthetic pathway for the functioning of secondary metabolites and other compounds. These have some complicated pathways which are controlled by various regulatory elements. Advanced clarification regarding biotechnology helps in understanding special metabolism in the plant system. Metabolic pathways of other alkaloids were also clarified due to a cytoplasmic study of the medicinal plants. For example, the metabolic pathways of indole alkaloids are assessed from iboga which is studied from its drug addiction effects (Dauncey and Howes 2020). These findings open the door for future advances in synthetic biology, particularly in the discovery of metabolic routes for medicinally essential indole alkaloids like the antimalarial quinine. Because the Himalayan mayapple (*Podophyllum hexandrum* Royle) contains more podophyllotoxin than the American mayapple (*Podophyllum peltatum* L.), it is the best source of lignin which is used for the semi-synthesis of anticancer medicines (e.g., etoposide) (Howes 2018). As the wild species of mayapple is a threat, its trades are restricted. The assessment of the genes which are responsible for the biosynthesis of podophyllotoxin in *Nicotiana benthamiana* Domin. and the formation of their metabolic pathway improves the sustainable production of the new plant species (Lau and Sattely 2015; Schultz et al. 2019). Bioactive chemical production in modified fungal strains has a lot of relevance in terms of sustainable development and health. The introduction of genetically modified food-grade fungus (e.g., *Aspergillus oryzae*) solves the problem of wild-type fungi creating dangerous mycotoxins during fermentation (Marič et al. 2019). To become more common and commercially feasible, the production of novel bioactive chemicals in fungal “cell factories” still requires tremendous effort. One major problem is to create technologies that enable precise biosynthesis pathways and enzyme specificity prediction. Integrating this understanding into semi-synthesis tactics that combine precursors generated by fermentation with chemical alterations might be a more cost-effective strategy (Sandargo et al. 2019).

9.7 Conclusion

As the different sets of conservation and sustainable use of medicinal plants are recommended, only a small portion of them reached at proper protection of plant species. The protection of medicinal plant species and resource availability is further pursued by conventional conservation and in natural habitats like botanical gardens. Medicinal plants play an important role in curing diseases and stimulating the well-being of the population. As the medicinal plants are decreasing, it can become a major issue in terms of the health of both man and animal in the coming times. Medicinal plants are a heritage from past generations. However, the current practice of illegally gathering medicinal herbs nearly exclusively from the wild is causing certain species' populations to decline. Therefore, immediate measures should be taken to conserve the resources and sustainable use of medicinal plants. The destruction or overexploitation of these plant species should be stopped, and good cultivation programs should be generated so that the demand of the population to use plant medicine for their daily healthcare could be satisfied. If no attention is given to the conservation of medicinal plants, there will be no more forest land or areas from where medicinal plant species can be harvested in the future. Moreover, in the present day, these plant species are either endangered or at the brink of extinction. So, there is a great need to conserve medicinal plants by using more advanced techniques and resources.

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Chapter 10

Scientific Databases for Conservation of Medicinal Plants



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Abstract Medicinal plants are one of the most important biological assets for human beings worldwide. The use of plants as part of a treatment system continues to increase in both traditional and modern medicine complete with its technology. Unfortunately, along with that, the threats to the existence of medicinal plants in nature have also increased. Therefore, the conservation of medicinal plants is unavoidable, and conservation efforts should also be in line with technological developments. One of the conservation plans for medicinal plants is the creation of a scientific database. The scientific database has important roles that are able not only to prevent information loss related to medicinal plants but also to encourage action for conservation and sustainable use. Many databases have been developed

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worldwide. Several database sources are presented in this chapter and have potential use as resource for developing database for medicinal plant conservation.

Keywords Database · Medicinal plants · Conservation · Sustainable use

Abbreviations

3DS	Three-dimensional structure (3DS)
APIs	Application programming interfaces
BGCI	Botanic Gardens Conservation International
CABI	Centre for Agriculture and Biosciences International
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CM	Chinese medicinal
DAFMPEP	Database for African Medicinal Plants
DBMS	Database management systems
DDBMS	Distributed database management system
DNA	Deoxyribonucleic acid
DQP	Distributed query processing
ENCODE	Encyclopedia of DNA Elements
FAO	Food and Agriculture Organization
GBIF	Global Biodiversity Information Facility
IMPPAT	Indian Medicinal Plants, Phytochemistry, and Therapeutics
IPNI	International Plant Names Index
IUCN	International Union for Conservation of Nature
KIPO	Korean Intellectual Property Office
MPDB	Medicinal plant database of Bangladesh
MPNS	Medicinal Plant Names Services
NAPRALERT	Natural PRoducts ALERT
NCBI	National Center for Biotechnology Information
NOAA	National Oceanic and Atmospheric Administration
NSERC	Natural Sciences and Engineering Research Council of Canada
POWO	Plants of the World Online
RAMP	Review of Aromatic and Medicinal Plants
TCGA	The Cancer Genome Atlas
TPL	The Plant List
UNEP-WCMC	The UN Environment Programme World Conservation Monitoring Centre
USDA	US Department of Agriculture
WHO	World Health Organization
WRI	World Resources Institute
ZSL	Zoological Society of London

10.1 Introduction

Humans worldwide have historically relied on biodiversity as a key source of food, shelter, and medicines, as well as for their spiritual and mental health. For thousands of years, medicinal plants have been a vital part of the human healthcare system by serving as an ingredient in traditional medications (Gaikwad 2014). They are commonly utilized as foods, as spices, and in cosmetics, fragrances, and aromatherapy. This position has allowed medicinal plants to play a significant role in human healthcare. According to WHO reports, more than 80% of the world's population still uses medicinal herbs as their major source of treatment (Devkota and Watanabe 2020).

Medicinal plants are an essential link between biodiversity and cultural diversity, comprising a substantial portion of biodiversity. More than 50,000 plants worldwide are estimated potentially to be medicinal plants (Schippmann et al. 2002), and the local communities play a crucial role in the conservation of these species and the preservation of traditional knowledge about their utilization (Marrelli 2021). However, their natural habitats are degraded, overexploited, damaged around the world, and also affected by climate change (Voek 2004; Hawkins 2008; Marrelli 2021), so they face extinction. Many of them are also wild plants that will be destroyed due to overharvesting and illegal trade (Ma 2010). In addition, skill and knowledge regarding medicinal use are often lessened in the younger generation (Hamilton 2004).

There are two fundamental issues that confront the development of global biodiversity knowledge, namely, how to provide reliable data and what is the usefulness of the data collected on biodiversity (Turnhout and Boonman-Berson 2011). Recording biodiversity requires not only an expert's knowledge in the identification of species but also a major investment of both time and financial resources (Turnhout and Boonman-Berson 2011). Biodiversity data are scattered, fragmented, not integrated between databases, and difficult to collect, and decision-makers are rarely able to access them (Stephenson and Stengel 2020). In addition, in terms of medicinal plants, the information on local knowledge on the utilization of medicinal plants is geographically dispersed across various indigenous tribes, institutions, and research organizations, making it difficult to obtain and limiting its utility (Gaikwad 2014).

Databases are an essential part of the study of biodiversity due to the fact that they provide a representation of the existence of biodiversity and information on it, both of which are required before biodiversity can be properly managed and maintained (Turnhout and Boonman-Berson 2011). The revolution in big data that occurred in the last two decades has a significant impact on the study of biodiversity (Heberling et al. 2021). A new discipline, biodiversity informatics, has evolved as a result of the growing integration of information technology and data mining; moreover, biodiversity informatics has developed rapidly, and a large number of open-access databases have arisen (Luo et al. 2021). Presently, diverse databases of medicinal plants must be accessible and provide information on the biodiversity of medicinal plants, particularly for ethnopharmacology studies, which aim to investigate the

potential efficacy, safety, and drug discovery of medicinal plants, for instance, can lead to repurposing (find new target) using docking analysis (Shawky et al. 2020) for the drug designing (Mumtaz et al. 2017).

There are numerous medicinal plant databases with a wide range of contents and representations. The purpose of a database should be not only to provide information for drug discovery and the preservation of traditional knowledge but also to contribute to plant conservation research. Medicinal plant data from various sources, both primary and secondary sources, should be collected into a standardized database, a scientific database, which is easily accessible and utilized by relevant users. This chapter reviews the role of a scientific database in the conservation of medicinal plants.

10.2 Definition of Scientific Database

Database is a computational archive to store and organize data to easily query (ask question to database)/retrieve data; consists of hardware (computer, storage) and software, to organize data in structured records; and allows the discovery to new information (data mining, machine learning, artificial intelligence), while a scientific database is a computerized collection of related data that is organized to be easily accessible for scientific investigation and long-term stewardship. Scientific databases allow the use of disparate data in new ways and integrate data even across disciplines (Synge and Heywood 1988).

Scientific research using information technology continues to increase in recent years. Databases are a means to obtain, collect, and investigate large amounts of data. This amount of data will not be easily accessed without the help of a computer (Hine 2006). Scientists have adapted to conducting research using data in different numbers, varieties, and distributions, especially for purposes that require an interdisciplinary approach. Unprecedented data exchange has now become necessary to support integrative analysis. This integration can be facilitated by scientific databases.

The advantages of developing and using scientific databases, among others, are that one of the databases will lead to an increase in the overall quality of the data. Database users will be so large that users could detect and fix problems in the data. Another advantage is related to costs. Data is generally cheaper to store than to recollect (Synge and Heywood 1988).

The collection of data and samples is the basis of a scientific database, where the dataset is a collection of samples, and each sample consists of an observation section, which describes certain scientific facts or events, and a metadata section, which represents the general nature of the sample. The structure (or schema) of the database is identical. Samples are associated with rich metadata, which describes the process of their creation, whereas metadata is used in scientific calculations only for initial data selection.

There are three kinds of data in the database (Lenz 1993), namely, microdata, macrodata, and metadata. Microdata is primary data on individuals, objects, or events that represent sample, census, or collection data. Macrodata is grouped or aggregated data that is cross-classified by a set of variable attributes, whereas metadata describes micro- and macrodata at semantic, structural, statistical, and physical levels so that they can be stored, modified, retrieved, and transmitted reasonably. Metadata describes the data life cycle, namely, collecting data from data sources, storing, processing and retrieving, and disseminating data in electronic data interchange (EDI), so metadata must be easily accessible.

Metadata is important to use in the development of concise data models for scientific visualization because metadata can capture syntactic and semantic information about data, structure, attributes, origins, and processing history. Therefore, metadata provides scientists with the additional high-level details needed for accurate data analysis. In other words, metadata describes data about data, from defining data structures to codifying knowledge about data. Any form of setting context for primary data can be considered metadata.

There are several different terms for metadata variants, each of which has a specific purpose. For example, structural purposes, or data characteristics, would describe the types and structures of data, primarily for use by access mechanisms for data storage and retrieval purposes. This information is the database schema which is usually defined by the developer. This can include information about primitive data types (e.g., float, integer) and structural information such as dimensions, ratings, positions, and connections. Additional data or information attributes describe the relationship between the data and the things it represents. The scientist will provide information including the name, unit, and range of the data fields, the spatial location of the data, calibration and instrumentation information, error statistics, and missing data indicators.

To identify facts about the data and their processing history, scientists use documentation metadata. Documentation metadata is usually textual which includes lab notes and text about a simulation or observation, a history of data processing, and scripts for future processing sessions. Additional information and documentation metadata are semantic, describing information associated with individual datasets rather than syntactic information associated with structural metadata.

Another purpose of metadata is semantic metadata which is domain and application specific, usually provided by scientists. The importance of metadata lies in its ability to help scientists understand the meaning and importance of data. Metadata provides scientists with the information to browse and search datasets and select data of interest. Dataset visualizations can even be thought of as metadata, which provides scientists with a mechanism for visual tracing. Semantic metadata allows scientists to express information about datasets in familiar and consistent terms. As a result, scientists can query their datasets more naturally and intuitively (Bergeron et al. 2005). The real feature of scientific databases is the ease with which the analysis of underlying hypotheses, scientific statements, rules, or implications (call it what you might) can be derived from the observed data (Pfaltz 2007).

Scientific database's settings are very different. Organizations in the fields of science, such as astronomy and biology, mostly carry out extensive and well-organized data storage for public use. Scientific data sources are data collection containers that involve many samples or various experimental conditions. Usually, this data is stored as a file and not in database form. Generally, samples are described with metadata, namely descriptive information about each sample's content and production process. Metadata helps select relevant experiment data using the user interface. The source exposes an API (application programming interface) or WEB interface, then the metadata associated with each sample will support data capture. As a result, data scientists need to select the best datasets and samples in sources after analyzing the metadata, but typically they do not further use them; in analyses, they retrieve and use scripting languages to query and analyze them (Pinoli et al. 2018).

Distributed database technology is a combination of distributed technology and database technology. A distributed database is conceptually a collection of data that is physically dispersed between nodes of a computer network and is logically linked to the same system. Its two main aspects are data synchronization and dissemination. In this system, node autonomy is more important than data centralization. The system must also be able to maintain the accessibility of data distribution so that during the programming process, data distribution is not considered (Pingzhong and Yongzhong 2014).

General-purpose database management systems are not used by most research databases. The fundamental problem that must be considered is the data structure and usage patterns that are not compatible with traditional database management systems. A distributed database management system (DDBMS) uses two or more database management systems to distribute data in a distributed system that appears as a single database to applications or users. Application tasks are distributed among the database and computers on the network using distributed query processing (DQP) technology. DDBMS technology is mainly concerned with data distribution. The basic purpose of DQP technology is to distribute computing processes. DQP is used in distributed database applications to access local and remote data in DDBMS, in addition to being used to edit global databases (Xiang 2013).

This chapter focuses on medicinal plants that have been catalogued for conservation. The databases that collect data in various contexts will provide information. All information has meaning and contributes to the growth of knowledge, insight, and wisdom. All data will generate information in accordance with the FAIR (findable, accessible, interoperable, and reusable) concept (Wilkinson et al. 2016).

10.3 Global Biodiversity Database Resources for Conservation

Biodiversity data in the world is spread across several organizations in the world, both managed by the private sector and managed by the national authorities of a country. Amount data was created and is maintained for quite specific purposes. These data include several things that are actually related to each other and often complement each other. Due to the ego of each data management institution, the data is fragmented and less than optimal. In addition, to obtain such data is not easy, especially for researchers and scientists from developing or underdeveloped countries. This could be due to the limitations of each country's policies or due to a technological gap between the countries that own the data and the countries that own the data sources. Most of the data is managed by modern industrialized countries spread across Europe or America, although the sources of the data are from developing countries. Stephenson and Stengel (2020) said that global databases serve as repositories for already-collected data and access to this information can aid in monitoring a project's progress. However, many conservationists are unable to locate or access these data.

Access to the best available biodiversity data, such as data on the status of endangered species or critical habitats (including biodiversity hotspots and protected areas), is critical for successful conservation and for any stakeholders who intend to ensure that development decisions consider potential impacts on biodiversity and the environment (Rodrigues et al. 2006). To date, very little has been done to identify and disseminate the sources of biodiversity data that are currently available. These have not specifically targeted data useful for monitoring, instead of concentrating on a single topic, like threats, or a single geographic area, like Europe. Integrating biodiversity data into decision-making and contributing to halting biodiversity loss would be able to conduct if the conservation and scientific communities made a greater effort to promote data sources and make the data freely and transparently available to those who need it the most (Stephenson and Stengel 2020). Meanwhile, to access the biodiversity health status of a species including plant, the IUCN Red List helps to provide all data needed and collected from worldwide, such as population, habitat and ecology, use, threats, and kind of conservation actions to decide (IUCN 2021).

These days, new techniques for information dissemination have been complemented by the use of electronic databases. The use of information technology has spurred this revolutionary shift in scientific research methodology. An effective method of managing data can be found in a computerized database. Researchers, planners, and other users can access coherent information, thanks to computerized databases that simplify data administration and analysis. Current tendencies toward online, open-access, and fully XML-enabled scientific publications are beginning to fully leverage the web's capabilities (Gjengedal 2008).

There is a wealth of information, but it is shared and presented in many ways. The IUCN/GWC study identified 145 sources of global data and 26 different types of

publications that regularly compile such data, highlighting the most helpful for monitoring. But they encountered a number of issues along the road. Although 86.9% of the data sources made at least some of their data instantly, freely, and accessible, the value of the data and the applicability of the formats were not determined (Stephenson and Stengel 2020).

BirdLife International, the CITES Secretariat, the United Nations Food and Agriculture Organization, the International Union for Conservation of Nature (IUCN), the US National Oceanic and Atmospheric Administration (NOAA), and the United Nations Environment World Conservation Monitoring Centre are some key players in biodiversity data management (Table 10.1).

IUCN, WCMC, WWF, and CITES are some of the well-known organizations that provide a lot of biodiversity data online and are free to use by anyone who needs it. Online information is also available, including those produced by global networks, one of which is the Global Biodiversity Information Facility (GBIF). The goal of GBIF, a global network and data infrastructure supported by governments around the world, is to give anyone, everywhere free access to information about all forms of life on Earth. All of the online biodiversity data that GBIF maintains is quickly becoming geospatially enabled. For organizing and searching map data held by specific conservation or scientific groups, the Conservation GeoPortal offers a metadata pane.

One of the databases that had become a reference for many scientists in the world, especially related to plants, was the web The Plant List (<http://www.theplantlist.org/>). This website was one of the most complete and very helpful for researchers and scientists as well as flora enthusiasts. A working list of all recognized plant species was known as The Plant List. It tries to be thorough for bryophytes and vascular plant species, including flowering plants, conifers, ferns, and their relatives (mosses and liverworts). The Plant List was made possible by cooperation between the Royal Botanic Gardens, Kew, and Missouri Botanical Garden, which combined several checklist datasets owned by these organizations and other contributors. The botanical community created The Plant List (TPL), a working list of all known plant species, in response to Target 1 of the 2002–2010 Global Strategy for Plant Conservation (GSPC). TPL hasn't changed since 2013; however, it served as the foundation for the Taxonomic Backbone of the World Flora Online (WFO), and the most recent data may be obtained there. In response to the 2011–2020 GSPC's amended Target 1: to produce an online flora of all known plants by 2020, a group of top botanical institutions from around the world is creating WFO.

Another important international institution is BGCI (Botanic Gardens Conservation International). BGCI has created a collection of searchable databases for botanic garden and plant conservation professionals. PlantSearch is the only global database of plant taxa in botanic gardens and related institutions, available online at <https://www.bgci.org/resources/bgci-databases/plantsearch/>. Beech et al. (2017) have published a plant dataset whose information comes from many scientists around the world. The total number of tree species currently recognized by science is 60,065, or 20% of all angiosperm and gymnosperm plant species, according to their thorough review of available data sources and expert opinion. Only 10 families

Table 10.1 List of general plant database

Authority	Description	Link
GBIF (Global Biodiversity Information Facility)	The Global Biodiversity Information Facility (GBIF) provides free and open Internet access to biodiversity information. The data includes records of species occurrence and taxonomic information	https://www.gbif.org/
IUCN Conservation Databases	The IUCN is the largest environmental organization in the world, providing databases, species conservation tools, and publications	https://www.iucn.org/
IUCN Red List of Threatened Species	The International Union for Conservation of Nature's Red List of Threatened Species is the world's leading information source since 1964 on the worldwide conservation status of all species, including plant species. It contains data on distribution, population size, habitat and ecology, usage and/or trade, threats, and conservation action to decide	https://www.iucnredlist.org/
UNEP World Conservation Monitoring Centre (UNEP-WCMC)	The UNEP-WCMC is a global center of excellence for biodiversity and nature contribution to society and the economy. Information with authority regarding biodiversity and ecological services. Datasets, instruments, maps, and reports	https://www.unep-wcmc.org/en
WWF International	Global organization for research and action on the environment. WWF covers information on climate change, biodiversity, endangered species, decreasing humanity's influence, and the health of the planet	https://wwf.panda.org/
BioTIME	BioTIME is a free and open resource for education, research, and conservation. Collected information on the species identification and its richness in ecological communities through time are shown in the BioTIME database	Dornelas et al. (2018)

(continued)

Table 10.1 (continued)

Authority	Description	Link
Molina-Venegas et al. (2021)	Full details on the dataset are provided in the latter publication and in the database paper: Molina-Venegas et al. (2021) A global database of plant services for humankind. PLOS ONE	https://figshare.com/articles/dataset/PLANT_USES_PLANT_BOOK_xlsx/13625546/3?file=28249554
USDA (US Department of Agriculture)	The PLANTS USDA Database provides standardized information about almost all the members of plant kingdom of the USA and its territories	https://plants.usda.gov/home
BGCI	GlobalTreeSearch is the first database to document all known tree species and their distributions at the country level. The database is the product of more than 2 years of labor to compile both the names and country-level distributions of tree species	https://www.bgci.org/resources/bgci-databases/globaltreesearch/
CITES	CITES has Trade Database comprising number of specific plants traded and nations exporting and importing data	
IUCN SSC Species Monitoring Specialist Group and Global Wildlife Conservation	There are five tables in the database:	https://www.speciesmonitoring.org/data-sources.html
	Table 1 shows prospective global biodiversity data sources for monitoring the condition of biodiversity	
	Table 2 shows global data sources that might be useful for monitoring biodiversity pressures and threats	
	Table 3 shows prospective global data sources for monitoring conservation responses to biodiversity loss	
	Table 4 shows global data sources with different applications in biodiversity monitoring	
	Table 5 shows reports that synthesize vast amounts of biodiversity data over time	
Kew, the Royal Botanic Gardens, the Harvard University	The International Plant Names Index (IPNI) is a database of	https://www.ipni.org/index.html

(continued)

Table 10.1 (continued)

Authority	Description	Link
Herbaria, and the Australian National Herbarium	names and associated basic bibliographical details of seed plants, ferns, and lycophytes. The data is standardized in taxonomically order with its references	
Board of Trustees of the Royal Botanic Gardens, Kew	Plants of the World Online (POWO) has 1,234,000 world-wide plant names, 140,400 thorough descriptions, and 366,000 photos	https://powo.science.kew.org/
National Library of Medicine, US government	The National Center for Biotechnology Information (NCBI) enhances research and health by making biomedical and genetic data available	https://www.ncbi.nlm.nih.gov/
Canada First; Apogee Canada; Ontario Ministry of Research, Innovation, and Science; Canada Foundation for Innovation; The Natural Sciences and Engineering Research Council of Canada (NSERC); University of Guelph	The Barcode of Life Data Systems (BOLD) is an informatics workbench that aids in the collecting, storage, analysis, and publishing of DNA barcode information. It spans a typical bioinformatics chasm by integrating molecular, morphological, and distributional data. BOLD is free to use for any researcher interested in DNA barcoding. It assists the construction of records that fulfill the requirements required for BARCODE designation in worldwide sequence databases by offering specialized services. It is particularly ideally positioned to assist initiatives involving large research partnerships due to its web-based delivery and flexible data security strategy	http://www.boldsystems.org Ratnasingham and Hebert (2007)

contain over half of all tree species (45%); Leguminosae, Rubiaceae, and Myrtaceae are the 3 families with the greatest diversity of tree species. The nations having the greatest number of tree species are Brazil, Colombia, and Indonesia. The majority of endemic tree species are found in nations, such as Brazil, Australia, and China, or islands where isolation has led to speciation (Madagascar, Papua New Guinea, Indonesia). Almost 58% of all tree species are indigenous to a single nation. GlobalTreeSearch (<http://www.bgi.org/globaltreesearch.php>) provides a comprehensive list of all known tree species, organized by scientific name and distribution

by nation. In order to assess, monitor, and manage the diversity of tree species, forests, and carbon reserves on a global, regional, and/or national scale, GlobalTreeSearch has been developed.

10.4 Scientific Databases of Medicinal Plants

Manzano et al. (2020) analyzed almost 100,000 articles in a bibliometric study of all works indexed in the Scopus database to 2019. On the one hand, many countries, institutions, and writers investigating this issue, as well as their evolution over time, have been recognized. The identification of communities, on the other hand, has been used to evaluate the linkages between the writers, the nations, and the issues under investigation. In terms of study themes, the last two eras, 2009–2014 and 2015–2019, have been investigated and concluded that the trend in worldwide research is more focused on the discovery of novel medicines or active substances than on crop cultivation.

There are already several databases on medicinal plants available for many countries (Table 10.2). Web-based databases further promote knowledge dissemination and feed the information exchange cycle between medicinal plant research and the general population. Despite the creation of several medicinal plant databases, there is still a lack of consistency.

The MPDB 2.0 database (<https://www.medicinalplantbd.com/>) contains a collection of over 500 Bangladeshi medicinal plants, as well as a list of their corresponding scientific, family, and local names, as well as their used parts, information about illnesses, active compounds, and PubMed IDs of related publications. Like MPDB 1.0, the MPDB 2.0 database (plants of 122 families, 381 genera, and 557 species) is a user-friendly interface dedicated to rendering information (including their scientific names, family names, local names, utilized parts, related elements, and active compounds) of plants around Bangladesh that have been suggested to have potent medicinal and/or nutritive properties, for both national and international researchers, health practitioners, drug manufacturers, and students (Hussain et al. 2021).

MPDB 3.1 is a professionally curated database of more than a thousand medicinal plant species in Bangladesh. This database contains information on plants' scientific names, families, growing habits, usable plant parts, and therapeutic applications in Bangladesh (<https://www.natureinfo.com.bd/mpb/database>). This database is useful to the scientific community for a rapid evaluation of plants for medicinal plant research, and it offers huge potential for the production of herbal medications employing ethnopharmacological knowledge (Uddin and Lee 2020).

Traditional Indian medical formulations are multi-component combinations whose therapeutic usage is based on empirical knowledge rather than a theoretical understanding of the mixture's active ingredients. Until recently, traditional Indian medicine knowledge, including essential medicinal plants and their formulations, was buried within texts like *Indian Materia Medica* and *Ayurveda Materia Medica*. The nondigital nature of this information hindered its usefulness in the development

Table 10.2 Other databases of medicinal plants available online

Authority	Description	Link
Journal: Natural Product Research	Database of Medicinal Plants for Drug Design (MPD3). The proposed MPD3 is a massive database that delivers the majority of knowledge on medicinal plants on a single platform	https://www.bioinformation.info/ Mumtaz et al. (2017)
The Hong Kong Polytechnic University	Chinese herbal medicine database. This database offers information on over 400 different varieties of regularly used Chinese medicinal (CM) herbs in Hong Kong. It includes images as well as a summary of the CM herbs' origin, fundamental nature, activity, application, and safety	https://herbaltcm.sn.polyu.edu.hk/
Royal Museum for Central Africa	Prelude database of medicinal plants. A database of traditional animal and human medicinal herbs used in sub-Saharan Africa The purpose of the PRELUDE data bank is to summarize the use of traditional medicinal plants in traditional human medicine and traditional veterinary care from about 1000 research articles	http://www.africamuseum.be/collections/external/prelude
Kew Royal Botanic Garden	Medicinal Plant Names Services (MPNS) is a global nomenclatural indexing and reference resource that allows health professionals and researchers to obtain data on plants and plant products related to pharmacological research, health regulation, traditional medicine, and functional foods	https://www.kew.org/science/our-science/science-services/medicinal-plant-names-services
American Botanical Council	HerbMed [®] is an interactive, electronic herbal database that gives hyperlinked access to the scientific research that supports the use of herbs for health. It is a source of evidence-based data for professionals, researchers, and the general public	https://www.herbalgram.org/resources/herbmedpro/
CABI (Centre for Agriculture and Biosciences International)	The Review of Aromatic and Medicinal Plants (RAMP) is a fully searchable abstracts library of worldwide published research	https://www.cabi.org/publishing-products/review-of-aromatic-and-medicinal-plants/

(continued)

Table 10.2 (continued)

Authority	Description	Link
	on culinary herb, spice, essential oil, and medicinal plant species, both cultivated and wild	
Beilstein-Institut zur Förderung der Chemischen Wissenschaften; Universität Passau	MedPhyt is a database of medicinal plants that have been confirmed to have pharmacological uses. The plant is the focus of the database content, with descriptions of its botanical features, history of medical application discovery, etymology, and synonyms. Aside from botanical characterization, there includes information on both medically relevant biochemical substances and their physical properties, as well as toxicological and pharmacological data	https://www.worldcat.org/title/medphyt-the-medicinal-plants-database/oclc/81006810 http://www.beilstein-institut.de/englisch/1024.htm
Muhimbili University College of Health Sciences	Database for African Medicinal Plants (DAFMEDP) can be requested through its web by the potential user. It has Tanzania medicinal plants database as well	http://hdl.handle.net/10625/32940
USDA	Phytochemical and ethnobotanical databases maintained by Dr. Duke. Ethnobotanical use and chemical activity in plants database	https://phytochem.nal.usda.gov/phytochem/search
University of Illinois Chicago	Natural PRoducts ALERT or NAPRALERT is a database system comprising ethnomedical data and therapeutic information regarding organism extracts from both non-clinical and clinical research. Also, secondary metabolites derived from natural sources are provided	https://pcrps.pharmacy.uic.edu/our-centers/uic-nih-center-for-botanical-dietary-supplements-research/napralert-database/
Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License	The useful tropical plants database offers information on the edible, medicinal, and several other applications of thousands of tropical plants. There are now 12,727 species listed in the database	https://tropical.theferns.info/
CUTS Centre for International Trade, Economics and Environment	Medicinal plants in the Himalayan states	www.cuts-international.org
Manosé Research Center		

(continued)

Table 10.2 (continued)

Authority	Description	Link
	The Thai medicinal plant recipe database “MANOSROI III” gathers Thai medicinal plant recipes from various districts of Thailand	https://www.manose.co/portfolio-item/work-2/
National Drug Information Center	The Philippine Herbs and Supplements Research Database is a library of research on Philippine medicinal plants and researches on health ingredients used in the nation. The site is organized into several sections, including a research databank, research tools, monographs and advisories, books and reviews, and links	http://www.tkdiph.com/
Korean Intellectual Property Office (KIPO)	The database was created between 2005 and 2007 and was focused on traditional Korean medicine. In December 2007, a database search function was launched	https://www.koreantk.com/ktkp2014/medicine/list-by-act-part.page
	KIPO’s traditional knowledge database contains a significant quantity of information derived from Korean traditional literature and research studies	
Annie’s Remedy Herbs for Self Healing	The web offers information on many traditional medicinal herbs and plants, but it represents just a small portion of the natural plants utilized for healing across the world. The emphasis is primarily on Western herbs; however, there includes a selection of the most prominent plants from Chinese and Ayurvedic herbal traditions, as well as other countries. The herb profiles include images, descriptions, medical applications, folklore, and references from today’s and ancient herbalists	https://www.anniesremedy.com/
Botanical.com, Mrs. Grieve	Botanical.com contains approximately 800 species of herbs and plants, as well as all related information like their botany, medicinal use, and cultivation. It may be searched using the	http://www.botanical.com/botanical/mgmh/mgmh.html

(continued)

Table 10.2 (continued)

Authority	Description	Link
	following terms: Plant and Herb Index (more than 800 types of herbs and plants), a word search of the 860 page content, a listing of 29 plants with recipes, and a listing of 44 toxic plants	
Henriette Kress, a herbalist in Helsinki, Finland	Henriette's herbal website is among the web's earliest and most comprehensive herbal information sites	http://www.henriettes-herb.com/

of novel drugs. As a result, digitizing this information and compiling it into a comprehensive database on Indian medicinal plants, phytochemistry, and therapeutics would allow researchers to use computational methods to pharmaceutical research (Mohanraj et al. 2018).

IMPPAT is a professionally curated database comprising thousands of Indian medicinal plants, phytochemicals, and therapeutic applications with a total of more than 22,000 plant-phytochemical associations and 11,514 plant-therapeutic linkages. There are almost a thousand about 960 possible drug candidate compounds within IMPPAT, the majority of which had no substantial similarity to existing FDA-authorized medications, making them the best candidates for future pharmaceuticals. The IMPPAT database is publicly available at <https://cb.imsc.res.in/imppat> (Mohanraj et al. 2018; Vivek-Ananth et al. 2022).

10.5 Databases for Medicinal Plant Conservation

Advances in technology today greatly affect various aspects of life, one of which is from the world of health. At this time, people in the world are fond of alternative medicine. Basically, alternative medicine that exists today is the same as traditional medicine, because the ingredients of the medicines are made based on recipes that have been around for a long time in the community. Even the components used in this ancient medicinal herb preparation are readily available in our area. People have used medicinal plants for ages and may possibly be regarded the basis of modern medicine. Plant-derived compounds have been and continue to be a key source of medicinal molecules. However, as time passes, more and more medicinal plants are becoming extinct in our environment, and younger generation does not have knowledge regarding medicinal plant and its tradition. As a result, the presences of knowledge on medicinal plants in database form are crucially required by the community.

10.5.1 Developing a New Database for Medicinal Plant Conservation

The existing medicinal plants database mostly have important information including local names, standardized botanical information (such as scientific name, family names, and genus names), plant habitats, plant distributions, plant photographs, and the uses. An increasingly modern era and increasingly sophisticated technology make medicines now easier to make by many drug companies as well as by traditional communities. The level of public demand for consuming modern medicines is also increasing. This is because modern medicines are faster and more practical to obtain. Knowledge about the benefits, efficacy of traditional medicinal plants that are still lacking makes the community's seeming lack of concern and interest in utilizing plants as medicine. Based on these problems, a solution is needed by creating a traditional medicinal plant information system based on information technology applications. The traditional medicinal plant database mostly had the same data with common medicinal plants, but they include the information about ethnic groups and the used by the ethnic group. Since the medicinal plants had many functions especially for maintaining health but lack of their localities information, it is important to know the occurrence of the plant. It is important to conserve the medicinal plants.

The database for conservation mostly have specific information including scientific name, plant habitats, plant photographs, plant distribution, red listed in IUCN, and biodiversity information. To develop the medicinal plants for conservation, the existing database of medicinal plant or traditional medicine plant database must include additional data that is needed for conservation. The additional data of the medicinal plants might consist of CITES Appendices, plant distribution, red listed in IUCN, and related legislation (Fig. 10.1).

In studying and searching for data regarding medicinal plants only through books, it is not easy practices as books are also easily damaged and lost. This can hinder and harm and make people no longer interested in studying and using herbal plants. Moreover, knowledge of herbal medicines is also only based on recipes or teachings from parents who sometimes have not proven the truth of the benefits of the medicinal plants used clinically. The younger generation today has difficulty learning about medicinal plants because medicinal plant gardens are becoming increasingly rare. In addition, the current generation prefers to use technology such as to play with smartphones or other similar gadgets. To facilitate information to the public and provide interesting learning related to medicinal plants using appropriate technology, some researchers have tried to create a web-based information system.

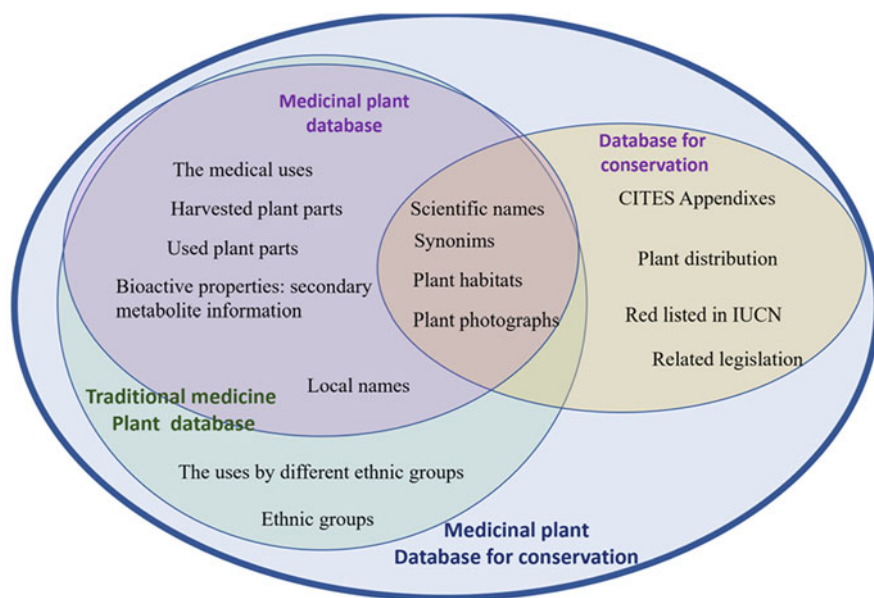


Fig. 10.1 Diagram of setting medicinal plant database for conservation

10.5.2 Challenges in Developing a New Database for Medicinal Plant Conservation

A new database for medicinal plant conservation must overcome challenges in merging all relevant and accessible information and data from multiple databases. Database merging is the process of physically or virtually integrating the data provided by multiple databases (Cholvy and Moral 2001). Merging databases for developing a new database for medicinal plant conservation is more than just merging plant names, and it may be difficult to do since relevant expertise is required, which involves time and cost. The requirement for cutting-edge data gathering and storage technologies are essential, so that stored data can be linked to other databases and safely stored in the cloud. However, if the merging process is managed correctly and all data used is standardized, the resulting database will be more organized and structured.

There are at least three obstacles in merging data, namely, language, inconsistencies between combined information, and validity (Cholvy 1998). In a database for medicinal plants, for example, language obstacles may arise from human error such as typos and different plant names entered in the database (Cahyaningsih et al. 2021a) depending on the source of the database and the technology of the database resource. Data from multiple database management systems with varying operational compatibility are kept in databases. This chasm will cause issues with data retrieval. During this stage, typos and duplicates should be discovered, while

inconsistencies between combined information and validity may arise from taxonomy and classification of stored plant names in new database, for example, scientific name, which is the Latin name from several sources such as POWO and GBIF that may differ from one another. One species may be accepted in one database resource, but it may be considered a synonym of another species or homonym, resulting in information and data about medicinal plants that differ (Allkin and Patmore 2018). A local name may also relate to some scientific names and vice versa. This plurality causes taxonomic errors in data of multiple types, each with its own set of standards, which can have an influence on data integrity. Standardization of what is the most appropriate taxonomic name and what local name to use, also should be decided during this stage.

10.5.3 Role of Scientific Databases for Conservation: Case of Indonesian Medicinal Plant Database

Indonesia, like numerous other nations with vast forest regions, has a richness of medicinal plants, but the majority of the information is still fragmented throughout several written records and even oral traditions. K. Heyne released four volumes of literature on the curative and preventative applications of plant material from Indonesia titled *De nuttige planten van Naderlansch-Indie* in the early twentieth century (1913–1922) (Hirschhorn 1983). Van Steenis-Kruseman released *Select Indonesian medicinal plants* in 1953, which was mostly based on historical resources (Elliott and Brimacombe 1987). In recent years, prestigious institutions all around the world have conducted extensive research on Indonesian medicinal herbs (Mitra et al. 2007).

Traditional herbal medicine of Indonesian people that has been performed for years is still commonly used for stamina maintenance and illness treatment since it is seen to be safer than chemical medications. Human health issues have been a source of worry since antiquity. This may be found in the depictions of plants and animals as sources of traditional remedies in temples, literature, and inscriptions. Indigenous science for medicinal plants is well-known to the people in China, India, and Brazil for millennia, and Ethiopia is incorporated in many elements of culture. Drinking traditional herbal medicine is still a common custom in various countries in Asia, Latin America, and Africa, including Indonesia and India (Sumarni et al. 2019).

There have been many studies related to ethnomedicine and medicinal plants, but the results are scattered, so it is necessary to think about building a national database portal for traditional medicinal herbs from Indonesia. With the availability of a national database containing knowledge of ethnopharmacology of traditional medicinal herbs, and the diversity of medicinal plants, as well as their utilization based on local wisdom, it will add to the repertoire of knowledge of Indonesian medicinal plants. This will increase confidence in the fact that Indonesia is one of the countries with high mega-biodiversity wealth and the ability to utilize medicinal plants since

ancient times. Various web databases of medicinal plants display detailed medicinal plant data in the form of plant names, scientific names, regional names of medicinal plants, descriptions of medicinal plants in the form of physical characteristics of medicinal plants, uses and benefits of medicinal plants, and also diseases that can be cured with these medicinal plants. All of this is very important knowledge for all levels of the general public as well as the drug and health industry in the present and future.

The database of Indonesian medicinal plants is still very limited. Some databases have been tried to be created by institutions, ministries, and universities. For example, IPB University has created a database of linkages between medicinal plants and their active ingredients, which can be traced at <http://ijah.apps.cs.ipb.ac.id/#/home>. Indonesian Herbal Herbs Analytics or IJAH Analytics is a website that provides prediction about herbal formulas that might be suitable for diseases. Likewise, in the University of Indonesia (UI), the database of Indonesian Medicinal Plant Active Compound provides three-dimensional structure (3DS) of chemical compounds. The database will be prepared by using MySQL format and is designed to be placed in <http://herbaldb.farmasi.ui.ac.id> website so that eventually this database can be accessed quickly and easily by users via the Internet (Yanuar et al. 2011). In addition, there is HerbalDB, a database of computational 3DS comprising almost 1500 of compounds of Indonesian herbal developed (Syahdi et al. 2019) and medicinal plants database on android basis (Putri et al. 2014).

Generally speaking, Cahyaningsih et al.'s studies regarding conservation planning have determined the priority of medicinal plants of Indonesia (2021a) and what conservation action for the short term and long term is required to conserve those plants not only in situ conservation but also ex situ conservation through gap analysis and climate change impact assessment (2021b, c). Those studies were desk study and mostly used some database online. However, before prioritizing the list and identifying what conservation planning needs to be done, setting the medicinal plant checklist was done with medicinal plant's name collecting and typing (digitating) them including any related information in it as most of them are in book form and scattered. They use data regarding origin, distribution, harvested part, threat status (based on IUCN Red List), and legislation related to each medicinal plant that are mostly available online from the global biodiversity database for conservation and database of medicinal plants.

10.6 Conclusion

The scientific database is useful for medicinal plants' conservation and may provide very essential foundation for further studies. Many database systems are created by institutions and provide different information. When they are merged or linked and combined, the information can complete to each other. Thus, it would be more useful for all related stakeholders who need it, such as herbalist, conversionist, industry, and government, to support conservation and sustainable use.

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Chapter 11

International Trade of Medicinal and Aromatic Plants (MAPs)



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Abstract Medicinal and aromatic plants (MAPs) have been used since ancient times for human health care and play an important role in the economic, social, cultural, and ecological aspects of local communities around the world. The MAPs are traded from the local market to regional, national, and international levels. Various commodities in trade consist of mainly dried plant parts, roots, leaves, bark, wood, flowers, or seeds or, sometimes, of several plant parts or even the whole plant. Several challenges were identified regarding the management of MAPs such as ambiguous policies, lack of resources, information, infrastructure, habitat degradation, and overexploitation.

Keywords Medicinal and aromatic plants · Trade of medicinal plant

11.1 Introduction

Medicinal and aromatic plants (MAPs) are plants containing aromatic compounds (Planta Europa 2010; Marshall 2011), which are used as raw materials for therapeutic purposes, food (Schippmann et al. 2006; Marshall 2011), and cosmetics

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(Schippmann et al. 2006). Mafimisebi et al. (2013) referred to MAPs as herbal, medicinal, and aromatic plants (HMAPs), namely sources of materials used in alternative medicine, new drugs, and other products used for health care, while Slikkerveer (2006) referred to them as medicinal, aromatic, and cosmetic (MAC) plants. In this study, MAC plants and HMAPs are combined into a single unit and hereinafter referred to as MAPs.

MAPs have been an important resource for human health care from prehistoric times to the present (Leamann 2006). The World Health Organization (WHO) estimates that more than 80% of the world's population fulfils their primary health-care needs through traditional medicine (Kalaichelvi and Swaminathan 2009), which mostly comes from plants. In various industries, chemical compounds from MAPs are also used as a source of dye and plant protection (Lubbe and Verpoorte 2011). MAPs are also used by humans as materials for toiletries, body care, incense, and ritual healing. Therefore, MAPs play an important role in the economic, social, cultural, and ecological aspects of local communities around the world. Various MAPs' products have been commercialized from raw materials to processed and packaged products such as medicines, herbal medicines, teas, alcoholic beverages, cosmetics, sweets, dietary supplements, varnishes, and insecticides (Lange et al. 2006).

The facts show that the demand for MAPs for household and industrial purposes continues to increase, resulting in trade at the local, regional, national, and international levels (Lange 2002; Lange et al. 2006; Barata et al. 2016; Vasisht et al. 2016). This is related to the fact that natural products derived from MAPs have fewer side effects than their synthetic counterparts (Lange 2002; Saeed and Samad 2017). The trade in MAPs not only serves to distribute various medicinal raw materials but also provides jobs and helps the economy. The increased use of MAPs internationally has resulted in the exchange of money, creating employment and income opportunities, increased food security, alleviated poverty, and increased regional development (Saeed and Samad 2017).

An estimated 400,000 tonnes of MAPs are traded annually, and this demand is likely to increase, but to date, more than 70% of crops are harvested from the wild (Saeed and Samad 2017). The estimated annual growth of the global MAPs' market is around 10–20% and is expected to reach \$5 trillion (Saeed and Samad 2017) to 7 trillion (Rathore and Mathur 2018) by 2050 (Saeed and Samad 2017; Rathore and Mathur 2018), but around 80% of imports and exports of MAPs in the world are dominated by 12 countries, mainly from Europe and Temperate Asia (Lange 2002). China is a major exporter of medicinal plants, accounting for 15% of the total exports of medicinal plants globally. India ranks second globally in exports contributing only 0.5% (Rathore and Mathur 2018). MAPs which are used by the industry in the form of extracts, phytopharmaceuticals, nutraceuticals, and cosmeceuticals are expected to grow faster than conventional drugs (Vasisht et al. 2016).

11.2 The History of Medicinal and Aromatic Plants' (MAPs') Trade

The beginning of MAPs' trade is difficult to estimate accurately because much of the local trade is either unrecorded or poorly classified. However, MAPs have been the historical mainstay of traditional health-care practices across major societies and are still used today by 75–80% of the world population for their basic health-care needs (Majaz and Khurshid 2016). Archaeological studies have shown that the practice of herbal medicine happened 2900 BC years ago in Egypt, 60,000 years ago in Iraq, and 8000 years ago in China (Pan et al. 2014; Majaz and Khurshid 2016). According to the World Health Organization, 60% (WHO 2015) to 80% (Kalaichelvi and Swaminathan 2009) of the world's populations are using traditional medicine for their primary health care and in some countries MAPs are extensively incorporated into the public health system (WHO 2015). All of the above information leads to the reason why the MAPs' trade has been running from ancient times to the present.

MAPs are offered in a wide variety of products on the market. The enormous demand for botanicals results in a huge trade from local to international level. In the 1990s, the reported annual worldwide importation of pharmaceutical plants amounted on average to 400,000 tonnes valued at USD 1224 million. The international trade is dominated by only few countries. For example, Japan and the Republic of Korea are the main consumers of pharmaceutical plants, and China and India are the world's leading producing nations; Hong Kong, the USA, and Germany stand out as important trade centres (Lange 2004).

Talking about MAPs certainly cannot be separated from “spices” which are medicinal and aromatic plants sourced from the archipelago of Indonesia. In fact, since 1224 BC, one species belonging to the medicinal and aromatic plant has been used to preserve the body of Pharaoh Ramses II by stuffing the Pharaoh's nostrils with peppercorns (Royyani et al. 2021). The history of spices is as old as human life. In the Middle Ages, Europeans looked for spices in the Indonesian archipelago and traded them on the European market. The spice trade routes involved the archipelago and other nations in one of the ancient trade routes, namely the spice route (Negeriempah.org 2022). Royyani et al. (2021) have given a detailed account of the history of spice trade since traces of olive oil, coriander seeds, and black pepper were detected in the ruins of the former headquarters of Roman Army (XI–XII AD). The silk route or land route of spice traded from the archipelago around indicated Damascus as the centre of trade. MAPs have been used by the Aryans since 1700 BC. The Malacca trade route, used by the Malays, could be traced through China, Malacca, India, and Sri Lanka, and China imported cloves from the Moluccas Islands. Spice was considered an aphrodisiac in Europe, and a Pepper Union was founded in England. The Portuguese started voyages to find sources of spices since 1453, arriving in Indian and Malaccan ports.

11.3 The MAPs' Trade Today

MAPs are traded from the local market to national and international levels (Barata et al. 2016). It is difficult to assess how many MAPs are commercially traded, on either a national or even an international level (Schippmann et al. 2002). Although detailed data are lacking, available information indicates that the world market for MAPs continues to grow. An estimated number of 70,000 plant species are used in folk medicine worldwide (Farnsworth and Soejarto 1991). The trading of MAPs in 1991–2003, recorded 320,550 tonnes (import) and 368,100 tonnes (export) for 12 leading countries (Lange 2006). The country-wise import and export of MAP materials of the top 12 countries (based on average trade volumes during 1991–2003) are represented in Figs. 11.1 and 11.2, respectively, based on the data given by Lange et al. (2006) which was collected from COMTRADE database, United Nations Statistics Division, New York. According to Fig. 11.2, Hong Kong is the leading importer of MAP materials with a yearly average import of approximately 59,950 tonnes with a value of 2.63 billion US\$, followed by the USA (51,200 tonnes equivalent to 1.39 billion US\$), Japan (46,450 tonnes with a value of 1.31 billion US\$), Germany (44,750 tonnes with a value of 1.04 billion US\$), and the Republic of Korea (33,500 tonnes with a value of 49 million US\$).

In case of global export of MAP materials, China is the leading country with average yearly exported quantity of 150,600 tonnes during 1991–2003, which represent more than 33% of the total global exportation (Lange 2006; Fig. 11.2). Hong Kong is the second leading nation with 55,000 tonnes annual export of MAP materials, followed by India (40,400 tonnes of a value of 61.7 million US\$), Mexico (37,600 tonnes with a value of 14.3 million US\$), Germany (15,100 tonnes with a

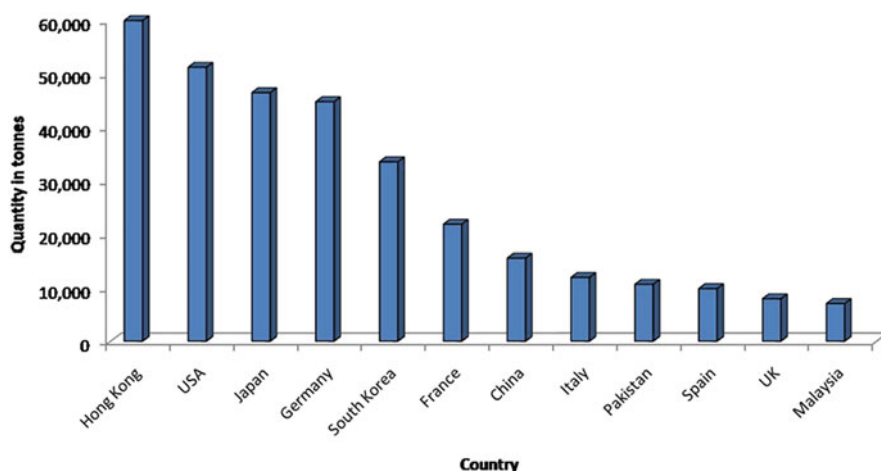


Fig. 11.1 The average import trade volumes of MAPs of the top 12 countries during 1991–2003 based on the data given by Lange et al. (2006) which was collected from COMTRADE database, United Nations Statistics Division, New York

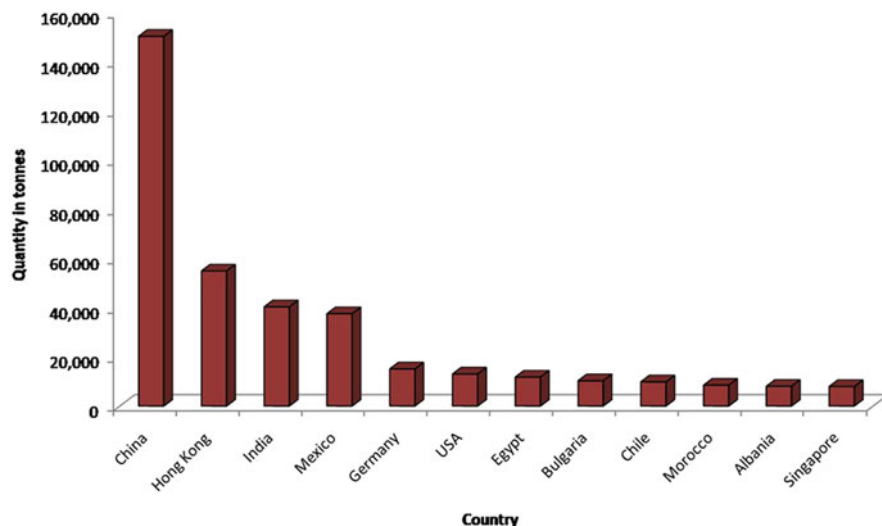


Fig. 11.2 The average export trade volumes of MAPs of the top 12 countries during 1991–2003 based on the data given by Lange et al. (2006) which was collected from COMTRADE database, United Nations Statistics Division, New York

value of 68.2 million US\$), and the USA (13,050 tonnes with a value of 104. five million US\$) (Fig. 11.2). The total export values of MAP materials in China and Hong Kong are 2.66 and 2.01 billion US\$, respectively (Lange 2006).

According to the International Trade Centre, world trade in oil seeds and oleaginous fruits; miscellaneous grains, seeds and fruit; industrial or medicinal plants; and straw and fodder was of the magnitude of US\$ 104,557,469 (US dollar thousand), on an average, based on import statistics for the period 2016–2020 (<https://intracen.org/resources/data-and-analysis/trade-statistics>, retrieved April 3rd, 2022). The global import and export values of MAPs were US\$ 94,579,786 (US dollar thousand) and US\$ 89,709,665 (US dollar thousand), respectively, in 2016 which increased to US\$ 114,128,966 (US dollar thousand) for import and US\$ 109,712,163 (US dollar thousand) for export in 2020 (<https://intracen.org/resources/data-and-analysis/trade-statistics>, retrieved April 3rd, 2022). The trade generally indicated an upward trend, except for 2019, probably due to the COVID-19 pandemic. China and India are the two major international players in Asia (Astutik et al. 2019). America was the leading exporter of MAPs in 2016 to 2020 (Fig. 11.3) whereas Asia was the leading importer (Fig. 11.4) during this period.

It is estimated that around 50,000–70,000 plant species have been used in traditional and modern medicine systems worldwide (Leamann 2006), but only about 3000 species are traded internationally (Saeed and Samad 2017), or about 4.3–6.3%. Various factors are suspected to be obstacles to MAPs' trade, including raw materials that have not been standardized and different policies/regulations for each country. It is estimated that only 5 to 15% of the 250,000–500,000 species of higher plants have been investigated for the presence of bioactive compounds

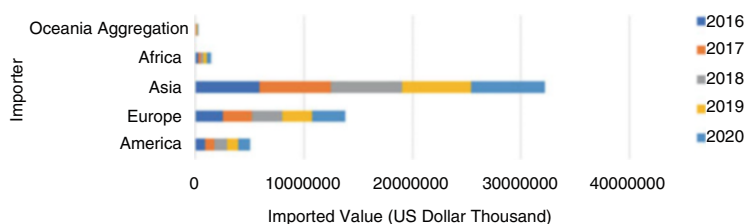


Fig. 11.3 Leading importers of MAPs from 2016 to 2020

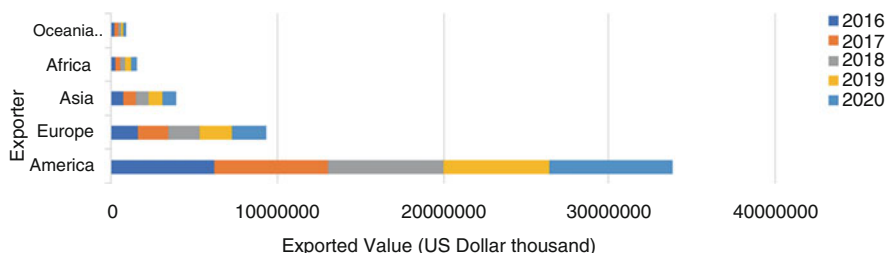


Fig. 11.4 Leading exporters of MAPs from 2016 to 2020

(Kalaichelvi and Swaminathan 2009), which directly or indirectly affects public confidence in their efficacy and bioactivity. On the other hand, comprehensive documentation between institutions and countries has not been found. However, it is suspected that the number of MAPs traded is increasing in line with the increasing number of researchers on local wisdom, bioassays, and their content of bioactive compounds, but comprehensive documentation has not been found.

The number of MAPs that have been used as medicine and for other functions varies greatly from one country to another in type and amount. This is thought to be related to the biodiversity of each country. For example, China is reported to have used about 5000 species, while India used 7000 species of medicinal plants in traditional medicine, which includes Ayurvedic medicine (1773 plant species), Siddha (1122 plant species), and Unani (751 plant species) (Kalaichelvi and Swaminathan 2009). It is estimated that on average 17% of the plants in each country have medicinal properties (Schippmann et al. 2006). According to Schippmann et al. (2006), China (4941), India (3000), and the USA (2564) are the three countries having highest number of medicinal plant species, followed by Thailand and Vietnam; both of them have 1800 plant species. Approximately 34.5% and 30.3% of the total flora of the Republic of Korea and Pakistan have medicinal properties, whereas 21% of the total flora of Bulgaria are used in therapeutics (Schippmann et al. 2006). According to this report, China has 15.3%, and India has 16.1% medicinal plants.

In addition to differing in the number of species that function as MAPS, the types of MAPs that are useful for serious diseases such as cancer are very limited in distribution. Several FDA (Food and Drug Administration) approved drugs are commercially produced from different medicinal plants (Table 11.1). These

Table 11.1 Plant-derived FDA-approved commercial drugs

Drug name (year of FDA approval)	Source of active photochemical	Therapeutic importance	Mode of action	Reference
Paclitaxel (1992)	Bark of <i>Taxus brevifolia</i>	Breast, ovarian, lung, pancreatic cancer	Disruption of microtubule dynamics	Gallego-lara et al. (2020)
Colchicine (2009)	Blub of <i>Colchicum autumnale</i>	Familial Mediterranean fever, acute gout flares	Tubulin disruption, inhibition of macrophage and inflammation	Yang (2010)
Vincristine (1963)	Leaf of <i>Catharanthus roseus</i>	Leukaemia	Inhibits the formation of microtubules and interferes with nucleic acid and protein synthesis by blocking glutamic acid utilization	Martino et al. (2018)
Vinblastine (1965)	Leaf of <i>Catharanthus roseus</i>	Lymphoma, choriocarcinoma, breast cancer	Inhibits microtubule formation resulting in cell cycle arrest	Martino et al. (2018)
Etoposide-semisynthetic analogues of podophyllotoxin (1983)	Rhizome of <i>Podophyllum peltatum</i> L.	Testes, lung	Inhibits type II DNA topoisomerase complex	Montecucco et al. (2015)
Temposide-semisynthetic analogues of podophyllotoxin (1990)	Rhizome of <i>Podophyllum peltatum</i> L.	Leukaemia	Inhibits type II DNA topoisomerase complex	Hirschfeld et al. (2003)
Camptothecin (1996)	Bark and stem of <i>Camptotheca acuminata</i>	Gastrointestinal, ovarian cancer	Inhibits DNA re-ligation through interaction with topoisomerase-type I DNA complex causing DNA damage	Li et al. (2017)
Rivastigmine	<i>Physostigma venenosum</i>	Alzheimer's disease	Inhibits acetylcholinesterase in the cortex and hippocampal region	Polinsky (1998)
Galantamine	Bulbs and flowers of <i>Lycoris radiata</i> , <i>Galanthus nivalis</i>	Alzheimer's disease	Reversible inhibition of acetylcholinesterase and allosteric potentiation of nicotinic acetylcholine receptors	Razay and Wilcock (2008)

(continued)

Table 11.1 (continued)

Drug name (year of FDA approval)	Source of active photochemical	Therapeutic importance	Mode of action	Reference
Homoharringtonine (2012)	Bark of <i>Cephalotaxus harringtonii</i>	Chronic myeloid leukaemia	Disables the elongation of peptide chain inhibiting protein synthesis	Li et al. (2020)
Topotecan-semisynthetic drug of camptothecin (1996)	Bark and stem of <i>C. acuminata</i>	Cancer	Inhibitor of DNA topoisomerase I	Brogden and Wiseman (1998)

medicinal plants have high demand in pharmaceutical industries for the production of bioactive therapeutic molecules for the treatment of various diseases, such as different types of cancers and Alzheimer's disease. However, these plants are not distributive evenly throughout the world. Trading of raw materials of these plants is very common.

Mostly dried plant parts, such as roots, leaves, barks, woods, flowers, seeds, etc., are the plant raw materials used in trade depending on the occurrence site of the economically demanded phytoconstituents in the plant. Several plant parts or even the whole plant is used in trade as raw plant material if the economically demanded phytoconstituents are concentrated in several plant organs or even in the whole plant body. To a small extent, the plant material is traded fresh or preserved in alcohol (Lange 2004). Medicinal and aromatic plants are extracted for their phytochemical content so that their use is even more complex.

Alkaloids, flavonoids, phenols, saponins, essential oils, tannins, etc. are the major groups of phytochemicals present in medicinal plants, which have great demand in global markets due to their diverse use in health sectors. Alkaloids, such as quinine, vindosine, vincristine, tabersonine, catharanthine, vindoline, yohimbine, vinblastine, and ajmalicine, are exploited in the treatment of diseases, which have antioxidant, analgesic, antileishmanial, antispasmodic, and bactericidal properties (Devkota et al. 2008; Barrales-Cureño et al. 2019; Kurek 2019). Plants are a very rich source of phenolic compounds and flavonoids, such as flavopiridol, quercetin, resveratrol, curcumin, rutin, epicatechin, etc., which are also in high demand due to their antiseptic, anti-inflammatory, anticancer, cardioprotective, immune-boosting, antimicrobial, and antitumor activities (Tungmunthum et al. 2018; Zhang et al. 2022).

Lavender oil, frankincense oil, peppermint oil, eucalyptus oil, lemongrass oil, cedarwood oil, and rosemary oil are examples of some essential oils derived from plants. These essential oils are also a good source of several bioactive compounds, which possess an aromatherapy and act as antioxidant, antimicrobial, antifungal, pain relievers, anxiety, and depression (Sindhu Sri 2020). They are also known for their antibacterial, antifungal, anti-parasitic, nematocidal, insecticidal, and antiviral activities (Sindhu et al. 2020). The compounds saponin and tannin used in different health-related issues and industries (Okigbo et al. 2008).

Medicinal and aromatic plants are traded both as commodities and, in some cases, as processed final products. The structure of global market of medicinal and aromatic plants is mostly competitive, growing exponentially, and developed countries are the most favoured beneficiaries such as Singapore, Japan, Germany, Malaysia, and the USA with the highest importing advantage (Roosta et al. 2017; Tripathi et al. 2017).

Marshall et al. (2006) explain activities associated with MAPs' supply chains: (a) domestication of the resource and on-farm cultivation, (b) collection of the wild resource, (c) management of the wild resource, (d) processing, (e) storage, (f) transport, and (g) marketing and sale. The supply chain of MAPs includes people and organizations in the flow of goods. It is started from collectors or processors, owners of the MAPs' resources, community-based traders, cooperatives, government departments, NGOs, and consumers lastly (Marshall et al. 2006). On the other hand, Astutik et al. (2019) explain two groups of actors in MAPs' product and

market chains which are upstream and downstream members. The upstream members encompass input suppliers, primary producers, processors, brokers, and traders. The downstream level comprises manufacturers, distributors, herbal doctors, retailers, and consumers (Astutik et al. 2019).

The MAPs trade of Indonesia can be seen from the demand side, which comes from the traditional medicines industry, food, beverage, pharmaceutical and cosmetic industries, household, and exports. Indonesia exports traditional plants, aromatic plants, and spices to Thailand, Iran, India, Bangladesh, and the USA, of which Thailand receives the biggest volume (Rizaty 2022). Most consumers of medicinal plants domestically are the medicine and pharmaceutical industry (63%), household consumers (23%), and exports (14%) (Utama 2017).

11.4 MAPs for Human Welfare

Since time immemorial, MAPs products have made a significant contribution to human health and well-being as well as contributed to the household income of generations of farmers through trade (Marshall 2011; Barata et al. 2016; Astutik et al. 2019). On the other hand, in fact, the trade in MAPs is one of the key factors in maintaining traditional knowledge about the use of medicinal plants (Salim et al. 2019). The contribution of medicinal plants to improved health, increased income, cultural identity, and livelihood security for many people in rural areas makes this resource extremely valuable (Upriety et al. 2011). Barata et al. (2016) explain that MAPs' genetic resources should be studied and preserved for the benefit of the present and future generations, as they are crucial to support human well-being by contributing also to the increase of income of the rural populations. In rural populations, trade of MAPs increases the household income, with women often playing a major role (Barata et al. 2016; Astutik et al. 2019). Women are often seen as decisions makers in the management of medicinal plants and men as traders and wholesalers (Astutik et al. 2019). People from other countries also increase their household income by playing different roles. They act as brokers of MAPs in Nepal and wild crafting in Central Himalaya and for local people in China, Bangladesh, and Vietnam (Astutik et al. 2019).

The large demand for medicinal plant materials has resulted in a very large trade both at the domestic and international levels. Various factors cause the supply of MAPs to vary from one region to another as well as from one country to another such as climate, plant distribution, topography, and different agricultural traditions. The MAPs trade is formed at the local, regional, and international levels.

If we investigate further, the local level of trade is actually much larger than the others, but the documentation is not very good. Silalahi et al. (2015) reported as many as 245 species of medicinal plants being traded in the traditional market of Kabanjahe, North Sumatra, Indonesia (Fig. 11.5). The local level MAPs trade is one of the economic sources for the community, especially for traders, suppliers, and collectors. Improved market relations between producers and buyers will result in



Fig. 11.5 Local level MAPs trading in Kabanjahe market, North Sumatra, Indonesia

increased economic benefits for local collectors, farmers, and dealers, enabling their communities to become significant hubs of economic activity with multi-dimensional impacts on economic development (Sher et al. 2014). The market chain of medicinal plants sold in Antananarivo, Madagascar, as reported by Randriamiharisoa et al. (2015) shows that the trade chain of MAPs to market from rural areas comes through collectors while rural and urban harvests may or may not involve collectors. Rathore and Mathur (2018) discussed the entrepreneurship development in MAPs and have summarized that the chain of MAPs trade flow until it reaches the consumer initiates from the cultivator and collector, via local collector at village level or local trader/middlemen trader (for larger zones of cultivation), to the wholesaler and regional traders at regional market, leading to retailers and ultimately available to the consumers.

Trading in MAPs also gave birth to new knowledge on their use. For example, the trade and use of medicinal plants in Pakistan is influenced by traditional Chinese medicine, Ayurveda, and Unani (Salim et al. 2019). Most of the harvesting and trade of MAPs thus makes a significant contribution to community livelihoods and local economic development (Belcher and Schreckenbergh 2007). In many regions harvesting MAPs is the main source of local cash income. While several studies document the challenges to the sustainability of MAPs presented by their trade, experts also argue that better market governance can contribute not only to sustainable harvests but also to increased profit sharing among social communities, actors, especially in poor communities (van Andel et al. 2015).

MAPs trade knowledge gaps and market structures hinder the development of public interventions, e.g. in supporting sustainable trade (He et al. 2018). The distribution and supply of MAPs varies greatly from region to region and from country to country. Trading in MAPs is the process of converting natural resources into income and leveraging social assets, and sustainable management increases their potential for success (Marshall 2011). MAPs trade is part of the local trade supply chain. Medicinal and aromatic plants are the main crops of domestic and industrial importance. Organic MAPs can increase profitability (Carrubba and

Militello 2013). About 42% of the sales of the 25 best-selling drugs worldwide are natural products or their derivatives (Kalaichelvi and Swaminathan 2009).

In the USA and Europe, the medicinal plant trade has grown at an average of 10% per year, among these plant medicines having 30% of the market. Global sales of herbal medicine/botany accounted for \$18.5 billion in 2000 and the main market is Europe and is estimated to account for 38% of the global market. Trade in Europe is dominated by Germany (42%), followed by France (25%), Italy (9%), and the UK (8%). New markets at the global level include Brazil, Argentina, Mexico, India, China, and Indonesia (Kalaichelvi and Swaminathan 2009).

Lubbe and Verpoorte (2011) have described several commercially used plant protection products from natural sources. Among pure compounds, Rotenone obtained from *Derris lanchocarpus* and *Tephrosia* spp.; Karanjin from *Derris indica*; Cinnamaldehyde from *Cassia tora* and several others are in commercial use. Plant-derived oil irritants to insects available on the market include oils extracted from *Pimpinella anisum*, *Glycine max*, *Eucalyptus* spp., *Thymus vulgaris*, *Tagetes* spp., *Gaultheria* spp. Plant extracts such as Milsana obtained from *Reynoutria sachalinensis* are reported to stimulate plant defense system against pathogen attack. Extracts from *Ryania speciosa* (Ryania extract) have been reported to be used to treat infected crops like codling moth, corn borer, citrus thrips, etc. Sabadilla extract from *Schoenocaulon officinale* is reported to contain alkaloids cevadine, veratridine, esters of alkamine and veracine and is used against thrips (Lubbe and Verpoorte 2011).

11.5 Source and Supply

The national, regional, and global markets for MAPs have been increasing. According to the FAO (2004), the largest markets for herbal medicines are found in Germany, China, Japan, the USA, France, Italy, the UK, and Spain. The major importing markets are the EU and USA. In these countries, increased demand for medicinal plants is being fuelled primarily by consumer interest in natural products and remedies, as well as by increasing concerns about the possible side effects of allopathic medicines. Major developing countries such as China and India are exporting medicinal plants, herbal tonics, cosmetics, perfumes, etc. There are therefore good prospects for export growth from LDCs in this market. However, markets in developed countries for herbal medicine especially in Europe and the USA are highly regulated and are very difficult to penetrate, particularly for developing countries and LDCs whose products have not undergone the stringent tests applied by developed country pharmaceutical manufacturers before mass production (Karki 2017).

Sales of herbal medicine alone were estimated to have exceeded US\$ 12.5 billion in 1994 and US\$ 30 billion in 2000, with annual growth rates averaging around

7.5%, depending on the region. Meanwhile, the herbal supplements market has had a higher annual average growth rate of 25% between 1990 and 1997. Rising global interest in medicinal plants has also created a sustained and largely “underground” trade in plant materials, many of which are being collected in LDCs in an unregulated manner, resulting in indiscriminate harvest of wild varieties and serious damage to bio-diversity (Karki 2017).

11.6 MAPs’ Conservation

Semwal et al. (2019) stated that the current herbal medicine market is estimated to be around US\$ 40 billion and will increase by 16% in the next 3–4 years. Between 50,000 and 80,000 flowering plants are used for medicine worldwide; many of them are collected from wild resources (Chen et al. 2016b). About 15,000 medicinal plant species are threatened with extinction worldwide because of overharvesting and habitat destruction (Chen et al. 2016b). It is estimated that only a few hundred species of MAPs have been cultivated out of tens of thousands of species of MAPs that have been reported and therefore need to increase their cultivation (Rao et al. 2004). MAPs cultivation also serves to ensure its supply and conservation. On the other hand, about 300 species of medicinal plants are included in the “IUCN red list” (Semwal et al. 2019). Population pressures in the wild are also due to increased commercial collection, largely unmonitored trade, and habitat loss (Lange 2006). Schippmann et al. (2006) stated that to avoid many wild MAPs species from being overexploited, it is necessary to cultivate wild species.

MAPs play a cultural, livelihood, or economic role in the lives of many people (Hamilton 2004); therefore their demand continues to increase from time to time. MAPs are found in the world’s richest biodiversity regions, most of which are economically less developed regions (He et al. 2018). Bentley (2010) states that the demand for natural resources has increased by 8–15% per year in Europe, North America, and Asia in recent decades (Bentley 2010). On the other hand, the supply of medicinal plants continues to decline, especially those harvested wildly. El-Wahab et al. (2008) reported in the North Sinai about 60% of medicinal plants are threatened due to intensive collection and other human activities. Hilonga et al. (2019) stated that monitoring the trade in illegally harvested medicinal plants is challenging because most of the ingredients are traded in various processed forms and most traders practice informal trade. We suspect the fact that the number of MAPs species that are at risk of extinction is much higher than the data previously reported; therefore research on the trade of wild plants from upstream to downstream is very crucial to be carried out in the future.

The European Plant Conservation Strategy (EPCS) states that 90% of Europe’s native MAPs species are still collected from the wild (Marshall 2011). Worldwide an estimated 9000 medicinal plant species are threatened (Lange 2002). Until now, most of the traded MAPs is harvested directly from nature or in the form of wild plants. The sustainable harvesting of MAPs is the most important conservation

strategy for most wild-harvested species, given its contribution to the local economy and its greater value to harvesters in the long term (Schippmann et al. 2006). Cultivation of medical plant species has various benefits over wild medicinal plant, which includes reduction of harvesting pressure on wild population of rare and vulnerable species, ensuring a steady supply of raw materials, and improving its genotypic quality (Chen et al. 2016a). The key concerns related to MAPs cultivation are the need for significant financial investment, narrowing of genetic diversity, possibility of genetic contamination of wild germplasm, and the harmful effects on ecosystems (Chen et al. 2016a).

There are two sources of supply of medicinal plants: (1) material collected from the wild, and (2) cultivated material (Kuipers 1997). Wild harvesting is the collection of plant material such as bark, leaves, fruits, herbs, flowers, woods, roots, and seeds from wild sources. The aromatic herbs are grown in home gardens, some are cultivated as field crops, either in sole cropping or in intercropping systems, and rarely as plantation crops (De Padua et al. 1999). Countries that do cultivate on a large scale are Hungary, Poland, India, China, Spain, and Argentina (Kuipers 1997).

Wild harvested material is considered qualitatively superior to cultivated stock because they grow in natural, more adverse environments and typically produce increased amounts of secondary metabolites (Peters 2018), and therefore, for example in China, wild material commands higher prices (Kuipers 1997). In Tanzania, wild-harvested plants play an important role in providing cures that are cheap, readily accessible, and available to the vast rural population (Hilonga et al. 2019). In Europe, prices for material from wild plants are much lower than for material of cultivated origin, with wild collection particularly prominent in Albania, Turkey, Hungary, and Spain. In terms of volumes, 30–50% of medicinal and aromatic plant material trade in Hungary is wild, 50–70% in Germany, 75–80% in Bulgaria and almost 100% in Albania and Turkey (Lange 1998).

The cultivation of medicinal plants traditionally collected from nature is essential both for the protection of plant species in their natural habitats and in response to the increasing demand for uniform sources of high-quality herbal medicine (Dudai and Yaniv 2014). To maintain the sustainability of MAPs, it is necessary to develop a conservation strategy. In general, conservation of MAPs can be carried out based on *in situ* and *ex situ* strategies (Fig. 11.6).

Some of the barriers to sustainable harvesting include poverty, breakdown of traditional controls, lack of community knowledge about sustainable harvesting rates and practices, unregulated land use, and lack of legislative and policy guidelines (Schippmann et al. 2006). Chen et al. (2016a) further stated that biotechnical approaches (e.g. tissue culture, micropropagation, synthetic seed technology, and molecular marker-based approaches) should be applied to increase yields and modify the potency of medicinal plants.

Several challenges were identified regarding the management of MAPs such as ambiguous policies, lack of resources, information, infrastructure, habitat degradation, and overexploitation (Upreti et al. 2011), which have to be addressed for sustainable management, use, and commercialization of medicinal plants.

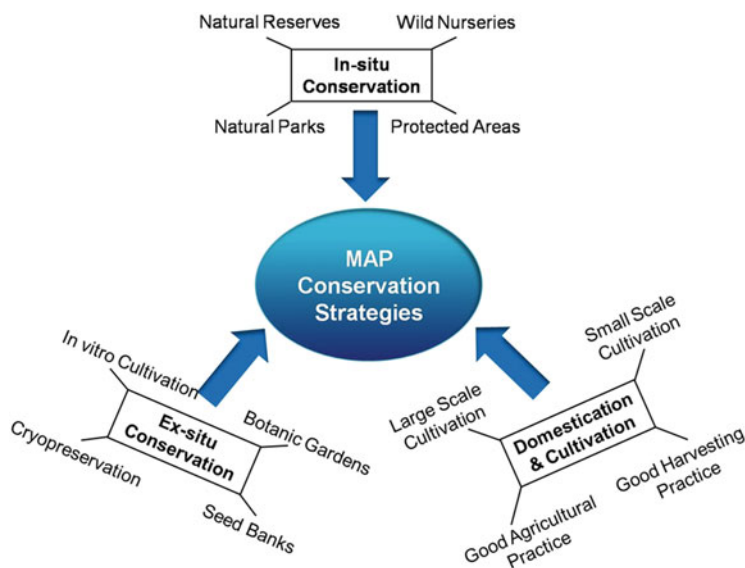


Fig. 11.6 Conservation strategies of MAPs

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Chapter 12

Inventorization of Ecology, Ethnobotany, and Conservation Status of *Dactylorhiza hatagirea*: Problems, Progress, and Prospects



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Abstract *Dactylorhiza hatagirea* (D. Don) Soo (Family-Orchidaceae), one of the important threatened species of medicinal value, has huge demand at the national and international market. However, low seed germination, narrow niche, low population size, and overexploitation have restricted its distribution range and availability in the region. The threat status and conservation efforts of *D. hatagirea* are ambiguous and therefore require clear understanding to suggest the gaps and possible conservation strategies. The synthesis of the study indicates the species is well explored for its ethno-botanical and medicinal usage all across the Himalaya; however, studies on availability (stock) in the wild, reproductive biology, efficient multiplication technique, and cultivation protocol are limited. Furthermore, isolation of bioactive molecules, their characterization, and use in nutraceuticals and pharmacological applications are largely overlooked. Considering the potential health benefits and huge demand, this species must be taken under special apprehension for conservation and sustainable utilization.

Keywords *Dactylorhiza hatagirea* · Threatened plants · Overexploitation · Conservation · Indian Himalayan Region

12.1 Introduction

Biodiversity loss across the globe has received widespread concern among biologists with fear to experience the sixth great extinction event (Swarts and Dixon 2009). The International Union for Conservation of Nature (IUCN) threat assessment report

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highlights that over 32,000 species are currently threatened, and of these over 12,500 species are plants (IUCN 2020). Among different groups, orchids are the most charismatic and diverse group of flowering plants in angiosperm and harbor nearly 32,464 species worldwide (www.theplantlist.org 2020). This immense species diversity comes with multiple challenges posed by intricate life histories and species rarity. The root cause of species rarity is mostly exacerbated by anthropogenic interferences that serve as a key precursor agent to extinction (Kaye et al. 2019; Singh et al. 2022b). As such, orchids have atypical biology requirement of specialized mycorrhizal association for germination, specific pollinators, habitat specificity, low seed set, strong dormancy, etc. further limiting their widespread distribution (Fay 2018). Considering the high aesthetic and medicinal values of orchids, these remain one of the most popularly traded non-timber forest products globally (Hinsley et al. 2018). Despite the fact that these plants are banned for trade under the Convention on International Trade in Endangered Species (CITES) (Appendix-II), illicit trade and unsustainable harvesting practices do not seem to halt their overexploitation.

Sustainable use and preventing the loss of bioresources have drawn wide attention with a series of standard operating procedures (SOPs). However, practical applicability of these SOPs across different genera needs consistent refinement considering ecological complexity and status of a particular species. In general, lack of information on species ecological niche requirements, reproductive biology, germination ecology, geographical distribution, and possible threats limits systematic conservation planning, especially of high-value threatened plants (Singh et al. 2022b). Among others, *Dactylorhiza hatagirea* (D. Don) Soo (Family Orchidaceae) is an important yet highly threatened medicinal orchid commonly known as “Salampanja” or “marsh orchid” (Singh et al. 2021), which has drawn much attention in recent years. The species is native and endemic to the Himalayan region. It is reported from temperate to alpine regions between 2500 and 4500 m asl in the Indian Himalayan Region (IHR) states (i.e., Himachal Pradesh, Uttarakhand, Sikkim, and Arunachal Pradesh) and Union Territories (Jammu and Kashmir and Ladakh), thriving most prevalently in moist grassy and undulating meadows (Singh 2021). Literature on the species indicates its properties to cure various diseases as diarrhea, dysentery, cough, stomachache, chronic fever, wounds and cuts, fractures, and general weakness through traditional herbal care system of medicine (Ballabh et al. 2008; Singh et al. 2021). The therapeutic effects of the species is due to its vital role in the treatment of spermatorrhea, impotency, in several neurodegenerative diseases such as hemiplegia, cerebropathy, phthisis, anxiety and depression, irritable bowel syndrome and as a memory enhancer (Thakur and Dixit 2007; Dhiman et al. 2019). The tuber of the species is reported to be a rich source of starch, mucilage, sugar, phosphate, glucoside-loroglossin (dactylorhins A to E), and polyphenolics (Fig. 12.1) (Kizu et al. 1999; Singh et al. 2022a).

The use of *D. hatagirea* in the treatment of various critical diseases makes its demand huge at national and international markets. For example, Goraya and Ved (2017) estimated an annual trade of 10–50 metric tons (MT) of the species, priced at Rs. 5000–6500 (US \$ 65.78–85.26) per kg (dry tuber). Recently, our recent study,

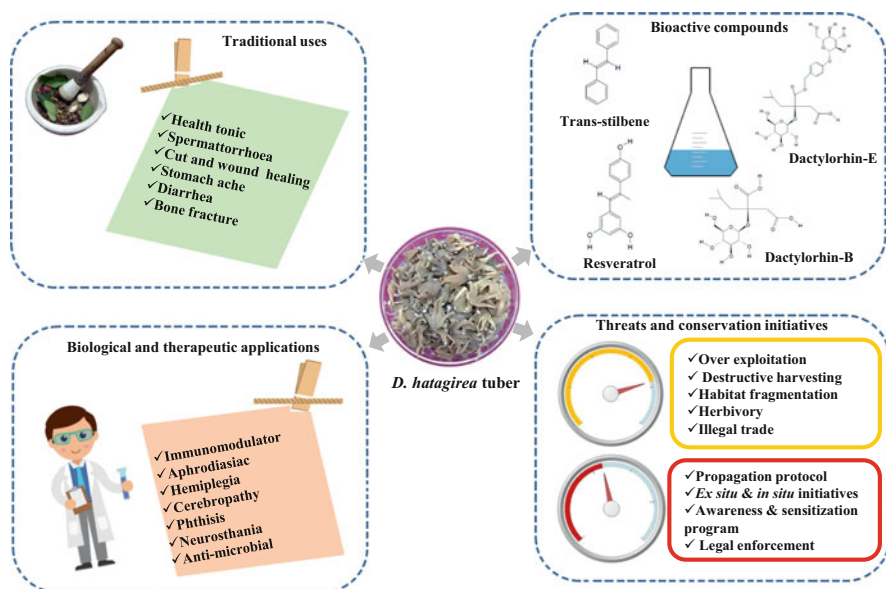


Fig. 12.1 An overview on *D. hatagirea* traditional and therapeutic applications, bioactive compounds, threats, and conservation implications

Singh et al. (2021) served as a point of reference to categorize the species in the IUCN list as ‘endangered’ (IUCN 2022). However, regional-level threat assessment categorizes this species under “critically endangered,” following IUCN guidelines (Ved et al. 2005; Adhikari et al. 2018). Low population density, isolated populations, and habitat specificity of the species amplify the impacts of anthropogenic threats such as habitat loss and fragmentation, destructive harvesting, climate change, etc. (Singh et al. 2021; Singh et al. 2022b). This chapter synthesizes the available information on *D. hatagirea*, including botanical description and distribution, ethnopharmacology, phytochemistry, propagation, and conservation strategies. Based on the information gathered, a framework for study gaps and improved conservation measures for the species, in particular, and vulnerable medicinal plants, in general, are provided.

12.2 Botanical Attributes

12.2.1 Botany and Taxonomy

Taxonomically, the genus *Dactylorhiza* is very complex and challenging (Pillon et al. 2006). The frequency of hybridization and resultant possible outcomes exacerbate this complexity (Averyanov 1990). The species is slender to robust plants

with palmately lobed tuberoids. The bracts are foliaceous mostly exceeding the ovary, with rose-purple, yellowish, and rarely white-colored flowers. Leaves are lanceolate or linear, with cylindrical inflorescence, which is mostly densely flowered. The flower lip is entire or 3 lobed, with the prominent spur, stigma slightly curved (concave), undivided and placed between anther cells. The ovary is sessile, glabrous, and twisted (Deva and Naithani 1986; Wani et al. 2020). A comparative morphometric description of the Himalayan *Dactylorhiza* species is presented (Table 12.1).

12.2.2 Distribution Patterns, Habitat Ecology, and Diversity

D. hatagirea is widely distributed in temperate to alpine meadows in IHR states, Pakistan, Nepal, Bhutan, Afghanistan, and Tibetan province (Bhatt et al. 2005; Bhattarai et al. 2014). The species is reported to have a wide coverage across the Hindu Kush Himalayan region (except Bangladesh and Myanmar), but is confined to small patches preferring habitats of high soil moisture. Soil physiochemical investigation reveals that the species thrives in a mildly acidic environment (pH 4.8–6.20), sandy loam soil with sufficient moisture (water-holding capacity 41.71% to 65.75%), organic matters, i.e., carbon-to-nitrogen ratio (C/N ratio) ranges in between 11.05 and 17.07 (Singh 2021). Undulating meadow, marshy region, open grassland, and the understory of *Rhododendron campanulatum* and *Berberis jaeschkeana* are some of the preferred habitats observed during various field visits. On account of reconnaissance surveys carried out across the IHR, Nepal and Bhutan reports population density to vary between 0.05 individual/m² and 4.05 individual/m², whereas frequency ranged between 20 and 90% (Bhattarai et al. 2014; Singh et al. 2021), with population distribution mostly being contagious. Status assessment of the species is limited, or studies undertaken are region-specific; thus, the information on the distribution patterns is still fragmentary. None of the studies has provided quantum availability (wild stock) in terms of biomass; therefore, it still remains a major gap area and needs priority attention.

12.2.3 Life Cycles and Challenges

D. hatagirea is a perennial species, roughly with a life expectancy of maximum 7 months and a brief dormant phase of 5 months. Being a tuberous species and not rhizomatous like most alpine species, the challenges to adhere and flourish are high. In the reproductive phase, a single new remnant tuber buds off from the mother tuber that subsequently develops into full flesh (or fresh) plants, whereas the older tuber starts to wither in the subsequent season (Singh 2021). The vegetative growth (bud sprouting) starts with the recession of snow cover in April, while flowering starts somewhere between the second week of June and continues up till August. Flowers

Table 12.1 Comparative morphometric descriptions of *Dactylorhiza* species reported from the Himalayan regions (Deva and Naithani 1986; Shapoo et al. 2016; Singh 2021)

S. no.	Morphometric character	<i>D. hatagirea</i>	<i>D. kafiriana</i>	<i>D. umbrosa</i>	<i>D. viridis</i>
1	Plant height	Up to 60 cm	20–35 cm	Up to 40 cm	Up to 25 cm
2	Tuber lobes	3–5 often with elongated tips	2–4, finger like	3–5 palmately lobed	2
3	Stem	Leafy upward, stout, and fistular	Straight or slightly flexuous	Solid or fistular	Leafy, solid, or fistular
4	Leaves	3–6	3–6	4–8	3–5
5	Leaf shape	Lanceolate or oblong-ligulate or elliptic	Lanceolate or linear	Lanceolate or oblong-lanceolate	Elliptic-oblong, acute or obtuse
6	Leaf apex	Obtuse to acuminate	Acuminate	Acuminate	Obtuse or acute
7	Inflorescence	Broadly cylindrical, densely flowered	Normally loosely few too many flowered	Densely flowered, cylindrical	Spike lax or densely many-flowered
8	Floral bract shape	Lanceolate	Narrowly lanceolate	Narrowly lanceolate	Linear to narrowly lanceolate
9	Floral bract color	Green	Reddish brown	Green, sometimes tinged with purplish red	Brownish green
10	Bracts type	Lanceolate	Narrowly lanceolate	Lanceolate	Lanceolate
11	Lip shape	Oval to sub-orbicular	Oblong or rhomboidal	Orbicular or reniform-rhomboidal	Deflexed, oblong-lanceolate
12	Shape of lip spots	Spoon-shaped	Dome-shaped	Heart-shaped	Spots absent, but a central longitudinal keel present
13	Spur type	Cylindrical more or less straight, equaling the ovary or slightly shorter	Cylindrical more or less equaling the ovary in length	Cylindrical, slightly curved towards apex, almost as long as ovary	Saccate-ovoid, straight, shorter than ovary
14	Flowering period	July–August	June–August	June–August	July–August

of the species lack nectar (food-deceptive orchid); therefore, specific pollinators (vectors) remain a gap area of research (Singh 2021). However, it is believed that bees, flies, and bumblebees pollinate the species. Like most orchid species, *D. hatagirea* too has specialized pollen grain with tetrahedral polyades that contains

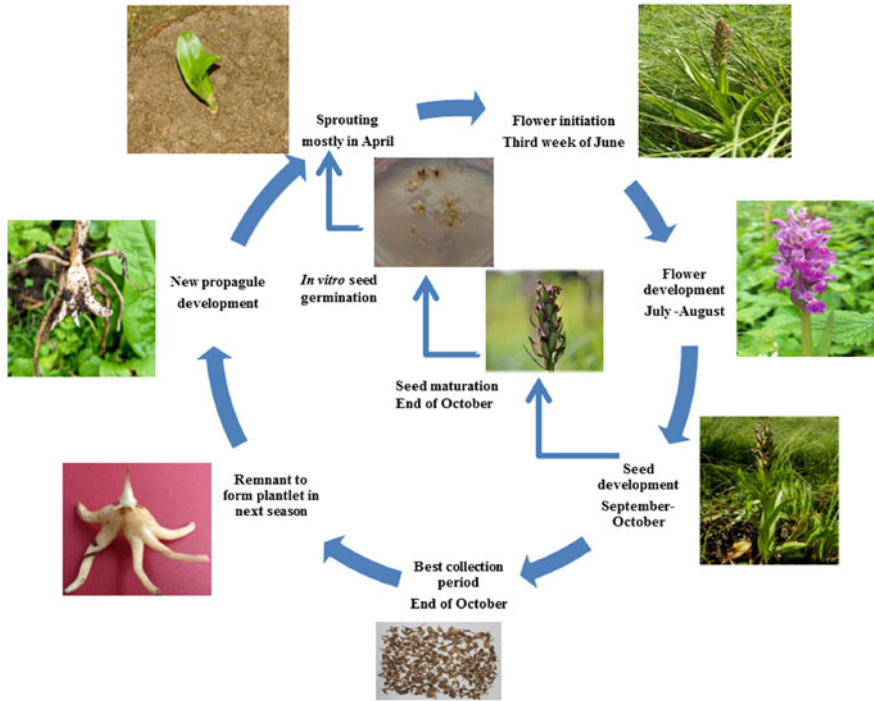


Fig. 12.2 A generalized life cycle with different growth phases (based on field observations) of *D. hatagirea*

a large number of pollen grains (Averyanov 1990). Seeds are similar to most orchids; however, they differ in terms of numbers, and roughly a single capsule approximately contains over 6000 seeds (Averyanov 1990). With the development of capsule in place (September–October), this embarks the completion of the growth cycle, and preparation starts for the senescence phase (Fig. 12.2). A major challenge before alpine species is to encompass freezing stress during the winter season. Interestingly, these species are reported to be generally opportunistic, i.e., they prefer to avoid freezing stress rather than to tolerate icy winters (Dhiman et al. 2019).

12.3 Propagation and Multiplication Approach

In nature, *D. hatagirea* propagates both through vegetative and sexual means. Sexual reproduction via seed germination is very poor (0.2–0.3%). This could be due to the fact that long seed dormancy and requirement of symbionts for establishment and nutrition, therefore, are less common in the species (Vij and Aggarwal 2003). Vegetative reproduction is most frequent by means of new daughter tuber emergence from mother tuber. Conventionally, this is most preferred over seed

germination, considering the plantlet success ratio. Several attempts have been made to propagate *D. hatagirea* by means of tuber fragmentation (Rajasekaran et al. 2009; Giri and Tamta 2012) and in vitro seed germination (symbiotically and asymbiotically) (Agarwal et al. 2015). However, results obtained are not replicable for mass multiplication. In vitro seed germination studies in different growth media (by varying concentrations) highlight the development of prominent protocorm-like bodies (PLBs) with shoot apical meristem. But further inoculation of PLBs in the same media concentration or organogenesis media response observed was very poor (even after 8–12 weeks) and ultimately perished on subsequent subculture. It is believed that *D. hatagirea* employs ecto-mycotrophic co-infection for seedling establishment and to fulfill its nutritional needs at this juncture. Therefore, future research on plant-microbe interaction identifying beneficial microbes is sought to benefit the organogenesis and/or planting establishment phase largely. Similarly, an effort to influence vegetative propagation of *D. hatagirea* has been reported. Giri and Tamta (2012) have segmented tuber into an apical, middle, and lower fragment and tested them against different types of auxins (1-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA)) at varying concentrations. Of this, only the apical segment was reported to exhibit sprouting and rooting. IBA (50.0 μ M) was found to be the most effective concentration (38.77%) for rooting whereas no response was found in control treatments (without plant growth regulators). Considering the slow and poor seed germination response, upscaling techniques via vegetative mode is suggested for future research, with emphasis on mass multiplication and agro-technique development.

12.4 Reproductive Barriers and Genetic Diversity

Genetic diversity of a species or population depends on either (i) intrinsic biological properties governing life form traits such as reproduction, pollination, seed formation, dispersal mechanism, etc., or (ii) extrinsic properties such as distribution, existence, or persistence. In addition, factors such as biogeographical zones and contemporary geographical range influence/determine the levels of genetic diversity. *D. hatagirea* is characterized by small, spatially isolated populations, mainly a consequence of ecological specialization and low reproductive success, thus making them vulnerable to genetic drift. Considering this, inbreeding depression and low genetic diversity in *D. hatagirea* have been actively reported. Microsatellite (SSR), inter-simple sequence repeats (ISSR), and random amplification of polymorphic DNA (RAPD) based studies highlight low to very high (i.e., 0.043 to 0.908%) level of polymorphism, with alleles ratio of 2 to 15 per locus (Warghat et al. 2012; Sharma et al. 2015; Singh 2021). Therefore, the selection of elite germplasm (with maximum genetic diversity) for conservation intervention (ex situ and in situ program) is suggested considering environmental perturbations and increasing anthropogenic pressure.

12.5 Phytochemistry

Despite *D. hatagirea* having huge ethno-botanical uses, little progress has been made to uncover the hidden bioactive properties of the species. Tubers are reported to contain metabolites like dactylorhin (A, B, C, D, E); dactyloses A and B monosaccharide derivatives, loriglossin, and millitarine (Kizu et al. 1999) (Fig. 12.3). Recently, Singh et al. (2022a) optimized an extraction process for the extraction of polyphenolic compounds based on ultrasonication technique. Under optimal extraction conditions, total phenolics (11.42 ± 0.48 mg GAE/g DW), flavonoids content (11.46 ± 0.28 mg QE/g DW), and antioxidant activity, i.e., DPPH (1.37 ± 0.008 mM AAE/g DW) and ABTS (1.06 ± 0.008 mM AAE/g DW) and anti-glycemic, i.e., alpha-amylase ($42.81 \pm 0.22\%$ inhibition) and alpha-glucosidase ($27.97 \pm 0.40\%$ inhibition) inhibition activity have been reported. Also, HPLC-PDA of the bioactive compounds were quantified at the optimized extraction condition. Based on the results obtained, we highlighted the decreasing order of their concentration: dactylorhin E (14.19 mg/g DW) > o-coumaric (3.22 mg/g DW) > chlorogenic acid (1.78 mg/g DW) > gallic acid (1.38 mg/g DW) > ascorbic acid (0.61 mg/g DW) > *p*-hydroxybenzoic acid (0.45 mg/g DW) (Singh et al. 2022a). Similarly, Dhiman et al. (2019) using de novo transcriptome studies picturized the biosynthesis of resveratrol and trans-stilbenes. This was subsequently confirmed through ultra-high-performance liquid chromatographic-diode array

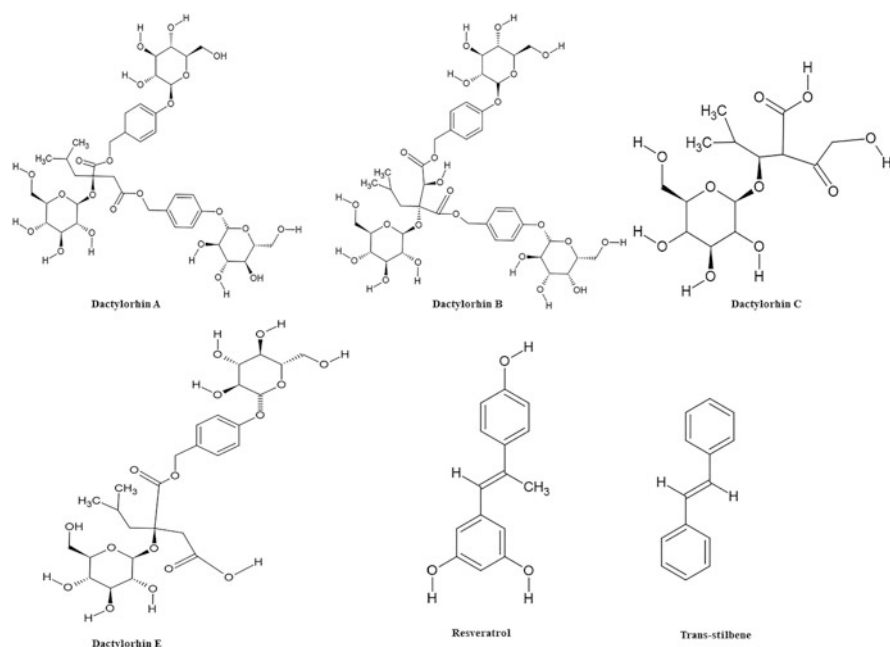


Fig. 12.3 Chemical structures of some identified major chemical compounds in *D. hatagirea*

(UHPLC-DAD) analysis and reported that tubers contain about 3.21 µg/100 mg resveratrol and 2.49 µg/100 mg trans-stilbene (on FW basis). In addition, an appreciable amount of soluble sugars (20.6–40.7 mg/g), starch (45.6–74.3 mg/g), and soluble protein (10.4–26.9 mg/g) is reported from tuber (Chauhan et al. 2014). Therefore, based on the bioactive compounds profile, further work related to nutraceuticals formulation and encapsulation needs attention for harnessing true therapeutics potential of the species.

12.6 Ethnopharmacological Relevance

It is well reported that *D. hatagirea* finds utility in various traditional systems of herbal medicine (i.e., Ayurveda, Siddha, Amchi, and Unani). Different kinds of formulations of the species are widely reported from various regions of the Himalaya. One of the common formulations reported is that powdered extract of the species is blended with milk and honey to serve as an energy drink to overcome general weakness (Singh 2021); this formulation is especially prescribed to debilitate women after delivery and to increase regenerative fluids in traditional system of medicine (Fig. 12.4). Likewise, formulations containing *Ficus religiosa*, *Cinnamomum zeylanicum* (leaves), *Elettaria cardamomum*, *Terminalia chebula*, *Spondias axillaris* (fruits), *Herminium monorchis* (bulbs), *Crocus sativus* (stigma), *Punica granatum* (seeds), *Rubia cordifolia* (roots), *Zingiber officinale* (rhizomes) and minerals, blended in the form of tablet, are given to patients (3 times a day) having urinary problems for 10–15 days (Ballabh et al. 2008). In addition, the raw tuber is consumed to overcome impotency, low sperm count, spermatorrhea, and to increase vigor (Wangchuk et al. 2009; Singh et al. 2021). In the traditional Chinese system of medicine, *D. hatagirea* is reported to benefit kidney problems, stomach and spleen disorder, improving appetite, also used as a cardiac stimulant, to treat dizziness, anemia, and irregular menstruation problems. The tuber yields a high quality “Salep,” extensively used as a health tonic to increase immunity (immunomodulator), and “Vajikaran drug” because of aphrodisiac property and astringent property (Thakur and Dixit 2007).

12.7 Threats to the Species

Prioritization of a particular species under any conservation program required understanding of the various level of threat to a particular species; this kind of information is pre-requisite for species conservation (Singh et al. 2022b). According to Ved (2003) a total of 880 medicinal plants are in trade in India, of which 66% are collected with destructive harvesting, i.e., harvesting of roots, bark, wood stem, or the whole plant. Overexploitation and destructive harvesting are the dominant exogenous factors declining populations of *D. hatagirea* in the wild. Habitat

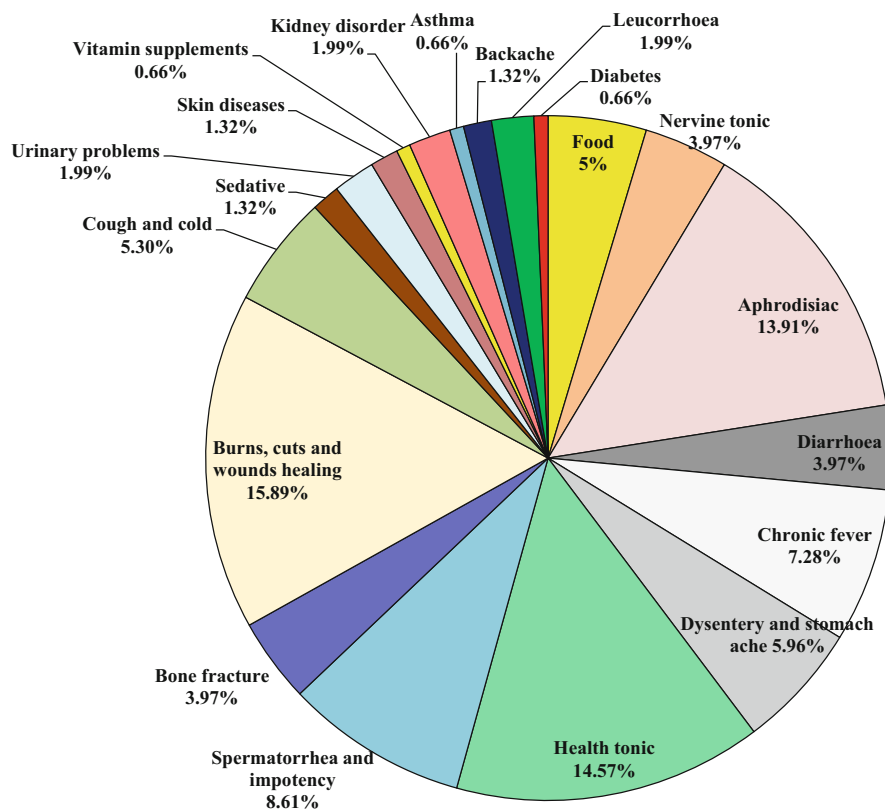


Fig. 12.4 Ethno-botanical studies cited against the use of *D. hatagirea* tuber

specificity, low seed viability and germination, low population size, and narrow niche are the major endogenous factors leading to population decline and restricted distribution of the species (Uniyal et al. 2002; Bhatt et al. 2005; Singh et al. 2021). *D. hatagirea* is facing acute threat of destructive harvesting; the tuber of the species is collected for medicinal purpose and consequently severely impacted the survival of species in the wild (Singh 2021). Since there is no cultivation practices developed so far, therefore the widening gap between demand and supply puts additional pressure on the wild population (Singh et al. 2021). Moreover, other than being grazed by the domestic livestock, the species is a preferred food of Himalayan monal pheasant (*Lophophorus impejanus*) and pyka (*Ochotona roylei*) (Dhyani and Kala 2005). Herbivores not only feed on the species but also destroy a sizeable amount of its population. Further, unprecedented widespread distribution of a native but aggressive habitat colonizer *Persicaria wallichii* in the alpine meadow has engulfed much of the habitats of high-value medicinal plants including *D. hatagirea* (Singh 2021). Furthermore, huge demand (5000 tons) of the species has resulted in

population decline and also increased mating chances between closely related individuals creating a state of genetic depression and bottleneck effect.

In view of the above studies, Shrestha and Shrestha (2012) and Singh et al. (2021) carried out rapid vulnerability and threat assessment for *D. hatagirea* and highlight the species to be in high threat category (I and II). The assertive remarks contributing maximum towards the vulnerability of the species include species habitat specificity, limited geographical distribution, and overexploitation. Thus, there is an urgent need for assessment of threat status of *D. hatagirea* using updated data on population size, area of occupancy, number of mature individuals, and geographic extent of occurrence. This is vital as the natural habitats, as well as populations of *D. hatagirea*, are being affected by increasing anthropogenic activities and endogenous factors, and now climate change. Some of the other factors that further limit species distribution and conservation concerns include: (1) low socio-economic profile and poverty, (2) livelihood opportunities inclined to traded commodities of which major junk is fulfilled by NTFTs, hence often results in unsustainable harvesting and illegal trade, (3) surplus demand, (4) basic gaps in ecological data and vague conservation status assessments, (5) inefficient in situ and ex situ propagation protocol, and (6) lack of awareness on sustainable resource utilization.

12.8 Research Gap

Low socio-economic profile and increasing ecological footprint are subject to MAPs habitat deterioration that ranges from partial to complete degradation in the Himalayas. Moreover, the region lacks uniformity in threat distribution irrespective of the biodiversity strata; hence it becomes imminent to work on species-level prioritization to minimize biodiversity loss. Previously many common causes of species rarity were reported; however, an intuitive multidimensional framework for research and development is lacking at the species level. Further, categorization of drivers that impede plant survivability is a much-needed research topic that needs to be addressed briefly. Doing this is thought to provide a better insight on core issues highlighting research gaps and way forward for future research (Fig. 12.5). Towards achieving this, bioresource mapping, development of medicinal plant conservation areas, studies on species reproductive biology, development of alternative propagation strategies, reducing dependency on wild populations and encouraging cultivation practices, and finally raising the profile of the species at various awareness drives would pave the way for sustainable resource utilization (Table 12.2).

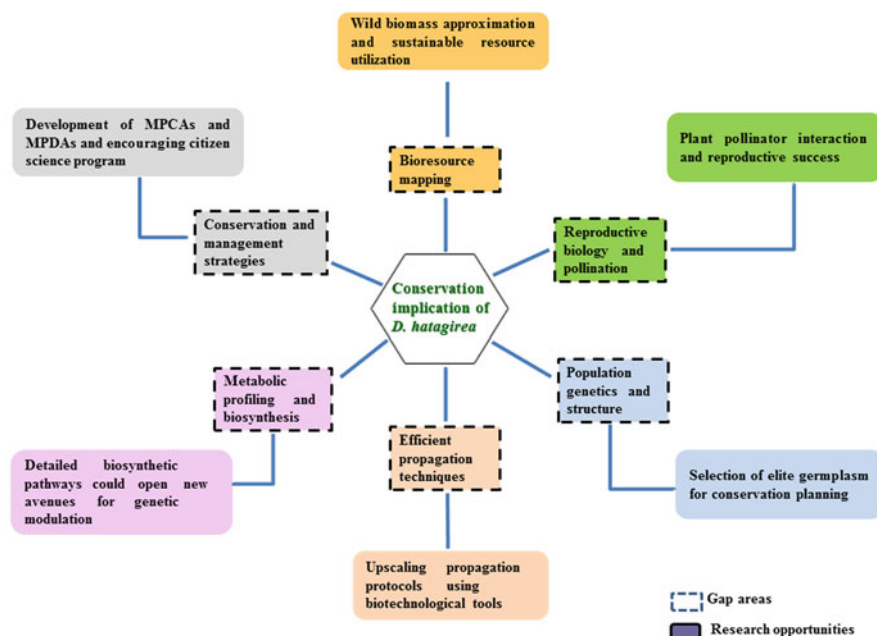


Fig. 12.5 Conservation implication of *D. hatagirea*, gap areas, and research opportunities

12.9 Conservation Challenge and Future Perceptive

Lack of efficient conservation strategies and prioritization thus calls for immediate concentrated efforts to formulate pragmatic ecological restoration model. Simultaneously, it is imperative to identify species-rich areas that can be taken up for in situ programs in collaboration with the local stakeholders. Based on this analysis, this study proposes a multistep conservation framework (Fig. 12.6), with an emphasis on citizen science program. It is expected to have a comprehensive ecological assessment drawing viable population size (area of extent) and demography, reproductive cycle, pollination biology and dispersal mechanism, germination ecology, mycorrhizal associates and types, predicting suitable habitats for reintroduction (using ecological niche model), etc., which are necessary for effective and efficient conservation strategies. These studies are critical but presently lack consideration for the species. Such approaches are sought to provide a better understanding of the species with the adoption of appropriate management plans.

Table 12.2 *D. hatagirea* research inventories, research findings, limitations, and future perceptive

Parameters	Research findings	Limitations	Opportunities	References
Seed germination	Standardization of protocorm-like bodies (PLBs) formation [2-iP (0.5 mg/L) + BA (1.0 mg/L) and 1/2 MS medium + P (1.0 g/L) + MES (1.0 g/L) + AC (0.1%)] has been achieved	- Seed developmental stage is overlooked - Understanding on organogenesis is missing - Reproducibility of the propagation protocol is limited	- Upscaling can be done with recent biotechnological tools - Isolation and characterization of mycorrhizal associates	Agarwal et al. (2015)
Macro-propagation	Apical segment exhibited sprouting and rooting. IBA (50.0 μ M) was most effective; maximum rooting percentage (38.88%), maximum average root number (1.51 roots/cutting)	- Only auxins (NAA, IBA, IAA) were used - Restricted concentration (10, 50, 100 μM) were used - Soil physiochemical properties is ignored	- Detailed understanding of soil characteristics is required - Range of PGRs can be used to enhance the rate of vegetative propagation - Isolated mycorrhizal associates could be used to enhance the plant growth	Giri and Tamta (2012)
Population assessment	Population density of <i>D. hatagirea</i> ranged from 0.15 to 0.87 individual/m ² along the altitudinal gradient (2500–4300 m)	- Habitat-specific information missing - Information on population density is limited to few areas - Details on associated species are missing	- Quantitative assessment across different habitat types and regeneration status using grid-based approach are needed - Delineating extent of occurrence and estimating available biomass in wild is required - Identification of species-rich areas for immediate conservation is needed	Singh et al. (2021)
Threat assessment	Rapid vulnerability assessment categorized the species under category II threats. Anthropogenic disturbances were identified as a major cause of species decline, in addition to	- Assessment report based on data interpretation of particular area - Lacks detailed comprehensive assessment involving use pattern, selection criteria for collection,	- Prioritization of population for conservation and management - Regulating grazing practices in consensus with the local communities - Strategies to check or contain the	Singh et al. (2021) and Shrestha and Shrestha (2012)

(continued)

Table 12.2 (continued)

Parameters	Research findings	Limitations	Opportunities	References
	restricted habitat distribution and poor germination rate	multiplication packages, etc. Threats due to widespread habitat engulfed by native species/invasive species or as a result of unchecked grazing that is unnoticed	wide spread of invasive or aggressive native species	
Active chemical constituents	Dactylorhins A, B, C, D, and E, dactyloses A and B, resveratrol, and trans-stilbene	- Reports on phyto-constituents remain largely ignored - Nutritional and anti-nutritional value remains undefined - Role of active constituents and possible bioactivities is missing	- Detailed chemical profile is needed - Salep (glucomannan water soluble polysaccharide) is prepared from the species; therefore isolation and characterization is suggested - Therapeutics potential could open up new avenues in biomedical sciences	Dhiman et al. (2019) and Kizu et al. (1999)
Medicinal property	Significant increase in mean sperm count (141.3 ± 9.6), seminal fructose concentration (5.79 ± 0.25), penile erection index (49.8 ± 1.1), and NO release ($12.9 \pm 1.7 \mu\text{m}$) was achieved	- Study limits investigation to a single dose (100 mg extract per kg body weight) concentration - Detailed mechanism of action is required	- Investigation across wide concentration range and experimental model is suggested - Possibility of controlled clinical studies with patients on sexual dysfunction	Thakur and Dixit (2007)
Climate change effect	<i>D. hatagirea</i> flowering and fruiting time advanced from June–October (Naithani 1984, Gaur 1999) to June–September based on perception studies	- Conclusion drawn from study is for a particular region - Scientific validation of the information with supporting environmental data is lacking	- In-depth survey across different regions - Long-term investigation with supporting climatic dataset is proposed	Maikhuri et al. (2018)
Species-specific micro-satellite marker	Species-specific 29 microsatellite markers developed, highlighting	Limited number of population (3 in nos.) undertaken for diversity analysis	Study related to population genetics and the extent of genetic diversity	Sharma et al. (2015)

(continued)

Table 12.2 (continued)

Parameters	Research findings	Limitations	Opportunities	References
	average allele per locus ranging in between 3.4 and 4.2 and level of polymorphism ranged from 0.043 to 0.908		can be undertaken • An important approach to identify elite populations for multiplication and conservation	
Traditional system of medicine	Traditionally used against aphrodisiac, cough, cuts, nervous disorder, tonic, wounds, kidney and urinary disorders	• Studies reports only used by indigenous communities without know-how • Studies pertaining to develop formulation are limited	• Validation of the traditional knowledge with the local practitioner is required • Detailed investigation on ethnopharmacological would draw true potential	Ballabh et al. (2008)

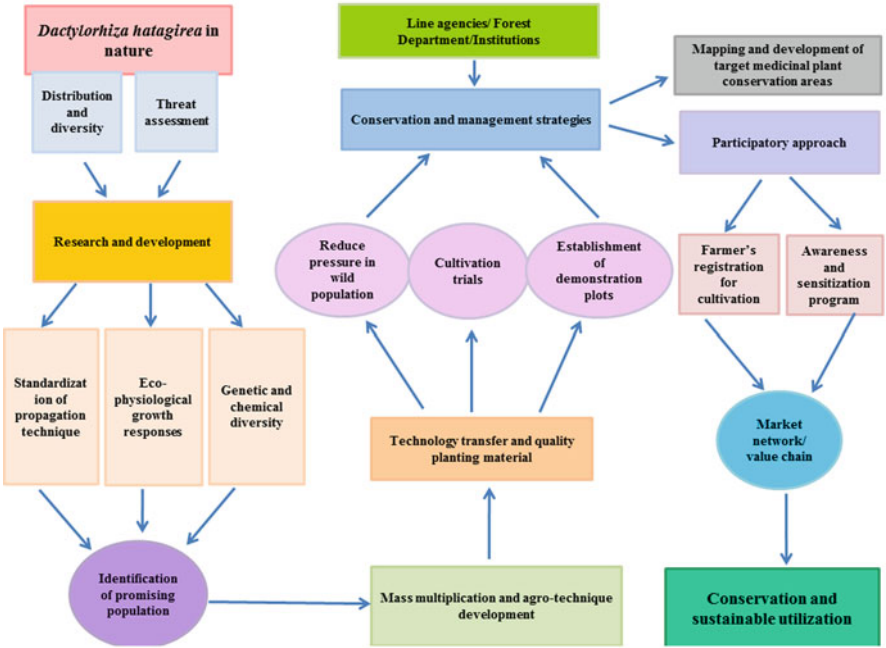


Fig. 12.6 Cumulative framework for conservation and sustainable harvesting practices plans for *D. hatagirea*

12.10 Conclusion

The information analyzed for *D. hatagirea* in this study is vital for conservation and management. Conservation through in situ approach is considered the best option, possibly through the identification of species-rich areas outside the protected areas network. However, in situ conservation is often not enough, while dealing with critically endangered species having a limited population, thus the creation of germplasm banks and promoting cultivation at farmer's field is important to sustain the species in the long run. In addition, awareness among the local people is an important means of conservation and management. The present work emphasized (1) *D. hatagirea* prefers moist grassy undulating meadows having mild acidic pH (4.8-6.20) soil, (2) ambiguity in botanical nomenclature and narrow geographical distributional range, (3) tubers are a rich source of bioactive active constituents (dactylorhins, resveratrol, trans-stilbene and polyphenolics compounds), and 'Salep' (a polysaccharide-rich extract) with prospects of role as an anti-inflammatory, anti-carcinogenic, cardio-protective, vasorelaxant, phytoestrogenic and neuroprotective agent, thereby confirming its use in folk medicine, (4) vegetative mode of reproduction is most prevalent, whereas seed germination is very poor, (5) habitat specificity, limited geographical distribution, overexploitation and habitat encroachment by *Persicaria wallichii* further limited its distribution, (6) a multistep conservation framework has been suggested for sustainable utilization and management plans.

Furthermore, studies must be carried out to develop efficient propagation protocols and isolation and characterization of mycorrhizal associates with emphasis on its mass multiplication. The inventories available on propagation and multiplication are grossly incomplete and lack reproducibility for mass multiplication. Plant authentication should be done through molecular and chemical-based approaches considering enormous herbal adulterant. Several phytochemical uses of these species are yet to be explored, providing opportunities for possible drug development. Implementation of such approaches will definitely help to promote conservation and sustainable utilization of the species.

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Chapter 13

Conservation and Sustainable Use of Medicinal Plants



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Abstract The research of natural products has reached, in the last years, a wide amplitude and diversification in the chemical area. Its importance is revealed by its application in the most diverse fields of knowledge, with studies directed to the understanding and use of biodiversity. Besides the economic interest, aiming at the use of these metabolites in the production of perfumes, dyes, medicines, pesticides, etc., one must consider the scientific knowledge that contributes to the development of several areas of science, such as pharmacology, botany, organic chemistry, evolution, and chemical ecology. In this chapter, we have discussed the sustainable use of medicinal plants in a conservation perspective focused on the use of plant resource, sustainable use of biodiversity, bioprospecting, adding value to biodiversity products, biological activities, essential oil, seasonality effect, socioeconomic approaches, and some future prospects.

Keywords Medicinal plants · Conservation · Sustainable use of biodiversity · Plant metabolites · Phytotherapies

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13.1 Use of the Plant Resource

The plant kingdom has been one of the richest sources of organic substances, contributing significantly to the supply of a great diversity of special classes and structures of metabolites. However, the current speed of biodiversity extinction is causing not only the loss of compounds with therapeutic and economic value but also the coding of interesting genes for enzymes with a potential use in plant breeding and biosynthetic studies of new natural products (Howes et al. 2020). Under this aspect, it is worth noting that Brazil, as the country with the largest plant genetic diversity in the world, needs to develop strategies for the sustainable use of a nature quite unexplored scientifically and technologically (Da Silva et al. 2019).

The importance of natural products in therapeutics has been increasingly remarkable. About 25% of prescriptions dispensed in developed countries during the last 25 years were related to drugs containing active ingredients of natural or semi-synthetic origin, usually from higher plants (Newman and Cragg 2020). In relation to the world market, about 80% of people use plants as a primary source of medicinal agents (Calixto 2019). It is important to emphasize that all research related to the chemistry of natural products should be developed in order to avoid the exploitation of nature without conservation of biodiversity.

Due to so many relevant discoveries from medicinal plants, it is clear that the search for longevity and improvement of human life has become one of the main motivators in the search for pharmacologically active natural substances. Different classes of substances can be cited, which have resulted in the discovery of important drugs of different therapeutic categories: quinine, the main component of *Cinchona officinalis* bark that inspired the synthesis of several antimalarial derivatives of the aminoquinoline group; vincristine and vinblastine, indole monoterpene alkaloids isolated from *Catharanthus roseus*, employed in the treatment of lymphomas and childhood leukemia; papaverine, widely used as a cerebral vasodilator, isolated from *Papaver somniferum*, also the source of the hypoanalgesics morphine and codeine; taxol, one of the most promising discoveries for the treatment of malignant neoplasms (Sofowora et al. 2013); and, more recently, camptothecin, an alkaloid extracted from the Chinese tree *Camptotheca acuminata* and derivatives (Martino et al. 2017).

13.2 Sustainable Use of Biodiversity

Medicinal plants are highly valued sources of herbal and bioactive compounds across the world, yet they are endangered owing to overexploitation, improper harvesting, and use. Additional issues such as population growth and climate change have put these valuable biological treasures at jeopardy. Furthermore, the burgeoning sector of herbal goods business has a lot of room for growth. As a result,

multiplication and cultural techniques must be designed and promoted at the earliest opportunity. Conservation is the process of managing the biosphere for the maximum benefit to the current generation while also preserving the potential for future generations. There are several actions that may be made in support of medicinal plant conservation and long-term use (Shafi et al. 2021).

To offer a credible reference for the conservation and sustainable use of medicinal plant resources, researchers looked at worldwide trends, advancements, and possibilities for methods and approaches. For the sustainable use of medicinal plant resources, both conservation measures (legislation, in situ conservations, and ex situ conservations), resource management (sound farming practices and sustainable use solutions), and the accompanying regulations should be fully taken into consideration (Chen et al. 2016). The future of natural medicines is changing as new threats to human health and biodiversity emerge. Advances in science and technology are paving the way for new compounds to be discovered in nature, as well as a variety of metabolic routes for their synthesis and more sustainable ways to get them, laying the groundwork for potential answers to global health concerns. These initiatives, which are based on biodiversity, provide hope for improving yields and securing future supplies of vital medications (Howes et al. 2020).

On the other hand, the high biodiversity in tropical forests has motivated several studies that seek to understand the mechanisms involved and effective solutions to stimulate development. Thus, it is important to study sustainable strategies for forest management, linked to the traditional knowledge of local communities, who live on the coast of Paraná and bring with them a vast history and interpretation of the use of plants. In this sense, it is important to analyze the system of representations, symbols, and myths that these populations build, because it is with these that they act on the environment. It is also with these representations and the accumulated empirical knowledge that they develop their traditional management systems (Tegegne et al. 2018).

In Brazil, there are traditional communities that, although not organized into a movement per se, recognize themselves and are recognized by the society for their specific identities, for example, caíçaras, indigenous guaraníes, quilombolas, and cipozeiros. All these people/communities make use of biodiversity for self-sustenance and/or income generation, from food and medicinal plants, small animals, and fish resources to, on a larger scale, forest ecosystems, restingas, mangroves, estuaries, and the marine environment.

These species and ecosystems are managed in various ways and intensities, which gives this biodiversity a peculiar character, academically called sociobiodiversity. The management of biodiversity carried out by these traditional communities not just makes the species and ecosystems a natural and “wild” resource but gives this biodiversity an additional element of human intervention, hence the prefix “socio.” This management ranges from the collection of fruits and plant parts, fishing, and extractivism in various natural ecosystems to cultivated areas such as backyards, vegetable gardens, and fields, the raising of small animals, and the use of techniques for capturing fish in addition to fishing, for example, the practice of “encircling.”

13.3 Atlantic Forest as a Source of New Biologically Active Substances

The Atlantic Forest is a complex of ecosystems of great importance, as it harbors a significant portion of the biological diversity of Brazil and the world. The richness of the Atlantic Forest is estimated at 20,000 vascular plant species, of which 8000 (40%) are endemic. These numbers constitute the benchmark of plant diversity found in this forest (Rezende et al. 2018).

The traditional use of plant species for food and medicinal treatment is common in Brazilian peasant communities, especially in communities inserted in highly complex forest environments such as the Atlantic Forest. The complexity of the environment, together with the great ethnic miscegenation, has encouraged the use of a considerable diversity of plants by the peasant communities living in these places. However, a large portion of the plant species traditionally used by peasants are not studied and are often unknown even by the younger generations of the community itself. The lack of knowledge about plant species that can be used leads to a dependence of peasant communities on industrialized products and contributes to a decrease in the diversity of foods and medicines accessible to peasants.

In this context, the Brazilian coastline presents itself as a laboratory due to the great diversity of plants that have not yet been identified from the chemical and biological point of view and that need to be studied and valued for their preservation in this biome. These initiatives can contribute to technological innovation and the introduction of new products with less impact from the socio-environmental point of view. However, they require strategic actions, technological skills, and collective efforts.

Thus, it is in this context that bioprospecting presents itself: first as a source of new compounds of natural origin with low toxicity, possibility of generating products with high added value that can be used as starting material in different branches of fine chemistry, and finally new products to improve human health and the environment. Besides counting on the natural resources available in the coastal region of Paraná, one can also count on the traditional knowledge of medicinal plants by the community for the discovery of bioactive compounds, mainly for income generation, since there is a history of use by the population with a wide list of species that need to be validated and, thus, become available for cultivation and commercialization.

Fišer et al. (2021) point out that the identification of native aromatic species is the first step toward this new knowledge and toward the preservation of these potential resources that can opportunely become a way to add income to the local population.

It is known that the therapeutic potential of Brazilian plants has been increasing every year and has aroused the interest of researchers around the world. However, the chemical and biological evaluation of the medicinal flora in Brazil is being carried out by a reduced number of research groups, considering the potential of the country's flora. Knowledge of the chemical composition of these species, besides providing data for the phytochemical knowledge of the national flora, will provide

criteria for the investigation and discovery of substances of pharmacological importance (Valli et al. 2018).

13.4 Bioprospecting

Bioprospecting is the systematic and coordinated search for valuable products obtained from bioresources such as plants, microbes, animals, and other species that may be developed further for commercialization and societal benefit (Oyemitan 2017).

Medicinal plants are gaining importance as bio-energy crops, industrial feedstock renewal, and bio-remedial sectors of the market and are benefitting from technical advancements produced for the food chain. For the sake of supporting rural communities and delivering healthy products, medicinal plant production should be given high attention. The absence of synergy between farmers, herbalists, and industry is a key difficulty in medicinal plant production following domestication. Around 25% of current medications are based on phytochemicals extracted from plants that are often used in traditional medicine. Phytochemical substances extracted from therapeutic plants have been used to create many synthetic equivalents.

Even if the cost of these natural products is expensive, the usage of natural products derived from plants has expanded, as seen by the rising popularity and acceptability of plant-derived healthcare and cosmetics/perfumery products. Medicinal plant cultivation is constantly in demand due to its dual purpose as a source of revenue and in healing. The cultivation and distribution of medicinal, nutraceutical, and fragrance herbs is a burgeoning agro-industry. According to recent global findings, wealthy nations are also incorporating these plants into their healthcare systems (Kewalanand and Prajapati 2018).

13.5 Adding Value to Biodiversity Products

People have been gathering plant and animal resources for their purposes since the dawn of humanity. Edible nuts; mushrooms; fruits; herbs; spices; gums; game; fodder; fibers used in shelter, clothing, or utensils; and plant or animal products for medical, cosmetic, or cultural purposes are all examples. Hundreds of millions of people in developing nations still rely on harvested plant and animal products for a substantial portion of their food and revenue. For cultural and commercial reasons, high-value goods such as mushrooms (morels, matsutake, truffles) and medicinal herbs (ginseng, black cohosh, goldenseal) are still collected in industrialized nations. Medicinal plants play a key part in these applications, not only as traditional remedies in many cultures but also as trade commodities that fulfill demand.

With the rise in human requirements, population, and economic commerce, demand for a wide range of wild species is expanding. With a growing awareness that certain wild species are being overexploited, a number of organizations are suggesting that wild species be introduced into agricultural systems. Cultivation, on the other hand, can have conservation implications that need to be better understood. Cultivation of medicinal plants, for example, can reduce the number of wild populations harvested, but it can also result in environmental degradation, genetic diversity loss, and a lack of incentives to conserve wild populations (Volenzo and Odiyo 2020).

Though new approaches to sustainable natural resource management have arisen throughout time, the growing desire for nature-based health remedies and the integration of endemic flora into global value chains may have negative consequences for ecosystems. The environmental concerns associated with the utilization of wild indigenous medicinal plant resources are compounded by a slew of agrotechnological risks and constraints that severely limit their domestication. As a result, the exploitation and commercialization of medicinal plants necessitates a thorough understanding of their demand and production systems, as well as a value chain analysis. As a result, novel techniques to their integration into global value chains are required (Tom and John 2020).

To secure a continuous and regular supply of medicinal plants for the pharmaceutical sector while also halting the deterioration of the natural resource base, it is necessary to develop adequate framework and technologies for medicinal plant production. In the context of socioeconomic development for sustainable livelihoods of subsistence rural people, the participatory action research framework method would be beneficial to farmers, merchants, scientists, and policy makers (Phondani et al. 2016).

13.6 Fiscalization

The environmental inspection is the exercise of the police power foreseen in the environmental legislation. It consists of the duty that the public authorities have to supervise the conduct of those who are potential or effective polluters and users of natural resources, in order to guarantee the preservation of the environment for the collectivity. Moreover, environmental inspection is necessary to repress and prevent the occurrence of conducts harmful to the environment. By punishing those who cause environmental damage, environmental inspection promotes deterrence. The application of fines, seizures, embargoes, and interdictions, among other measures, aims to prevent environmental damage, punish violators, and avoid future environmental violations. Within this context, what is the challenge? The challenge is to identify activities that cause negative impact on biodiversity and act to align them with the precepts of sustainable development. On the other hand, the obstacles are the inoperability of the enforcement agencies, coupled with the lack of interest and/or knowledge of society, reinforced by the divergence of interests among economic groups.

Some ideas emerge in this approach, among which we highlight:

The strength of the federal Conservation Units lies in the employees who work constantly to motivate the interest of society in these areas, not only as a tourist attraction, but as an asset to be valued more and more in light of the socio-environmental challenges faced by Brazil. This strength also lies in the mobilization of social players, communities, and residents from the surrounding areas—who depend directly or indirectly on the units—social and environmental organizations, the school community, companies, and other government agencies. More and more, these players, in partnership, have been making a difference, proving the high potential of these areas in the social, environmental, and economic development of territories. In this sense it is important to strengthen the System of Conservation Units—UC (public and private), increasing the quantity and improving the quality, especially with regard to management and supervision with the preparation and implementation of management plans in all Units. Another important focus is to ensure the conservation and, where appropriate, the preservation and recovery of fragile areas, especially in coastal areas such as restingas, estuaries, and mangroves. Evaluate qualitatively and quantitatively the ecosystem resources, as well as the ecosystem services, for a better management. And last but not least, ensure the application of financial resources for research on natural resources and biodiversity, enforcement, and biological activity.

13.7 Biological Activity

Herbal medicine has been an issue of increased worldwide relevance in the last decade, affecting both global health and international trade. Traditional medicinal plants continue to play a vital role in the healthcare systems of huge segments of the world's population in terms of global health. This is especially true in underdeveloped nations, where conventional medical systems have a long and continuous history of practice. Traditional medicinal plants are increasingly being recognized and developed for their medical and economic advantages in both developing and developed nations; however, this varies widely by location (Zank and Hanazaki 2017). Plants having a long history of ethnomedicine usage can be a rich source of chemicals for the treatment of a variety of maladies and infectious diseases in the pharmaceutical environment. Medicinal plants are thought to be a storehouse of a variety of bioactive chemicals with various medicinal qualities. Anti-inflammatory, antiviral, anticancer, antimalarial, and analgesic qualities are among the many therapeutic benefits linked with medicinal plants (Aye et al. 2019).

They have the advantage of providing patients with single natural specific compounds (e.g., alkaloids, glycosides, saponins, polyphenols, and flavonoids), are smoother in action, and are better tolerated than synthetic drugs; additionally, they have fewer cumulation problems and can thus be used for a longer period of time. As a result, there is a surge in interest in medicinal plants and their active components (Table 13.1).

Table 13.1 Biological activities of compounds isolated from selected medicinal plants

Source	Compound	Biological activities	Reference(s)
<i>D. culrata</i> (stem bark)	Dalberatin A, dalberatin B	Cancer chemopreventive activity	Ito et al. (2003)
<i>E. chinense</i> (roots)	Khonklonginol A, khonklonginol B, lupinifolinol, dehydrolupinifolinol, flemichin D, eriosemaone A, lupinifolin, 3-epi-lupinifolinol, 2'-dihydroxy lupinifolinol, 3,5,2',4'-tetrahydroxy-6'',6'' dimethylpyrano (2'',3'':7,6)-8-(3''',3'''-dimethylallyl) flavone, tectorigenin, genistein, kaempferol, kaempferol-7-O- β -D-glucopyranoside, astragalin	Cytotoxicity, antimycobacterial activity, antimicrobial activity, anti-oxidant activity	Sutthivaiyakit et al. (2009) and Prasad et al. (2013)
<i>E. suberosa</i> (stem bark)	4'-Methoxy licoflavanone, alpinumisoflavone	The cytotoxic effects on apoptosis in human leukemia HL-60 cells and their potency to induce cancer cell death	Kumar et al. (2013)
<i>E. suberosa</i> (stem bark, flower)	Erysodine, erysotrine	The anxiolytic effects in the elevated plus-maze and the light-dark transition model	Serrano et al. (2011)
<i>M. pendula</i> (timber)	Millettilone A, 3R-claussequinone, pendulone, secundiflorol I, 3,8-dihydroxy-9-methoxypterocarpan, 3,10-dihydroxy-7,9-dimethoxypterocarpan	Leishmanicidal activity	Takahashi et al. (2006)
<i>S. grandiflora</i> (roots)	Isovestitol, medicarpin, sativan, betulinic acid	Antituberculosis activity against <i>M. tuberculosis</i> H37Rv	Pari and Uma (2003)
<i>T. triquetrum</i> (whole plant)	Tadehaginosin, 3,4-dihydro-4-(4'-hydroxyphenyl)-5,7-dihydroxycoumarin	Hypoglycemic activity in vitro by HepG2 cells	Wu et al. (2015)
<i>T. triquetrum</i> (aerial part)	Tadehaginosides C-J, tadehaginoside	Antidiabetic activity	Zhang et al. (2016)
<i>A. echioides</i> (whole plant)	Dihydroechioidinin-5,7,8-Trimethoxyflavone	Anti-inflammatory activity	Shen et al. (2013)
<i>J. gendarussa</i> (stem and bark)	Justiprocumin B, patentiflorin A	Anti-HIV activity	Zhang et al. (2017)
<i>P. integrifolia</i> (stem bark)	10-O-trans-p-Methoxycinnamoyl catalpol, 4''-Hydroxy-E-globularinin, Premnosidic acid, Premnadimer, 4- β -Hydroxyasarinin-1-O- β -glucopyranoside	Antioxidant activity	Yadav and Gupta (2013)

(continued)

Table 13.1 (continued)

Source	Compound	Biological activities	Reference(s)
<i>V. trifolia</i> (aerial part)	Agnuside, negundoside, 6- <i>p</i> -hydroxybenzoyl mussaenosidic acid	Antioxidant activity	Tiwari et al. (2012)
<i>V. trifolia</i> (leaves)	Methyl- <i>p</i> -hydroxybenzoate	Mosquito larvicidal activity against <i>Culex quinquefasciatus</i> and <i>aedes aegypti</i>	Kannathasan et al. (2011)
<i>A. pennata</i> (leaves)	Taepeenin D, (+)-Drim-8-ene, quercetin 3- <i>O</i> - β -D-glucopyranosyl-4- <i>O</i> - β -D-glucopyranoside	Hedgehog/GLI-mediated transcriptional activity, cytotoxic against pancreatic cells and prostate cells	Rifai et al. (2010)
<i>C. auriculata</i> (leaves)	Pseudosemiglabrin, (2 <i>S</i>)-7,4-dihydroxy flavan (4 β $\rightarrow$ 8)-catechin, (2 <i>S</i>)-7,4-dihydroxy flavan (4 β $\rightarrow$ 8)-gallocatechin, (–)-epigallocatechin	Hepatoprotective effects	Rajagopal et al. (2003)

13.8 Essential Oils

Essential oils are natural materials obtained from aromatic plants that have been used for disinfection, anti-inflammatory, soothing, and stimulating substances for thousands of years across the world and now have the potential to be employed in clinical care. Ibn al-Baitar (1188–1248) is said to have made the first written reference of essential oil production techniques and processes. Geographic location, plant origin, genetics, bacterial endophytes, and extraction processes all influence the chemical makeup of essential oils. In the culinary and cosmetic sectors, essential oils are widely employed.

They can be used to prevent and cure infectious infections as natural alternatives to pharmaceutical medications. Other ailments such as respiratory tract, digestive system, gynecological, andrological, endocrine, cardiovascular, nervous system, and skin infections have historically been treated with them. Many of them have also showed anticancer properties (Firenzuoli et al. 2014). Various techniques are used to extract essential or volatile oils from the flowers, barks, stems, leaves, roots, fruits, and other parts of the plant (Babar Ali et al. 2015).

In addition, from aromatic plants, essential oils can be extracted, compounds considered volatile, natural, and complex, having in the composition the mixture of several phytoconstituents, chemicals produced from the secondary metabolite. Essential oils present several types of biological effects, such as antimicrobial, analgesic, sedative, and anti-inflammatory actions, being used in traditional medicine (Simões et al. 2017; Sharifi-Rad et al. 2017) (Table 13.2).

Table 13.2 Functional properties of essential oils and their active compounds

Sl. no.	Medicinal plant essential oil	Functional properties	Compounds	Reference
1	<i>Lippia alba</i> , bushy matgrass	It is used as food supplement, for seasoning and as a drink, digestive and respiratory conditions	Myrcene, linalool, β -ocimene, α -guaiene, germacrene, carvone, limonene, eucalyptol, camphor, caryophyllene, neral, geranial, and piperitone	Sonia Malik et al. (2021)
2	<i>Piper</i>	Chronic diseases such as diabetes, atherosclerosis, and osteoporosis; antioxidant and anticholinesterase properties	Dillapiol, γ -terpinene, <i>p</i> -cymene, and α -terpinene	Midia et al. (2021)
3	<i>Annona acutiflora</i>	Against <i>Aedes aegypti</i> larvae; anticholinesterase effect	α -Santalene, bicyclogermacrene, and α -zingiberene	Folly et al. (2021)
4	<i>Ipomoea alba</i> , <i>I. indica</i> , <i>I. amnicola</i> , <i>I. tiliacea</i> , <i>I. carnea</i> , <i>Ipomea pes-caprae</i> , <i>I. batatas</i>	Against snakebite and filariasis	β -Caryophyllene, <i>cis</i> -cadina-1(6),4-diene, and germacrene D	Tenório et al. (2021)
5	<i>Valeriana jatamansi</i>	Cytotoxic activity against human lung carcinoma and human skin carcinoma cancer cells	Patchouli alcohol, β -bisabolol, maaliol, α -bulnesene	Maurya et al. (2021)
6	<i>Zanthoxylum armatum</i>	Against various diseases like asthma, bronchitis, indigestion, varicose veins, and diarrhea	Monoterpenes and oxygenated monoterpene hydrocarbons with β -phellandrene, sabinene, β -myrcene, limonene, linalool, terpinen-4-ol, 1,8-cineole, undecan-2-one, and <i>cis</i> -sabinene hydrate	Vashisath et al. (2021)
7	<i>C. camphora</i>	Effective in controlling <i>A. gemmatilis</i>	Linalool	Chamali et al. (2021)
8	<i>E. intertexta</i> and <i>E. diversifolia</i>	Anti-inflammatory and anti-Alzheimer activities	1,8-cineole and α -pinene and 1,8-cineole, caryophyllene oxide, and <i>p</i> -cymene	Chamali et al. (2022)
9	Rosemary leaves	Strong antioxidant effect	α -Pinene, camphor, borneol, and (<i>E</i>)-caryophyllene	Meziane-Assami et al. (2021)

13.9 Seasonality

Seasonal variations are a periodic sequence that affects the availability of active ingredients in medicinal plants, hence affecting therapeutic efficacy. This is supported by a review of various scientific papers published in prestigious international magazines. Essential oil component indicates a cyclical increase in oil production during the growing season and then a gradual fall toward the winter, according to research; thus, late summer is the optimal time to collect. Other study, on the other hand, determined that the optimal season for gathering essential oils containing plant components is during the winter. Seasons have a tremendous impact on not only essential oils but also other key elements such as polyphenol, flavonoids, glycosides, alkaloids, and others. There is no common guideline regarding when to harvest secondary metabolites for a higher yield. Although various researches have been published on the influence of seasons on secondary metabolites, there is no comprehensive data on the best season for collecting pharmaceutically essential components from various plant sources (Soni et al. 2015).

Plants exist on a world with days and seasons, and their phytoconstituents are affected by this. Due to seasonal variations in the availability of active principles in medicinal plants, their dosing pattern for therapeutic effectiveness is also altered. The optimal harvesting time for certain secondary metabolites in order to maximize production is unknown. Changes in key elements such as polyphenol, flavonoids, glycosides, alkaloids, and essential oil are influenced by the seasons. The optimal time to harvest essential oil components is in the late summer. The optimum time to collect secondary metabolites containing plant components is during the winter (Palshikar and Ambavade 2019).

Drug gathering has been advised in Vedic and Ayurvedic literature for different portions of the plant in different seasons, asterisms (akatra), on the basis of vryas and medicinal purposes. Climate, temperature, rainfall, length of day light, altitude, cultivation methods, lunar cycle effect, collection from wild area, soil condition, and methods of collection, processing, and storage all have an impact on the plant's secondary metabolites, which in turn affect the drug's therapeutic efficacy (Tavhare et al. 2016).

13.10 Socioeconomic Approaches

It is important to highlight that the constitution of networks is an important way to consolidate projects for the conservation and sustainable use of natural resources. Such networks can be structured on socioeconomic, environmental, and scientific pillars. We can highlight the following aspects:

Economic: development of new products.

Scientific: production of patents and various publications, contributing to the advancement of knowledge in the area, and training of human resources at the

scientific initiation, master's and doctorate levels, contributing to the overcoming of regional inequalities in S&T in the country.

Social: generation of job and income in the industry and in family agriculture (cultivation of the selected species, providing raw material for the industry).

Environmental: contribution to the conservation of species through cultivation techniques and propagation to be studied and the appreciation by the community, which has in the selected species an opportunity for work and improvement of living conditions (sustainable use of native species).

13.11 Conclusions and Future Prospects

Due to the process of fragmentation and destruction of the forests, there has been a decrease in the patterns of biodiversity, resulting in changes in the ecosystem and its elements. Among the immense biodiversity of the biome and the innumerable plant species, several families such as Myrtaceae, Lauraceae, and Piperaceae can be highlighted. Thus, one can consider that studies of chemical composition, yield, and biological activities of these families can provide important subsidies to the knowledge of their medicinal potential. The knowledge of sustainable use practices provides better use of species without degrading the natural environment, promotes development strategies, and contributes to the preservation of local biodiversity.

In view of the above and due to the plant diversity, research that considers the association between the genetic resource available in the forest and the sustainable use of natural resources becomes very relevant. However, it is necessary to formulate conservation strategies and develop research with native species, in order to guarantee that the pressure suffered by extractivism is replaced by sustainable agricultural management. Such actions aim to subsidize family farmers, enabling the emergence of work and income from the cultivation of medicinal plants, but in a perspective of sustainable territorial development.

On the other hand, selective sampling of biodiversity from ethnobotanical and traditional medicine information can significantly reduce research costs. However, the use of associated traditional knowledge can involve additional layers of political and contractual complexity. Thus, the use of natural raw materials for the production of medicines can contribute to the dissemination of environmental conservation practices in the field. The strict quality control, the reduced need for scale, and the higher added value inherent in medicines make environmental conservation more profitable for local communities or farmers, providing incentives for the exploitation of natural resources to become sustainable.

In conclusion, the bet on compounds inspired by biodiversity, with potential to generate new molecules, can contribute to the learning and consolidation of a productive chain of medicinal plants in developing countries.

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Chapter 14

Traditional Practices of Ethnomedicinal Plants in the North-Eastern Region of India and Their Conservation for Sustainable Utilization



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Abstract Medicinal plants are valuable resources for drugs, and the major population in developing countries depends on herbal drugs. Increasing demands on the use of medicinal plants as primary therapeutics put pressure on the conservation strategies of wild medicinal plants. The north-eastern region is considered a reservoir of many medicinal plants accounting for approximately half of the available medicinal plants in India. Many medicinal plant species are endemic to this region, and many growing in the wild are left unaware, so there is a need for identification and conservation. National policy needs to be framed and implemented to conserve the population of medicinal plant species. In this modern world, it is essential to adopt both ex situ and in situ methods as complementary techniques for the conservation of the medicinal plant population.

Keywords Conservation · Medicinal plants · North-eastern region · Wild · Herbal drugs

14.1 Introduction

Maintaining biodiversity is essential to the supply of ecosystem services (Pereira et al. 2013). Despite a global effort for protection, the loss of biodiversity is not decreasing (Butchart et al. 2010). The major causes of biodiversity loss include habitat change and their over-exploitation, pollution, the introduction of invasive

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species, and climatic changes (Marchese 2015). While climatic changes may force biodiversity to adapt through either the change of habitat and life cycles or the development of new physical traits (Berteaux et al. 2010), the threats of pollution and over-exploitation of habitats are often irreversible (Marchese 2015).

An effective strategy to preserve biodiversity is therefore setting conservation priorities through the declaration of biodiversity hotspots (Marchese 2015). India is one of the biodiversity-rich countries in the world and is included in at least four geographically distinct hotspot regions (Mittermeier et al. 2011). The north-eastern region of India is included in two biodiversity hotspot zones, namely, the Himalayas (Sikkim, Assam, and Arunachal Pradesh) and the Indo-Burma hotspot (other north-eastern states). The reported endemism of plant species in the two hotspots falls between 31 and 52%, indicating a rich floral diversity (www.cepf.net).

North-East India has India's richest reservoir of plant diversity and supports around 50% of India's total plant diversity, including 40% of India's endemic plant species (Hedge 2000; Mao and Hynniewta 2000). The unique environmental conditions in the region comprising tropical, subtropical, temperate, and alpine climatic conditions support the growth of a range of plant varieties. The region is also a source of many of the medicinal plant bioresources, which are used in traditional healthcare practices (Deb et al. 2015). *Maiba* and *Maibis* (local healers in Manipur) have practised traditionally healing methods using locally available herbs and plants (Singh 2015). Many of these medicinally important plants and their uses have been documented in several literatures (Singh et al. 2001; Singh 2015; Panmei et al. 2019; Chakraborty et al. 2020). In this chapter, we highlight the ethnomedicinal properties of 11 medicinal important plants available in the north-eastern states of India. Threats to the population of such medicinal plants and their conservation strategies, in general, are also discussed.

14.2 Ethnomedicinal Plants of the North-Eastern States: Botanical Description, Distribution, and Their Traditional Practices of Ethnomedicinal Uses

People in the rural areas largely depend on locally available medicinal plants for their basic healthcare needs. The following is the documentation of 11 medicinal plant species belonging to 9 families. The scientific name, its taxonomic affiliation, and its therapeutic uses are listed in Table 14.1.

14.2.1 *Allium hookeri*

Allium hookeri or hooker chives (Fig. 14.1a) belongs to the Alliaceae family and has the characteristics of two different families of monocot: Amaryllidaceae and

Table 14.1 Classification and therapeutic uses of the 11 selected ethnomedicinal plants of the north-eastern states

Scientific name	Local vernacular name (North-East India)	Family	Therapeutic uses
<i>Allium hookeri</i>	Common name: Hooker chives Manipur: Maroi napakpi Meghalaya: Ja-ut Mizoram: Runphek	Alliaceae	Anti-asthmatic (Bok et al. 2019) Anti-hypertension activity (Tong et al. 2021)
<i>Alpinia galangal</i> Linn.	Common name: Greater galangal Arunachal Pradesh: King pang Assam: Jernao Manipur: Kanghoo Meghalaya: Phlang saw Mizoram: Aichal Nagaland: Awsu Tripura: Kurchi vaca or Paale	Zingiberaceae	Anesthetic (Khumpirapang et al. 2018) Anti-inflammatory (George et al. 2021) Antimelanogenic (Panich et al. 2010) Antimicrobial (Hsu et al. 2010) Antiviral (including anti-HIV) (Tamura et al. 2009) Gastroprotective (Matsuda et al. 2003) Neuroprotective (Singh et al. 2011)
<i>Capsicum annuum</i> L	Common name: Pepper, bell pepper Arunachal Pradesh: Solu Assam: Jolokia Manipur: Morok atekpa Meghalaya: Sohmynten Mizoram: Hmarcha Nagaland: Chusi Sikkim: Khorsani Tripura: Moso	Solanaceae	Analgesic (Jordt et al. 2000) Anti-inflammatory (Jang et al. 2011) Antimicrobial (Dos Santos et al. 2017) Anti-obesity (Tan et al. 2014) Hepatoprotective (Zhang et al. 2019) Immunomodulatory (Yang and Song 2021)
<i>Curcuma amada</i> Roxb.	Common name: Mango ginger Manipur: Yai-heinou nambi Mizoram: Aiengpui Nagaland: Amra Haridra Sikkim: Phacheng	Zingiberaceae	Analgesic activity (Mujumdar et al. 2004) Antiallergic activity (Pushpangadan et al. 2006) Antibacterial activity (Siddaraju and Dharmesh 2007) Anticancer potential (Syiem et al. 2010) Antifungal activity (Singh et al. 2002) Anti-hyperglycaemic activity (Syiem et al. 2010) Anti-inflammatory activity (Mujumdar et al. 2000) Antioxidant activity (Prakash et al. 2007) Antitubercular activity (Singh et al. 2010)

(continued)

Table 14.1 (continued)

Scientific name	Local vernacular name (North-East India)	Family	Therapeutic uses
<i>Curcuma caesia</i> Roxb.	Common name: Black turmeric Arunachal Pradesh: Chongkah, Homen Assam: Kala haladhi Manipur: Yaimu Mizoram: Aihang Nagaland: Narkachura	Zingiberaceae	Platelet aggregation inhibitory activity (Policegoudra and Aradhya 2008) Biopesticide activity (Ahmad and Ahmad 1991) Brine shrimp lethal activity (Krishnaraju et al. 2006) CNS depressant (Mujumdar et al. 2004) Cytotoxicity (Policegoudra and Aradhya 2008) Enterokinase inhibitory activity (Syiem et al. 2010) Hypotriglyceridemic activity (Srinivasan and Chandrashekhara 1992) Analgesic activity (Kaur and Satija 2013) Anti-asthmatic activity (Paliwal et al. 2011) Antibacterial activity (Rajamma et al. 2012) Anti-convulsant (Karmakar et al. 2011) Antifungal activity (Banerjee and Nigam 1976) Anthelmintic activity (Gill et al. 2011) Anti-inflammatory activity (Rajamma et al. 2012) Antioxidant activity (Chirangini et al. 2004) Antitumorogenic activity (Das et al. 2012) Anxiolytic (Karmakar et al. 2011) CNS depressant activity (Karmakar et al. 2011) Locomotor depressant (Karmakar et al. 2011) Muscle relaxant effects (Karmakar et al. 2011) Smooth muscle relaxant activity (Arulmozhi et al. 2006) Antibacterial (Sasikumar et al. 2017)
<i>Haematocarpus validus</i>	Common name: Blood fruit Meghalaya: Te pattang, Sohnsam Mizoram: Theichhung-sen, Rosco Tripura: Thoyphal	Menispermaceae	
<i>Houttuynia cordata</i> Thunb.	Common name: Lizard's tail Arunachal Pradesh: Mumbre, Siya hamang, Muchandariin Assam: Ashundary, Tengalai, Mojoukhmo,	Saururaceae	Antibacterial (Wu et al. 2021) Anti-diabetic (Wu et al. 2021) Anti-inflammatory activity (Wu et al. 2021) Antimutagenic (Chen et al. 2003)

	Aithong, Jarmendo Manipur: Toningkhok Meghalaya: Ja-myr-doh, Tmyr-doh, Machha-turi Mizoram: Uithinhang Nagaland: Nuichua, Nokana Sikkim: Ghandhay jhar		Antioxidative activity (Ng et al. 2007) Antitumour (Liu et al. 2020) Antiviral activity (Das et al. 2022) Immunomodulatory properties (Wu et al. 2021)
<i>Meriandra bengalensis</i>	Common name: Bengal sage Manipur: Kanghuman	Lamiaceae	Anti-diabetic (Kidane et al. 2018) Antihypertension (Rana and Blazquez 2009)
<i>Phlogacanthus thyriformis</i> (Roxb. Ex. Hardw.) Mabb.	Common name: Nongmangkha Arunachal Pradesh: Teeta vasa, Phikhip, ran-hing, Taksirma momnang sing Assam: Tita phool Manipur: Nongmangkha Meghalaya: Dieng-soh kajut, allot, jia merembut Mizoram: Khumtiangkohha Nagaland: Titi vasaka Tripura: Basakpata, Ravan basak	Acanthaceae	Analgesic (Mukherjee et al. 2009) Anti-arthritis (Das et al. 2017) Anticancer activity (Chanu et al. 2021) Anti-diabetic activity (Koushik et al. 2020) Anti-diarrhoeal activity (Paharia and Pandurangan 2013) Anti-hyperglycaemic effect (Chakravarty and Kalita 2012) Anti-inflammatory (Das et al. 2015) Antimicrobial activity (Singh and Singh 2010) Antioxidant activity (Chanu et al. 2012) Cytotoxic activity (Tiwarly et al. 2015)
<i>Polygonum posumbu</i> Buch-ham. Ex D. Don	Common name: Oriental lady's thumb Manipur: Fakpai	Polygonaceae	Antibacterial (Ishwori et al. 2014) Antipyretic (Yunnam and Tripathi 2012) Dyspepsiac (Yunnam and Tripathi 2012) Reduce heartbeat (Singha et al. 2016)
<i>Zanthoxylum armatum</i> DC	Common name: Winged prickly ash, toothache tree Assam: Zabrang Manipur: Mukthubi Mizoram: Arhikreh Meghalaya: Dieng-sohkhiam, Dieng-jaiur Sikkim: Warekpa, bale timur, Nangri Tripura: Chaingslong	Rutaceae	Anti-diabetic (Khan et al. 2018) Anti-inflammatory (Bhatt and Upadhyaya 2010) Antimicrobial (Srivastava et al. 2013) Antinociceptive (Guo et al. 2011) Antioxidant (Karmakar et al. 2015) Antispasmodic (Barkatullah et al. 2013) Cytotoxicity (Mukhija et al. 2015) Hepatoprotective (Ranawat et al. 2010) Insecticidal (Zhang et al. 2018) Phytotoxicity (Barkatullah et al. 2011)

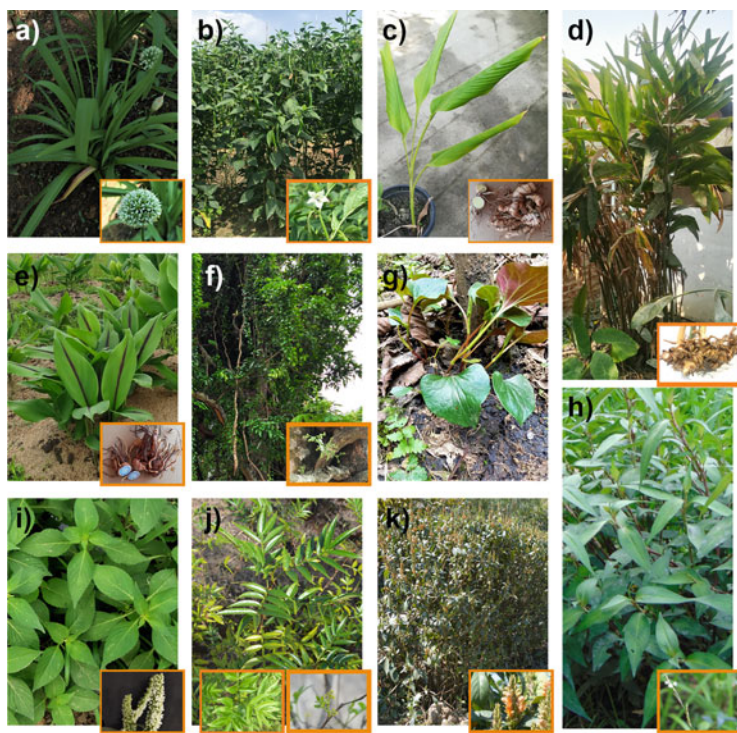


Fig. 14.1 The various ethnomedicinal plants identified in the north-eastern part of India; flowering parts/rhizomes are shown in the insight of each figure. (a) *Allium hookeri*, (b) *Capsicum annum* L., (c) *Curcuma amada* Roxb., (d) *Alpinia galanga*, (e) *Curcuma caesia* Roxb., (f) *Haematocarpus validus*, (g) *Houttuynia cordata* Thunb., (h) *Polygonum posumbu* Buch-Ham. ex D. Don., (i) *Meriandra bengalensis*, and (j) *Zanthoxylum armatum* DC

Liliaceae. The genus has the feature of Amaryllidaceae in having scape umbel inflorescence with membranous bracts and Liliaceae in having a superior ovary. The plant comprises roots, green-flattened leaves, and scapes bearing umbel inflorescence (Devi et al. 2014a). The roots of *A. hookeri* are thick, fleshy, and whitish.

The plant species has been reported from most parts of India including the north-eastern states of Assam, Meghalaya, and Manipur. Globally, it is distributed across Southwest Asia, Mediterranean, Bhutan, South-Central China, East Himalaya, Myanmar, Sri Lanka, and Tibet (Barooah and Ahmed 2014).

Leaves and roots are important tissues that are not only used as spice condiments but are also reported to have medicinal properties (Jang et al. 2017; Swarnalata and Sharma 2016). In Manipur, the leaves are used to treat ulcers and stomach ailments and to reduce body temperature and blood pressure (Singh et al. 2016; Swarnalata and Sharma 2016). In pharmacopoeias and traditional systems of medicine, it is documented to cure cough, cold and vomiting, wounds, skin diseases, body aches,

Table 14.2 Bioactive compounds identified from different plant parts of *Allium hookeri* Linn

Sl. no.	Plant parts	Extract/oils	Bioactive compounds	References
1	Root and stem	Methanol	Dimethyl thiosulfinate, allyl methyl thiosulfinate, 1-propenyl methyl thiosulfinate, methyl 1-propenyl thiosulfinate, methyl propyl thiosulfinate, propyl methyl thiosulfinate, diallyl thiosulfinate, allyl propyl thiosulfinate, 1-propenyl allyl thiosulfinate, 1-propenyl propyl thiosulfinate	Rhyu and Park (2013)
2	Root	Methanol	Ferulic acid-4-O- β -D-glucopyranoside	Jun et al. (2015)
		Ethanol	Benzoic acid, tetradecanoic acid, hexadecanoic acid, ferulic acid, cinnamic acid, octadecanoic acid, hexanedioic acid	Kim et al. (2016)
3	Whole plant	Water	Alliin, S-propyl-L-cysteine sulfoxide, L-tryptophan, N- γ -glutamyl-S-(1-propenyl)cysteine, N- γ -glutamyl-S-propylcysteine, N- γ -L-glutamyl-L-phenylalanine, sinapic acid, ferulic acid, N-feruloyltyramine	Park et al. (2016a)
		Water and ethanol	Gallic acid, protocatechuic acid, hydroxybenzoic acid, catechin, vanillic acid, caffeic acid, chlorogenic acid, syringic acid, <i>p</i> -coumaric acid, ferulic acid, sinapic acid, rutin, cinnamic acid, quercetin	Park et al. (2016b)

etc. (Devi et al. 2014a). The list of phytochemicals/bioactive compounds reported from *A. hookeri* is given in Table 14.2.

14.2.2 *Capsicum annuum* L.

Capsicum annuum is the oldest and most widely cultivated species among the five domesticated *Capsicum* species. *C. annuum*, *Capsicum chinense*, and *Capsicum frutescens* are categorized under the white-flowered group and have similar morphological traits as they share an ancestral gene pool. *C. annuum* has white flowers with pale purple anther and greenish-white style and filament. Leaves are lanceolate with broad shoulders and dark green. The shape and size of the fruits varied from spherical to blocky. The flesh of the fruit is a little hard and thick (Csilléry 2006). The colour of the fruits varies from green, yellow, orange, red, to purple, and the fruit may be pungent or non-pungent.

C. annuum originates from central-east Mexico (Kraft et al. 2014). North-East India is blessed with a rich biodiversity of *Capsicum* species and can be distinguished based on their morphological appearance, flavour, and pungency levels (Bhutia et al. 2019). Two varieties of *C. annuum* have been awarded the geographical indication (GI) tag from the Department of Industry Promotion and Internal Trade, India. These include 'Dalle Khursani' (*Capsicum annuum* var. *cerasiforme*) of Sikkim and Hathei chilli of Manipur.

Table 14.3 Bioactive compounds identified from different plant parts of *Capsicum annuum* L

Sl. no.	Plant parts	Extract	Compound	References
1	Fruits	Methanol	Capsaicin, dihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, nordihydrocapsaicin	Todd et al. (1977)
		Ether	Lutein, α -carotene, β -carotene, neoxanthin, 9-cis-neoxanthin, α -cryptoxanthin, violaxanthin, capsanthin, capsanthone, zeaxanthin, capsorubin, capsochrome, capsanthin 5,6-epoxide, quercetin, apigenin, luteolin, myricetin, apigenin, and kaempferol	Deli et al. (2001)
2	Seeds	Methanol	Capsicoside E, capsicoside F, and capsicoside G	Iorizzi et al. (2002)

C. annuum has wide ranges of photochemical compounds (Table 14.3), of which capsaicinoids are the class of compounds responsible for the pungency (hotness) of chilli. Carotenoids, flavonoids, vitamins, minerals, and essential oil are the other photochemical constituents beneficial to human health. Fruits of Hathei chilli (Fig. 14.1b) can be used green or red. Dry chilli is extensively used as a spice in curried dishes. It is also used as an ingredient in curry powder and seasonings. Dehydrated green chilli is a good source of vitamin 'C'.

Dalle Khursani (*C. annuum* var. *cerasiforme*) also known as red cherry pepper is a local cultivar of Sikkim. As the name suggests, it bears a round bright red chilli with a nearly round shape which is approximately 2–2.8 cm. *C. annuum* var. *cerasiforme* contains capsaicin which is responsible for its pungency. Its pungency level is very high with a Scoville rating of 100,000 to 350,000 SHU (Scoville heat units) (Bhutia et al. 2016). Dalle Khursani is also grown in hilly regions of Nepal and the Himalayan range, and the local tribes consumed it for curing duodenal diseases (Sen et al. 2016).

14.2.3 *Curcuma amada* Roxb

The plant is a perennial and rhizomatous herb (Fig. 14.1c). The plant measures 1 m with long petiolate, oblong-lanceolate 5 to 6 leaves. The rhizomes are fleshy, pale yellow inside with lighter colour outside, 5–10 cm long, and with well-defined nodes and internodes. The rhizomes are branched and its branching is sympodial (Policegoudra et al. 2011).

It is found in the wild in parts of West Bengal and is cultivated in Gujarat, Uttar Pradesh, Kerala, Karnataka, Tamil Nadu, and the north-eastern states. They originated in the Indo-Malayan region and distributed widely in the tropics from Asia to Africa and Australia (Sasikumar 2005).

Traditional healers of Manipur use fresh rhizome for treating intestinal worm infection. Rhizomes are carminative, are stomachic, and are used on contusions and

Table 14.4 Bioactive compounds identified from rhizomes of *Curcuma amada* Roxb

Plant part	Extract	Composition	Reference
Rhizome	Acetone	Curcumin, phytosterol, pinene, camphor, curcumene, and ar-turmerone	Jain and Mishra (1964)
		Curcumin, demethoxycurcumin, bis-demethoxycurcumin	Gupta et al. (1999)
	Ethanol	Free phenolic acids, caffeic acid, gentisic acid, ferulic acid, gallic acid, cinnamic acid, protocatechuic acid, syringic acid, and p-coumaric acid, and bound phenolic acids, ferulic acid, cinnamic acid, p-coumaric acid, syringic acid, caffeic acid, gallic acid, and gentisic acid	Siddaraju and Dharmesh (2007)
	Chloroform	Difurocumenonol, amadannulen, amadaldehyde	Policegoudra et al. (2007)
	Volatile oil	α -pinene, β -pinene, myrcene, β -ocimene (E and Z), perillene, limone, camphor, curzerenone, 1,8-cineole, camphene, limonene, β -elemene, isoborneol, β -curcumene, ar-curcumene, lin-alool, zingiberene, linyol acetate, safrole	Dutt and Tayal (1941), Choudhury et al. (1996), Srivastava et al. (2001) and Singh et al. (2003)

sprains (Devi et al. 2017). The Mishing tribe of Golaghat district, Assam, uses the rhizome for cough, cold, and diabetes (Das and Hazarika 2015). *Bawm* traditional practitioner of Mizoram applied the rhizome poultice externally for chronic ulcer and inflammation (Lalramnghinglova 2016). Traditional practitioners in Nagaland used the rhizome for cough and digestion (Shankar and Devalla 2012). Various tribal communities of Nagaland also use the rhizome as deworming, anti-inflammatory, and antifungal agents (Zhasa et al. 2015). In Sikkim, fresh rhizomes are cut and rubbed over the whole body to cure skin allergy and body itching. Rhizome paste is also applied externally to sprained joints (Badola and Pradhan 2013). The bioactive compounds reported from *Curcuma amada* are listed in Table 14.4.

14.2.4 *Alpinia galanga*

The species is a rhizomatous herb (Fig. 14.1d) that grows up to 7 feet. The leaves are long, oblong-lanceolate, acute, and glabrous, and the ligules are short and rounded beneath with acute apices (Chouni and Paul 2018). The matured leaf measures around 10–40 cm. The inflorescence bears greenish-white flowers with compound

panicle, flowers with short pedicel, corolla-lobes linear-oblong, lip obovate-clawed emarginate, white-veined with lilac, staminodes reduced at the base of the lip; stamen arcuate, shorter than the lip (Sharma et al. 2011). The rhizomes are cylindrical which measures up to 6–10 cm, fleshy cream in colour, and covered with brown scales. The fruit is orange to red in colour, ellipsoidal, and capsule-shaped.

Alpinia galanga is believed to be originated in Sumatra and Java (Roxburgh 1810). It is common in the parts of the South Asian countries, Sri Lanka, and the Arabic Gulf. Its distribution within India is restricted to the north-eastern states, West Bengal, and South Indian states.

Traditional healing practices using *A. galanga* involve extracts derived from rhizomes, leaves, and seeds. Rhizomes were used for regulating blood circulation, as antiparasitic, and in the treatment against the cold in Manipur (Yumnam and Tripathi 2012). Fresh rhizome paste is mixed with *Euphorbia neriifolia* Linn. and used to treat local inflammation and skin allergy caused by microbes or insect bites (Tag et al. 2007). In Nagaland, galangal rhizomes are used in treating rheumatic pain, catarrhal infections, fever, leucoderma, piles, and respiratory troubles (Sumi and Shohe 2018). The Chakma tribe from Tripura uses fresh rhizome juice orally to ease painful menstruation and at the time of childbirth to induce labour pain and to promote expulsion of the placenta (Bora et al. 2014). Table 14.5 lists the bioactive compounds that have so far been reported from the different plant parts of this species.

14.2.5 *Curcuma caesia* Roxb

Curcuma caesia Roxb. is commonly known as black turmeric because of its bluish-black rhizome (Fig. 14.1e). The plant is normally 0.5 to 1 m high with leaves having deep violet patches that run throughout the lamina. It has a tuberous rhizome with a diameter of 2–6 cm and has a camphorous odour. The inflorescence is 15–20-cm-long dense spike, the bracts are green, and the bracts of coma are deep red and turn crimson when old. The flower is smaller than bracts with pale yellow and reddish borders. The rhizomes are bluish-black in colour, emit a characteristic odour, and had a bitter taste (Pandey and Chowdhury 2003; Das et al. 2013).

It is native to North-East and Central India and is also widely cultivated as a medicinal plant in Southeast Asian countries (Das et al. 2013). The species thrives well in moist deciduous forest areas in clayey soil.

The rhizome is widely used in the north-eastern states as a medicinal plant because of the presence of an array of bioactive compounds (Table 14.6). The paste of the rhizome is applied on wounds, sprain, bruises, rheumatic pain, and cough in Manipur and Nagaland (Ningombam et al. 2014; Behura and Srivastava 2004). In Mizoram, the rhizome infusion is taken internally for cough, cold, and diarrhoea (Lalramnghinglova 2016). In Arunachal Pradesh, the fresh decoction of the rhizome is used as anti-diarrheic, while the fresh paste of rhizome is applied over snake and scorpion bites (Chadalavada and Budala 2017). The rhizomes are also

Table 14.5 Bioactive compounds identified from different plant parts of *Alpinia galanga* Linn

Sl. no.	Plant parts	Oil/extract	Bioactive compounds	References
1	Rhizomes	Essential oil	α -Pinene, β -pinene, myrcene, limonene, (Z)- β -ocimene, β -caryophyllene, β -bisabolene, trans- β -farnesene, caryophyllene, 1,8-cineole	Charles et al. (1992)
			Phenylpropanoids, eugenol acetate, terpinen-4-ol, α -terpineol, (E)- β -farnesene, β -sesquiphellandrene	Raina et al. (2014)
		Methanol	Galangoflavonoside	Jaju et al. (2009)
		Acetone	p-Methane-1,8-epoxy-acethoxychavicol acetate, (1'S)-1'-acetoxyeugenol acetate, 1'-acetoxyeugenol acetate, (1'S)-1'-Acetoxychavicol acetate, 1'-Acetoxychavicol acetate	Khairullah et al. (2020)
			Alpinine, kaempferide, galangin, pineol, 3-dioxy 4-methoxy flavone	Divakaran et al. (2013)
2	Flowers	Oil	Catechin, pinocembrin, galangin 3-methyl ether, kaempferol, isorhamnetin, quercetin 3-methyl ether, quercetin	Tang et al. (2018)
		Methanol	(E)-whiskey lactone, T-muurolol, cubenol, and β -eudesmol	
3	Leaves	Oil	α -Pinene, β -pinene, myrcene, limonene, (Z)- β -ocimene, β -caryophyllene, β -bisabolene, trans- β -farnesene, caryophyllene, 1,8-cineole, camphor, (E)-methyl cinnamate, bornyl acetate, guaiol	Charles et al. (1992) and Jirovetz et al. (2003)
4	Stem	Oil	1,8-cineole, camphor, β -pinene, (E)-methyl cinnamate, bornyl acetate, guaiol, α -fenchyl acetate	Jirovetz et al. (2003)
5	Seeds	Methanol	1'-Acetoxychavicol acetate, 1'-acetoxyeugenol, caryophyllene oxide, caryophyllenol-I, caryophyllenol-II, n-7-heptadecene, n-pentadecane, fatty acid methyl ester	Mitsui et al. (1976)
		Ethanol	Alpinone, (2R,3S)-pinobanksin-3-cinnamate, isokaempferide, kumatakenin, pinocembrin, galangin 3-methyl ether	Bian et al. 2014
6	Roots	Oil	α -Fenchyl acetate, 1,8-cineole, borneol, bornyl acetate, elemol	Jirovetz et al. (2003)
		Ethanol	Galangin 3-methyl ether	Nair and Gunasegaran (1982)

Table 14.6 Bioactive compounds identified from rhizomes of *Curcuma caesia* Roxb

Plant part	Extract ^a	Composition	Reference
Rhizome	Volatile oil	Camphor, ar-turmerone, (Z)-ocimene, ar-curcumenone, 1,8-cineole, elemene, borneol, bornyl acetate, curcumenone	Pandey and Chowdhury (2003) Vairappan et al. (2013)
	Ethanol (hexane/ethyl acetate)	Furanodiene, germacrone, isofuranodienone, curzerenone, aerugidiol	
	Ethanol (chloroform/hexane)	Zederone, curcumenone, germacrone 4,5-epoxide, curcumenol	
	Ethanol	Diarylheptanoids (curcuminoids) and sesquiterpenoids	

^aCompounds in the parenthesis represent elution buffer used in the fractionation of the extract

used traditionally for the treatment in stomachache, typhoid, and wounds in the tongue by the Monpa community of Dirang (West Kameng) in Arunachal Pradesh (Namsa et al. 2011). The powdered rhizome is orally administered with water to treat gastric disorder (Borah et al. 2020). Fresh juice is mixed with mustard oil for the treatment of dysentery in cattle in Assam (Saikia and Borthakur 2010).

14.2.6 *Haematocarpus validus*

The plant is a large creeping woody climber, perennial growing under extreme conditions from withered to highly acidic soils (Fig. 14.1f). Morphologically, the plant leaves are oval shape, alternate, petiolate, acute, entire, and deciduous which spread on the tall trees. Leaves are 7.3–10.2 cm long, 3.4–5.2 cm wide, dull green when living, and pale grey or slightly whitish and shining when dried. The plant stems are somewhat rough and fibrous; the bark is grey to white when living and greyish-brown when dried. Seeds are curved, non-endospermic, radical short, cotyledons thick and long (Momin et al. 2018). *Haematocarpus validus* species are dioecious where male and female flowers are borne separately. The flowering time varies depending on the place. In Singngat hill of Manipur, these species give flower biennial in the middle of September to October and gradually develop into fruits. The season of fruit ripening and harvesting is from April to June. In the Garo Hills condition of Meghalaya, the vine comes into flowering from October to December, and fruits are available in the local markets from the last week of March till June.

Blood fruit plants are native to the limited geographical distribution of the Asian continent such as India, Bangladesh, Indonesia, Singapore, Thailand, and Sri Lanka. In India, this fruit is found growing wild in the Andaman and Nicobar Islands, Arunachal Pradesh, Mizoram, Tripura, Assam, Meghalaya, and Manipur (newly identified location).

Table 14.7 Bioactive compounds identified from different plant parts of *Haematocarpus validus*

Sl. no.	Plant parts	Extract	Bioactive compound	Reference
1.	Fruit	Methanol	Anthocyanin, beta-carotene, tannins, flavonoids, iron	Singh et al. (2014)
			Alkaloids, saponin	Alex et al. (2017)
2.	Leaf	Methanol	Choline, ecgonine, sinomenine	Alex et al. (2017)

Local tribes of the north-eastern part of India generally use this fruit for its medicinal properties. In Meghalaya, this iron-rich fruit is highly valued by the Garo tribe to treat blood-related disorders. Phytochemicals detected from the extract of this plant are listed in Table 14.7. They also used this fruit for wine preparation (Momin et al. 2018). Blood fruit contains a high amount of anthocyanin, and when fully ripe, it gives a blood-red colour which is used as a natural colouring agent and added to food products to enhance its flavour and appearance (Singh et al. 2014). These fruits are being used as a colouring dye for handicrafts in Tripura. Products like pickles and chutneys are also prepared from immature green fruits (Momin et al. 2018).

14.2.7 *Houttuynia cordata* Thumb

Houttuynia cordata Thumb. is a flowering herb belonging to the Saururaceae family (sometimes referred to as ‘lizard’s-tail family’). The white flower-like structure shown in Fig. 14.1g is the inflorescence which is a terminal spike bearing four (sometimes six) white basal bracts. The flowers are very tiny, numerous in number, without petals, and greenish-yellow in colour. The green leaves are heart-shaped and borne alternately, while the stem usually produces adventitious roots. The plant bears horizontal rhizomes, which connect the erect aerial stems. The odour is fishy on rubbing, and the taste is slightly astringent.

It is widely distributed in different parts of Asia like Vietnam, China, Japan, North-East India, and Korea. It grows well in moist and shady areas with its distal parts protruding vertically.

The plant is widely used in the north-eastern states of India for various ailments. The Apatani tribe in the Ziro valley of Arunachal Pradesh uses the shoot for freshness and good sleep (Tilling et al. 2015). In Assam, leaves are used as chutney and are also used for curing dysentery in Cachar areas of the state. The Khasi, Jaintia, Garo, and other tribes in Meghalaya use the juice from its young shoots for treating against cholera, dysentery, and blood deficiency conditions (Hynniewta and Kumar 2008). Local healers of Manipur called *Maibas* use the plant for dysentery, muscular sprain, stomach ulcer, anaemia, gastritis, and tuberculosis (Khumbongmayum et al. 2005). Tribes in Manipur and Nagaland use the plant extract to cure intestinal worm infection and as an anthelmintic (Yadav and Temjenmongla 2011). Implications of its culinary usage are observed since both the leaves and the roots of this plant are

Table 14.8 Bioactive compounds identified from different plant parts of *Houttuynia cordata* Thunb

Sl. no.	Plant parts	Extract/oils	Bioactive compounds	References
1	Aerial parts	Methanol	Aristolactam B, piperolactam A, aristolactam A, norcepharadione B, cepharadione B, splendidine	Kim et al. (2001)
		Chloroform	Aristololactam	Ma et al. (2017)
2	Whole plant	Essential oil	Bornyl, β -myrcene	Lu et al. (2006)
		Methanol	Houttuynoside, houttuynamide	Chou et al. (2009)
		Ethanol	Houttuynoids A, B, C, D, and E	Chen et al. (2012)
		Volatile oil	Houttuynia	Chen et al. (2014)
3	Stems and leaves	Ethanol	Rutinin, hyperoside	Xu et al. (2006)
		Ethyl acetate	Quercitrin, quercetin, hyperoside	Tian et al. (2012)
4	Dry leaves	Methanol	Neochlorogenic, chlorogenic, 4-dicaffeoylquinic	Nuengchamnong et al. (2009)
5	Aerial parts and under-ground stem	Essential oil	Ethyl caprate, ethyl dodecanoate, 2-undecanone, 2-tridecanone	Verma et al. (2017)

used fresh as an herbal garnish. The unusual stingy taste of the leaf is often described as ‘fishy’ (thus named as ‘fish mint’). The list of phytochemicals/bioactive compounds reported from *H. cordata* is given in Table 14.8.

14.2.8 *Polygonum posumbu* Buch-Ham. Ex D. Don

Polygonum posumbu is a flowering plant (Fig. 14.1b). The plant is 30–70 cm tall; stems are light green to reddish-brown, glabrous, and round in circumference. The branches in the stems are extensively creeping at the base and have decumbent branches in all directions. Leaves are hairless, ovate, and arranged alternately. This plant has clusters of pink or rosy pink flowers which are racemoid of 2 cm long on the spike-like termination of stems. The blooming period of the flowers of this plant happens during the early fall of summer and lasts up to 2–3 months. The seeds of the plant possess three-angled seeds which are black and shiny.

The plant is widely distributed in Asian countries such as Indonesia, Japan, Korea, the Kuril Islands, Bangladesh, Primorye, Myanmar, Java, Nepal, the Philippines, Thailand, and China. The plant is also commonly grown in North-East Indian states (Barooah and Ahmed 2014).

Tender shoots are consumed as spices for flavour and leaves are used as mouth freshener (Devi et al. 2011) and it was effective against gastric problems, indigestion, cough, fever, etc. (Devi and Das 2010; Yumnam et al. 2012; Devi et al. 2014b; Singh et al. 2015). As a folk medicine, *P. posumbu* is used for curing dyspepsia and fever and eaten raw as a supplement in the diet as antipyretic (Yumnam and Tripathi

2012). The juice or the tender shoot and the fresh leaves are used to reduce heartbeat (Leishangthem and Sharma 2014; Singha et al. 2016).

14.2.9 **Meriandra bengalensis**

Meriandra bengalensis is an annual herbaceous and aromatic shrub (Fig. 14.1i) and 3–6 ft. high, with densely pubescent branches. Leaves are petioles, oblong-lanceolate, 2–3 inches long, minutely crenate, with a scent resembling *Salvia officinalis*, but stronger. Whorls are very dense, globose, forming long racemose panicles at the end of the branches; lowest bracts as long as the flowers. The calyx is densely pubescent; the corolla is white with a tube as long as the calyx; the limb is small.

M. bengalensis is reported to be found in Saudi Arabia, Yemen, and Eritrea (Mothana et al. 2019). In India, it is reported in West Bengal and Manipur (Rana and Blazquez 2009). Fresh leaves of *M. bengalensis* are used as a tonic for sore throat; it is also used for reducing high blood pressure (Rana and Blazquez 2009). Though the plants have been cultivated since time immemorial for their use in traditional folklore medicine, the information available is still limited (Ningombam et al. 2014). Some of the biochemicals reported from this plant are listed in Table 14.9.

14.2.10 **Zanthoxylum armatum DC**

This medicinal plant is a large spiny shrub or small tree which grows up to 6 m (Fig. 14.1j). Leaves are distinctively trifoliolate with the leaf-stalk winged. Minute yellow flowers arise in leaf axils. Flowers have six to eight acute sepals. Petals are absent. Male flowers have six to eight stamens and large anthers because of which the flowers look yellow. Female flowers have one- to three-celled ovary, are pale red,

Table 14.9 Bioactive compounds identified from different plant parts of *Meriandra bengalensis*

Plant part	Extract/oil	Bioactive compounds	References
Aerial parts and roots	Oil	Sesquiterpenes, diterpenes, triterpenes	Rana and Blazquez (2009)
Leaves	Essential oil	Camphor, linalool, 1,8-cineole, α -terpineol, d-terpineol	
	Methanol	Alkaloids, flavonoids, tannins, phytosterols	Kidane et al. (2018)
	Ethanol	Alkaloids, flavanoids, tannins, saponins	
	Oil	Camphene, 1,8-cineole, camphor, borneol, thymol, β -caryophyllene 1, α -selinene	Ali et al. (2012)
Roots	Methanol	Cryptotanshinone 17, hydroxycryptotanshinone, tanshinone IIA, and przewaquinone A	Mothana et al. (2019)

and split into two when ripe. Seeds are round and shining black. The flowering occurs from March to April (Singh and Singh 2011).

The plant is distributed in many parts of Asia including China, Taiwan, Nepal, Bhutan, the Philippines, Malaysia, Pakistan, Japan, the Republic of Korea, India, and Indonesia (Singh and Singh 2011; Xu et al. 2019). It is also prominently grown in the north-eastern states of India (Singh and Singh 2011).

Different communities of Manipur use this plant for various ailments and are also consumed as spices. The decoction prepared from the leaves is used to treat cough (Panmei et al. 2018, 2019), and leaves are also used as spices or condiments (Gangte et al. 2013). In Nagaland, the leaves and fruits of the plant are used for the treatment of diarrhoea, diabetes, cough, fever, dental problems, skin diseases, leucoderma, leprosy, hepatopathy, gout, convulsions, and paralysis. The crushed fruit is used in fish poison (Dominic and Ramanujam 2012). The seed and bark are reportedly used as a tonic and in indigestion in Arunachal Pradesh (Shankar and Rawat 2008). During fever, cholera, and stomach disorders, the seed and bark are used as a tonic (Perme et al. 2015). Tribals of the western part of Mizoram used the leaves to ward off fowls and lice (Lalfakzuala et al. 2007). In Tripura, the root paste of the plant is used for the treatment of acute gastric pain (Shil and Choudhury 2009), besides its use for cough and asthma, chronic fever, and dyspepsia. The seed is used in toothache, and its oil is applied in rheumatism (Guha et al. 2018). It is used for the treatment of pneumonia, typhoid, and gout by different ethnic groups in Assam (Barua et al. 2007; Medhi et al. 2014). In Sikkim, the fruit is used in toothache, for treating gum and dental disorders, and also as dyspepsia and lotion for scabies (Purohit et al. 2014). In West Sikkim, the fruit is used in stomach pain (Mukhia and Mukhopadhyay 2012). Fruits are consumed as a spice by the Khasi, Jaintia, and Garo tribes of Meghalaya (Kayang 2007). The many medicinal properties of the plant will be attributed to the array of phytochemicals reported from the parts of this plant (Table 14.10).

14.2.11 *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb

Phlogacanthus thyrsiformis is an evergreen shrub growing up to 2–4 m high (Fig. 14.1k). Leaves are oblanceolate to elliptic-oblong. Calyx are 5-partite, split towards the base, linear-lanceolate, and pubescent outside. The corolla is yellow, pubescent outside, slightly two-lipped, and minutely curved. Stamens are inserted near the tube base, while the stigma is extended. The capsule is elongated, clavate, obtuse, long, and glabrous. The flowering and fruiting period is from December to April (Ningombam and Singh 2014).

The subtropical Himalayas, the Upper Gangetic Plain, Bihar, North Bengal, and Assam are described as their habitat (Khare 2007). The plant grows at an altitude of 650–1300 m above sea level covering the moist tropical region and sometimes the

Table 14.10 Bioactive compounds identified from different plant parts of *Zanthoxylum armatum* DC

Sl. no.	Plant parts	Extract/oil	Phytochemicals/constituents/major compounds	References
1.	Fruits	Essential oil	Linalool, limonene, methyl cinnamate, myrcene, α -thujene, 1,8-cineole, p-cymene, cis-ocimene, γ -terpinene, camphor, α -fenchol, carvone, α -terpineol, tagetonol, β -caryophyllene, Allo-aromadendrene, sesquiterpene alcohol	Ahmad et al. (1993)
2.	Seeds	Essential oil	α -Thujone, α -pinene, sabinene, β -pinene, myrcene, o-cymene, limonene, 1,8-cineole, α -terpinene, Z-sabinene hydrate, Z-linalool oxide, E-linalool oxide, linalool, Z-pinene hydrate, terpinen-4-ol, α -terpineol, E-carveol, nerol, cuminaldehyde, carvone, piperitone, geraniol, phellandral, E-methyl cinnamate, E-nerolidol, methyl palmitate, palmitic acid, oleic acid	Tiway et al. (2007)
3.	Leaves	Essential oil	2-Undecanone, 2-tridecanone, 1,8-cineole, linalool, terpinen-4-ol, α -terpineol, trans-caryophyllene, α -humulene, germacrene D	Bisht and Chanotiya (2011)
4.	Stems	Ethanol	Dihydrophaseic acid 4'-O-[6''-O-(4'''-hydroxy-3''', 5'''-dimethoxy)]- β -D-glucopyranoside, dihydrophaseic acid 4'-O-[6''-O-(3'''-methoxy-4'''-hydroxy) benzoyl)]- β -D-glucopyranoside	Liu et al. (2019)
5.	Bark	Methanol	Asarinin, fargesin, horsfieldin, eudesmin, planispine A, planispine B, yangambin, acuminatolide, salicifoliol, magnolone, magnone A	Zhang et al. (2018)

temperate region. The plant is reported from the north-eastern region and West Bengal in India and Burma, Bhutan, Malaya Peninsula, and Indonesia (Ningombam and Singh 2014; Dutta et al. 2016).

P. thyrsoformis has been widely used in the traditional system of medicine in North-East India by local healers. In Manipur, it is used as a folk medicine for cough, cold, burns, influenza, smallpox, boils, skin problems, malaria, diarrhoea, dysentery, cholera, sprains, body ache, high blood pressure, constipation, irregular menstruation, easy delivery of childbirth, and abortion (Ningombam and Singh 2014). In Nagaland, leaves' decoction is used for treating fever, diarrhoea, dysentery, bronchial diseases, and stomach trouble and as a diuretic, expectorant, and sedative (Ozukum et al. 2019). It is also used for relieving cough and joint pain (Shankar and Devalla 2012). In Arunachal Pradesh, it is used for treatments of cough, asthma, fever, stomachache, loose motion, malaria, and paralysis (Shankar and Rawat 2008; Tangjang et al. 2011; Perme et al. 2015). In Mizoram, the root of the plant is used in treating ulcers by forming a combination with other plants (Rai and Lalramnghinglova 2010). The Chakma community of Tripura used leaf juice as an expectorant (Das and Choudhury 2009). In Assam, it is used as a traditional medicine for jaundice treatment (Barua and Handique 2002) and allergy (Kalita and Bora 2008). In South Sikkim, it is reported that the inflorescence of the plant is

Table 14.11 Bioactive compounds identified from different plant parts of *Phlogacanthus thyrsoformis* (Roxb. Ex. Hardw.) Mab

Sl. no.	Plant parts	Extract/oil	Phytochemicals/constituents/major compounds	References
1	Flower	Ethyl acetate	N-hexadecanoic acid; 3-fluorobenzoic acid; 2-thiopheneacetic acid; β -sitosterol; eicosapentaenoic acid; methyl 9,12-heptadecadienoate; ethyl-9,12,15-octadecatrienoate; ethyl-9.cis.,11.Trans-octadecadienoate	Das et al. (2017)
2	Leaf	Methanol	Phenol, 2,6-dimethoxy; dispiro [2.2.2.2] deca-4,9-diene N, N-dimethyltrimethylsilamine; 1, 8-diamino-3,6-dioxaoctane; benzoic acid, 4-hydroxy-3,5-dimethoxy-, hydrazide; orcinol; 2,6,6-trimethyl-bicyclo[3.1.1]heptane, trans; hexadecanoic acid, methyl ester; 9-octadecenoic acid (Z)-, methyl ester; phytol; methyl stearate; cyclopropane, isothiocyanate; 7H-purin-6-amine, 7-methyl; squalene; alloaromadendrene; stigmasterol; 5-methyl-2-phenyl-1H-indole; 2-ethylacridine	Suchiang and Kayde (2017)
			2-Ethylhexane; decade; dodecane; tetradecane; nonadecane; hexanoic acid, 2-ethylhexyl ester; 2,4-dimethyl-3-hexanone; neophytadiene; n-hexadecanoic acid; phytol; linoelaidic acid; 9,12,15-octadecatrienoic acid, (Z,Z,Z); octadecanoic acid; chalepin; 1,3,5(10)-estratrien-3,17.beta,-diol, 17-acetate(ester); 5-hydroxymethyl-1,1,4a-trimethyl-6-methylenedecahydronaphthl	Kripasana and Xavier (2020)
			Saponins, phenols, tannins, diterpenes	Saikia et al. (2018)
		Water	Alkaloids, saponins, phytosterols, phenols, tannins, flavonoids, coumarins, diterpenes	
		Ethanol	Saponins, phenols, tannins, coumarins	Barua et al. (1985)
			Phlogantholide-A	

used to control blood pressure and blood sugar levels and is highly effective in bronchitis and asthma (Bhutia et al. 2021). Tribal communities of Meghalaya used it for the treatment of fever (Jaiswal 2010) and diabetes (Parkash 2021), and leaves and flowers are used to cook with fish and meat (Kayang 2007). The list of phytochemicals/bioactive compounds reported from *P. thyrsoformis* is given in Table 14.11.

14.3 Needs for Conservation

The reported number of plants used as medicine and in traditional medicinal formulations in India is ~7500 species which is nearly 44% of the total numbers of plant species reported in the country (Hamilton 2003). Plant families with a higher

number of medicinal plants usually have higher proportions of threatened species (Huang 2011). Many species have depleted to the brink of extinction by over-harvesting of the wild plants because of high value of herbal medicines. For instance, in 2008, the fresh leaves of *Croton caudatus* Geiseler (locally known as ‘Chawilien Damdawi’) and other plant parts were in high demand for the preparation of crude herbal extracts over the claim of the miraculous cure of cancer by a local therapist. With too much media hype, extensive exploitation to the brink of extinction occurred in the natural forest reserve at Saikot, Churachandpur, Manipur (<http://e-pao.net/GP.asp?src=9..200608.jun08>).

C. amada and *C. caesia* have a long history of usage in traditional medicine, cosmetics, and culinary elements among many ethnic groups of the Indian subcontinent (Nayak 2002; Sasikumar 2005). Natural habitats of these plants are destroyed due to indiscriminate collection of its rhizome and various human interferences leading to the threat of extinction. *Curcuma caesia* has been listed as one of the critically endangered species as per the IUCN list of threatened species (Das and Saikia 2019). *Haematocarpus validus* (Miers) Bakhf. ex Forman (Menispermaceae), a critically endangered species, was rediscovered after a gap of 100 years from India and added a new location to the Indo-Burma biodiversity hotspot region (Singh and Bedi 2016). Increasing anthropological activities and climatological factors are the major causes responsible for its threat of extinction. Most of the fruit is harvested from the wild, and no systematic efforts for domestication have been made so far (Bohra et al. 2018). Moreover, fruit predation by forest fauna for many underutilized plants growing in wild hinders the regeneration process (Sundriyal and Sundriyal 2001).

The high endemism of certain medicinal plants is in many cases influenced by climatic conditions. For example, Hathei chilli is native to only Sirarakhong village of Ukhrul district, Manipur. The chilli is known for its unique flavour and has an extremely high American Spice Trade Association (ASTA) colour value of 164. It has been granted the geographical indication (GI) tag by the Government of India in September 2021. The unique characteristics of this chilli are lost when the same plantlet or seedling is sown in the neighbouring villages. It is very crucial to protect the cultivation areas as any adverse climatic fluctuations will have a direct impact on the cultivation and harvesting. This makes it one of the major challenges in the conservation policy of the Hathei chilli. Additionally, the limited areas of growth of the endemic chilli make it hard to meet the ever-increasing demands, thereby resulting in over-exploitation.

14.4 Conservation Methods

No single sector, private or public, can undertake the conservation of medicinal plants alone. It requires extensive team efforts in preparing a strategy for the conservation and sustainable use of its medicinal plants. The initial process should begin with a regional discussion with experts of different fields such as agronomists,

ecologists, horticulturists, plant breeders, taxonomists, traditional health practitioners, etc. to assess the scenario and draw an action plan. Firstly, ethnobotanical surveys of the medicinal plants should be documented and re-examine the systems of traditional medicine and practice (Lipp 1989). Secondly, correct identification of the medicinal plants and assessing their distributions and abundance are necessary.

A good practice of conservation of the medicinal plants is to cultivate the medicinal plants wherever possible (Hamilton 2004). This practice not only preserved wild stocks but also ensures that the survival of rare and over-exploited medicinal plants is not endangered. Besides it also reduces the possibility of misidentification and adulteration during wild species collection. In modern practice, botanical gardens play an important role in ex situ conservation (Havens et al. 2006). The programme of botanical gardens may include the development of propagation and cultivation techniques for ecologically diverse flora and grow them under a common condition (Li and Pritchard 2009). They are therefore inherently well-placed to respond to the very specific local conservation needs of medicinal plants and the people who rely on them for health and livelihood. In the north-eastern region, two well-maintained botanical gardens are the Jawaharlal Nehru Botanical Garden, Gangtok, and Assam State Zoo cum Botanical Garden, Guwahati.

Alternatively, threatened medicinal plants may also be conserved through seed banks, in vitro storage methods, pollen banks, and DNA banks. The resources provided by these collections are necessary for in situ recovery programmes. While ex situ conservation may be appropriate for the restocking of locally depleted plant species, the range of genetic variation of medicinal plants used in such methods is distinctively lower than its wild counterpart.

In an ideal situation, the conservation of medicinal plants as an evolving population in nature is the preferred way. Ensuring such in situ conservation can be achieved by setting protective research areas such as national parks. However, managing such national reserves goes beyond removing external threats. It requires an executive policy for recording and monitoring the medicinal plants and a technical mechanism for the collection of medicinal plants within the protected area. In other words, conservation and exploitation of medicinal plants are a necessary component of the management plans of the reserve. Species that may have become nearly extinct by over-collection may also be attempted to be re-introduced either to their original habitats or into a similar habitat wherever feasible.

In the case of *H. validus*, domestication by promoting homestead cultivation of this underutilized fruit could help in reducing the burden on natural stock where harvesting is done from wild-grown, thereby replenishing the wild stock. Many of the wild edible plants of the north-eastern region of India are underutilized or unknown to the modern world. Therefore, wild edible plants which are on the verge of extinction due to ignorance, unawareness, and extensive deforestation have been a focus for many ethnobotanists in recent decades (Bharucha and Pretty 2010).

Religious beliefs and traditional folklores also hold importance in the preservation of many medicinal plants. Since time immemorial, maintenance of sacred groves by dedicating a patch of forest to deities has been practised. Sacred groves

are reported in many of the north-eastern states including Manipur, Meghalaya, Sikkim, Arunachal Pradesh, and Assam. Such sacred groves harbour a rich diversity of medicinal plant species which has been used by traditional healers to treat different ailments (Khumbongmayum et al. 2005). In the case of *Phlogacanthus thyrsoformis*, traditional myths of not plucking the plant on Sunday and mid-noon of any day are still practised today in the state of Manipur. This practice is another form of a traditional way to prevent the over-exploitation of the plants that have been passed on for generations (Ningombam and Singh 2014).

Conservation methods inducted in our institution include several ex situ techniques. For this purpose, the Institute has set up National Bioresources Park at Haraorou, Imphal East, Manipur, with the sole vision of producing disease-free planting materials of medicinal and horticultural plant species. Since micropropagation (tissue culture) is one of the most accepted methods for cell proliferation and introduction to nature, the Institute has established a state-of-the-art modern Tissue Culture Facility at the park to propagate plants of economic value. At present, the emphasis of micropropagation is laid on the development of plantlets of orchid species and members of the Zingiberaceae family. Apart from the initiation of the National Bioresources Park, the Institute also maintained a garden for selected medicinal and ornamental plants as well as horticultural crops on its campus.

14.5 Conclusion

Sustainable use of medicinal plants should be made to practise for all medicinal plants and more particularly for those with low abundance (Larsen and Olsen 2007). Regenerating plant parts such as leaves can be considered over whole plants or roots if they offer similar pharmacological activities (Wang et al. 2009). Alternative practice for sustainable use can also be the use of biological products from the associated endophytic microorganisms of the said medicinal plants (Venieraki et al. 2017). This practice had an added advantage in that microbes can be grown on a larger scale at a much lesser time than that is required for the cultivation of the medicinal plant while ensuring that a myriad of the chemical diversity of the host plant is maintained (Salam et al. 2017).

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Chapter 15

Occurrence and Diversity of Major Naphthoquinones in Higher Plants: Their Distribution and Conservation Strategies



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and Biswajit Ghosh

Abstract Naphthoquinones (NQs) are quinone derivatives with remarkable functional properties and occur naturally in higher plants and bacteria, algae, fungi, and insects. They have a magnitude of utility in treating various types of cancer, inflammation, HIV and malaria. Besides, they have a considerable role as dyes to colour clothes and foods commercially. Higher plants from diverse taxonomic groups are a potential source of several NQs. These plants are distributed in different parts of the world, having different ecological preferences. Owing to the noteworthy applications of NQs in human welfare, it is essential to look after their sources and their diversity across the globe. Many significant sources of NQs are endemic and grow in a very restricted region. Moreover, a large number of species are now threatened in their natural habitat. Therefore, this chapter focused on the occurrence and distribution of different NQs in higher plants. In addition, the diversity, distribution, and threatened status of the higher plants containing distinct NQs have also been discussed. The comprehensive data on those plants will benefit their future management and determine proper conservation strategies, particularly for those threatened.

Keywords Conservation · Diversity · Naphthoquinones · Plumbagin · Threatened

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15.1 Introduction

Naphthoquinones (NQs) are the naturally occurring group of compounds having diverse distribution throughout the biological world. They can be obtained from bacteria, algae, fungi, lichens, insects, and plants (Hook et al. 2014). The remarkable application of NQs in human welfare increases researchers' attention to this compound. Its utilisation covers a broad spectrum, especially for their potential biological activities. Plumbagin, lapachol, shikonin, juglone, 7-methyljuglone, and lawsone are well-known NQs for their distinct commercial values and remarkable applications. Shikonin and its enantiomer alkannin found in the root of *Lithospermum erythrorhizon* and *Alkanna tinctoria* are red pigments with a promising application in food and cosmetic industries as natural colourants (Huu Tung et al. 2013; Hook et al. 2014). Lawsone is located in the leaves of the henna plant (*Lawsonia inermis*) and has been used for centuries as a natural colouring agent for hair, skin, and leather (Alem et al. 2020). Plumbagin is another incredible NQ principally found in the roots of the genus *Plumbago* with a noteworthy implementation in cancer research for their antiproliferation activities (Dissanayake et al. 2022). Juglone, a dark orange-coloured NQ isolated mainly from walnut used to colour cloths and fabrics, is also occasionally used as herbicides (Islam and Widhalm 2020). NQ is structurally similar to the naphthalene with a core benzene ring attached to a conjugated cyclic diketone with carbonyl groups. Interestingly, phyloquinone (vitamin K1) and menaquinone (vitamin K2) are also 1,4-NQ and essential for photosynthesis in plants, green algae, and cyanobacteria, functioning as an electron transporter in Photo system-I. In addition, several known NQs are produced by plants and released into their surroundings, considered to play essential roles in the plant to microbes and plant-to-insect interaction, even in plant-to-plant competition (Widhalm and Rhodes 2016).

Naphthoquinones are found in different groups of plants within the plant kingdom (Table 15.1). Fern viz., *Lygodium japonicum* is, also known as Japanese climbing fern, endemic to Southern and South-Western China and contains, 6-hydroxy-2-isopropyl-7-methyl-1,4-naphthoquinone in their roots (Chen et al. 2010). Two new NQs have recently been discovered, known as goniothalamine A and B, isolated from the roots of *Goniothalamus scortechinii*, a monocotyledonous species belonging to the family Annonaceae native to Peninsular Malaysia and Thailand (Prawat et al. 2012). Plumbagin is found in the rhizomes of another monocotyledonous species, *Aristea ecklonii* or 'blue-eyed iris', under the Iridaceae native to Central and Southern Africa. Besides these, most NQs are distributed in Eudicots, and nearly 200 plant species belong to different families containing different NQs. Plant species containing valuable NQs show enormous diversity and distribution in a global scenario. The NQ plumbagin, which is famous for its potential bioactivities, is principally found in the root of *Plumbago* or 'Chitrak', and for thousands of years, the plant has been used as a remedy for chronic menstrual disorders, viral warts, chronic diseases of the nervous system, obesity, piles, worms, colitis, ascites and liver diseases in India and China (Shukla et al. 2021). However,

Table 15.1 Naphthoquinones from different higher plants

Family	Plants name	Naphthoquinones	Distribution	References
Iridaceae	<i>Aristea ecklonii</i>	Plumbagin	Cameroon, Mozambique, Rwanda, Tanzania, Uganda, Zaïre, Zimbabwe	Mabona et al. (2013)
	<i>Cipura paludosa</i>	Eleutherine, isoeleutherine, 11-hydroxyeleutherine	Paraguay, South of Brazil, central Mexico, Cuba, Bolivia, India, Honduras, Bangladesh, Venezuela	Tessele et al. (2011)
	<i>Eleutherine americana</i> (Syn. <i>E. bulbosa</i>)	Eleutherinone, eleutherin, iso-eleutherin, eluthinone-A, 11-hydroxyeleutherine	Argentina Northwest, Bolivia, Brazil Southeast, Brazil West-Central, Colombia, Cuba, Dominican Republic, Ecuador, French Guiana, Guyana, Haiti, Jamaica, Paraguay, Peru, Venezuela, Cambodia, India, New Caledonia, Trinidad-Tobago, Vietnam	Chen et al. (2020), Alves et al. (2003)
	<i>Eleutherine plicata</i>	Eleutherin, Isoeleutherin	Argentina Northwest, Bolivia, Brazil, Colombia, Cuba, French Guiana, Guyana, Haiti, Jamaica, Leeward Is., Paraguay, Peru, Puerto Rico, Suriname, Venezuela, Cambodia, India, New Caledonia, Trinidad-Tobago, Vietnam	Vale et al. (2020)
Proteaceae	<i>Conospermum incurvum</i>	Conocurvone	Western Australia	Dai et al. (1994)
	<i>C. sphacelatum</i>	Teretifolione-B, Concurvone	Queensland	Butler et al. (1999)
	<i>C. stoechadis</i>	Stoechadone	South-Western Australia and Western Australia	Aguinaldo et al. (1996)
	<i>Lomatia hirsuta</i>	2-Methoxyjuglone	Argentina South, Chile Central, Chile South, Ecuador, Peru	Simonsen et al. (2006)
	<i>Lomatia tasmanica</i>	Juglone	Tasmania	Deans et al. (2018)
	<i>Lomatia polymorpha</i>	Juglone	Tasmania	(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
Leguminosae	<i>Caesalpinia sappan</i> (Syn. <i>Biancaea sappan</i>)	5-Hydroxy-1,4-naphthoquinone	Assam, Bangladesh, Cambodia, East Himalaya, Laos, Myanmar, Nepal, Sri Lanka, Thailand, Vietnam, Nigeria, Uganda, West Himalaya, Mauritius	Lim et al. (2007)
Ulmaceae	<i>Ulmus davidiana</i>	Mansonone-E	Amur, Buryatiya, China, Mongolia, Japan, Korea, Kuril Is., Manchuria, Myanmar	Lee et al. (2012)
Juglandaceae	<i>Juglans mandshurica</i>	5-Hydroxy-1,4-naphthalenedione (juglone), Juglone	Amur, China, Japan, Korea, Manchuria, Sakhalin, Taiwan	Colaric et al. (2005)
	<i>J. nigra</i>	Juglone	USA, Austria, Belgium, Bulgaria, Czechoslovakia, Denmark, Germany, Hungary, Italy, New Mexico, North Caucasus, Romania, Tadzhikistan, West Himalaya	Islam and Widhalm (2020)
	<i>J. regia</i>	Juglone, 1,4-naphthoquinone	Afghanistan, Iran, Iraq, Lebanon-Syria, North Caucasus, Pakistan, Transcaucasus, Turkey, West Himalaya, China, Korea, Morocco, Great Britain	Ramezani et al. (2020)
	<i>Juglans cathayensis</i>	Juglone, 5,8-dihydroxy-1,4-naphthoquinone, 2-ethoxy juglone, 2-methoxy juglone, 3-methoxy juglone, engelhardtquinone	Amur, China North-Central, China South-Central, China Southeast, Khabarovsk, Korea, Manchuria, Taiwan	Gan et al. (2015)
	<i>Pterocarya fraxinifolia</i>	Juglone, 3,30-Bijuglone, cyclotrijuglone	Iran, North Caucasus, Transcaucasus, Turkey	Ebrahimi and Gashri (2015)
Lythraceae	<i>Lawsonia inermis</i>	2-Hydroxy-1,4-naphthoquinone (lawsone), Lawsone	India, Kenya, Oman, Egypt, Ethiopia, Somalia, Sudan, Sri Lanka, Cote D'Ivoire, Gambia, Ghana, Guinea, Liberia, Mali, Nigeria, Senegal, Sierra	Bakkali et al. (1997), Alem et al. (2020)

Droseraceae	<i>Aldrovanda vesiculosa</i>	Plumbagin	Leone, Togo, South Africa, North and East Africa, Arabian Peninsula, Liberia, Libya, Madagascar, Maldives, Mali	Adamec (2018)
			Extinct in Austria, Baltic States, Bulgaria, Greece, Italy, Korea, Uzbekistan	
			Native in Bangladesh, Belarus, Botswana, Cambodia, Ukraine, Poland, Cameroon, Central European Rus, Chad, Czechoslovakia, France, Germany, Ghana, Hungary, India, Western Australia, Yugoslavia, Zambia	
	<i>Dionaea muscipula</i>	Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), droserone, 3-chloroplumbagin	North Carolina, South Carolina	Krychowiak-Mańska et al. (2021)
	<i>Drosera aliciae</i>	7-Methyljuglone	Cape Provinces, South Africa	Krolicka et al. (2009)
	<i>D. adela</i>	7-Methyljuglone	Queensland	Culham and Gomall (1994)
	<i>D. andersoniana</i>	Plumbagin	Western Australia	Schlauer et al. (2005)
	<i>D. anglica</i>	7-Methyljuglone	Austria, America, Czechoslovakia, Denmark, France, Germany, Great Britain, Romania, Spain, Sweden, Switzerland, Ukraine	Länger et al. (1995)
			Extinct in Belgium	
	<i>D. auriculata</i>	Plumbagin, 7-methyljuglone	New South Wales, New Zealand North, New Zealand South, South Australia, Tasmania, Victoria	Devi et al. (2016)
	<i>D. admirabilis</i>	7-Methyljuglone	Cape Provinces	
				(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
	<i>D. burmanii</i>	Plumbagin, 7-methyljuglone	China Southeast, Japan, Korea, New Guinea, Australia, New Zealand, Philippines, Sumatra, Taiwan, Tasmania	Boonsongcheep et al. (2019)
	<i>D. binata</i>	Plumbagin, 7-methyljuglone	New Zealand North, New Zealand South, Queensland, South Australia, Tasmania, Victoria, Western Australia	Siatkowska et al. (2021)
	<i>D. burkeana</i>	7-Methyljuglone	Angola, Botswana, Burundi, Cape Provinces, Congo, Free State, Kenya, Lesotho, Madagascar, Malawi, Mozambique, Northern Provinces, Swaziland, Tanzania, Uganda, Zambia, Zaire, Zimbabwe	Kalema et al. (2016)
	<i>D. communis</i>	7-Methyljuglone	Argentina Northeast, Bolivia, Brazil North, Brazil Northeast, Brazil South, Brazil Southeast, Brazil West-Central, Colombia, Paraguay, Venezuela	Devi et al. (2016)
	<i>D. cistiflora</i>	Plumbagin, 7-methyljuglone	Cape Provinces	Devi et al. (2016)
	<i>D. capensis</i>	Plumbagin, 7-methyljuglone	Cape Provinces, California, New South Wales	Hatcher et al. (2021)
	<i>D. filiformis</i>	Plumbagin, 7-methyljuglone	USA	Egan and van der Kooy (2012)
			Extinct in: Connecticut, Delaware, Rhode I.	
	<i>D. erythrorhiza</i>	Plumbagin	Western Australia	Schlauer et al. (2005)
	<i>D. gigantea</i>	Plumbagin, Droserone, 5-O-glucoside, Hydroxydroserone, hydroplumbagin-4-O-glucoside,	Western Australia	Krychowiak-Masnicka et al. (2021)

	Hydroxydroserone 5- <i>O</i> -glucoside, Hydroxydroserone 8- <i>O</i> -glucoside			
<i>D. hamiltonii</i>	Ramentaceone or 7-methyljuglone (5-hydroxy-7-methyl-1,4-naphthoquinone)	Western Australia	Devi et al. (2016)	
<i>D. indica</i>	Plumbagin, Ramentaceone or 7-methyljuglone (5-hydroxy-7-methyl-1,4-naphthoquinone)	India, Angola, Bangladesh, China, Cambodia, Cameroon, Africa, Chad, Congo, Ethiopia, Gambia, Ghana, Guinea, Ivory Coast, Japan, Java, Kenya, Laos, Liberia, Madagascar, Mozambique, Myanmar, Namibia, New Guinea, Nigeria, Philippines, Senegal, Sri Lanka, Sudan, Sumatra, Tanzania, Thailand, Uganda, Vietnam, Zimbabwe	Boonsongcheep et al. (2019)	
<i>D. intermedia</i>	Plumbagin, Ramentaceone or 7-methyljuglone (5-hydroxy-7-methyl-1,4-naphthoquinone)	North America, Spain, Ukraine, Turkey, Trinidad & Tobago, Sweden, Switzerland, Britain, Romania Extinct in Hungary	Grevenstuk et al. (2012)	
<i>D. natalensis</i>	Plumbagin	Cape Provinces, Madagascar, Mozambique, Northern Provinces, Swaziland, Zimbabwe	Budzianowski (2000)	
<i>D. macrophylla</i>	Plumbagin	Western Australia	Schlauer et al. (2005)	
<i>D. microphylla</i>	Plumbagin	Western Australia		
<i>D. modesta</i>	Plumbagin	Western Australia		
<i>D. madagascariensis</i>	7-Methyljuglone	Madagascar, Botswana, Burundi, Cameroon, Nigeria, Ethiopia	Paper et al. (2005)	
<i>D. prolifera</i>	Plumbagin	Queensland	Schlauer et al. (2005)	
<i>D. peltata</i>	Plumbagin, 7-methyljuglone, Droserone, Droserone-5- <i>O</i> -glucoside	New South Wales, New Zealand North, South Australia, Tasmania, Victoria	Wongsa et al. (2018)	

(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
Drosophyllaceae	<i>D. platypoda</i>	Plumbagin	Western Australia	Šamaj et al. (1999)
	<i>D. ramentacea</i>	Plumbagin	Madagascar	
	<i>D. regia</i>	Plumbagin, 7-methyljuglone	Cape Provinces	Rivadavia et al. (2003)
	<i>D. rotundifolia</i>	Plumbagin, 7-methyljuglone, Rossolide, Droserone	Southeast China, Belarush, Italy, Japan, Korea, North America, Europe	Tienaho et al. (2021)
	<i>D. slackii</i>	Plumbagin	Cape Provinces, India	Biswal et al. (2017)
	<i>D. spathulata</i>	7-Methyljuglone	Southeast Asia, Australia	Gaddam et al. (2021)
	<i>D. stolonifera</i>	Plumbagin, 7-methyljuglone	Western Australia	Cook et al. (2018)
	<i>D. trinerva</i>	Plumbagin	South Africa	Egan and van der Kooy (2013)
	<i>D. venusta</i>	Plumbagin, 7-methyljuglone	South Africa	
	<i>D. villosa</i>	Plumbagin, 7-methyljuglone	Brazil	
	<i>D. whitakeri</i>	Plumbagin, Droserone, Hydroxydroserone	Australia	
	<i>D. collinsiae</i>	Plumbagin, 7-methyljuglone	South Africa	
	<i>Drosophyllum lusitanicum</i>	Plumbagin	Morocco, Portugal, Spain	Goncalves et al. (2009)
Nepenthaceae	<i>Nepenthes khasiana</i>	Plumbagin, Droserone, 5-O-methyle droserone	India, Bangladesh	Joshi et al. (2022)
	<i>N. insignis</i>	Plumbagin, 7-methyljuglone, Rossolide, Hydroplumbagin 4-O-glucoside	New Guinea	Murphy et al. (2020)
	<i>N. thorelii</i>	Plumbagin, Droserone	Cambodia, Vietnam	Likhitwitayawuid et al. (1998)
	<i>N. rafflesiana</i>	Plumbagin, Droserone, Hydroxydroserone	Borneo, Malaya, Sumatra	Zulkapli et al. (2021)
	<i>N. gracilis</i>	Plumbagin		Gwee et al. (2014)

			Borneo, Malaya, Sulawesi, Sumatra, Thailand	
<i>N. bicalcarata</i>	Plumbagin		Borneo	Shah et al. (2018)
<i>N. mirabilis</i>	Plumbagin		Borneo, Cambodia, China Southeast, Hainan, Java, Laos, Lesser Malaya, Maluku, Myanmar, New Guinea, Philippines, Sumatra, Thailand, Vietnam	Miguel et al. (2020)
<i>N. gymnamphora</i>	Plumbagin		Java	Schlauer et al. (2005)
<i>N. bongso</i> (Syn. <i>N. talangensis</i>)	7-Methyljuglone		Sumatra	
<i>N. adnata</i>	7-Methyljuglone		Sumatra	
<i>N. eustachya</i>	Plumbagin		Sumatra	
<i>N. dubia</i> (Syn. <i>N. tenuis</i>)	Plumbagin		Sumatra	Cheek et al. (2017)
<i>N. inermis</i>	Plumbagin		Sumatra	Schlauer et al. (2005), Gronemeyer et al. (2016)
<i>N. jacquelineae</i>	7-Methyljuglone		Sumatra	
<i>N. ovata</i>	7-Methyljuglone		Sumatra	
<i>N. rhombicaulis</i>	Plumbagin, 7-methyljuglone		Philippines, Sumatra	
<i>N. longifolia</i>	Plumbagin		Indonesia, Malaysia	Schlauer et al. (2005)
<i>N. sumatrana</i>	Plumbagin		Sumatra	Gronemeyer et al. (2016), Yudaputra (2021)
<i>N. tobaica</i>	Plumbagin		Sumatra	Schlauer et al. (2005)
<i>N. spathulata</i>	7-Methyljuglone		Sumatra	Gronemeyer et al. (2016)
<i>N. singalana</i>	Plumbagin		Java and Sumatra	Gronemeyer et al. (2016), Schlauer et al. (2005)
<i>N. densiflora</i>	Plumbagin		Sumatra	Gronemeyer et al. (2016)

(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
	<i>N. diatas</i>	Plumbagin	Sumatra	Schlauer et al. (2005)
	<i>N. albomarginata</i>	7-Methyljuglone	Borneo, Peninsular Malaysia, Sumatra	Gronemeyer et al. (2016)
	<i>N. rajah</i>	Plumbagin	Borneo, Sumatra	Greenwood et al. (2011)
	<i>N. smilesii</i> (Syn. <i>N. anamensis</i>)	7-Methyljuglone	Cambodia, Laos, Thailand, Vietnam	Kováčik et al. (2012)
	<i>N. boschiana</i>	Plumbagin	Borneo	Gilbert et al. (2020)
	<i>N. villosa</i>	Plumbagin	Borneo	Badwaik et al. (2019)
	<i>N. macrophylla</i>	Plumbagin	Borneo	Moran and Clarke (2010)
	<i>N. macfarlanei</i>	Plumbagin	Malaya	Schlauer et al. (2005)
	<i>N. benstonei</i>	Plumbagin, 7-methyljuglone	Malaya, Thailand	Sanusi et al. (2017)
	<i>N. sanguinea</i>	7-Methyljuglone	Malaya, Thailand	
	<i>N. gracilima</i>	Plumbagin	Malaya	Schlauer et al. (2005)
	<i>N. northiana</i>	Plumbagin	Borneo	
	<i>N. reinwardtiana</i>	Plumbagin, 7-methyljuglone	Borneo, Sumatra	De et al. (2019)
	<i>N. miki</i>	Plumbagin	Sumatra	
	<i>N. lowii</i>	Plumbagin	Borneo	
	<i>N. ephippiata</i>	Plumbagin	Borneo	
	<i>N. alata</i>	Plumbagin	Philippines	Sanusi et al. (2017)
	<i>N. philippinensis</i>	Plumbagin	Philippines	
	<i>N. maxima</i>	7-Methyljuglone	Maluku, New Guinea, Sulawesi	Schlauer et al. (2005)
	<i>N. fusca</i>	Plumbagin, 7-methyljuglone	Borneo	
	<i>N. spectabilis</i>	Plumbagin	Sumatra	
	<i>N. stenophylla</i>	Plumbagin	Borneo	

	<i>N. faizaliana</i>	Plumbagin	Borneo	Sanusi et al. (2017)
	<i>N. pilosa</i>	Plumbagin	Borneo	
	<i>N. eymae</i>	Plumbagin	Sulawesi	
	<i>N. glabrata</i>	Plumbagin	Sulawesi	
	<i>N. hamata</i>	Plumbagin	Sulawesi	
	<i>N. tentaculata</i>	Plumbagin	Borneo, Sulawesi	
	<i>N. multensis</i>	Plumbagin	Borneo	
	<i>N. mira</i>	Plumbagin	Philippines	
	<i>N. bellii</i>	Plumbagin, 7-methyljuglone	Philippines	
	<i>N. sibuyanensis</i>	7-Methyljuglone	Philippines	
	<i>N. burkei</i>	Plumbagin,	Philippines	Schlauer et al. (2005)
	<i>N. ventricosa</i>	Plumbagin, 7-methyljuglone	Philippines	
	<i>N. neoguineensis</i>	Plumbagin	Philippines, New Guinea	
	<i>N. papuana</i>	Plumbagin	Philippines, New Guinea	
	<i>N. lamii</i>	Plumbagin	New Guinea	
	<i>N. tomoriana</i>	Plumbagin	Sulawesi	
	<i>N. danseri</i>	Plumbagin	Maluku, New Guinea	
	<i>N. viillardii</i>	Plumbagin	New Caledonia	
	<i>N. masoalensis</i>	Plumbagin	Madagascar	
	<i>N. madagascariensis</i>	Plumbagin	Madagascar	Dávila-Lara et al. (2021)
Ancistrocladaceae	<i>N. pervillei</i>	Plumbagin, 7-methyljuglone	Seychelles	
	<i>N. distillatoria</i>	Plumbagin	Sri Lanka	
	<i>N. ventrata</i>	Plumbagin	Philippines	
	<i>Ancistrocladus abbreviatus</i>	Plumbagin, droserone, malvone A, nepenthone A, ancistroquinones B–F	Cameroon, Ghana, Guinea, Ivory Coast, Liberia, Nigeria, Sierra Leone	Bringmann et al. (2008)
	<i>A. heyneanus</i>	Plumbagin	India	Bringmann et al. (2000)

(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
Dioncophyllaceae	<i>A. korupensis</i>	Michellamines D-F, korupensamine E	Cameroon, Nigeria	Hallock et al. (1997)
	<i>Triphyophyllum peltatum</i>	Dioncoquinones-A, -B, -C	Guinea, Ivory Coast, Liberia, Sierra Leone	Simoben et al. (2018)
	<i>Ceratostigma minus</i>	Plumbagin, plumbasides B, C	China, Tibet	Yue et al. (1994)
	<i>Dioncophyllum thollonii</i>	cis-, trans-Isoshinanolone	Congo, Gabon	El Sayed and Egbuna (2021)
Plumbaginaceae	<i>Plumbago auriculata</i>	Plumbagin	Africa, Portugal, Tunisia, Venezuela, Vietnam, Ethiopia, Bangladesh, North-East India	Nabil (2022)
	<i>P. indica</i>	Plumbagin, 3,30-biplumbagin, elliptinone	India, Bangladesh, Philippines, Sulawesi, Sumatra, Vietnam, Peru	Dissanayake et al. (2022)
	<i>P. zeylanica</i>	Plumbagin, neoisoshinanolone, 1-epi-neoisoshinanolone, Plumbagin, isoshinanolone, Plumbagin, epi-isoshinanolone	India, Nepal, Bangladesh, Vietnam, Guatemala, Sri Lanka, Paraguay, Brazil, Pakistan, Thailand, Kansas, Ghana, Italy, Ethiopia, Madagascar, Netherlands, Galapagos, China, Kenya, Liberia	Kakade et al. (2022)
	<i>Reynoutria japonica</i> (Syn. <i>Fallopia japonica</i>)	2-Methoxy-6-acetyl-7-methyljuglone (2-methoxystypandrone)	South Korea, Japan, China, North America and Europe	Khalil et al. (2019)
Polygonaceae	<i>Rheum officinale</i>	6-Methoxy-2-acetyl-3-methyl-1,4-naphthoquinone-8-O- β -D-glucopyranoside, 2-Methoxy-6-acetyl-7-methyljuglone	China, Czechoslovakia, Great Britain, Laos, Vietnam	Gao et al. (2017)
	<i>Polygonum aviculare</i>	6-Methoxyplumbagin	Europe, North America	Al-Hazimi and Haque (2002)

Balsaminaceae	<i>Impatiens balsamina</i>	Lawson	India, Sri Lanka, Austria, Bangladesh, Benin, Brazil South, France, Galápagos, Guatemala	Sakunphueak and Panichayupakaranant (2010, 2012)
	<i>Impatiens glandulifera</i>	Lawson, 2-methoxy-1,4-naphthoquinone	Nepal, Pakistan, Argentina, Denmark, Western Himalaya	Ruckli et al. (2014)
	<i>Impatiens capensis</i>	Lawson, 2-methoxy-1,4-naphthoquinone	North America, Europe, Mexico	Motz et al. (2012)
	<i>I. noli-tangere</i>	Lawson, 2-methoxy-1,4-naphthoquinone	Northwest European Region, East Himalayan region, Italy, Japan, Albania	Szewczyk (2018)
	<i>Impatiens parviflora</i>	Lawson	Afghanistan, Kazakhstan, Kirgizstan, Manchuria, Mongolia, Tadzhikistan, Turkmenistan, Uzbekistan, Austria, Belgium, Romania, Sweden, Switzerland, Ukraine	Lobstein et al. (2001)
	<i>Diospyros melanoxylon</i>	Droserone	India, Sri Lanka, Bangladesh	Patil et al. (2017)
Ebenaceae	<i>Diospyros anisandra</i>	Plumbagin	Cuba, Guatemala, Mexico Southeast	Flota-Burgos et al. (2020)
	<i>Diospyros sylvatica</i>	2-Methyl-anthraquinone, plumbagin, diosindigo, diospyrin, isodiospyrin and microphyllone	India, Sri Lanka	Ganapaty et al. (2004)
	<i>Diospyros batocana</i>	Biramentaceone, rotundiquinone and mamegakinon, 7-Methyljuglone	Angola, Botswana, Caprivi Strip, Zambia, Zaïre, Zimbabwe	Alves et al. (1983)
	<i>Diospyros ebenum</i> (Syn. <i>D. assimilis</i>)	Plumbagin, diospyrin,	India, Sri Lanka, Florida	Ganapaty et al. (2006)
	<i>Diospyros canaliculata</i>	Diospyrone, plumbagin	Angola, Cabinda, Cameroon, Central African Repu, Congo, Gabon, Ghana, Ivory Coast, Liberia, Nigeria, Zaïre	Dzoyem et al. (2011)
	<i>Diospyros chamaethamnus</i>	7-Methyljuglone, biramentaceone, mamegakinone, xylospirin, diospyrin,	Angola, Botswana, Caprivi Strip, Namibia, Zambia	Costa et al. (1999)

(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
		isodiospyrin, diosquinone, 2-methylnaphthazarin		
	<i>Diospyros chloroxylon</i>	Isodiospyrin	Bangladesh, India	Rauf et al. (2017)
	<i>Diospyros crassiflora</i>	Crassiflorone	Madagascar	Deblauwe (2021)
	<i>Diospyros dichrophylla</i>	Isodiospyrin	Cape Provinces, KwaZulu-Natal, Mozambique, Northern Provinces, Swaziland	Du et al. (2009)
	<i>D. mespiliformis</i>	Plumbagin, Diosquinone	Angola, Botswana, Cameroon, Caprivi Strip, Central Africa, Chad, Eritrea, Ethiopia, Gambia, Ghana, Guinea, Ivory Coast, Kenya, Mali, Mauritania, Mozambique, Namibia, Nigeria, Northern Provinces, Senegal, Sudan, Swaziland, Tanzania, Uganda, Yemen, Zambia, Zimbabwe	Olanlokun et al. (2021)
	<i>Diospyros elhretoides</i>	Isodiospyrin, isodiospyrol A	Cambodia, Hainan, Myanmar, Thailand	Prajoubklang et al. (2005)
	<i>Diospyros kaki</i>	Plumbagin, shinanolone, marinone	India, Bangladesh, Taiwan, Vietnam, China, Korea, Japan	Bawazeer and Rauf (2021)
	<i>Diospyros lycioides</i>	Juglone, 7-methyljuglone	Angola, Botswana, Cape Provinces, Mozambique, Namibia, Northern Provinces, Swaziland, Zambia, Zaire, Zimbabwe	Maroyi (2018a, b)

<i>Diospyros mafiensis</i>	Diosquinone	Mozambique, Tanzania	Mmongoyo et al. (2017)
<i>Diospyros mainqayi</i>	Shinanolone	Borneo, Malaya, Sumatra	Nematollahi et al. (2012)
<i>Diospyros maritima</i>	Droserone	Borneo, Cambodia, Sumatra, China, Philippines, Australia, Jawa, Sulawesi, Florida	Ue-Cachón et al. (2013)
<i>Diospyros mespiliformis</i>	Diosquinone	Angola, Botswana, Cameroon, Central Africa, Chad, Eritrea, Ethiopia, Gambia, Ghana, Guinea, Ivory Coast, Kenya, Mali, Mauritania, Mozambique, Namibia, Nigeria, Northern Provinces, Senegal, Sudan, Swaziland, Tanzania, Uganda, Yemen, Zambia, Zimbabwe	Olanlokun et al. (2021)
<i>D. melanoxylon</i> (Syn. <i>D. montana</i>)	Diospyrin	India, Sri Lanka, Bangladesh	Sharma (2017)
<i>Diospyros morrisiana</i>	Shinanolone	Japan, China, Taiwan	Nematollahi et al. (2012)
<i>Diospyros piscatoria</i>	Diospyrin, isodiospyrin, bisisodiospyrin	Cabinda, Cameroon, Congo, Gabon, Ghana, Guinea, Ivory Coast, Liberia, Nigeria, Sierra Leone, Zaïre	Adeniyi et al. (2000)
<i>Diospyros samoensis</i>	Martinone	Fiji, New Guinea, Samoa, Tonga	Nematollahi et al. (2012)
<i>Diospyros usambarensis</i>	7-Methyljuglone, isodiospyrin, mamegakinone	Mozambique, Northern Provinces, Tanzania, Zimbabwe	Marston et al. (1984)
<i>Diospyros virginiana</i>	Shinanolone	USA	Wang et al. (2011)
<i>Diospyros Wallachian</i>	5,50-Dihydroxy-2,20-dimethyl-7,70-binaphthalen-1,10,4,40-tetraone	Borneo, Malaya, Myanmar, Sumatra, Thailand	Salae et al. (2010)
<i>Diospyros zombensis</i>	Diosquinone	Kenya, Malawi, Mozambique, Tanzania, Zambia	Gafner et al. (1987)

(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
	<i>Euclea crispa</i>	7-Methyljuglone, diospyrin, neodiospyrin	Angola, Botswana, Cape Provinces, Lesotho, Malawi, Mozambique, Northern Provinces, Swaziland, Zimbabwe	Maroyi (2018a, b)
	<i>Euclea dewinteri</i>	Isodiospyrin, neodiospyrin, 7-methyljuglone	Northern Provinces	Joubert et al. (2006)
	<i>Euclea divinorum</i>	7-Methyljuglone, isodiospyrin, neodiospyrin	Angola, Botswana, Ethiopia, Kenya, Malawi, Mozambique, Namibia, Northern Provinces, Rwanda, Somalia, Sudan, Tanzania, Uganda, Zimbabwe	Al-Fatimi (2019)
	<i>Euclea crispa</i> subsp. <i>linearis</i> (Syn. <i>Euclea linearis</i>)	7-Methyljuglone, diospyrin, neodiospyrin	Cape Provinces, Northern Provinces, Zimbabwe	Alayande (2017)
	<i>Euclea natalensis</i>	7-Methyljuglone, diospyrin, isodiospyrin, neodiospyrin	Angola, Botswana, Cape Provinces, Kenya, KwaZulu-Natal, Malawi, Mozambique, Northern Provinces, Somalia, Swaziland, Tanzania, Zambia, Zaire, Zimbabwe	Oosthuizen and Lall (2020)
	<i>Euclea pseudobenus</i>	7-Methyljuglone, diospyrin, isodiospyrin, neodiospyrin	Angola, Cape Provinces, Namibia	Hook et al. (2014)
	<i>Euclea racemosa</i> subsp. <i>schimperi</i>	7-Methyljuglone, diospyrin, isodiospyrin, neodiospyrin, mamegakinone	Cape Provinces, Comoros, Egypt, Ethiopia, Kenya, Mozambique, Northern Provinces, Oman, Rwanda, Somalia, Sudan, Swaziland, Tanzania, Uganda, Yemen, Zambia, Zaire, Zimbabwe	Gebremariam et al. (2015)
	<i>Euclea tomentosa</i>	Neodiospyrin	Cape Provinces	Joubert et al. (2006)

Ericaceae	<i>Euclea undulata</i>	Diospyrin, 7-methylpt	Botswana, Cape Provinces, Mozambique, Namibia, Northern Provinces, Swaziland, Zimbabwe	Joubert et al. (2006)
	<i>Chimaphila umbellata</i>	2,7-Dimethyl-1,4-naphthoquinone (chimaphilin)	Austria, Belarus, Columbia, California, Czechoslovakia, Delaware, Denmark, Russia, Finland, France, Germany, Guatemala, Haiti, Hungary, Idaho, Illinois, Indiana, Irkutsk, Japan, Kazakhstan, Khabarovsk, Krasnoyarsk, Maine, Manchuria, Mexico, Poland, Romania, South Dakota, Sweden, Switzerland, Ukraine, Utah, West Siberia, Yugoslavia	Das et al. (2022)
	<i>Pyrola rotundifolia</i>	2-Methyl-7-hydroxymethyl-1,4-naphthoquinone (chimaphilin)	Afghanistan, Albania, Belarus, Belgium, Bulgaria, Buryatia, China, Czechoslovakia, Denmark, Finland, France, Germany, Great Britain, Greece, Russia, Hungary, Iran, Ireland, Irkutsk, Italy, Japan, Kirgizstan, Mongolia, Myanmar, Netherlands, North Caucasus, Norway, Poland, Romania, Spain, Sweden, Switzerland, Tibet, Turkey, Ukraine, Vietnam, West Siberia, Yugoslavia	Szewczyk et al. (2020)
	<i>Pyrola japonica</i>	5,8-Dihydro-2,7-dimethyl-1,4-naphthoquinone (5,8-dihydrochimaphilin)	China North-Central, China Southeast, Inner Mongolia, Japan, Khabarovsk, Korea, Kuril Is., Manchuria, Primorye, Taiwan	Lee et al. (2001)
	<i>Pyrola asarifolia</i> subsp. <i>asarifolia</i> (Syn. <i>Pyrola incarnata</i>)	Chimaphilin	Alaska, Alberta, India California, China North-Central, Chita, Inner Mongolia, USA, Irkutsk, Japan,	Duan et al. (2015)

(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
Boraginaceae			Kamchatka, Kazakhstan, Korea, Manchuria, Mongolia, Montana, Nepal, Nevada, New Brunswick, West Siberia	
	<i>Alkanna tinctoria</i>	Alkannin	Albania, Algeria, Bulgaria, Cyprus, Czechoslovakia, Egypt, France, Greece, Hungary, Italy, Lebanon-Syria, Libya, Morocco, Palestine, Romania, Spain, Tunisia, Turkey, Ukraine, Yugoslavia	Huu Tung et al. (2013)
	<i>Alkanna graeca</i> subsp. <i>graeca</i> (Syn. <i>Alkanna calliensis</i>)	Alkannin	Albania, Greece, Yugoslavia	Sahakyan et al. (2019)
	<i>Alkanna cappadocica</i>	Alkannin	Turkey	Abdel-Gelil et al. (2019)
	<i>Alkanna corcyrensis</i>	Acetyl-, isobutyl-, angelyl-, b, b-dimethylacryl-alkannin	Albania, Greece	Sahakyan et al. (2019)
	<i>Alkanna orientalis</i>	Angelyl-, b, b-dimethylacryl-alkannin	East Aegean Is., Iran, Lebanon-Syria, Palestine, Saudi Arabia, Transcaucasus, Turkey, Yemen, Algeria, Tunisia	Yagloglu et al. (2022)
	<i>Alkanna haussknechtii</i> (Syn. <i>Alkanna primuliflora</i>)	Acetyl-, propionyl-, isobutyl-, angelyl-, b, b-dimethylacryl-alkannin	Turkey	Semerdjieva et al. (2020)
	<i>Arnebia benthamii</i>	Shikonin, acetylshikonin, b-acetoxylisovalerylshikonin	India, China, Pakistan	Ganie et al. (2012)
	<i>Arnebia densiflora</i>	b, b-Dimethylacrylalkannin, tetracylalkannin,	Greece, North Caucasus, Transcaucasus, Turkey	Yuzbasioglu Baran et al. (2020)

Boraginaceae	<i>Arnebia euchroma</i>	Shikonin, acetylshikonin, b,b0-dimethylacrylshikonin, amebiabinone, acetylalkannin, b,b0-dimethylacrylalkannin, b-hydroxyisovalerylalkannin	Afghanistan, Iran, Kazakhstan, Kirgizstan, Nepal, Pakistan, Tadzhikistan, Tibet, Turkmenistan, Uzbekistan, West Himalaya, Xinjiang	Liao et al. (2020)
	<i>Arnebia hispidissima</i>	Alkannin, acetylalkannin, isovalerylalkannin, b-hydroxyisovalerylalkannin, shikonin, acetylshikonin, b-acetoxyisovalerylshikonin	Cameroon, Chad, Djibouti, Egypt, Ethiopia, India, Iran, Nigeria, Oman, Pakistan, Palestine, Saudi Arabia, Somalia, Sudan, Tibet.	Kamil et al. (2021)
	<i>Arnebia guttata</i>	Shikonin, acetylshikonin, b-acetoxyisovalerylshikonin	Afghanistan, China, Kirgizstan, Mongolia, Pakistan, Tadzhikistan, Tibet, Turkmenistan, Uzbekistan, West Himalaya	Gao et al. (2021)
	<i>Arnebia speciosa</i> (Syn. <i>A. nobilis</i>)	Alkannin, acetylalkannin, b,b0-dimethylacrylalkannin	Afghanistan, Pakistan	Arora et al. (2012)
	<i>Bothriospermum secundum</i>	Bothriodumin	China North-Central, China South-Central, China Southeast, Korea, Manchuria	Liu et al. (2009)
	<i>Varronia polycephala</i> (Syn. <i>Cordia polycephala</i> syn. <i>Cordia corymbosa</i>)	Cordiaquinones	Bolivia, Brazil, Colombia, Costa Rica, Dominican Republic, Galápagos, Guatemala, Guyana, Haiti, Honduras, Mexico, Panamá, Paraguay, Peru, Puerto Rico, Trinidad-Tobago, Venezuela	Freitas et al. (2012)
	<i>Varronia leucocephala</i> (Syn. <i>Cordia leucocephala</i>)	Cordiaquinone L, cordiaquinone M	Brazil	Diniz et al. (2015)
	<i>Varronia curassavica</i> (Syn. <i>Cordia curassavica</i>)	Cordiaquinones A and B	Argentina, Brazil, Mexico, Costa Rica, Colombia, Venezuela, Guatemala, Uruguay	Ioset et al. (2000)
				(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
	<i>Varronia linnaei</i> Syn.	Cordiaquinone B	Colombia, Costa Rica, Jamaica, Cuba, Haiti, Mexico	Hook et al. (2014)
	<i>Cordia linnaei</i>			
	<i>Echium italicum</i>	Shikonin	Algeria, Morocco, Spain	Jin et al. (2020a)
	<i>E. flavum</i>			
	<i>Echium lycopsis</i>	Shikonin	Algeria, Syria, Liberia, Cyprus, France, Egypt, Italy, Turkey, Spain, Saudi Arab, Morocco, Tunisia	Zhu et al. (2016)
	Syn. <i>E. planragineum</i>			
	<i>Echium rubrum</i> Syn.	Shikonin	Algeria, Balears, Corse, France, Sardegna, Spain, Tunisia	Zhu et al. (2016)
	<i>E. creticum</i>			
	<i>Echium vulgare</i>	Shikonin, acetylshikonin, isobutyrylshikonin, isovalerylshikonin, b,b0-dimethylacrylshikonin	Albania, Finland, France, Germany, Great Britain, Greece, Hungary, Ireland, Norway, Poland, Portugal, Romania, Belarus, Belgium, Bulgaria Turkey, Ukraine, Turkmenistan	Wang et al. (2022)
	<i>Eritrichium incanum</i>	Shikonin, acetylshikonin, a-methylbutyrylshikonin, b,b0-dimethylacrylshikonin	Amur, Chita, Inner Mongolia, Khabarovsk, Korea, Manchuria, Primorye	Papageorgiou et al. (1999)
	<i>Eritrichium sichotense</i>	Shikonin, acetylshikonin, isobutyrylshikonin, b,b0-dimethylacrylshikonin	Korea, Primorye	
	<i>Lappula squarrosa</i> (Syn. <i>Lappula consanguinea</i>)	Shikonin, acetylshikonin, a-methylbutyrylshikonin, b,b0-dimethylacrylshikonin	Eastern Europe, South Asia, Southeast Asia, Middle Europe	
	<i>Lithospermum erythrorhizon</i>	Shikonin	Afghanistan, Altay, China, Kirgizstan, Mongolia, Pakistan, Tadzhikistan, Tibet, Turkmenistan, Uzbekistan, West Himalaya	Hook et al. (2014)

Gentianaceae	<i>Maharanga bicolor</i>	Deoxyalkannin, alkannin, acetylalkannin	East Himalaya, Nepal, Tibet	Rajbhandari et al. (2007)
	<i>Onosma boryszenica (arenaria)</i>	Hydroxyisovalerylalkannin, acetylalkannin	Ukraine	Olenikov et al. (2020)
	<i>O. caucasica</i>	Shikonin	North Caucasus, Transcaucasus, Turkey	Kiling (2021)
	<i>O. conferta</i>	Shikonin, isobutyl-, acetylshikonin, dimethylacryl-, isovaleryl-	China	Ahmad et al. (2018)
	<i>O. echioides</i>	Alkannin/shikonin, deoxyalkannin/shikonin, arnebin-6, etc.	Albania, Bulgaria, Italy, Sicilia, Spain, Turkey-in-Europe, Yugoslavia	Shoaib et al. (2019)
	<i>O. hookerii</i>	Shikonin, isobutyl-, acetylshikonin, dimethylacryl-, isovaleryl-	East Himalaya, Nepal, Tibet	Wu et al. (2021)
	<i>O. paniculata</i>	Shikonin, isobutyl-, acetylshikonin, dimethylacryl-, isovaleryl-, a-hydroxyisovaleryl-	China, East Himalaya, Tibet	Kretschmer et al. (2012)
	<i>O. waltonii</i>	Shikonin, isobutyl-, acetylshikonin, dimethylacryl-, isovaleryl-	Tibet	Hu et al. (2006)
	<i>O. lhokaensis</i>	Shikonin	Tibet	He et al. (2018)
	<i>Sweritia calycina</i>	2-Methoxy-1,4-naphthoquinone	Burundi, Ethiopia, Kenya, Rwanda, Tanzania, Uganda, Zaïre	Goel et al. (2020)
	<i>Mitracarpus frigidus</i>	Psychorubrin	Brazil, Colombia, French Guiana, Guyana, Paraguay, Peru, Venezuela	Lemos et al. (2018)
	<i>Psychotria hoffmannseggiana</i> (syn. <i>rubra</i>)	Psychorubrin	Bolivia, Brazil, Costa Rica, Cuba, Mexico, Ecuador, Venezuela, Peru, Guatemala, Panama, Trinidad and Tobago	Gontijo et al. (2021)
Rubiaceae	<i>Palicourea hoffmannseggiana</i>			
	<i>Notopleura camponutans</i> (Syn: <i>Psychotria camponutans</i>)	Pentalongin; Psychorubrin	Colombia, Costa Rica, Panamá	Martins and Nunez (2015)
(continued)				

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
Calceolariaceae	<i>Gynochthodes parvifolia</i> (Syn. <i>Morinda parvifolia</i>)	Morindaparvin C-G	China, Philippines, Taiwan, Vietnam	Kang et al. (2016)
	<i>Rubia cordifolia</i>	2-Carbamoyl-3-hydroxy-1,4-naphthoquinone	Angola, Assam, Bangladesh, Borneo, Cape Provinces, China, Japan, Pakistan, Java, Taiwan, Vietnam, India, Bangladesh, Ethiopia	Kumari et al. (2021)
	<i>Rubia tinctorum</i>	Morindone	Afghanistan, Albania, Cyprus, East Aegean Is., Greece, India, Iran, Iraq, Italy, Lebanon-Syria, Pakistan, Palestine, Turkey, Turkmenistan, Ukraine, Uzbekistan, Vietnam, West Himalaya, Yugoslavia	Ahmadi et al. (2020)
	<i>R. oncotricha</i>	Rubioncolins A, B, C; 2-carbomethoxy-2,3-epoxy-3-prenyl-1,4-naphthoquinone, 3-carbomethoxy-4-hydroxy-1,2-naphthoquinone	China	Lumb and Trauner (2005)
	<i>R. yunnanensis</i>	Rubinaaphthins A-D	South Central China	Suyama et al. (2017)
	<i>Dolichopentas longiflora</i> Syn. <i>Pentas longiflora</i>	Quinones; pentalongin; mollugin	Kenya, Malawi, Rwanda, Tanzania, Uganda, Zaire	Kagisha et al. (2021)
	<i>Calceolaria andina</i>	2-(1,1-Dimethylprop-2-enyl)-3-hydroxy-1,4,1,4-naphthoquinone	Central Chile	Khambay et al. (1999)
	<i>Calceolaria sessilis</i>	2,3,3-Trimethyl-2,3-dihydronaphtho [2,3-b] furan-4,9-quinone, 2-hydroxy-3-(1,1-dimethylallyl)-1,4-naphthoquinone	Central Chile	Morello et al. (1995)
	<i>Primulina eburnea</i> Syn. <i>Chirita eburnea</i>		China South-Central, China Southeast	Cai et al. (2005)
Gesneriaceae				

		7-Hydroxy-a-dunnione, 8-hydroxy-a-dunnione, 7,8-dihydroxy-a-dunnione, 7-methoxy-6,8-dihydroxy-a-dunnione			
	<i>Sinningia aggregata</i>	Aggregatin D		Brazil, Paraguay	Souza et al. (2015)
	<i>Streptocarpus dunnii</i>	Dunnione		Northern Provinces, Swaziland	Hook et al. (2018)
	<i>Capraria biflora</i>	Biflorin (6,9-Dimethyl-3-(4-methyl-3-pentenyl)naphthol[1,8-bc]-pyran-7,8-dione)		Argentina, Brazil, Guatemala, Colombia, Costa Rica, Cuba, Paraguay, Peru, Mexico, Venezuela	Wisintainer et al. (2015)
Lamiaceae	<i>Tectona grandis</i>	Lapachol, Tectonaquinone A-C		India, Myanmar, Vietnam, Bangladesh, Cambodia	Vyas et al. (2022)
	<i>Salvia columbariae</i>	Miltionone II, cryptotanshinone, tanshinone IIA		USA, Mexico	Wu et al. (2012)
Paulowniaceae	<i>Paulownia tomentosa</i>	Methyl 5-hydroxy-dinaphtho [1,2-20,30] furan-7,12-dione-6-carboxylate		China, Korea	Park et al. (1992)
Pedaliaceae	<i>Sesamum indicum</i>	2-Chloro-5,8-dihydroxy-3-(3 methyl-2-butenyl)-1,4-naphthoquinone, (chlorosessamone), hydroxysesamone, 2,3-epoxysesamone		India, Bangladesh	Mili et al. (2021)
	<i>Cyrtax antisiphilitica</i>	Lapachol		Argentina Northwest, Bolivia, Brazil, Ecuador, Paraguay, Peru, Suriname	Hussain et al. (2007)
Paulowniaceae	<i>Catalpa ovata</i>	Catalpol, Catalpalactone		China	Kim et al. (2019)
Bignoniaceae	<i>Dolichandra unguiscati</i>	Lapachol		Argentina Bahamas, Belize, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, Guatemala, Guyana, Haiti, Honduras, Jamaica, Mexico, Panamá, Paraguay, Peru, Puerto Rico, Trinidad-Tobago, Uruguay, Venezuela	Hook et al. (2014)

(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
	<i>Fernandoa adenophylla</i>	Dilapachone, adenophyllone	India, Cambodia, Bangladesh, Myanmar, Thailand, Vietnam	Alhumaydhi et al. (2021)
	<i>Handroanthus guayacan</i> (Syn. <i>Tabebuia guayacan</i>)	Lapachol, dehydro-a-lapachone, a-Lapachone, b-Lapachone	Colombia, Costa Rica, Guatemala, Honduras, Mexico, Nicaragua, Panamá, Peru, Venezuela	Grose and Olmstead (2007a, b)
	<i>Handroanthus impetiginosus</i> (Syn. <i>Tabebuia avellanedae</i>)	(a-Lapachone), lapachol	Argentina, Brazil, Colombia, Costa Rica, Guatemala, Honduras, Mexico, Nicaragua, Panamá, Peru, Venezuela	Hamed et al. (2020)
	(Syn. <i>Tabebuia impetiginosa</i>)			
	<i>Handroanthus incanus</i> (Syn. <i>Tabebuia incana</i>)	B 5- and 8-8-hydroxy-2-(10-hydroxyethyl) naphtho[2,3-b]furan-4,9-dione	Bolivia, Brazil North, Colombia, Ecuador, Peru	Morais et al. (2007)
	<i>Handroanthus serratifolius</i> (Syn. <i>Tabebuia serratifolia</i>)	Lapachol, dehydro-lapachone	Bolivia, Brazil, Ecuador, French Guiana, Peru, Trinidad-Tobago, Venezuela	Segoloni and Di Maria (2018)
	<i>Phyllanthron comorense</i>	Dehydro-a-lapachone, a-lapachone	Comoros	Hussain and Green (2017)
	<i>Kigelia africana</i> (Syn. <i>Kigelia pinnata</i>)	Kigelinone, isopinnata, dehydro-a-lapachone, lapachol, 2-(1-hydroxyethyl)-naphtho[2,3-b]furan-4,9-dione	Angola, Botswana, Cameroon, Chad, Congo, Eritrea, Ethiopia, Gabon, Gambia, Ghana, Guinea, Ivory Coast, Kenya, Liberia, Mozambique, Namibia, Niger, Nigeria, Northern Provinces, Rwanda, Senegal, Sierra Leone, Somalia, Sudan, Swaziland, Tanzania, Togo, Uganda, Zimbabwe	Gupta and Jain (2019)
	<i>Mansoa alliacea</i>	9-Methoxy- α -lapachone, 4-hydroxy-9-methoxy- α -lapachone	Bolivia, Brazil North, Brazil Northeast, Colombia, Ecuador, French Guiana, Guyana, Peru, Trinidad-Tobago	Salazar et al. (2017)

<i>Markhamia lutea</i>	Lapachol, a-lapachone, dehydro-iso-a-lapachone	Burundi, Cameroon, Congo, Gabon, Ghana, Gulf of Guinea Is., Ivory Coast, Kenya, Nigeria, Rwanda, Sudan, Tanzania, Togo, Uganda	Ibrahim et al. (2016)
<i>M. stipulata</i>	Hydroquinone: Dehydro- α -lapachone (43); lapachol (44); dehydro-iso- α -lapachone (45); β -lapachone (46); tectol (47)	Bangladesh, Cambodia, China South-Central, China Southeast, Laos, Myanmar, Thailand, Vietnam	
<i>M. obtusifolia</i>	Hydroxynaphthoquinones	Angola, Botswana, Central African Republic, Kenya, Malawi, Mozambique, Namibia, Rwanda, Tanzania, Zimbabwe	
<i>M. tomentosa</i>	2-Acetyl-naphtho[2,3-b] furan-4,9-dione (49); 2-acetyl-6-methoxynaphtho[2,3-b] furan-4,9-dione (50)	Angola, Benin, Burkina, Cabinda, Cameroon, Congo, Ghana, Guinea, Guinea-Bissau, Ivory Coast, Nigeria, Senegal, Sierra Leone, Togo, Zaïre	
<i>Newbouldia laevis</i>	Lapachol, dehydro-a-lapachone, 3-hydroxy-dehydroiso-alapachone	Angola, Benin, Burkina, Cameroon, Congo, Gabon, Gambia, Ghana, Guinea, Ivory Coast, Liberia, Mauritania, Nigeria, Senegal, Togo, Zaïre	Okagu et al. (2021)
<i>Radermachera sinica</i>	Lapachol, dehydro-iso-a-lapachone	India, China, Malaya, Vietnam, Taiwan	Wang et al. (2016)
<i>Stereospermum chelonoides</i>	Lapachol, Stereochenols A and C, sterekunthal B, sterequinone C	India, Cambodia, Nepal, Laos, Sri Lanka	Shanta et al. (2018)
<i>S. kunthianum</i>	Stereokunthals A and B, pyranokunthones A and B	Benin, Burkina, Burundi, Cameroon, Central African Republic, Chad, Djibouti, Eritrea, Ethiopia, Gambia, Ghana, Guinea, Ivory Coast, Kenya, Mali, Mauritania, Mozambique, Niger, Nigeria, Senegal, Sudan, Tanzania, Togo, Uganda, Zimbabwe	Wangso et al. (2021)

(continued)

Table 15.1 (continued)

Family	Plants name	Naphthoquinones	Distribution	References
	<i>Tabebuia heterophylla</i> (syn. <i>T. pentaphylla</i>)	Tecomaquinones-I,-II,-III	Bahama, Jamaica	El-Hawary et al. (2021)
	<i>T. rosea</i>	Lapachol, dehydro-a-lapachone, dehydro-iso-a-lapachone	Colombia, Costa Rica, Ecuador, Guatemala, Guyana, Honduras, Mexico, Nicaragua, Panamá, Venezuela	Pagaza-Straffon et al. (2021)
	<i>Handroanthus ochraceus</i> (Syn. <i>T. ochracea</i>)	Lapachol, 2-(1-hydroxyethyl)-naphthol[2,3-b] furan-4,9-dione 2-Acetyl-6-methoxynaphthol[2,3-b] furan-4,9-quinone, 5,8-Dihydroxy-2-(10-hydroxyethyl) naphthol[2,3-b] furan-4,9-dione, 2-acetyl-7-methoxy-8-hydroxy naphthol[2,3-b] furan-4,9-dione	Argentina Northwest, Bolivia Brazil, Colombia, Costa Rica, Ecuador, El Salvador, Guyana, Honduras, Nicaragua, Panamá, Paraguay, Peru, Trinidad-Tobago, Venezuela	Hamed et al. (2020)
	<i>Tecomella undulata</i>	Lapachol, dehydro-a-lapachone	Afghanistan, India, Iran, Oman, Pakistan	Valizadeh et al. (2020)
	<i>Handroanthus billbergii</i>	Lapachol	Aruba, Colombia, Cuba, Ecuador, Peru, Venezuela	Gómez-Estrada et al. (2012)
Acanthaceae	<i>Tabebuia aurea</i> (Syn. <i>T. argentea</i>)	Lapachol	Argentina Northeast, Argentina Northwest, Bolivia, Brazil North, Brazil Northeast, Brazil South, Brazil Southeast, Brazil West-Central, Paraguay, Peru	Nirupama et al. (2011)
	<i>Avicennia marina</i>		Aldabra, Bangladesh, Borneo, Cape Provinces, China Comoros, Egypt,	El Dohaji et al. (2020)

		Avicequinone A and C, stenocarpoquinone B, avicennones D, E	India, Iran, Java, Kenya, Madagascar, Malaya, Mozambique, Mozambique, Myanmar, New Guinea, Australia, New Zealand, Oman, Pakistan, Philippines, Saudi Arabia, Somalia, Sri Lanka, Sudan, Sulawesi, Sumatra, Taiwan, Tanzania, Tasmania, Thailand, Vietnam, Australia, Yemen	Ito et al. (2000)
	<i>Avicennia alba</i>	Avicequinone A, B	Bangladesh, Borneo, India, Java, Malaya, Marianas, Myanmar, New Guinea, Philippines, Sulawesi, Sumatra, Thailand, Vietnam	Williams et al. (2006)
	<i>Mendoncia cowanii</i>	Avicequinone-C, stenocarpoquinone B, avicequinones D and E	Madagascar	Boonyaketgason et al. (2018)
	<i>Rhinacanthus nasutus</i>	Rhinacanthone, rhinacanthins-A, -B, -C, -N, Rhinacanthin-Q, Rhinacanthins-C, -D, -N	India, Laos, Malaya, Myanmar, Philippines, Sri Lanka, Thailand, Vietnam, Bangladesh, Cambodia	Carballo-Arce et al. (2015)
	<i>Marcgravia nervosa</i>	2-Methoxy-1,4-naphthoquinone	Colombia, Costa Rica, Ecuador, Nicaragua, Panamá	Fan et al. (2019)
Asteraceae	<i>Xanthium strumarium</i>	5-Hydroxy-3,6-dimethoxy-7-methyl-1,4-naphthalenedione, 7-(4-hydroxy-3-methoxyphenyl)-1-phenylhept-4-en-3-one	Afghanistan, Albania, Algeria, Bulgaria, Cambodia, China Czechoslovakia, France, Greece, Hainan, Hungary, India, Iran, Italy, Kriti, Laos, Lebanon-Syria, Mongolia, Morocco, Myanmar, Caucasus, Pakistan, Palestine, Portugal, Spain, Sri Lanka, Switzerland, Taiwan, Thailand, Tibet, Turkey, Ukraine, Yugoslavia	
	<i>Xanthium sibiricum</i>			

Plumbago is not the only genus that contains plumbagin. It can also be found in endangered, carnivorous aquatic plant *Aldrovanda vesiculosa*. Several terrestrial carnivorous plants of Droseraceae and Nepenthaceae also produces plumbagin, moreover a large number of species in Nepenthaceae are endemic and endangered. Like the diverse nature of the plants that produces plumbagin, the amount of plumbagin content in those plants is also a subject to great variation. Lawsone is another important NQ also used traditionally for hair colouring and to make temporary tattoos on skin in cultural events in Egypt and India. Although lawsone is predominantly found in a tree species, *Lawsonia inermis* or Henna belong to the family Lythraceae, again it is also found in several species of ornamental herbaceous plant genus *Impatiens*. These two lawsone containing genera are broadly differs in their nature, habitat and global distribution. The production of juglone is restricted in the Juglonaceae family, and majorly it is found in black walnut. Juglone is a toxic substance released from the roots of Black Walnut to suppress the growth of other species around it, the phenomenon known as allelopathy (Tattar 1989). Plant species that contain NQs have a widespread distribution throughout the world. They are diversified in the nature of NQ they contain, their habitat, and their occurrence. Many of them are widely found to a larger extent, while several others are endemic and threatened. Some are cultivated economic expansion globally while others are extensively cannibalised from their natural habitat. Owing to the usefulness of NQ, it is, therefore, essential to look after the plants that contain major NQ. This chapter pays sufficient attention to the distribution, diversity and conservation strategies of major NQ-containing plants.

15.2 Bioactivity of Naphthoquinones

Naphthoquinones are naturally occurring widespread secondary metabolites that generally appear in different forms, such as juglone, lawsone, plumbagin, phylloquinone, shikonin, alkannin, diospyrin, and lapachol (Figs. 15.1a–l and 15.2a–h). They naturally occur in some actinomycetes (*Streptomyces*), fungi (*Fusarium*, *Marasmius*, and *Verticillium*), algae (*Cyanidium caldarium*, *Landsbergia quercifolia*, *Chlamydomonas reinhardtii*, etc.), lichen (*Trypethelium eluteriae*, *Squamarina cartilaginea*, *Astrothelium* sp.), and plants (*Plumbago* sp., *Juglans* sp., *Nepenthes* sp., *Drosera* sp., etc.) (Perry et al. 1991; Himmelreich et al. 1994; Yoshida et al. 2003; Sun et al. 2010; Ozawa et al. 2012; Basnet et al. 2019). The most noteworthy naphthoquinones-containing plants are associated with phylogenetically heterogeneous plant families such as Droseraceae, Avicenniaceae, Balsaminaceae, Asteraceae, Lamiaceae, Boraginaceae, Ebenaceae, Juglandaceae, Nepenthaceae, Plumbaginaceae, Bignoniaceae, etc. (Babula et al. 2009). Phylloquinone, such as vitamin K₁, Menaquinones and other naphthoquinones, are found in several bacteria, fungi, and plant families (Hook et al. 2014). Naphthoquinones can be found free or as glycosides within the plants. They are usually coloured yellow, orange, and brown and may serve as a source of

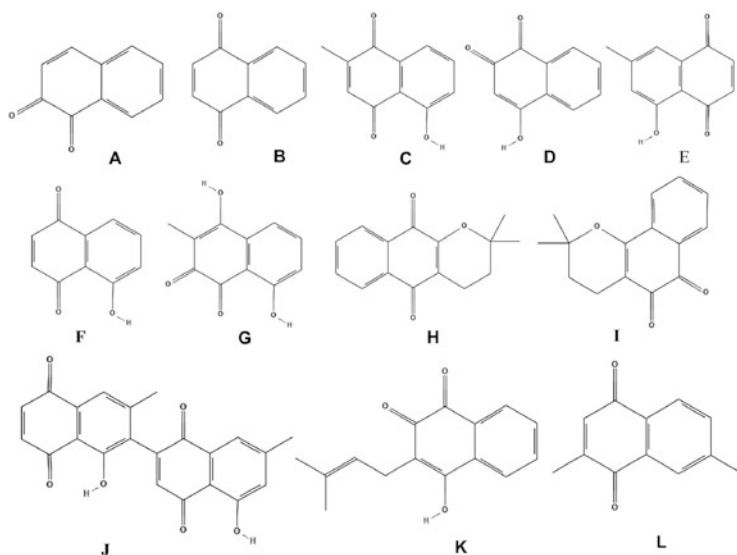


Fig. 15.1 Structure of some important naphthoquinones found in higher plants. (a) 1,2 Naphthoquinone; (b) 1,4 Naphthoquinone; (c) Plumbagin; (d) Lawsonone; (e) 7-methyljuglone; (f) Juglone; (g) Droserone; (h) α -Lapachone; (i) β -Lapachone; (j) Diospyrin; (k) Lapachol; (l) Chimaphilin

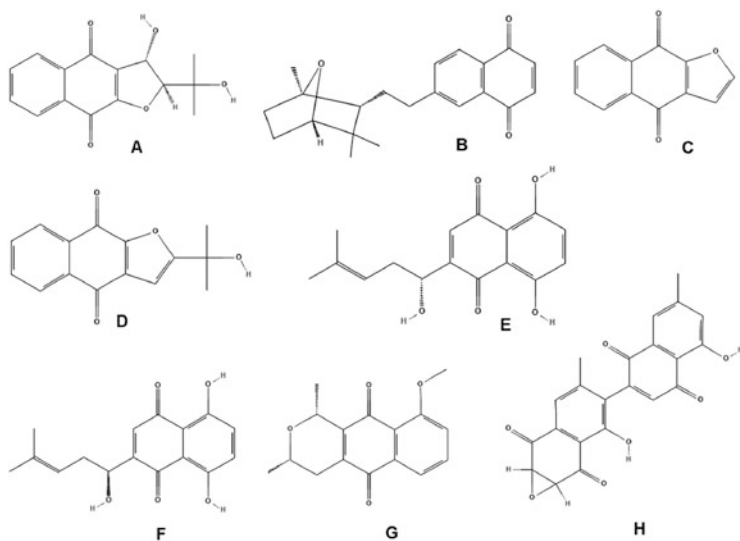


Fig. 15.2 Structure of some important naphthoquinones found in higher plants. (a) Avicequinone A; (b) Cordiaquinone; (c) Avicequinone B; (d) Avicequinone C; (e) Shikonin; (f) Alkannin; (g) Eleutherin; (h) Diosquinone

pigmentation in some plants (Seigler 1998). The quinine derivatives such as benzoquinones, anthraquinones, and naphthoquinones are derived by acetate-malonate and shikimic acid pathways. Naphthoquinones are naphthalene diones that exist in two isomeric forms. In 1,4-naphthoquinones, the naphthalene skeleton is substituted in positions C(1) and C(4) and in 1,2-naphthoquinones, it is replaced in C(1) and C(2) (Fig. 15.1a, b).

Naphthoquinones have been used in the medicinal field for their anticancer, antimicrobial, cardioprotective, and neuroprotective properties since ancient times. The compound also has trypanocidal, fungicidal, and anti-viral activity (Aminin and Polonik 2020). In ethnomedicinal practices of several Asian and South American countries, naphthoquinones-containing plants have a wide range of therapeutic applications, most notably in treating various types of cancer (Cao et al. 2018). It can also be crucial in ecological relationships among higher plants and microbes. Several naturally occurring naphthoquinones, such as juglone, plumbagin, lawsone, lapachol, shikonin, alkannin, droserone, etc. have been studied by many investigators (Hook et al. 2014; Devi et al. 2016). This compound portrays an array of bioactivity directly linked with its mechanism of action. NQ serves as vital links in the electron transport chains in several metabolic pathways. Several studies have shown that NQ can inhibit topoisomerase I and II, which play a crucial role in the regulation of replicative DNA synthesis (Widhalm and Rhodes 2016).

15.2.1 Mechanism of Action

The development of medicinally important drugs for a new era by using the naphthoquinone compound could be achieved only by knowing the compound's mode of action. Therefore, the synthesis of natural naphthoquinones-derived compounds has been studied in recent years. Adding several functional groups in the ring of 1,4-naphthoquinone, the minimum inhibitory concentrations (MICs) show better than many commercial drugs (Ravichandiran et al. 2019). In addition, NQs have displayed potent antibacterial activity against a broad spectrum of microorganisms (Dissanayake et al. 2022). Some authors have attributed the inhibition activity to competitive interaction between the compound and the electron transport (Hennig et al. 1998). Other authors have demonstrated that the cytotoxicity activity of naphthoquinones is due to the production of reactive oxygen species (ROS) that interact with the electron transference in the mitochondrial respiratory chain (Zheng et al. 2020; de Almeida et al. 2021).

15.2.2 Anticancer Activity

Naphthoquinones show anticancer effects by inhibiting cell growth, inducing apoptosis, inhibiting DNA topoisomerases, and decreasing angiogenesis. Most

plant-derived NQs exhibited significant cytotoxicity against several potent cancer cell lines. Plumbagin substantially affects myeloma and leukaemia (Wu et al. 2016). Plumbagin also inhibits cell proliferation, induces apoptosis and causes cell cycle arrest by preventing NF- κ B in cancer cells (Xu et al. 2013). It also inhibits tumour angiogenesis and growth by interfering with VEGFR2-mediated Ras signalling (Lai et al. 2012). The application of plumbagin in a very minimal amount significantly inhibits the cell invasion in Her2-overexpressing breast cancer and inducing apoptosis (Kawiak and Domachowska 2016). Plumbagin also inhibits tubulin polymerisation leading to the disruption of microtubule formation (Acharya et al. 2008). Juglone has demonstrated the ability to interfere with processes of mitosis by preventing the activation of cysteine-rich proteins (Chuang et al. 2021). Some investigations show plumbagin, and other 1,4-naphthoquinone derivatives such as juglone, lawsone, and lapachol displayed high cytotoxicity against keratinocytes (HaCaT) and strongly induced ROS formation and depleted cellular glutathione (Klaus et al. 2010). The cytotoxic effects of alkannin in higher on the HEp-2 cell line ($IC_{50} = 4.96 \text{ mg mL}^{-1}$) and MCF-7 and DU-145 cell lines (Yuzbasioglu Baran et al. 2020). Shikonin exhibited potent cytotoxicity against MRC-5 lung fibroblasts and melanoma cell lines, including SBcl2, WM35, WM9, and WM164 (Kretschmer et al. 2012). The cytotoxic effects of Rhinacanthin have been demonstrated in three cancer cell lines, namely, KB for oral cavity cancer, HepG2 for hepatocellular cancer and HeLa for cervical cancer (Kumar et al. 2017).

15.2.3 Antimicrobial Activity

The global market value of antibiotics is increasing daily after the discoveries of important antibiotics such as penicillin, streptomycin, etc. However, a massive threat of multidrug-resistant pathogenic microbes has severely targeted the public health sector. Multidrug-resistant (MDR), called ‘superbugs’, comprises *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Enterococcus faecium* and *Enterobacter* spp. The high morbidity rate due to these microbial infections has drawn attention in present days. NQs have been used for decades as potential antibacterial agents that inhibit the growth of Gram-positive and Gram-negative bacteria (Lawrence and Jeyakumar 2013; Widhalm and Rhodes 2016)

7-methyljuglone, diospyrin and its derivatives were tested against *Mycobacterium tuberculosis* H37Rv. The MICs values of isodiospyrin ($10.0 \text{ } \mu\text{g mL}^{-1}$), diospyrin ($8.0 \text{ } \mu\text{g mL}^{-1}$), 7-methyljuglone ($0.5 \text{ } \mu\text{g mL}^{-1}$) and neodiospyrin ($10.0 \text{ } \mu\text{g mL}^{-1}$) (Van der Kooy et al. 2006). The naphthoquinones performed well compared to some antimycobacterial drugs, such as ethambutol, isoniazid and rifampicin (Halicki et al. 2018). Plumbagin shows inhibitory activity against the resistant strain of *M. tuberculosis* H37Rv at 12.5 mg mL^{-1} (Kuethe et al. 2009). Plumbagin has shown antimicrobial activity against *Staphylococcus epidermidis*. *Enterococcus faecalis* shows inhibition zones ranging from 17.67 to 43.00 mm with

MIC values ranging from 15.6 to 250 $\mu\text{g mL}^{-1}$ (Gonçalves et al. 2009). Another investigation on the antimicrobial effects of plumbagin has been studied in *Bacillus subtilis* by detecting differentially expressed proteins suggesting that the 1,4-NQ could repress the tricarboxylic acid cycle along with the electron transport chain and the fatty acid synthesis. Shikonin showed antibacterial activity against gram-positive bacteria, including *Staphylococcus aureus*, *Enterococcus faecium* and *Bacillus subtilis*, whereas the minimum inhibitory concentration (MIC) of these compounds ranged from 0.3 mg mL^{-1} to 6.25 mg mL^{-1} . Juglone, another important natural NQ, can inactivate the *E. coli* PPIase by covalent modification of cysteine residues. It modifies sulphhydryl groups to inhibit the ESS1, PPIase homolog, and RNA polymerase II, in *Saccharomyces cerevisiae* (Widhalm and Rhodes 2016).

Fungal infections pose significant threats that cause morbidity and mortality in humans, particularly in patients with compromised immune systems due to issues such as HIV infections, organ transplants, leukaemia, etc. (Dellièvre et al. 2018). Moreover, the resistance characteristics of the causal organisms toward antifungal drugs make the situation more dreadful and demand the discovery of new antifungal compounds. *Candida albicans* is the most recognisable fungal pathogen as it causes almost 80% of *Candida*-mediated infections (Gnat et al. 2021). Several natural naphthoquinones have shown anti-*Candida* activity. Plumbagin expressed an antifungal activity with MICs of 0.8 $\mu\text{g mL}^{-1}$ and MFC of 1.56 $\mu\text{g mL}^{-1}$ against *C. albicans*. Shikonin showed activity against *C. krusei* with a MIC of 4.0 $\mu\text{g mL}^{-1}$ and *C. glabrata* with a MIC of 8.0 $\mu\text{g mL}^{-1}$. These results are comparable to those with the well-known drug fluconazole (Futuro et al. 2018). Lapachole has been shown to have antifungal activity against *Candida* infection (Ahmadi et al. 2020). Plumbagin shows inhibitory action against *Penicillium citrinum*, *Aspergillus flavus* (CCAM 080001), *A. oryzae* (CCAM 080006), *A. niger* (CCAM 080002), and *Rhizopus oryzae* (CCAM 080011) (Tian et al. 2014). Plumbagin also exhibits antifungal activity against several plant pathogenic fungi such as *Aspergillus niger*, *Alternaria alternata*, *Fusarium oxysporum*, *Bipolaris oryzae*, etc., with MIC values ranging from 4.8 to 56.6 $\mu\text{g mL}^{-1}$ (Shin et al. 2007). Droserone and 5-*O*-methyldroserone also show antifungal activity against various plant and human pathogens such as *Aspergillus* spp. and *Candida* spp. (Eilenberg et al. 2010). Plumbagin showed inhibitory activity against different yeast strains with prominent inhibition zones ranging from 23.67 to 42.23 mm and MIC values ranging from 31 to 63 $\mu\text{g mL}^{-1}$ (Devi et al. 2016). Shikonin has been proven more effective than other herbal medicines commonly used in the anti-HIV-1 activity (Mandal et al. 2020). The studies on shikonin showed significant antiamoebic activity against *Entamoeba histolytica* (Hook et al. 2014). Juglone shows potent inhibition on SARS-CoV-2 with its IC_{50} value of 15.75 ± 8.22 (Jin et al. 2020b). 1,4-NQs are often considered powerful antimalarial agents. Lapachol and plumbagin showed high activity when evaluated against *Plasmodium berghei* ($\text{IC}_{50} = 0.002 \mu\text{g mL}^{-1}$). Lapachol also showed activity against *Plasmodium falciparum* with IC_{50} lower than 1.0 $\mu\text{g mL}^{-1}$ (Gomes et al. 2021).

15.2.4 Anti-inflammatory Activity

Naphthoquinones have been investigated as a notable compound in treating several immunological disorders such as autoimmune, inflammatory and vascular diseases (Bawazeer and Rauf 2021). In addition, the anti-inflammatory effects of NQs have been demonstrated by several *in vitro* studies (Melzig et al. 2001; Rauf et al. 2017). NF- κ B is one of the key elements of different vital cellular processes such as inflammation, apoptosis and proliferation. Plumbagin inhibits the activation of NF- κ B to modulate the functions of lymphocytes and immune responses (Checker et al. 2009). Plant-derived NQs substantially suppressed the Kelch-like enoyl-CoA-hydratase (ECH) associated protein 1 (Keap1), which directly stimulates the accumulation of NF-E2-related factor-2 (Nrf2) transcription factor that causes and induction of antioxidant response elements (AREs). AREs are responsible for several enzymes related to oxidative stress, heat stress, etc. (Son et al. 2010; Ahmed et al. 2012; Abed et al. 2015). Several 1,4-NQ derivatives such as plumbagin, shikonin, etc. have shown miraculous effects against heat-induced oedema, carrageenan-induced paw oedema, human neutrophil elastase activity, etc. (Aminin and Polonik 2020). When applied in a minimum dose, alkannin and shikonin inhibit the vascular permeability in rats with heat-induced oedema on their epidermis (Hayashi and Zasski 1977). The extract of *Cipura paludosa*, *Diospyros kaki* and *Onosma paniculate* has shown a significant reduction in the carrageenan-induced paw oedema model and histamine-induced paw oedema model (Bawazeer and Rauf 2021). Recent studies have shown that the anti-inflammatory effects of naphthoquinones are mainly inhibition of leukotriene B₄ (LTB₄) (Chen et al. 2002). The other modes of this activity could be inhibiting neutrophils, mast cell granulation suppression, and consecutive degeneration of the phosphatidylinositol signalling pathway. The expression of TNF- α , IL-1 β and IL-6 has been down-regulated while treating with juglone in rat hypothalamus. Juglone treatment also significantly reduces the expression of TLR4/NF- κ B that prevents the expression of proinflammatory cytokines, including IL-1 β , IL-6, IL-12, IL-23, and TNF- α (Chen et al. 2019).

15.3 Lawson

Lawsone (2,2-Hydroxy-1,4-naphthoquinone), or hennotannic acid, is a naturally occurring naphthoquinone found in the plant *Lawsonia inermis*, and several species of the genus *Impatiens*. It is an essential derivative of hydroxynaphthoquinone with distinct chemical and pharmacological properties (Lamoureux et al. 2008). The compound was isolated for the first time in 1959 from the plant *L. inermis* also called henna (Jain et al. 2010). Lawsone in higher plants has derived from *O*-succinylbenzoate (OSB) pathway. The conversion of chorismate to 1,4-dihydroxy-2-naphthone (DHNA) is the central core part of the OSB pathway. 1,4-naphthalenoid

ring of most phylloquinones is derived from DHNA (Grotzinger and Campbell 1972). Earlier investigations on lawsone biosynthesis indicate shikimic acid as the precursor as the [U-14C]-shikimate retrieved in the benzene moiety of lawsone. However, later studies on *Impatiens balsamina* with [2-14C]-glutamate³³ and [U-14C]- α -ketoglutarate labelled lawsone show that the quinone moiety in 1,4-NQs originated from the OSB. Further findings established that OSB is an intermediate and that DHNA is the product from which the OSB pathway branches toward producing various 1,4-NQs, including lawsone (Widhalm and Rhodes 2016). Lawsone is the key component responsible for the red orange pigment extracted from the henna trees and broadly used as a skin colouring dye. In addition to its cosmetic application lawsone also plays a role in skin protection by blocking ultraviolet radiation when applied on the skin (Munt et al. 2015). Furthermore, it is also used to inhibit steel corrosion, applied as antiaging additive to vulcanised natural rubber (Doong and Chiang 2005; Ostovari et al. 2009).

L. inermis (L.), or 'henna' is a monotypic species of the genus *Lawsonia* belonging to the family Lythraceae. Since ancient times, immense use of henna or 'mehndi' in Hindu and Muslim ritualistic and religious customs in south Asian countries such as India, Pakistan, Iran and Middle East has been evident (Singh et al. 2015). The traditional use of henna for the embellishment of skin, hair, and fingernails in social functions among Hindu and Muslim women symbolise a deep cultural sense (Singh et al. 2015). When mixed with the water and applied to the skin, the lawsone present in the henna leaves chemically reacts with the keratin via a Michael addition, forming a brilliant red orange colour that lasts for months (López et al. 2014). The plant is a perennial shrub or small tree that grows wild and is native to the tropical region of Northern Africa, Southern Asia, Australia, the Middle East and the Arabian Peninsula. Although the plant is found in a wide range of habitats, they prefer a relatively hot and dry climate with prolonged droughts. Therefore, henna is commercially cultivated in Morocco, Yemen, Iran, Sudan, Libya, Somalia, Bangladesh, Western India and Pakistan, considering a relatively warm climate (Wilkinson and Elevitch 2000). India is one of the major producers of henna, cultivated mainly in Rajasthan, Gujarat, Uttar Pradesh, Tamil Nadu, Madhya Pradesh, and Punjab. Morphometric, genetic and biochemical variation appears to be a fundamental fact in *L. inermis*, those cultivated in different agroclimatic conditions with diverse hydrous and thermal profiles (El Massoudi et al. 2019; Kalia et al. 2019). Therefore, selecting the desirable traits among those variations is a superior way to improve the cultivation protocol and upgrade the production quality (Shiji and Siril 2017).

Several species of the genus *Impatiens* belong to the family Balsaminaceae and produce lawsone. It has been determined that lawsone is generally localised in their leaves during the plant's growth, but during flower development, it is mainly present in the flowers (Lobstein et al. 2001). Approximately 480 species of the genus *Impatiens* are distributed in tropical and subtropical humid forests and near rivers of India, Southwest Asia, Africa and Southern China (Szewczyk 2018). The native old-world plant is also introduced into Argentina, Bolivia, Brazil, Caroline, Colombia, Cuba, Dominican Republic, Mexico Central, Morocco, Tasmania,

Trinidad-Tobago, and Venezuela (<https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:325983-2>). It prefers to grow on humid soil in the forest margins, specifically at 4000 m altitude from sea level (Vrchotová et al. 2011; Yu et al. 2016). Although lawsone has been detected in a few members of the genus, for instance, *I. capensis*, *I. balsamina*, *I. glandulifera* but others have potential values in food, medicine and ornamental plants (Motz et al. 2012; Block et al. 2019; Pires et al. 2021). Despite the wide distribution of *Impatiens*, nearly 32 species are threatened, according to the red list of the IUCN.

15.4 Plumbagin

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a yellow crystalline compound initially isolated from the genus *Plumbago* hence the name suggested. The compound is a hydroxynaphthoquinone with 1,4-naphthoquinone skeleton that contains methyl and hydroxy groups at 2 and 5 positions (i.e. an aromatic methyl *p*-quinone moiety) (Kapadia et al. 2005). The roots of *Plumbago* have been used for several hundred years to treat dyspepsia, piles, diarrhoea, and skin diseases in Indian Ayurveda (Chopra et al. 1956). It has also been scripted in the ayurvedic text ‘Charaka Samhita’ as the earliest reference that flourished nearly the second century BC (Padhye et al. 2012). Therefore, the simplest hydroxynaphthoquinone has been well known for a long time to humankind for its remarkable therapeutic values even before the discovery of the structural chemistry of the compound. In the modern era, several potent pharmacological activities of plumbagin were uncovered, including anticancer, antimicrobial, anti-inflammatory, antidiabetic, and neuroprotective (Nair et al. 2016; Rajalakshmi et al. 2018; Ono et al. 2022). Particularly in cancer research, the compound received incredible attention as it can suppress malignant activities by inhibiting cancer cell growth, invasion, and metastasis (Aziz et al. 2008; Manu et al. 2011; Cao et al. 2018). However, the biosynthetic pathway of the plumbagin is not well established, and very few genes have been identified for the key enzymes involved in the regulatory steps (Nayak et al. 2014; Muralidharan et al. 2020). Biosynthesis of plumbagin most likely follows the acetate-polymalonate pathway, also called polyketide pathway where the oxygen atom is eliminated from the third acetate unit by polyketide reductase before the initial crystallisation. The pathway involved the conversion of malonyl CoA and acetyl CoA through a series of unknown reaction before the production of plumbagin (Muralidharan et al. 2020).

Although plumbagin is primarily isolated from the genus *Plumbago* and belongs to the family Plumbaginaceae, it is also found in several members of Droseraceae and Ebenaceae families. The main plumbagin producing plant, *Plumbago* comprises 17 species mainly found in the tropical to subtropical region and occasionally in the temperate zones (<http://www.worldfloraonline.org/taxon/wfo-4000030425#C>). Members of the genus are shrubs or perennial herbs, and the presence of a hairy calyx is a prominent characteristic feature of the genus. *Plumbago indica*, commonly known as Scarlett Leadwort (Agnichita), is an evergreen perennial herb that attains a



Fig. 15.3 Plants containing plumbagin. (a) *Plumbago zeylanica*; (b) *Plumbago auriculata*. Scale bar = 10 mm

height 0.5–2.0 m. Dark pink or scarlet coloured flowers developed during winter (Rafa et al. 2016). The plant is native to the Indian subcontinent and is also found in other parts of Southeast Asia, Bangladesh, Philippines, Sulawesi, Sumatra, Thailand, Vietnam, Assam, Cambodia, China South-Central, East Himalaya, and Hainan. It is also introduced in Leeward Is., Peru, Puerto Rico, Sri Lanka, and Trinidad-Tobago. In India, the plant is distributed specifically in the states of the southern part and is also indigenous to Sikkim and Khasi Hills (Drury 1866). Another species of the genus *Plumbago* is *P. zeylanica*, a major source of plumbagin found in their roots and a small amount in leaves and stems. The plant is widely spread in tropical and subtropical regions of Southeast Asia, Australia and Africa, favourably growing from sea level up to 2000 m altitude in savannas, deciduous woodland, and scrublands. In India, the plant has a wide range of distribution from Central India to West Bengal, Assam, Bihar, Maharashtra, Rajasthan, Tamil Nadu, and Uttar Pradesh. The plant is a shrub that attains a height of about 0.5–2.0 m (Fig. 15.3a). Unlike *P. indica*, this plant develops white flowers in winter and hence commonly called ‘Swet chita’. *P. indica* mostly grows in the southern parts of India and is therefore used by the Hakim (physician) of South Indian states, while in Northern and Eastern India, the application of *P. zeylanica* is more flourished in Ayurveda (Babu and Austin 2016). The phytochemical contents of a plant depend on various factors such as genetic makeup, environmental influence, etc. It is found that the plumbagin content and other phenolics and flavonoid contain can greatly vary based on plant genetic makeup and agroclimatic zones (Roy et al. 2022). The morphological traits such as flowers and other vegetative characters of *P. zeylanica* tend to be larger in size in the geographic locations where annual precipitation is higher (Panda et al. 2015). Numerous genetic markers for RAPD and ISSR were used to analyse the genetic diversity of *P. zeylanica*. Considerable genetic variation is found among and within the population in different sub-geographic regions; moreover, the diversity is higher in eastern and northern Indian populations and the southern parts (Panda et al. 2015). The genetic variation in this species is maintained by outcrossing; therefore, they show higher inter-population genetic diversity than intrapopulation. A significant

amount of plumbagin is also found in the root of *P. auriculata*, while it is also a small amount in the stem and leaves (Mallavadhani et al. 2002). Like the other *Plumbago* species *P. auriculata* is also a bushy evergreen shrub that attains a height of about 3 m. However, it is recognised for its blue flowers, so it is commonly called ‘Nil Chitrak’ (Fig. 15.3b) (Singh et al. 2018). This plant is native to South Africa and has been introduced into different tropical and subtropical regions worldwide such as India, Bangladesh, China, Somalia, Spain, Florida, Mexico, Vietnam, etc. It is widely cultivated as an ornamental garden plant. Considerable genetic variations are found by selective DNA markers in these three different *plumbago* species and it is also evident that *P. indica* and *P. zeylanica* are closely related than *P. auriculata* (Gadge and Nathar 2014).

The family Droseraceae is predominantly carnivorous herbaceous and consists of only three genera (<http://www.worldfloraonline.org>). The genus *Aldrovanda* and *Dionaea* are monotypic, while the largest genus, *Drosera*, comprises about 219 species. Several members of the family contain plumbagin in their aerial parts. *Aldrovandra vesiculosa* or waterwheel plant is the only species of the genus *Aldrovandra* and it also contained plumbagin in their tissue (Culham and Gornall 1994). This aquatic herb is free-floating, rootless, attains a height up to 20 cm long develops snap-tarp 2.5–6.0 mm in length to capture prey, generally zooplanktons (Poppinga et al. 2019). The genus was named after Ulisse Aldrovandi, an Italian naturalist (Kundu et al. 1996; Cameron et al. 2002). *Aldrovandra* shows a broad tolerance level in different climatic zones; therefore, it is widely distributed in tropical regions of Asia, Australia and Africa and also in extreme temperate sites such as Russia and Poland (Elansary et al. 2010). Although the plant covers a wide range, its distribution is patchy and the increasing extinction makes it ‘critically endangered’ in the IUCN red list (San Martín et al. 2003). *Dionaea muscipula* or ‘Venus flytrap’ is a terrestrial carnivorous plant captured interest since the Darwin’s period. The plant is native and endemic to the Coastal plain of North and South Carolina, New Jersey and Florida, where nutrients are sorely limited (Luken 2005; Böhm et al. 2016). In the nutrient-poor environment, the plant has adapted by modifying the leaves into active traps to capture small insects such as ants and flies (Escalante-Pérez et al. 2014). The abrupt declination of mature individuals in the habitat severely affects the population, and as a consequence, the plant is enlisted as ‘vulnerable’ on the IUCN red list. Members of the genus *Drosera* are famous carnivorous plants with tentacles that produce glue-like exudate that help in the prey-catching mechanism (Nakamura et al. 2013). The plant is also known as sundew because the glue on the tentacles looks like a dew droplet. Nearly 219 species of the genus are distributed throughout the world. Besides the plumbagin, the members of the genus also contain several other important NQs, viz. droserone, 7-methyljuglone, Hydroxydroserone, etc. (Culham and Gornall 1994). Plumbagin is found in the roots of several species such as *D. andersoniana*, *D. auriculata*, *D. burmanii*, *D. erythrorhiza*, *D. intermedia*, *D. natalensis*, etc. *Drosera gigantea*, the giant sundew, contains plumbagin is an endemic and native to Western Australia. According to the IUCN, more than 150 species of this genus are endemic.

Another carnivorous genus *Nepenthes* of the monotypic family Nepenthaceae, mainly found in Southeast Asia, is a potential source of plumbagin (Likhitwitayawuid et al. 1998). The plants are typical climbers and produce pitfall traps or pitchers to capture prey, particularly arthropods. They primarily acquire nutrients from the prey and therefore, can be sustained in sunny, moist and nutrient deficient habitat. *Nepenthes* is considered the largest pitcher plant genus with more than 140 recognised species. The species are mainly distributed into three geographical centres viz. Borneo, Sumatra and Philippines (Robinson et al. 2009). It has been suggested that the period of the origin and evolution of the genus was nearly 80 MYA during the association of the Indian subcontinent to Australia (Danser 1928). Then *Nepenthes* spread and survived in India and later diversified northward and eastward into Indochina. Despite the immense diversification, the geographical distribution of the genus has extreme patchiness and endemism. *Nepenthes khasiana* contains a high amount of plumbagin (1.33%) in their roots (Raj et al. 2011). It is the only native Indian pitcher species endemic to the Meghalaya and enlisted as endangered according to the IUCN red list. *Nepenthes insignis* is an endemic species and is only found in the forest and rocky areas of New Guinea (Rischer 1995). Several species of this genus viz. *N. dubia*, *N. eustachya*, *N. adnata*, *N. sumatrana*, *N. tobaica*, *N. singalana*, *N. densiflora*, *N. Rafflesiana*, etc. contain plumbagin and are endemic to the mountains of Sumatra and Peninsular Malaysia (Cheek et al. 2017; Batoro and Wartono 2017; Nainggolan et al. 2020). The widespread fruit tree genus *Diospyros* or persimmon belongs to the family Ebenaceae and contains several NQs such as droserone and diospyrin, including plumbagin (Patil et al. 2017; Flota-Burgos et al. 2020; Ganapaty et al. 2006). *Diospyros kaki* is the most cultivated species of the genus, also known as Japanese persimmon, which contains plumbagin in the roots (Park and Lee 2018). It mainly originated in China, and latter distributed to Japan and South Korea. It is largely cultivated in East Asia and the Mediterranean regions (Martínez-Calvo et al. 2013).

15.5 Juglone

Juglone (5-hydroxy-1,4-naphthoquinone) is an aromatic organic compound that belongs to the redox-active compound class 1,4-naphthoquinone. Juglone is widely found in nature especially in several members of Juglandaceae. Juglone is also responsible for the walnut tree's yellow pigmentation (Inbaraj and Chignell 2004). According to several studies, juglone is well-known for secondary metabolite and allelochemical, which often has a cognisable biological effect on other living organisms (Duke 2003). Juglone has a distinct phenyl group with the quinone moiety of 1,4-naphthoquinone. Biosynthesis of juglone takes place through the *O*-succinylbenzoate pathway with seven enzymatic reactions starting from the end product of the shikimate pathway, chorismite (Widhalm and Rhodes 2016). The radiotracer study suggests that the carboxyl group of shikimic acid is present on the quinone moiety of 1, 4-NQ, and that particular radiolabelled carbon is also found in

1 and 4 position carbon of juglone. This observation indicates that the formation of juglone is a symmetrical process from 1,4-NQ through the decarboxylation of 1,4-dihydroxy-2-naphthoate (DHNA), which can be derived from the phylloquinone pathway (McCoy et al. 2018).

Juglandaceae or walnut family is the leading natural source of juglones. It is one of the most minor but significant plant families having only 12 genera and 90 species (<http://www.worldfloraonline.org/taxon/wfo-7000000309>. Accessed on 27 June 2022). The walnut, hickory and pecan are some significant timber and nut-producing trees of the walnut family, and they also have several pharmaceutical properties (Kozłowski et al. 2018). Juglone is found in most of the members of Juglandaceae, but mostly it has been found in *Juglans regia*, *J. nigra*, *J. cineraria*, *J. cathayensis*, *J. mandshurica*, *Carya olivaeformis*, *C. illinoensis*, *C. ovata*, *Pterocarya fraxinifolia* and *P. stemoptera* (Thakur and Cahalan 2011; Hook et al. 2014). This bioactive compound is also isolated from *Diospyros lcyioides*, and some species of Proteaceae such as *Lomatia hirsuta*, *L. tinctoria*, *L. tasmanica*, *L. polymorpha*. (Deans et al. 2018). Juglone is found in almost every organ of the producing plant. High amount of juglone has been extracted from root, husks, leaves, and stem barks (Chudhary et al. 2020). The distribution of the Juglandaceae species mainly covers six biogeographic regions, viz. North-West-East Asia, South Asia, Central and South America, North America, Malay Archipelago and Europe, but they widely spread in the humid subtropical and warm-temperate forest regions of the eastern Asian region and northern America (Song et al. 2020).

Juglans regia (Persian walnut or English walnut) is a monoecious and dichogamous tree species. The plant is considered to be located in Central Asia, but it is currently widely distributed in several countries in Europe, America, Africa, Australia and New Zealand (Aradhya et al. 2006). The use of walnut by humans has been well documented since ancient times. As a nut-producing plant, walnut has ranked in the second position after almonds, and the global production of walnut is nearly 1.5 million tons, which are principally produced by China, followed by the USA (Frederick et al. 2014). Walnuts contain omega-fatty acid and several other essential components with numerous health benefits, so the global demand is increasing daily (Delaviz et al. 2017). Black walnut or *J. nigra* is also a vital juglone producing plant native to Eastern and Western America, but it is also found in several European countries, New Zealand, Russia and some Southeast Asian countries (Grant et al. 2011). The woods from black walnut have demanding value due to their high durability and shock-resistance attributes. Several tribes from different regions of the world have used several parts of black walnut for toothache, diarrhoea, urinary tract infection, controlling blood pressure, dysentery, etc. (Paudel et al. 2013). Japanese walnut (*J. ailanthifolia*) is a deciduous tree native to Japan. It is widely distributed in some European countries, New Zealand, and Australia (Tamura and Hayashi 2008). *Juglans mandshurica*, also known as Manchurian walnut, is a perennial and fast-growing deciduous tree native to Asia and widely distributed throughout the temperate and subtemperate zone of India, China, Japan, some parts of the Siberia Russia, and the Korean Peninsula (Luan et al. 2021). Butternut or *Juglans cinerea* is another ancestral species with several medicinal values native to

North America and Canada (Parks et al. 2013). Among other genera, hickories (*Carya* sp.) are the second-best producer of juglone in nature. Their native distribution is throughout eastern Canada to some parts of the United States and Mexico (Grauke 2003).

15.6 Lapachol

Lapachol (2-hydroxy-3-(2-methyl-1-propenyl)-1,4-naphthoquinone) is a derivative of lawsone first isolated from the Lapacho tree (*Tabebuia impetiginosa*) by an Italian scientist in 1882. The compound lapachol is potent in fighting metastasis and reducing cancer growth (Balassiano et al. 2005). The first trial of the anticancerous effect on rat in 1968 was successful. However, the phase-I clinical trial was not fruitful, and consequently, further clinical research was terminated (Block et al. 1974). Later, it was reported that the application of lapachol on patients with various cancers such as liver, breast, kidney and prostate tremendously reduces tumour growth (Hussain et al. 2007). Although lapachol is primarily found in several genera of the family Bignoniaceae, they have also occurred in the other families of order Lamiales.

The genus *Tabebuia* belongs to the family Bignoniaceae, distributed throughout its native range, Mexico to Tropical America (Grose and Olmstead 2007a, b). It is the largest genus of the family comprising of 128 species mainly large shrubs or small trees. Traditionally, species of the genus are used to cure syphilis, malaria, and stomach disorder in the Amazonian territory, specifically in Brazil. Consequently, stem bark and wood of different *Tabebuia* species, viz. *T. impetiginosa*, *T. rosea* and *T. serratifolia* become demanding across the board (Gordon et al. 2010). *Tabebuia ochracea* is distributed mainly in the seasonal Savannas, Central Brazil Cerrado vegetation, and Costa Rica in Central America. Analysis of microsatellite loci in different populations of *T. ochracea* showed the low level of polymorphism and genetic diversity in seasonal dry forest due to fragmentation and disturbance (Moreira et al. 2009). Lapachol is also isolated from the dried inner bark of *T. impetiginosa*, distributed in the Amazon rainforest and several other parts of Latin America. *Radermachera xylocarpa* is another member of the Bignoniaceae, mainly found in Deccan, Konkan, Khandesh and Western Ghats of India that produces lapachol in the stem bark (Shetgiri et al. 2001). The species is rare and considered as endangered (Khanna et al. 2021). Besides Bignoniaceae, several other species from different families also produce lapachol. A famous example is *Tectona grandis* belongs to the family Verbenaceae and is one of the most tropical timbers yielding plants also has lapachol in the heartwood (Vyas et al. 2022). The plant is native to Southeast Asia, mainly India, Burma and Bangladesh.

15.7 Shikonin and Alkannin

Shikonin is a novel natural naphthoquinone compound found abundantly in the roots of *Lithospermum erythrorhizon* and several other members of Boraginaceae (Auber et al. 2020). Shikonin and alkannin are two enantiomeric naphthoquinones studied since ancient times. Shikonin, the R enantiomer, has been studied widely from the root extract of *L. erythrorhizon* and is considered the major constituent of the red pigment obtained from that particular plant (Papageorgiou et al. 2008). The S enantiomer alkannin has been isolated from several plants such as *Arnebia densiflora*, *Arnebia euchroma*, *Echium rubrum*, etc. (Zhu et al. 2016; Liao et al. 2020). The presence of alkannin in the plant's root extract is the primary reason behind its red colouration. The structure of shikonin could easily be ambiguous as it has structural similarities to other quinones. It contains a naphthazarin moiety and a chiral six-carbon side chain (Papageorgiou et al. 1999). 4HBA/MVA pathway is well known and shared by many species from Boraginaceae to synthesise isohexenylnaphthazarins, a type of 1,4-NQs (Widhalm and Rhodes 2016). The biosynthetic pathway of alkannin and shikonin have quite similarities to ubiquinone biosynthesis has been demonstrated from the callus culture of *L. erythrorhizon* (Inouye et al. 1979). Tracer experiments showed that geranyl-para-hydroxybenzoic acid and geranyhydroquinone are the biosynthetic intermediates of shikonin (Papageorgiou et al. 2008). The biosynthetic pathway of shikonin proposed by Inouye's group was later supported by the isolation of these intermediates from shikonin-producing cells of the strain M18 (Yazaki et al. 1986).

Shikonin is found in several members of Boraginaceae family. Nearly twenty species of six genera of Boraginaceae are found to be contained shikonin (Bagheri et al. 2018). The distribution of the members of the family is largely spread in Southeast Asia, South-Central China, the Western Himalayan region, Northern America, Central Europe, Africa and other regions of Asian continent. *Lithospermum erythrorhizon* is native to China, Japan, Korea and other parts of Asia (Wen et al. 2012). *Arnebia guttata*, another member of the family contains shikonin in the roots and native to China but also found in Afghanistan, southwest Siberia, and some parts of Pakistan, Kazakhstan and Tibet (Ambrish and Srivastava 2014). The root extract of *L. erythrorhizon*, *A. guttata* and *A. euchroma* is used to prepare a herbal medicine, Zicao which has been used by Chinese civilisation since ancient times as it contains numerous beneficial values including antibacterial, antiviral, antitumourigenic, anti-obesity and others (Chen et al. 2018). Acetylshikonin from Zicao prevents obesity in rats on a high-fat diet by inhibiting lipid accumulation and inducing lipolysis (Su et al. 2016). *Echium italicum* is a native species to Eastern Europe, Afghanistan, Turkey, Lebanon (Özcan 2013). *Echium plantagenium*, also known as Paterson's curse, is native to Lybia, Syria, some parts of central Europe, Western Australia, Turkey and South America (Konarzewski et al. 2012). *Onosoma* is a large genus with about 150 species distributed in India, China, Iran, Pakistan and other south Asian countries. Some species of *Onosoma* are considered endemic due to the gradual detrimental environmental changes (Kumar et al. 2013).

15.8 Diversity and Conservation of Naphthoquinone-Containing Plants

Naphthoquinones have a greater structural diversity as well as various functional properties. The plants that produce NQ are also very diverse in nature. Some of them are aquatic, while others are terrestrial. Some prefer to grow in semi-desert climates to tropical areas, and some are found in high rainfall areas. Several of them depend on the soil for their nutrition requirements, while others develop carnivorous characteristics and grow in nutrient-poor soil. Plumbagin is produced by several plant families such as Plumbaginaceae, Droseraceae, Ebenaceae, etc. and members of these families have greater diversity and distribution (Adamec 2018; Bawazeer and Rauf 2021; Dissanayake et al. 2022). The genus *Plumbago* is mainly distributed in tropical regions to temperate regions. *Plumbago auriculata* is native to South Africa while *P. indica* and *P. zeylanica* are originated in India. Another great source of plumbagin is the members of the Droseraceae and Nepenthaceae family, and both are carnivorous but with different prey capturing mechanisms. Several members of these families are endemic and threatened. As a whole carnivorous plants are highly specific to their niches in which they occur and often the habitats are highly sensitive (Brewer and Schlauer 2018). The plants have complex biological mutualism and a very specialised nutrition requirement therefore certain changes in global climate possess imminent threat to them. Threatened status of the species in the genera *Drosera* and *Nepenthes* are 9% critically endangered, 12% endangered, 11% vulnerable and 18% critically endangered, 10% endangered, 12% vulnerable respectively (Cross et al. 2020). The monotypic genus *Aldrovenda* has a patchy distribution and enlisted as critically endangered (San Martin et al. 2003). *Dionaea muscipula* is endemic to the South and North Carolina, and it is also vulnerable (Miernicka et al. 2021). Several plant species of the genus *Drosera* are endemic namely, *D. adelae* endemic to Queens Island, *D. andersoniana* has restricted distribution to western Australia (<https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:321769-1>). *Nepenthes khasiana* is an endangered plant having restricted distribution in Meghalaya, India (Joshi et al. 2022). Many species of *Nepenthes* are endemic to the Sumatra region, and some of them are also threatened. *Nepenthes dubia*, *N. rajah*, *N. mikei*, *N. ephippiata*, *N. Spectabilis* are critically endangered to vulnerable species restricted to Sumatra (Adam et al. 1992). A very popular fruit tree genus *Diospyros*, also known as persimmon, contains plumbagin and other important NQs such as diospyrone, 7-Methyljuglone, Crassiflorone, Diospyrin, etc. (Ganapaty et al. 2004). Several species of *Diospyros* are largely cultivated in different parts of the world, but some of them are only found in some specific areas (Deblauwe 2021). Another important NQ is lawsone, primarily used as a dye mostly found in *Lawsonia inermis* (Jain et al. 2010). This tree prefers to grow in dry and semi-arid habitats and is cultivated in several parts of the world. A large number of plants of the genus *Impatiens* are trendy for their ornamental flowers under the family Balsaminaceae also produce Lawsone (Lobstein et al. 2001). Juglone is a very significant NQ mainly obtained from the members of *Juglans* or walnut belonging to the family

Juglandaceae. *Juglans nigra* (black walnut), *J. regia* (English walnut), *J. ailanthifolia* (Japanese walnut), *J. cinerea* (butternut) produce a large amount of juglone. Walnut is the most popular tree nut consumed worldwide and therefore cultivated globally. *Pterocarya fraxinifolia*, or Caucasian wingnut also having juglone, is assessed as vulnerable by the IUCN, mainly distributed to Iran, North Caucasus, Transcaucasus, and Turkey. Besides walnut, juglone is found in other genera such as *Lomatia*, belonging to the family Proteaceae (Simonsen et al. 2006). The leaves of three endemic species of Tasmania viz. *L. polymorpha*, *L. tasmanica*, *L. tinctoria* contain juglone (Deans et al. 2018). Another genus *Conospermum* of the family Proteaceae contains some essential NQs. *Conospermum incurvum* and *C. stoechadis* are native and distributed to Western Australia and contain conocurvone and stoechadone, respectively (Dai et al. 1994; Aguinaldo et al. 1996).

Many NQ-containing plants are endemic, have a patchy distribution, are rapidly decreasing in their natural population, and are threatened to become extinct. Therefore, immediate attention is required for their conservation. Among 123 species of *Nepenthes* assessed by the IUCN, 15 have been classified as endangered, while ten are critically endangered. Several strategies have been considered by the different institutes, national parks, and botanical gardens for their conservation (Miguel et al. 2018). International carnivorous plant society encourages a series of actions to promote the conservation of threatened *Nepenthes*, involving *ex-situ* conservation, maintenance and cultivation of population to support their pollination and reproduction (Nongrum et al. 2009; Miguel et al. 2018). Biotechnological approaches, particularly *in vitro* propagation, are successfully applied to *N. khasiana* as a part of their production and conservation (Latha and Seeni 1994; Devi et al. 2013; Joshi et al. 2022). *Aldrovanda vesiculosa*, the free-floating carnivorous plant of the family Droseraceae is now critically endangered and vanishes from several sites it previously appears. Ecological disturbance is the primary cause of extinction of this plant from more than 90% of sites over the last 150 years (Walters 1979; Cross 2012). The purposeful introduction of this plant to new suitable sites, particularly in European regions, appears to be an efficient conservation strategy to rescue the population from inevitable extinction (Adamec 2005, 2018). Plant tissue culture has been exploited in *A. vesiculosa* with the aim of conservation and reintroduction of the species to their natural habitat (Parzymies 2021). Micropropagation techniques have also been optimised for near threatened species *Drosera intermedia* for conservation purposes (Grevenstuk et al. 2010).

15.9 Conclusion

This chapter summarised the occurrence of different naphthoquinones in higher plants and their roles in medical science and other human welfare. Plants that contain NQs not only belong to a diverse taxonomic group but also broadly differ in their habits and habitats. The diversity of the habits is too enormous, from a tiny aquatic

floating herb to a large evergreen tree, even the carnivorous plants that explore different prey-catching mechanisms for their nutrient requirements. Several groups of carnivorous plants such as *Aldrovanda* sp., *Drosera* sp., *Nepenthes* sp. etc. are considered excellent sources of NQs. Moreover, the same NQ can be found in different plant groups that may appear in different ecozones and countries. Several valuable NQ-containing plants such as *Lawsonia* sp., *Impatiens* sp., *Diospyros* sp., *Juglans* sp., etc. are largely cultivated in different parts of the world and prefer different habitat. Nevertheless, many are still found only in their natural habitats, for instance, *Plumbago* sp. *Nepenthes* sp. *Drosera* sp. *Aldrovanda* sp., *Dionaea* sp., *Nepenthes Khasiana*, *N. dubia*, *N. rajah*, *Dionaea muscipula*, *Drosera Adela*, etc. They are some examples of NQ-containing plants which are endemic, having only a restricted distribution in some specific parts of the globe. Endemism and patchy distribution in specific sites often become a potential threat to several NQs-containing plants that make them ‘endangered’ even ‘critically endangered’. Therefore, the present chapter focused on the occurrence of NQs in higher plants, along with those species’ distribution diversity and conservation strategies. Finally, it will be facilitative in designing management strategies and conservation programmes for valuable NQ-containing plants, especially those challenged by inevitable natural threats. The dossier on the occurrence of the NQs helps uncover the underutilised potential sources of NQs that will extend the application of this valuable compound in human welfare, particularly in medical research.

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Chapter 16

Astragalus fridae: Genetic Source, Applications, and Conservation



Fardad Didaran, Ali Akbar Ghasemi-Soloklui, and Mojtaba Kordrostami

Abstract The Fabaceae genus *Astragalus fridae* contains medicinal, nutritional, and industrial qualities. This plant is endemic to Semnan Province, Iran, with Aftar village in the northwest serving as its primary habitat, and is presently on the endangered species list. *Astragalus* includes polysaccharides and saponins, as well as poisonous chemicals such as indolizidine alkaloids, nitroaliphatic compounds, and selenium. Antioxidants, immune system stimulants, liver defenders, antiviral and bacterial agents, and compounds that impact the heart arteries have all been isolated from diverse species. *Astragalus* has a wide range of applications due to its species richness, including pharmacological and industrial usage, as well as soil stability and fodder production. Antioxidant substances found in the root inhibit hepatic glycogen loss and boost protein and total serum albumin levels. In this regard, the most recently recognized therapeutic capabilities of *Astragalus* are in the field of anti-AIDS and anti-cancer activities.

Keywords *Astragalus fridae* · Enzyme · Antioxidant · Species in danger of extinction

16.1 Introduction

After the Orchidaceae and the Asteraceae, the Fabaceae (Leguminosae) family of flowering plants is the third-largest family of flowering plants, with 800 different genera and 20,000 different species (Abberton and Thomas 2011). It is a very varied family that may be found all over the globe. It includes a vast variety of living forms,

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ranging from annual xerophytes and equatorial gigantic trees to arctic-alpine herbs and perennial shrubs found in temperate or tropical regions. While some legumes are considered to be unwanted weeds in cereal farming, others are important grain crops in their own right. The emphasis of this study is on grain legumes, often known as pulses, as well as two pasture and forage legumes. These latter species are known as pulses. The Fabaceae family gets its name from the distinctive fruit that is produced by its members. This fruit is known as a legume and is responsible for giving the family its name. The structure of the flower may vary greatly; nevertheless, the butterfly-like blossom, also known as a papilionoid flower, is virtually always present in the Papilionoideae subfamily (which has over 14,000 species). The family Fabaceae is comprised of several commercially significant and adaptable plants, the bulk of which are sources of grains and pulses. Some of the earliest crop plants cultivated by humans were legumes, such as soybeans and mung beans in East Asia; fava beans, lentils, chickpeas, and peas in the Fertile Crescent of the Near East; and common bean or lupin in Central and South America. Grain crops were domesticated approximately 10,000 years ago. The relationship that legumes have with bacteria that can fix nitrogen not only adds value to agricultural production but also has a significant impact on the functioning of natural ecosystems. In addition, the pea, which belongs to the genus *Pisum sativum* L. and is a kind of legume, served as the primary experimental organism for Mendel's groundbreaking work in determining the fundamental foundation of heredity (Alaniya et al. 2007; Adegbite and Amusa 2008; Amri et al. 2008; Arbabian et al. 2009; Arbaoui and Link 2008).

It is vital to reconstructing the phylogenetic connection of the Fabaceae to have a knowledge of the beginnings of this family as well as its subsequent diversity. The plastid *rbcL* gene was used to initiate phylogenetic studies of the Fabaceae family (Benchadi et al. 2013; Delgado and Gómez 2016), which were then followed by analyses that included the more variable *matK* gene (Moghanloo et al. 2019a; Naseri et al. 2017; Rabizadeh et al. 2019). Both of these locations are now recognized as valid barcodes for plants (Kruckeberg and Rabinowitz 1985; Linnek et al. 2011). However, the picture is by no means comprehensive since a great number of species either has not yet had their genomes sequenced or are only represented by one or two accessions each. Despite this, significant advancements have been achieved in the cultivation of chickpeas, common beans, cowpea, and soybean, in addition to the model legumes *Medicago truncatula* Gaertn. and *Lotus japonica* (Regel) K. Larson. Molecular systematics has provided several examples that support the conclusion that the family is monophyletic (Özipek et al. 2005; Moghanloo et al. 2019a; Naseri et al. 2017). Based on morphological characteristics, the following three major groups are recognized and considered to be subfamilies at this time: the mimosoid legumes, also known as Mimosoideae (sometimes regarded as family Mimosaceae with four tribes and 3270 species); the papilionoid legumes, also known as Papilionoideae (or family Fabaceae/Papilionaceae with 28 tribes and 13,800 species); and the ca (Abbo et al. 2008, 2011). Estimates for the period of origin and early history of the legumes differ, but a rich Eocene macrofossil record demonstrates that certain lineages of the family existed around 50 million years ago (Mya). The oldest known remnants of legume pollen date back to around 60–75 million years ago

(Klimaszewska and Keller 1985; Amri et al. 2008; Avunduk et al. 2008), making them older than the macrofossils.

There are over 3300 species in the genus *Astragalus*, all of which are classified as members of the legume family Fabaceae and the subfamily Faboideae. Iran is the primary source and one of the centers of species variety in the ancient world for this genus, which is extensively scattered across the temperate parts of the planet (Ambrose 1995; Amri et al. 2008; Maassoumi 1998). Iran is home to a peculiar and indigenous species of the plant known as *Astragalus fridae*. This plant is deemed to be endangered both by the International Union for the Conservation of Nature's (IUCN) classification system and by the resources that are now accessible in Iran (Ali et al. 2007; Jafari et al. 2018; Safar et al. 2014). On the other hand, the majority of the species are used as feed for livestock and wildlife, and 32 of its species are utilized in the production of food, medicine, cosmetics, tea, coffee, and herbal gums, respectively. *A. fridae* has a wide variety of primary and secondary metabolites, including polysaccharides and saponins, as well as poisonous chemicals, including indolizidine alkaloids and nitroaliphatic compounds (Adegbite and Amusa 2008). In addition to these antioxidant and liver-protective actions, the plant also has antiviral and antibacterial properties, as well as chemicals that have an impact on the heart's blood arteries (Jafari et al. 2018; Lock and Simpson 1991; Maassoumi 1998). The antioxidative components that were extracted from the root stopped the decline in the amount of glycogen stored in the liver, which led to a rise in both the serum's total protein and albumin levels.

16.2 In Situ Conservation of the Wild Relatives of Cultivated Plants

It is possible to describe in situ genetic reserve conservation as “the location, designation, management, and monitoring of genetic diversity in natural wild populations inside specified regions designated for active, long-term conservation.” This is how the phrase is usually written out (Palacio et al. 2007; Rabizadeh et al. 2019; Tian and Xi 1989). A genetic reserve is actively managed, even if the management only involves regular monitoring of the target CWR taxa; as long as the target population levels are maintained above the minimum viable population of approximately 5000 individuals, no additional conservation action may be required; however, if the target population levels drop below the minimum viable population, additional conservation action may be required (Abberton and Thomas 2011; Zhang et al. 2011). Importantly, in situ conservation action is a commitment to a long-term strategy since large resources need to be committed to developing a genetic reserve. This is the case because in situ conservation action must be carried out. There is a preference for in situ conservation, primarily because of the overall need to maintain ecosystem health, but also because it has the advantage of maintaining the dynamic evolution of the CWR diversity itself concerning parallel biotic and abiotic changes.

Although the goal of conservation is to always implement complementary conservation, which involves the parallel application of in situ and ex situ conservation techniques, this preference exists even though the goal of conservation is to always implement complementary conservation. Additionally, in situ conservation is strongly recommended due to the sheer number of CWRs that are involved, the need to maintain an effective genetic representation, and the difficulty in precisely identifying which CWR or traits are required by plant breeders currently and in the future. This is the case even though the primary access route for breeders to diversity is via backup in situ samples deposited in ex situ gene banks (Yu et al. 2007; Zarre and Podlech 2001).

However, without monitoring and active management, the genetic diversity within and between individual CWR populations could be eroded, and entire populations could even go extinct if no action is taken to protect them. All species that are found in protected areas are said to be passively conserved if the surrounding ecosystem or habitat is unaltered. Nevertheless, Upadhyaya et al. (2006) highlight the fact that many protected areas already play an essential role in the conservation of CWR species. This is true although many managers may be ignorant that the land under their care includes valuable agricultural genetic diversity. However, if we want to ensure that CWR taxa retain the greatest possible genetic diversity, we will need to conduct research on and keep an eye on the genetic diversity of CWR populations, as well as the natural dynamics of those populations. If we do not do this, our efforts to create protected areas for these taxa may be in vain. It is also important to highlight that the in situ management of CWR may need quite a different approach than what is necessary for the maintenance of more typical protected areas, the primary goal of which is to maintain climax communities. For instance, CWR of major crop plants are frequently found in pre-climax communities (*Vicia johannis* Tamamsch, *Lathyrus cicera* L., *Pisum sativum* subsp. *elatius* Asch. & Graebn.), which require relatively intensive site management. Alternatively, CWR may be closely associated with traditional farming practices (*Vicia johannis* Tamamsch, *Lathyrus cicera* L.), in which case genetic reserve management would (Rabizadeh et al. 2019; Safar et al. 2014; Tian and Xi 1989).

Iriondo et al. (2008) give comprehensive guidance on how to go about carrying out in situ CWR conservation. They also establish minimum requirements for the management of CWR genetic reserves. Both sets of guidelines may be found on their website (2012). Particularly, there has been relatively little amount of effort put forward to preserve the in situ variety of legume CWR.

This has been in part caused by two related disconnects: (a) the disconnect between academic studies identifying where genetic reserves or less formal in situ management activities should be established and their actual implementation, and (b) the lack of collaboration between the communities that work with plant genetic resources and protected areas. Both of these disconnects have contributed to this situation (Abberton and Thomas 2011; Safar et al. 2014; Tian and Xi 1989; Zarre and Podlech 2001). Consequently, native populations of legumes are at risk of experiencing genetic erosion or potentially going extinct (Lock and Simpson 1991; Maassoumi 1998). Maxted (1995) made what may have been the first

recommendation for the establishment of in situ genetic reserves for the diversity of legume CWR. He suggested four locations to conserve Fabae species in Syria and Turkey. This may have been the first recommendation for the establishment of in situ genetic reserves. Ceylanpinar (Ambrose 1995; Amri et al. 2008; Asfaw et al. 2009; Du et al. 2003), one of the three reserves that were subsequently formed in Turkey as part of the Global Environment Facility initiative, places a premium on the in situ conservation of cereal and legume CWRs. Within the borders of Syria, the province of Sweida is home to one of the locations that Maxted (1995) suggested should be used for in situ legume conservation (Jafari et al. 2018; Lock and Simpson 1991; Moghanloo et al. 2019a). Additional genetic reserves to conserve legume CWR have been established in the following locations: for *Lathyrus grimesii* Barneby in Nevada, the United States of America (Abbo et al. 2008; Alaniya and Gigoshvili 2012; Arbaoui and Link 2008); for *Vavilovia formosa* (Stev.) Fed. at AknaLich, on the Geghama mountain ridge, Yerevan Province, Armenia, and other legumes within the Erebus Reserve near Yerevan, Armenia (Harden and Zolfaghari 1988; Jafari et al. 2018; Niu et al. 2011; Rabizadeh et al. 2019; Yu et al. 2007). Although the in situ conservation that is presently in place is a significant step forward, it is crucial to note that none of these genetic reserves currently satisfy the basic parameters for maintaining CWR genetic reserves that were established by Lloret et al. (2012). There is a strong likelihood that wild soybean (*Glycine soja* Willd.) and farmed soybean descended from the same progenitor (Ambrose 1995).

In addition to ex situ conservation, an in situ conservation method is also utilized to preserve wild soybean. This is because populations of *G. soja* are often characterized by high levels of genetic variation. More than 40 in situ conservation sites, spread over 15 provinces and regions, have been constructed in China (Du et al. 2003, 2006). Genotyping 40 people at 20 SSR marker sites for each community allowed for the identification of their genetic variation, and the findings revealed that at least 90% of the overall genetic diversity was present in each population (Abbo et al. 2008; Avunduk et al. 2008).

16.3 *Astragalus* L. Phytochemistry

In recent years, researchers have looked at the chemical components of a total of 46 different species of the genus *Astragalus*. Additionally, more than 200 different components have been extracted from them thus far. Although the investigations were conducted on several species, the chemical compositions of the *Astragalus* genus seemed to be quite consistent. These chemical compositions mostly comprise terpenoids, flavonoids, and polysaccharides. Always in their free or glycosidic forms, intriguing chemicals, such as terpenoids and flavonoids, may be found in plants. In the meanwhile, we discovered that around 40% of the study on *Astragalus*'s composition was concentrated on the plant's aerial portions. In the *Astragalus* genus, saponin is the primary kind of chemical ingredient that may be

found. Saponins of the cycloartane- and oleanane-type were isolated from it, and they exhibited some fascinating biological features.

It has been shown that the plants belonging to the genus *Astragalus* are the largest source of cycloartane-type saponins. These saponins have been shown to contain cardiotonic, hypocholesterolemic, antidepressant, and anticlastic activities, in addition to immunomodulatory activity. Researchers continued their hunt for structurally intriguing cycloartane glycosides derived from the genus as a result of the attractive range of pharmacological effects found in the plant. There have been around 140 different forms of cycloartane-type saponins discovered up to this point. The primary substituted sugar groups in them are β -D-glucopyranosyl (Glc), β -D-xylopyranosyl (Xyl), α -L-rhamnopyranosyl (Rha), or α -L-arabinopyranosyl (Ara). In cycloartane glycosides derived from the *Astragalus* genus, other groups such as-D-glucuronopyranosyl (GlcA), -D-fucopyranosyl (Fuc), -D-apiofuranosyl (Api), and acetyl (Ac) were discovered.

16.3.1 The Biological Processes Involved in the Genus Astragalus

In recent years, the majority of the species in this genus have mostly been utilized in folk medicine for their anti-inflammatory, immunostimulant, antioxidative, anticancer, antidiabetic, cardioprotective, and hepatoprotective characteristics. They have also been shown to have antiviral effects. It was determined that the polysaccharides, saponins, and flavonoids were the active components that were responsible for the aforementioned benefits.

16.3.1.1 Activity That Reduces Inflammation

Both in vitro and in vivo testing revealed that an extract of *astragalus*, together with the plant's polysaccharides and saponins, have anti-inflammatory properties. Kim et al. discovered that the extract of *A. membranaceus* not only improved the skin lesions caused by atopic dermatitis in mice that had been induced with 1-chloro-2,4-dinitrobenzene, but it also suppressed the expression of Th2 cytokines and significantly reduced the amount of TNF-. These results were found in mice that had been induced with the chemical 1-chloro-2,4-dinitrobenzene. The researchers, therefore, concluded that *A. membranaceus* was successful in the treatment of atopic dermatitis because it regulated cytokines (Abberton and Thomas 2011; Zhang et al. 2011). *Astragali Radix* was shown to have an anti-inflammatory effect by Ryu et al., and this effect was mediated by the MKP-1-dependent inactivation of p38 and Erk1/2, as well as the suppression of NF kappa B-mediated transcription (Du et al. 2003; Zarre and Podlech 2001). *Astragalus* polysaccharides, the plant's primary component, have been shown to successfully mitigate the palmitate-induced pro-inflammatory

responses in RAW264.7 cells by activating AMPK (Kruckeberg and Rabinowitz 1985; Fathiazad et al. 2010; Linnek et al. 2011). In lipopolysaccharide-infected Caco2 cells, they demonstrated anti-inflammatory efficacy in addition to structure-protective capabilities (Alaniya et al. 2007). On the other hand, research was conducted to investigate the anti-inflammatory properties of saponins.

Astragaloside IV, meanwhile, was found to be a promising natural product with both healing and anti-scarring effects for the treatment of wounds (Fathiazad et al. 2010), could be used as a novel anti-inflammatory agent, and attenuated diabetic nephropathy in rats by inhibiting NF- κ B mediated inflammatory gene expression (Abbo et al. 2012; Mehrabian et al. 2007; Zhang et al. 2011). These findings were published in the Journal of Medicinal Chemistry.

Qin et al. (2012), examined the impact of *A. membranaceus* extracts on the advanced glycation end product-induced inflammatory response in α -1 macrophages. The findings revealed that it might block advanced glycation end product-induced inflammatory cytokine production to down-regulate macrophage-mediated inflammation through p38 mitogen-activated protein kinase and nuclear factor's (NF)- κ B signaling pathways (Mehrabian et al. 2007). The potential adjuvant impact of astragalus polysaccharides on humoral and cellular immune responses to hepatitis B subunit vaccination was examined by Du et al. (2003). This result suggested that it was a potent adjuvant for the hepatitis B subunit vaccine and that it could enhance both humoral and cellular immune responses via activation of the Toll-like receptor 4 signaling pathway and inhibit the expression of transforming growth factor (Yu et al. 2007). In addition, the results showed that it could inhibit the expression of transforming growth factors. Using male Swiss albino mice that were 6–8 weeks old, the research team of Nalbantsoy et al. (2012) investigated the in vivo effects of two different astragalus saponins on the immune response cytokines. According to the findings, astragaloside VII and macro-phyllsaponin B exhibited potent immunoregulatory effects in mice without inducing the production of inflammatory cytokines and did not have a discernible impact on inflammatory cellular targets in vitro (Avunduk et al. 2008). Astragaloside IV was revealed to be capable of competing with the inhibiting impact of high mobility group box 1 protein on the immunological function of regulatory T cells via a dose-dependent in vitro experiment conducted by Huang and colleagues (Kucherbaev et al. 2002).

16.3.1.2 Activity Against Cancer

Polysaccharides, saponins, and flavonoids found in *Astragalus* have recently been shown to have anti-tumor activity, according to the findings of several active screenings conducted in recent years. Tian and Xi (1989) investigated the adjunct anticancer effect of *Astragalus* polysaccharides on H22 tumor-bearing mice and found that it exerted a synergistic anti-tumor effect with adriamycin. They also found that it alleviated the decrease in the sizes of the spleen and thymus that was induced by adriamycin in H22 tumor-bearing mice (Horo et al. 2010). Astragaloside IV, which has the potential to act as an anti-tumorsaponin, has been shown to inhibit the

expression of Vav3.1 in a way that is both doses- and time-dependent (Tian and Xi 1989). Astragaloside II, on the other hand, could inhibit the expression of the P-glycoprotein and the *mdr1* gene, which led researchers to conclude that it was a powerful multidrug resistance reversal agent and that it had the potential to be an adjunctive agent in the treatment of hepatic cancer (Niu et al. 2011). On the other hand, the results of the experiments demonstrated that the total flavonoids of *Astragalus* and calycosin could suppress the growth of K562 cells (Delgado and Gómez 2016).

16.3.1.3 Activity Concerning Cardioprotection

Using a variety of different rat models, Ma et al. (2013) investigated the cardioprotective effect of the extract of *Radix Astragali* on myocardial ischemia and its underlying mechanisms in ROS-mediated signaling cascade in vivo. They concluded that the cardioprotection was due to the protection of tissue structure and a decrease in serum markers of the ischemic injury (Du et al. 2006). This conclusion was reached after the researchers studied the cardioprotective effect of the extract of *Radix Astragalin*.

16.3.1.4 Antidiabetic

The research conducted by Liu et al. (2010) indicated that *Astragalus* polysaccharide could regulate part of the insulin signaling in insulin-resistant skeletal muscle and that it could be a potential insulin sensitizer for the treatment of type 2 diabetes (Delgado and Gómez 2016). *Astragalus* polysaccharide was discovered to be able to up-regulate the expression of galectin-1 in the muscle of mice with type I diabetes mellitus, according to Du et al. (2006). Saponins and astragaloside IV may be able to prevent the development of peripheral neuropathy in rats with diabetes that has been caused by streptozotocin (Horo et al. 2010). Astragaloside V was found to inhibit the formation of N-(carboxymethyl)lysine and pentosidine during the incubation of bovine serum albumin with ribose, which suggested that it might be a potentially useful strategy for the prevention of clinical diabetic complications by inhibiting advanced glycation end products (Du et al. 2006). In addition, astragaloside V was found to inhibit the formation of N-(carboxymethyl)lysine and pentose.

16.3.1.5 Antioxidant Capacity and Activity

Researchers have looked at the flavonoids and saponins found in *A. mongholicus* and found that they have anti-oxidative properties. In vitro studies have shown that the antioxidants formononetin, calycosin, and calycosin-7-O-D-glucoside are capable of scavenging 1,1-diphenyl-2-picrylhydrazyl free radicals. It was discovered that both formononetin and calycosin effectively prevent the cell damage caused by xanthine/

xanthine oxidase. In both the cell-free system and the cell system, calycosin demonstrated the most powerful antioxidant activity compared to the other compounds.

16.3.1.6 Anti-Aging

Lei et al. (2003) found that astragalosides had an anti-aging effect, and their investigation showed that the mechanism could be connected to the enhancement of brain function and immunomodulatory actions (Maassoumi and Ranjbar 1998; Delgado and Gómez 2016). *Astragalus* polysaccharides were found to have anti-aging effects after Gao et al. (2008) concluded that they could increase the lifespan of mice, improve the activity of superoxide dismutase, and decrease the malonaldehyde content in mice blood serum in comparison to the control group (Avunduk et al. 2008; Niu et al. 2011). These results suggested that *Astragalus* polysaccharides can slow the aging process.

16.4 *Astragalus fridae*: A Species That Is Threatened with Extinction

It belongs to the hardwood group and is a subcategory of the dicotyledons, dialypetale, and calciflore plants, *A. fridae* Rech.F (Aubert et al. 2006; Abbo et al. 2008; Podlech 2008). It is a species of perennial plant that belongs to the Fabaceae family, the genus *Astragalus*, and the order Rosales. The majority of this species' range may be found in the northeastern region of Iran, namely in Sorkheh and Semnan (Lock and Simpson 1991; Amri et al. 2008; Safar et al. 2014; Fig. 16.1). According to the International Union for the Conservation of Nature (IUCN), this species can only be found in Iran, making it an endemic species. It is also considered to be an endangered species (Maassoumi 1998; Palacio et al. 2007; Amri et al. 2008; Abbo et al. 2012; Safar et al. 2014).

The phenological phases that were shown in Fig. 16.2 were vegetative, blooming, seed development, maturity, regrowth, and winter hibernation.

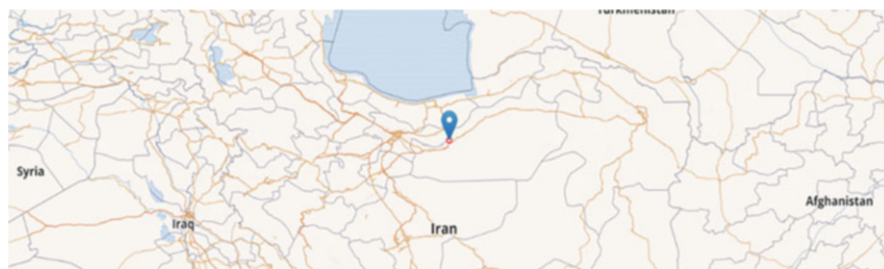


Fig. 16.1 The map of the site location in Semnan Province, Iran

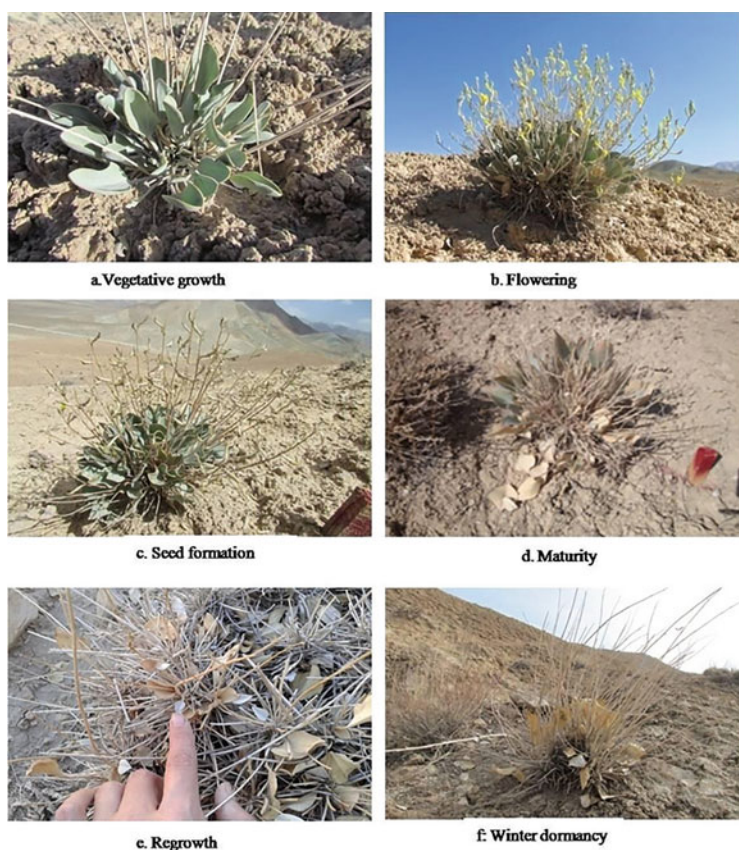


Fig. 16.2 Phenological stages of *A. fridae*. (a) Vegetative, (b) Flowering, (c) Seed setting, (d) Maturity, (e) Regrowth, (f) Winter dormancy (Naseri et al. 2017)

The World Union for the Conservation of Nature and the reports compiled by the Environment Organization both include the nation of Iran that is home to endangered plant species. The habitat of this species has been subjected to abnormal changes as a result of unprincipled exploitation for livestock grazing on the one hand, and a lack of attention to genetic resources in pastures and their protection, in granting various uses, including mines. These factors have combined to cause these changes. Therefore, because of the unique conditions that this species faces in the country and around the world, as well as the significance of preserving the genetic resources of the country, it was necessary to carry out several studies to investigate the possibility of reproduction and re-establishment of gypsum species in their natural environments. These studies were necessary because of the special conditions that this species faces in the country and around the world.

16.5 Conclusion

Astragalus fridae, *Gypsophila mucronifolia*, *Astragalus semnanensis*, *Centaurealachnopus*, *Euphorbiaypsicola*, and *Nepeta eremokosmos* are the six endemic gypsophytes species that have been discovered in western Semnan. The biosystematics research that was conducted on gypsophytes plants in the western part of Semnan was able to differentiate between the groups of gypsophytes and non-gypsophytes plants. This zonation is influenced to a large extent by environmental variables such as climate and soil. Additionally, it was discovered that although the conditions of the substrate are influenced by climate, they still affect the adaptation of the plant species. This was discovered by studying the adaptability of the *Astragalus fridae* species as well as investigating the morphological, micro-morphological, and anatomical changes of the species. The genetic variety of species over time creates circumstances that are unique to certain substrates, such as gypsum substrates (Klimaszewska and Keller 1985; Lock and Simpson 1991; Eftekhari and Asadi 2001; Amri et al. 2008). In a broad sense, the rate at which calcium and sodium are exchanged is directly proportional to the permeability of the soil. Salts move at varying speeds and may be found in a variety of soil layers depending on whether or not there is sufficient moisture deep. Typically, carbonates can be found on the top, sulfates can be found in the center, and chlorides can be found at the bottom (in the depth).

The rise in calcium and the decrease in sodium in these layers led to an increase in the permeability of the soil. The electrical conductivity of the soil and plant is impacted by the degree to which this permeability is present. In contrast to what we had anticipated, the results of soil pH and EC analyses revealed that gypsum soils increase the conditions of the soil in which some plants may be grown (Horo et al. 2010; Abbo et al. 2012; Moghanloo et al. 2019b). Higher elevations, such as the region of Aftar (northwest of Semnan), which has higher attitudes and humidity than the other sites that were researched, have a greater abundance of the *A. fridae* plant. When compared to other species, such as *Dorema ammoniacum* and *Stachy inflata*, which thrive in environments with high levels of CaCO_3 , these two plants are seen far less often.

In the scientific fields of phytochemistry and analysis, researchers have looked at the chemical components of a total of 46 different types of *Astragalus* species. Although more than 200 chemicals, such as flavonoids and cycloartane-type saponins, were extracted from them, a systematic phytochemistry study has to be enhanced. In addition, the researchers conducted several qualitatively and quantitatively analytical experiments for flavonoids and saponins from the radix of *A. fridae* and *A. mongholicus* using different methods; however, the simultaneous analysis of two different kinds of constituents was only found in very rare instances. The bioactivity evaluation of extracts and isolated compounds in the field of pharmacology focused on anti-inflammatory, immunostimulant, antioxidative, anticancer, antidiabetic, cardioprotective, hepatoprotective, and antiviral effects, but research on the anti-inflammatory activity of flavonoids was uncommon. Although a large

number of pharmacological investigations were performed using crude extracts of *Astragalus* species, the connection between the plant's chemical contents and its action is still not completely understood. In addition, there is a need for further data to be gathered about pharmacokinetics and bioavailability, particularly concerning the target tissue.

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Chapter 17

Tinospora cordifolia as a Potential Candidate for Health Care of Post-Menopausal Women



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Abstract In women, biological aging is accelerated with reproductive phase cessation which is mainly attributed to the decline in biological reserves of sex hormones. During the midlife transition, various deleterious neurological or physiological health outcomes are evident in aging women that result in their increased disability-adjusted life years (DALYs) count. Although these events are not common amongst all transiting women but the majority of them, owing to their different lifestyle factors such as overweight and obesity and associated disease conditions are susceptible to these menopause-driven outcomes later in their life. In routine clinical practices, hormone replacement therapy (HRT) is recommended to withstand the compromised health during and/or post midlife transition period, however, the conflicting reports of increased susceptibility to coronary heart diseases, heart attack, and breast cancer in women during and/or post-HRT compels to explore safer alternatives with least or no side effects. Preference for complementary therapies and natural products is increased in recent years in aging women, mainly due to the presence of phytoestrogens in them. *Tinospora cordifolia*, an important herb in the Ayurvedic system of medicine is capturing the interest of various research groups worldwide, owing to different ethano-pharmacological activities of this plant such as anti-inflammatory, anti-diabetic, anti-cancer, immune-modulatory, anxiolytic, and neuroprotective reported in various in vitro and in vivo model systems. Along with these reported activities, recently some active ingredients of *T. cordifolia* are also reported to act as selective estrogen receptor modulators (SERMs). The current book chapter is aimed to ascertain and discuss the role of *T. cordifolia* as a potential candidate to manage the various menopause-associated health problems in aging women.

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Keywords Menopause · Anxiety-like behaviour · Vasomotor symptoms · Estrogen · Mood disorders · Cognitive impairments · *Tinospora cordifolia*

17.1 Introduction

Menopause or complete cessation of menstrual periods is an outcome of reproductive aging, accompanied by gradual loss of ovarian follicles. Globally, 1.2 billion women are speculated to be menopausal or postmenopausal by 2030, with an increase of 47 million new entrants every year (Sussman et al. 2015). Women, as they pass through the mid-life transition period, become more vulnerable to the combined consequences of menopause, obesity, and inflammation as a result of estrogen deprivation which leads to disruption of metabolic homeostasis and a myriad of adverse health outcomes in women (Freeman et al. 2010). An association of obesity with multiple factors such as hypertension, depressive symptoms, sedentary lifestyle, diabetes mellitus, sleep deprivation, vasomotor symptoms (VMS), and anxiety has been observed in middle-aged women (Blümel et al. 2015). Similarly, a sedentary lifestyle during middle-age has been reported to be directly proportional to the severity of menopausal outcomes and obesity (Blümel et al. 2016).

Hormone replacement treatment (HRT) is often recommended to manage the troublesome menopause symptoms, but older women generally do not opt for HRT due to health risks associated with it, which include breast cancer, pulmonary embolism, and coronary heart disease (Kargozar et al. 2017). Moreover, HRT is not recommended for women who have or ever had breast cancer, arterial thromboembolic illnesses, estrogen-sensitive malignancies, liver disease, untreated hypertension, endometrial hyperplasia, or hypersensitivity to treatment-related active components (Goodman et al. 2011). To manage menopausal symptoms, it is necessary to investigate new complementary and alternative therapies which have few or no adverse effects. Women prefer complementary and alternative medicine (CAM) to treat these symptoms to HRT. A prediction states that about 51% of women choose complementary and alternative medicine (CAM) over HRT and that 60% of them consider it to be a better alternative for treating menopausal symptoms (Posadzki et al. 2013). According to a recent report, CAM methods are based on nutritional (herbs, dietary supplements, and probiotics), psychological (spiritual practices, mindfulness, and psychotherapy), physical practices (massage, therapy, acupuncture, yoga, spinal manipulation), and combinations of these, such as psychological and nutritional or psychological and physical (<https://nccih.nih.gov/health/integrative-health>, National Center for Complementary and Integrative Health n.d.).

In developing countries such as China, Nigeria, Ghana, and India, herbal medicines account for almost 80% of primary healthcare; they are the most commonly used supplemental or adjunctive therapy globally (Elufioye et al. 2017). The ‘WHO Global Report on Traditional and Complimentary Medicine 2019’ further illustrated the substantial rise in traditional and complementary medicine’s acceptability around the globe (WHO Report 2019). Herbal and other natural products are more likely to

contain pharmacophores and have a diversity of stereochemical properties, which increase the number of hits for targets with intricate protein interactions in drug screening. The high bioavailability and metabolite-like characteristics of herbal medications are also beneficial for these medicines' functional assay-based evaluation and target site delivery (Harvey et al. 2015). The ancient Indian medical system known as Ayurveda has been practiced for nearly 5000 years. Ayurveda's all-encompassing therapeutic strategy strives to maintain the balance between the body, mind, and spirit to achieve a state of health (Basnyat and Kolasinski 2014). In Ayurveda, *Tinospora cordifolia*, vernacularly known as Guduchi or Giloy, an indigenous Indian medicinal herb, is known for its vast ethano-pharmacological properties. Since medieval times, *T. cordifolia* is known for its rejuvenating properties. Scientific validation of its antipyretic (Ashok et al. 2010; Hussain et al. 2015), anti-inflammatory (Ghatpande et al. 2019; Philip et al. 2021), anti-cancer (Palmieri et al. 2019; Patil et al. 2021; Sharma et al. 2019), and osteoprotective (Abiramasundari et al. 2017; Kapur et al. 2008) roles has further added to its medicinal value. Similarly, immunomodulatory (Aranha and Venkatesh 2020; Sachdeva et al. 2014), anxiolytic (Singh et al. 2017, 2021), anti-obesity (Singh et al. 2020; Bhandari et al. 2022a) and neurotherapeutic (Birla et al. 2019; Mishra et al. 2016; Sharma and Kaur 2018) activities have attracted the attention of various research groups globally. However, its therapeutic potential to deal with menopausal transition and related pathophysiology is much less explored. This book chapter is a compilation of various reported ethano-pharmacological activities of *T. cordifolia* that can recommend a natural product from *T. cordifolia* as a potential alternative candidate for the management of menopause-associated health outcomes.

17.2 *Tinospora cordifolia* and Its Effect on Vasomotor Symptoms

Vasomotor symptoms or hot flashes and night sweats are known as primary symptoms of menopause onset. Ovarian hormones fluctuations during menopause results in dysfunctions of the tightly regulated temperature of the circulatory system (including the supply to the brain, internal body cavity, and peripheral vasculature) that results in intense triggering of heat dissipation stimuli leading to peripheral vasodilation and sweating (Deecher 2005). According to the 'Study of Women's Health Across the Nation (SWAN),' around 80% of women experience VMS at some point during the midlife transition, with varying prevalence rates among racial/ethnic groups (Soules et al. 2001). Vasomotor symptoms are reported to occur in the perimenopausal period in synchrony with a hormonal fluctuation and last till attaining the postmenopausal phase (Rödström et al. 2002). In a recent clinical trial, during and post-withdrawal effect of *Guduchi Satva* along with *Ksheera* treatment was assessed in 27 perimenopausal women using clinical care proforma and Greene climacteric scale. The above combination of herbal medicine was

reported to improve vasomotor (hot flushes, night sweats, and loss of libido), psychological (irregular heartbeat, nervousness, sleeping issues, excitability, anxiety), and physical (dizziness or fainting, pressure or tightness in head, numbness in different body parts, muscles or joint pain) symptoms in these women (Thulasi et al. 2022). In a questionnaire-based self-evaluation study on 117 women aged between 40–65 years, a combination of *T. cordifolia*, *Asparagus racemosus*, *Withania somnifera*, and *Commiphora mukul* was reported as safer and more effective management of menopausal symptoms in healthy menopausal women over 12 weeks. The study revealed that with aforesaid polyherbal intervention, the menopausal symptoms related to vasomotor, psychosocial, physical, and sexual domains were significantly improved in these women (Steels et al. 2018). In addition to these reports, a polyherbal formulation including *T. cordifolia* as an active ingredient was reported to reduce palpitations, joint pain, constipation, hot flashes, and headache in aged women possessing menopausal syndrome (Singh et al. 2008).

17.3 Management of Sleep and Mood Disorders with *T. cordifolia* in Menopausal Women

Sleep deprivation is a state of partial or total loss of sleep in an organism that leads to deleterious health problems (Kushida 2004). In women, sleep deprivation is common amongst more than 40–60% of transiting women, and as per the 2005 National Institutes of Health State-of-the-Science Conference Statement, sleep disturbance is recognised as a core menopausal symptom (Lampio et al. 2016). Additionally, 31–42% of women are reported to develop chronic insomnia as they attain the menopausal phase (Ciano et al. 2017). The menopause stage is also associated with mood disorders such as depression and anxiety in aging women. The incidence of developing anxiety and depression symptoms is greater amongst perimenopausal and postmenopausal women compared to premenopausal women (Mulhall et al. 2018). Also, poor health status, early awakening, and trouble falling asleep were independently associated with anxiety symptoms, whereas, poor health, night sweats, low education status, and higher body mass index showed an independent association with the incidence of depression symptoms (Tang et al. 2019). Evidence-based studies have previously reported the therapeutic effect of *T. cordifolia* in the management of sleep and mood disorders using different model systems. Butanol extract of *T. cordifolia* (B-TCE) was shown to ameliorate anxiety and cognitive deficits in glutamate-induced excitotoxicity in young female rats (Sharma et al. 2020). In another study, young animals fed on a 30% high-fat diet supplemented with dry stem powder of *T. cordifolia* (TCP) were reported to be less anxious than that on a high-fat diet alone fed animals (Singh et al. 2021). Ethanol extract of *T. cordifolia* (TnCE) was reported to possess prominent anxiolytic activity in post-traumatic stress disorder (PTSD), as evident from the behavioural, histopathological, and protein expression studies in mice model system (Ranjan et al. 2022). However,

the effect of *T. cordifolia* in the management of menopause-associated sleep and mood disorders is less explored. Previously our group has reported the beneficial effects of different extracts of *T. cordifolia* in sleep-deprived middle-aged female rats. Fifty percent ethanolic extract of *T. cordifolia* (TCE) was reported to improve anxiety, locomotor, and cognitive functions in 11–12 months old sleep-deprived female rats (Mishra et al. 2016). In another study, pre-treatment with butanol extract of *T. cordifolia* (B-TCE) improved exploratory behaviour and neuromuscular coordination in middle-aged female rats along with modulation of neuroinflammation, cell survival, apoptosis, and synaptic plasticity pathways in hippocampus and piriform cortex regions of the brain (Bajaj et al. 2022). Further, B-TCE pre-treatment was shown to act as an anxiolytic candidate in sleep-deprived middle-aged female rats. Expression study of intermediates associated with neuro-inflammation, synaptic plasticity, and heat shock proteins along with cell survival and apoptosis pathways (Bajaj and Kaur 2022). Supplementation of TCP in feed was shown to improve cognitive functions and exert anxiolytic activity in middle-aged acyclic female rats, as evident from different behavioural tests. Further, a molecular expression study of potent intermediates showed alleviation of neuro-inflammation and apoptotic responses in the hippocampus and PFC regions of the brain (Bhandari et al. 2022c). These findings provide evidence supporting the therapeutic potential of this miraculous herb in the management of sleep and mood disorders later in life.

17.4 Management of Menopause-Driven Metabolic and Liver Dysfunctions with *T. cordifolia*

Drastic changes occur in the expression of metabolic indicators and hepatic lipid metabolism during and/or post midlife transition in aging women. With aging, a variety of morphological alterations happen within the liver such as alterations in liver regeneration capacity, reduced blood flow, and liver volume, that are expected to evolve during menopause. It has been reported that there is an annual decrease in liver blood flow, volume, and functions by approximately 1% after the 40 s. Further, a 20–40% decline in liver volume was reported in elderly people and this reduction was more profound in women (Brady 2015). In several clinical studies, high levels of total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-c), lipoprotein(a), and low levels of high-density lipoprotein cholesterol have been reported in postmenopausal women compared to their pre-menopausal phase (Li et al. 1996; Ushioda et al. 2006). During the menopausal transition, the fat accumulation and bodyweight distribution shift from peripheral to central body parts, independent of bodyweight gain (Poehlman et al. 1997). During menopause, the ability to utilise circulatory low-density lipoprotein cholesterol (LDL-c), a substrate for E2 synthesis decreases which results in reduced estrogen production (Thaung Zaw et al. 2018). In another cross-sectional study, the association between early menopause and diabetes was reported in Chinese women, however, the effect of abdominal obesity was not

significant (Li et al. 2020). Further, early menopause with postmenopausal obesity was reported to influence the risk factor for the development of type 2 diabetes mellitus in aging women (Jiang et al. 2019). In overweight middle-aged women with abdominal obesity, aging and progression of leptin resistance were associated with impairment of body composition, energy expenditure, mental health, and insulin sensitivity (Naufel et al. 2019). This suggests that excessive visceral abdominal fat and alterations in fatty acid metabolism at menopause onset may predispose aging women towards metabolic and hepatic disorders.

Evidence-based studies have demonstrated the management of metabolic and hepatic dysfunctions with *T. cordifolia*. Dry stem powder of *T. cordifolia* (TCP) was reported to modulate the expression of intermediates involved in hepatic lipid metabolism and effectively managed bodyweight over 12 weeks in 30% HFD-induced obesity in young female rats (Singh et al. 2020). In an earlier study, aqueous extract of *T. cordifolia* has been shown to act as an adjuvant for the management of insulin resistance and related abnormalities in carbohydrates and lipid metabolism in high-fructose-fed male rats (Reddy et al. 2009). Similarly, *T. cordifolia* was reported to be more effective than the insulin sensitiser pioglitazone and the anti-hyperlipidaemic medication atorvastatin at treating rats with high fat and high sugar-induced dyslipidemia (Munshi et al. 2014). Hypertriglyceridemia, an indicator of disrupted lipid metabolism is a metabolic condition with elevated plasma levels of triglycerides. The water extracted (1:5 w/v) *T. cordifolia* formulation was provided to volunteers ($n = 24$) with hypertriglyceridemia for 14 days. Administration of *T. cordifolia* in hypertriglyceridemia patients ($n = 24$) has been shown to reduce triglycerides, LDL, and VLDL levels. Along with this, they found that *T. cordifolia*'s potential to inhibit oxidative damage and regulate the metabolism of amino acids, butanoate, bioppterin and vitamins resulted in its ability to reduce hypertriglyceridemia (Shirolkar et al. 2020).

The anti-oxidative activity of *T. Cordifolia* was tested using swaras and hima extracts of *T. Cordifolia* which exhibited the potential to alleviate the paracetamol-induced hepatotoxicity and oxidative stress in the rodent model system (Kaushik et al. 2017). Methanolic extract of *T. cordifolia* was also shown to possess anti-oxidative and hepatoprotective activities against cadmium-induced hepatotoxicity in rats (Baskaran et al. 2018). Recently, *T. cordifolia* in a multi-herbal combination with *Punica granatum* L., *Putranjiva roxburghii* Wall., *Swertia chirata* Buch.-Ham., and *Trigonella corniculata* L. has also been shown to ameliorate the paracetamol-induced acute hepatotoxicity and oxidative stress in female Wistar rats (Kaur et al. 2021). Similar hepatoprotective potential against paracetamol-induced hepatotoxicity was reported in another polyherbal combination of *T. cordifolia*, *Andrographis paniculata*, and *Solanum nigrum* in a mice model system (Singh et al. 2015). *T. cordifolia* has also been reported to mediate the hepatoprotective and immunostimulatory responses in carbon tetrachloride (CCl₄) intoxicated rats (Bishayi et al. 2002). Potential bioactive from *T. cordifolia* have been recognised based on their interactions with multiple protein targets associated with the pathogenesis of diabetes and obesity. Isocolumbin showed interaction with

adiponectin, β -sitosterol with cholesteryl ester transfer protein, isocolumbin with pancreatic α -amylase, isocolumbin with α -glucosidase, tinocordiside with lamin A/C, berberine with JNK1, β -sitosterol and isocolumbin with PPAR- γ , berberine with suppressor of cytokine signaling 3, and β -sitosterol with aldose reductase (Mandar et al. 2021). Further based on the analysis of the drug-likeness score, acute oral toxicity, rat acute toxicity, fish toxicity and aqueous solubility results, Mandar et al. (2021) suggested the probability of lesser side effects of *T. cordifolia* for human consumption.

Previous findings from our lab have provided the first-ever pre-clinical evidence of the anti-obesity potential of dry stem powder of *T. cordifolia* (TCP) in middle-aged acyclic female rats. Animals fed on either TCP-supplemented normal chow or a high-fat diet (HFD) showed better bodyweight management effects than their age- and diet-matched control group animals. Amelioration of age-driven oxidative stress, liver functions, and lipid profile with TCP supplementation further provides evidence for the anti-obesity potential of TCP supplementation in the diet. Mechanistic study of intermediates in liver tissue revealed that TCP supplementation alleviated hepatic dyslipidemia via modulation of proteins associated with lipogenesis, lipolysis, biological sterol synthesis, and β -oxidation of fatty acids (Bhandari et al. 2022a).

17.5 *T. cordifolia* and Management of Osteoporosis in Menopausal Women

The menopause transition is also accompanied by a change in bone mineral density that enhances the risk to develop osteoporosis and fall fractures later in life (Riis et al. 1996). Fluctuating estradiol and follicular stimulating hormone (FSH) levels during menopausal transition correlate with these alterations in bone mass. During the transmenopause phase, a doubling of FSH levels corresponds to an additional 0.3% loss per year in bone mineral density in both the femoral neck and lumbar spine (Crandall et al. 2013). A study on 40 peri/postmenopausal women undergoing hormonal therapy has reported that women with HT gained about 2% in bone mineral density, while in the control group there was a 6% loss in bone mineral density over 2 years (Naessén et al. 1992). In addition to these reports, 0.4% less loss per year in bone mineral density was reported in women enrolled in SWAN, who started hormone therapy during the menopausal transition (Solomon et al. 2015). These reports highlight the role of hormonal reserves in deciding the rate of osteoporosis during the menopausal transition in women.

Treatment with ethanolic stem extract of *T. cordifolia* (TC) was shown to exert an osteoprotective effect in ovariectomised (OVX) rats. The rate of bone loss in tibiae was slower in TC treated group than in OVX rats and showed a significant reduction in serum osteocalcin and cross-laps levels. Also, it was observed that the effects of TC treatment were much milder than those produced by E(2) treatment, thus

suggesting the estrogen-like effect of TC on the bone density of OVX rats (Kapur et al. 2008). Similarly, β -ecdysone, an active ingredient present in *T. cordifolia* was shown to ameliorate the rate of reduction in bone mineral density in OVX rats in a dose-dependent manner similar to that of the E2 treated group (Seidlova-Wuttke et al. 2010). Also β -ecdysone was tested for binding capacity with ER receptors and was reported to exert an osteoprotective effect independent of binding with estrogen receptors. β -ecdysone and E(2) were shown to significantly increase the thickness of joint cartilage along with a percentage increase in the trabecular area in the metaphysis of the tibia (Kapur et al. 2010). They also reported that *T. cordifolia* and other β -ecdysone producing plants or pure β -ecdysone can help in the prevention and treatment of osteoporosis in aged and obese individuals. Alcoholic extract of *T. cordifolia* was reported to exert a pro-stimulatory effect on osteoblast cells and rat primary osteoblasts, and further phytochemical analysis of the extract revealed β -ecdysone as an osteoprotective principle of *T. cordifolia*. The non-estrogenic effect of *T. cordifolia* was suggested due to its possible role as a selective estrogen receptor modulator (SERM) to control the osteoporosis in absence or depletion of estrogen reserves (Abiramasundari et al. 2018). Recently, our group has reported that TCP mediated improvement in locomotor coordination and grip strength in middle-aged acyclic female rats (Bhandari et al. 2022b). With TCP supplementation, grip strength and locomotor functioning of acyclic female rats were improved in both normal chow and HFD-fed groups. TCP supplementation also modulated the expression of proteins associated with neuroinflammation (GFAP and Iba1), synaptic plasticity (BDNF/Trk β and p38-MAPK/ERK), and cell survival (AP1, p-BAD, and Bcl-xL) pathways in the cerebellum region from middle-aged acyclic female rats (Bhandari et al. 2022b).

17.6 Conclusion

Midlife transition in women may serve as an important therapeutic window of opportunity to intervene in slowing down/preventing the progression of the various menopausal symptoms. Considering contradictory reports of HRT, authors have compiled information about the therapeutic claims of *T. cordifolia* for the management of menopausal symptoms in aging women. Further, crosstalk between lifestyle factors, i.e. overweight and obesity, with the prevalence and severity of different menopausal symptoms is also reviewed for a better understanding of the relationship between lifestyle and postmenopausal health outcomes. Despite the limited number of literature reports, the available literature addressing the beneficial effect of *T. cordifolia* supplementation alone or in polyherbal formulations has provided strong evidence for the management of various primary (vasomotor symptoms) and secondary (sleep deprivation, osteoporosis, obesity, dyslipidemia, anxiety, and diabetes) health issues associated with the menopausal onset and aging women (as depicted in Fig. 17.1). However, further clinical studies are required before

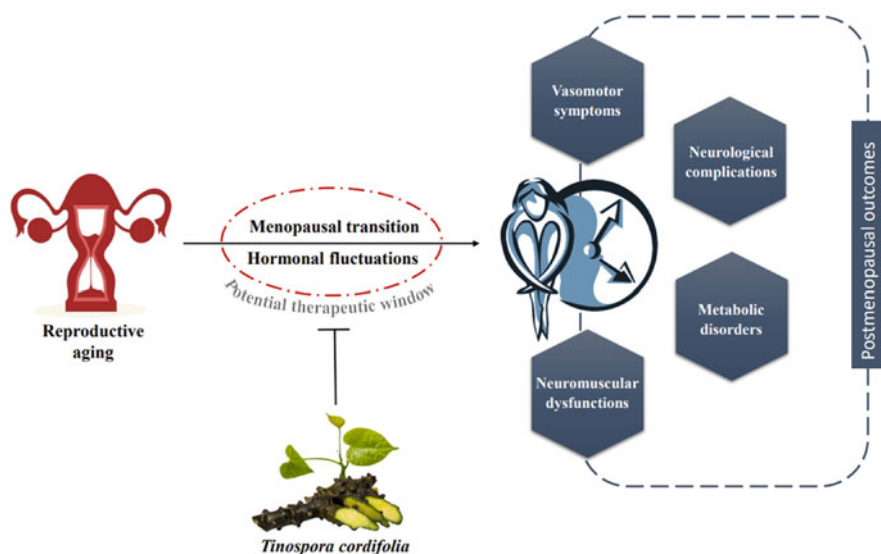


Fig. 17.1 A pictorial representation of menapausal transition associated health outcomes and their management with *Tinospora cordifolia*

recommending the usage of this miracle herb for the management of menapausal symptoms.

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Chapter 18

The Potential Role of Medicinal Plants, Traditional Herbal Medicines, and Formulations to Overcome SARS-CoV-2 Induced Health Crisis



Mihir Halder , Rahul Bose, and Sumita Jha

Abstract Since December 2019, the infection of Severe Acute Respiratory Syndrome-related Coronavirus 2 (SARS-CoV-2), also known as novel coronavirus, has been responsible for the biggest global public health crisis in recent history, which infected more than 645,925,544 individuals with 6,635,798 deaths in 228 countries and territories. Although few re-purposed anti-COVID-19 drugs and WHO-approved vaccines are now available, side effects, limited potency and efficacy toward new virus variants, public acceptance, availability, and affordability to entire global populations are the major limitations of presently available treatment against this disease. Thus, the demand for medicinal plant-based therapy for this life-threatening disease is still high. Many medicinal plants, traditional herbal therapies, and formulations have been employed and screened on an emergency basis from the onset of the COVID-19 pandemic to find effective and safer potent plant-derived anti-COVID-19 drug-based treatments against the new COVID-19 pandemic. Besides vaccination programs, several clinical trials of traditional herbal medicines and formulations have also been underway in India, China, and other countries, considering medicinal plants are a viable source of several therapeutic molecules, including antiviral compounds. In this chapter, the potential role of medicinal plants, traditional herbal medicines, and formulations have been reviewed based on literature review-, in silico-, in vitro- and clinical trial-based research, which shows a bright future in the management and/or treatment of this disease following adequate standardization and optimization. The results of such investigations might lead to the identification and characterization of therapeutic molecules or formulations which helps in new drug development in the near future.

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Keywords SARS-CoV-2 · COVID-19 · Traditional herbal medicine · Medicinal plant · Herbal formulation · In silico study · Clinical trial

18.1 Introduction

The Severe Acute Respiratory Syndrome-related Coronavirus 2 (SARS-CoV-2), also known as a novel coronavirus (COVID-19), is the third novel coronavirus to emerge in the last two decades following acute respiratory syndrome (SARS)-CoV and the Middle East respiratory syndrome (MERS)-CoV. The first incident of infection with SARS-CoV-2 was registered on December 31, 2019, in Wuhan city of China, and on March 11, 2020, the World Health Organization (WHO) declared the disease (COVID-19) caused by SARS-CoV-2 a global pandemic (Cucinotta and Vanelli 2020). The rapid global transmission and severity of the disease have wreaked havoc on global financial markets, economies, and societies (Habibi et al. 2020). Since the influenza pandemic, the COVID-19 pandemic is considered the current greatest public health crisis as the number of cases expanding at an exponential rate and caused over 676,500,802 incidents of COVID-19 infection with 6,773,753 deaths in 228 nations and territories throughout the world as of February 7, 2023 (<https://www.worldometers.info/coronavirus/>, accessed on February 7, 2023). The United States (104,509,261), India (44,683,543), France (39,543,118), Germany (37,842,223), and Brazil (36,878,774) are the top five countries in terms of total COVID-19 cases (<https://www.worldometers.info/coronavirus/>, accessed on February 7, 2023).

Some re-purposed anti-COVID-19 drugs, such as Lopinavir/Ritonavir, Ribavirin, Arbidol, Amantadine, Azithromycin, Favipiravir, Remdesivir, Oseltamivir, Chloroquine, Hydroxychloroquine, etc., (De et al. 2021; Zadeh et al. 2021) and few WHO-approved vaccines, such as COVOVAX, Novavax, Spikevax, Comirnaty, Convectia, Jcovden, Vaxzevria, Covishield, Covaxin are now available (Noor 2021; Zhang et al. 2022). However, several issues such as side effects, limited potency, and efficacy toward new virus variants, public acceptance, availability, and affordability to entire global populations, are the major limiting factors of currently available weapons against COVID-19 (Jiang et al. 2022). In addition to the vaccination programs, there is always an urgent need to find effective and safer antiviral drug-based treatments, preferably of herbal origin, to ensure comprehensive preventative and therapeutic aims against the new COVID-19 pandemic from the start of the COVID-19 to date. Unfortunately, therapeutic drugs based on plant-derived products for the treatment of SARS-CoV-2 are still unavailable after more than one year of the pandemic. Development of an effective antiviral drug against SARS-CoV-2 is challenging due to the virus infectivity, disease course, and high rate of mutation.

Considering medicinal plants as a viable source of solution to the current SARS-CoV-2-induced health crisis, several investigations have been conducted to screen the phytochemical diversity of medicinal plants to develop an anti-SARS-CoV-2

herbal medicine. Medicinal plants are gaining popularity in the current situation due to the scarcity of effective synthetic drugs against COVID-19.

The goal of this study is to summarize the present status of medicinal plant-based research and the possible usefulness of medicinal plants and their phytoconstituents, traditional medicines, and formulations to find out the answer to COVID-19, which includes ethnobotanical uses by different local communities, in silico investigation, in vitro assays and clinical trials.

18.2 Common Features of SARS-CoV-2

SARS-CoV-2 falls to the genus Beta-coronavirus of the *Coronaviridae* family, which includes four genera Alpha (α -CoV), Beta (β -CoV), Gamma (γ -CoV), and Delta-coronaviruses (δ -CoV) (Yesudhas et al. 2021). It is an enveloped, non-segmented positive-sense, single-stranded RNA beta-coronavirus with symmetric helical nucleocapsid (Khan et al. 2020; Al-Kuraishy et al. 2022). SARS-CoV-2 has ~28,882 base pairs genome that encodes viral proteins in up to 14 open reading frames (Adhikari et al. 2021). The virus encodes 20 different proteins, including four major structural proteins (S: spike; E: envelope; M: membrane; N: nucleocapsid), and several nonstructural proteins, such as RNA-dependent RNA polymerase (RdRp), coronavirus main protease (3CLpro), and papain-like protease (PLpro) (Chen et al. 2020; Yesudhas et al. 2021). SARS-CoV-2 infects human cells and produces respiratory disease with severe, mild, or no symptoms through angiotensin-converting enzyme 2 (ACE2) receptor-mediated binding and entry in respiratory epithelial cells via spike proteins of SARS-CoV-2 (Walls et al. 2020). These proteins are the main target sites of potent drug development. The viral 3-chymotrypsin-like cysteine protease (3CLpro) or the main protease (Mpro) of SARS-CoV-2 is highly conserved cysteine hydrolases, critically required for coronavirus replication and polyprotein processing (Tripathi et al. 2021).

The most common COVID-19 symptoms include headache, mild fever, dry cough, congestion or runny nose, sore throat, fatigue, muscle or body ache, to severe life-threatening pneumonia with shortness of breath, chest pains, loss of taste and smell, diarrhea, nausea, and vomiting (Adhikari et al. 2021), which appear after an average incubation period of approximately 4.8 ± 2.6 days (Liu et al. 2020). The higher age and occurrence of co-morbidities due to preexisting chronic diseases, such as cardiovascular, pulmonary, cerebrovascular, immunodeficiency, digestive, and endocrine disorders, act as positive triggering factors to develop complications or severe symptoms of the disease (Lam et al. 2020; Sanyaolu et al. 2020). COVID-19 generates a strongly impaired acquired immune response and uncontrolled innate inflammatory responses in COVID-19 severity, which may be related to cytokine storm (Hu et al. 2021). Cytokine storm is considered a primary cause of the severity of disease and mortality in COVID-19 patients (Mehta et al. 2020).

SARS-CoV-2 is a highly pathogenic and transmissible coronavirus that spreads predominantly by respiratory droplet transmission and close contact in a

human-human mode when infected individuals cough and sneeze (Rothan and Byrareddy 2020). Based on 96% shared genomic identity between SARS-CoV-2 and bat-coronavirus (Zhou et al. 2020) and 91.02% between SARS-CoV-2 and Pangolin-Coronavirus (Zhang et al. 2020a), it has been predicted that SARS-CoV-2 virus originated in a local seafood market of China as a consequence of zoonotic transmission (Ahmad et al. 2020) possibly from bats to human via an intermediate host pangolin (Zhao et al. 2020; Zhou et al. 2020). Despite this prediction, there is still much debate. The recent extraordinary efforts of several vaccine developers were truly remarkable in the development of numerous vaccines with the magnitude of potential efficacy against COVID-19.

18.3 Current Status of Global SARS-CoV-2 Therapeutic and Associated Problems

The application of many pre-existing conventional antiviral drugs is repurposed emergency basis based on their previous efficacy, virus biology, and pathology for the treatment against SARS-CoV-2 in the current global health crisis that causes millions of deaths worldwide. In contrast to new drug development against COVID-19, drug repurposing gives an instant solution through cell-based or computational screening, avoiding effective dosage, safety, and mechanism of action-related issues. Several large- and small-scale randomized controlled clinical trials have also been conducted to validate the effectiveness and efficacy of several antiviral and immunomodulatory drugs aiming at reducing disease severity. Lopinavir/ritonavir, Ribavirin, Arbidol, Amantadine, Azithromycin, Favipiravir, Remdesivir, Oseltamivir, Chloroquine, and Hydroxychloroquine, etc., are the commonly used anti-COVID-19 drugs (De et al. 2021; Zadeh et al. 2021). Casirivimab/Imdevimab monoclonal antibodies and convalescent plasma prevent virus entry; Remdesivir, Molnupiravir, and Paxlovid nucleotide analogs prevent viral replication, and a variety of repurposed JAK-STAT signaling pathway inhibitors, corticosteroids, and recombinant agonists/antagonists of cytokine and interferons have been found to provide clinical benefits in terms of mortality and hospitalization (Jiang et al. 2022).

Furthermore, some side effects, such as allergic reactions, vomiting, insomnia, headache, dizziness, gastrointestinal problems, immune suppression, irregular or absent menstrual periods, etc., are associated with the application of these drugs (Zadeh et al. 2021). In most cases, scientific evidence about their efficacy, effectiveness, and safety is limited or controversial due to a lack of reliable, unbiased, high-quality optimal clinical trial reports (Zadeh et al. 2021; Zhao et al. 2021). Moreover, the limited potency and efficacy of antiviral therapy toward new virus variants is another burning issue in the treatment of COVID-19 (Jiang et al. 2022). The development of broad-spectrum antiviral drugs and a combination of antiviral therapies with a distinct mode of action is critical for controlling the ongoing COVID-19 pandemic and preventing future outbreaks of new viruses.

The development of immunotherapies approaches demands large-scale clinical trial-based validation for the creation of reliable information and clinical experience, as well as the discovery of the best patient-directed strategy via validated disease severity biomarkers due to the immune heterogeneity of clinically similar COVID-19 patients (Jiang et al. 2022).

As of October 9, 2022, 11 vaccines, namely COVOVAX, Novavax, Spikevax, Comirnaty, Convidecia, Jcovden, Vaxzevria, Covishield, Covaxin, Covilo, and Sinovac, have been approved for Emergency Use Listing against COVID-19 by the WHO (<https://covid19.trackvaccines.org/agency/who/>) which have efficacy ranging from 50% to 95% (Noor 2021; Zhang et al. 2022). Anti-SARS-CoV-2 vaccination programs were launched globally with great enthusiasm immediately after the official approval by the WHO for emergency use to control the global spread of COVID-19 (Abdulla et al. 2021). According to current data, 69.4% of the world population has received a minimum of one dose of a COVID-19 vaccine, and 26.4% of people in low-income countries have received a single dose (Mathieu et al. 2020), implying that the vaccination rates in economically less developed countries are significantly lower.

Vaccination is, without a doubt, the most effective way for decreasing hospitalization and mortality associated with SARS-CoV-2 infection, as demonstrated by clinical studies and real-world evidence (Zhang et al. 2022). However, several problems remain concerning this disease, including the rapid increase in the number of post-vaccinated reinfection incidents in different countries (Shastri et al. 2021; Vitale et al. 2021); vaccines not demonstrating equal effectiveness and efficacy against new emerging highly transmissible variants of SARS-CoV-2, such as delta (Yang and Shaman 2022) and Omicron (Pulliam et al. 2022); variation in availability, affordability, and accessibility of this vaccine at the individual and a country level (Ali et al. 2021); public vaccine hesitancy (Coustasse et al. 2021); and adverse effects of post-vaccination (Patone et al. 2022). Moreover, after the first dose of immunization, 0.5% of the population in the United Kingdom found positive for SARS-CoV-2, whereas 0.2% found positive after the second dosage. In addition, early findings suggest that the B.351 South African variation is a neutralizing escape mutation to convalescent sera, which is problematic (Tegally et al. 2020).

Furthermore, public acceptance of COVID-19 vaccinations is not the same throughout the globe (Al-Mohaithef and Padhi 2020), which is influenced by confidence, convenience, complacency, and attitudes. Significant demographic variation was observed in vaccine acceptability (Malik et al. 2020; El-Elimat et al. 2021). On the other hand, the distribution of vaccine equity among all the countries and to each of the people of the globe is still challenging, especially the developing country and poor people, which may need a long duration to cover the entire population.

18.4 The Potential Role of Medicinal Plants to Treat COVID-19

Since prehistoric times, medicinal plants and their diverse bioactive constituents have served as a highly plausible platform for a treasure trove of phytochemicals that have demonstrated promising benefits against various human viruses, including SARS-CoV, MERS-CoV, H₁N₁, H₉N₂ influenza viruses, respiratory viruses, etc. The lack of specific treatment for COVID-19 forced researchers to search for phytotherapeutic agents or drug candidates from the plethora of medicinal plants and traditional herbal medicine from the first day of this pandemic. Although currently there are a few WHO-approved vaccines and re-purposed drugs on the markets, these therapies are suffering from several issues, such as side effects, low potency, and poor efficacy against newly emerging strains of COVID-19, public acceptance, availability, affordability, and accessibility to the entire global population. Thus, the demand for medicinal plant-based therapy for this life-threatening disease is still high.

Previous experiences with new diseases and the statistic of about 50% of all approved drugs sold between 1981 and 2014 were derived from natural products (Newman and Cragg 2020), suggesting ample opportunity to find potent weapons against novel SARS-CoV-2 from medicinal plants. This might be a game changer due to their advantages in the context of biocompatibility and toxicity compared to synthetic drugs. In addition, SARS-CoV-2 has resemblances with SARS-CoV, MERS-CoV, and other RNA viruses, raising the possibility of discovering effective anti-COVID-19 phytochemicals from pre-existing traditional herbal medicine/formulations or bioactive phytochemicals already employed for the treatment of these RNA viruses or prior coronaviruses. Medicinal plant-based traditional herbal medicine might help on several fronts, including alleviating COVID-19 symptoms and providing raw materials for strong antiviral drugs. As an essential field of investigation in present COVID-19 research, careful large-scale exploration of various herbal compounds and medicinal plants should be included. In contrast, the uniqueness of COVID-19 makes it difficult to determine the efficacy of existing phytotherapeutic products against it.

Several publications have been published on the possible medicinal plant-based targeted therapeutics and clinical management of SARS-CoV-2 infection based on a systematic re-evaluation of the previously published articles related to medicinal plants, phytochemicals, and traditional herbal medicines and formulations having an antiviral activity or used to treat viral respiratory infections (Jalali et al. 2021; Adhikari et al. 2021; Khan et al. 2021). Medicinal plants, traditional medicinal formulations, or phytochemicals with antiviral, anti-inflammatory, and immunomodulatory effects are cited frequently in such studies. For example, Jalali et al. (2021) suggested 30, 18, and 17 potential medicinal plants or bioactive components that interfere with COVID-19 pathogenesis, sign and symptom therapy, and prevention and support therapy of COVID-19. The potential medicinal plants or bioactive components that interfere with COVID-19 pathogenesis, includes *Alnus japonica*

(Betulaceae), *Andrographis paniculata* (Acanthaceae), *Artemisia annua* (Compositae), *Asparagus racemosus* (Liliaceae), *Camellia sinensis* (Theaceae—Rutin, theaflavin-3,30-digallate, tannic acid, [–]-epigallocatechin gallate), *Cibotium barometz* (Cibotiaceae), *Citrus* spp. (Rutaceae—Hesperetin, hesperidin, Rhoifolin, Neohesperidin), *Cynara scolymus* (Compositae—Cynaroside), *Dioscorea batatas* (Dioscoreaceae), *Erigeron abajensis* (Compositae—Scutellarin), *Equisetum hyemale* (Equisetaceae—Herbacetin), *Glycyrrhiza glabra* (Leguminosae—Glycyrrhizin), *Hibiscus sabdariffa* (Malvaceae—Anthocyanins, Delphinidin-3-Osambubioside, Cyanidin-3-Osambubioside), *Lindera aggregata* (Lauraceae), *Linum usitatissimum* (Linaceae), *Hancornia speciosa* (Apocynaceae—Chlorogenic acid), *Houttuynia cordata* (Saururaceae), *Isatis indigotica* (Brassicaceae—indigo, sinigrin, aloe emodin, hesperetin, Sinigrin, 2,2-Di(3-indolyl)-3-indolone, Phaitanthrin D), *Psoralea corylifolia* (Fabaceae—Bavachinin, neobavaisoflavone, isobavachalcone, 40-Omethylbavachalcone, psoralidin, corylifol A), *R. officinale* (Polygonaceae—Emodin), *Scutellaria baicalensis* (Lamiaceae—Baicalin Cosmosiin), *Paulownia tomentosa* (Paulowniaceae), *Pyrrosia lingua* (Polypodiaceae), *Toona sinensis* (Meliaceae), *Torreya nucifera* (Taxaceae), *Tribulus terrestris* (Zygophyllaceae Cinnamic amides), *Tripterygium wilfordii* (Celastraceae), etc. (Jalali et al. 2021). The possible medicinal plants/components may be used for sign and symptom therapy of COVID-19 includes, *Aloe barbadensis* (Asphodelaceae—HF1Z polysaccharide), *Althaea officinalis* (Malvaceae—Mucilage-polysaccharides), *A. paniculata* (Acanthaceae—Andrographolide Arabinogalactan), *Angelica decursiva* (Apiaceae—Columbianadin), *Artemisia capillaris* (Compositae—Capillarisin (flavone), *Cinnamomum cassia* (Lauraceae—7-Hydroxycoumarin, 4-allylanisole, Cinnamic acid Ethylester, acetic acid cinnamylester, 20-Hydroxyacetophenone, 2-Hydroxycinnamic acid), *Cissampelos pareira* (Menispermaceae), *Citrus* spp., *Eriobotrya japonica* (Rosaceae), *Euphorbia hirta* (Euphorbiaceae) *Cuminum cyminum* (Apiaceae), *G. glabra* (Leguminosae—Arabinogalactan protein), *Salvia officinalis* (Lamiaceae—Polysaccharide), *Malva sylvestris* (Malvaceae—Polysaccharides), *Paederia foetida* (Rubiaceae—Radix Puerariae Puerarin (Isoflavonoid), *Tetrastigma hemsleyanum* (Vitaceae—Galacturonic acid, glucose, mannose, arabinose, galactose, and rhamnose), *Ziziphus abyssinica* (Rhamnaceae), etc. (Jalali et al. 2021). Jalali et al. (2021) also suggested 17 potential medicinal plants or bioactive components for the prevention and supportive therapy of COVID-19, which includes *Aloe vera* (Xanthorrhoeaceae—Acemannan), *Allium sativum* (Amaryllidaceae), *A. paniculata* (Acanthaceae), *Astragalus aaronii* (Leguminosae—Polysaccharide), *Althaea officinalis* (Malvaceae), *Cinnamomum zeylanicum* (Lauraceae), *Curcuma longa* (Zingiberaceae), *Echinacea purpurea* (Compositae—Chicoric acid 0.5%), *Echinacea* spp. (Compositae), *G. glabra* (Leguminosae), *Lonicera japonica* (Caprifoliaceae—Chlorogenic acid 25%), *Nelumbo nucifera* (Nelumbonaceae), *Panax ginseng* (Araliaceae—Polysaccharide), *Rheum officinale* (Polygonaceae—Anthraquinones (0.1–0.2%)), *T. vulgaris* (Lamiaceae), *Urtica dioica* (Urticaceae), *Zingiber acuminatum* (Zingiberaceae), etc.

Khan et al. (2021) also cited several medicinal plants and their phytochemicals, such as *Artemisia vulgaris*, *Boerhavia procumbens*, *Capparis spinosa*, *Cistanche tubulosa*, *Zingiber officinalis*, *Euphorbia hirta*, *Carum copticum*, *Hyoscyamus niger*, etc., which commonly applied against respiratory problems. *G. glabra*, *A. annua*, *I. indigotica*, *Lindera aggregate*, *Pelargonium sidoides* have shown promising potential against the previous epidemic SARS-CoV.

Withania somnifera and its several secondary metabolites, such as withanoside V, withanone, somniferine, etc. have been shown to play an important role in maintaining immune homeostasis of the body, inflammation regulation, pro-inflammatory cytokines suppression, protection of multiple organs, antiviral, anti-stress, and anti-hypertensive properties (Singh et al. 2022). Available data demonstrated antiviral efficacy in Withanoside V of *W. somnifera*, which may be investigated as an herbal treatment against SARS-CoV-2 infection following the standardization of drug development and formulation characteristics in the near future (Singh et al. 2022).

Foka et al. (2022) conducted a systematic review and analysis of literature that suggested the biomedical and antiviral properties of *Azadirachta indica* extracts that could be of importance in the design of potential anti-SARS-CoV-2 nanoformulations based on several studies that are highlighting the antiviral effect of *A. indica* plant extracts on this pathogen.

These articles have offered vital information and data on a variety of medicinal plants and their numerous bioactive compounds, which may aid in the management, prevention, and treatment of SARS-CoV-2 infection. Although such studies do not contain any sort of direct in vitro, in vivo, or clinical evaluation of their effectiveness and efficacy against the SARS-CoV-2, this type of information opens up a new channel for planning future in vivo, in silico and clinical investigations in the present pandemic situation.

18.5 Some Medicinal Plant-Based Ethnobotanical Study Related to COVID-19

Different indigenous populations and tribal groups of developing countries have a long history of use of traditional herbal medicinal plants to cure diseases. To combat the present threat of global health crisis, these populations greatly depend on their traditional medicinal plant-based therapeutics. Several ethnobotanical studies have also been published during the last two years that highlight the use of different medicinal plants to cure the various symptoms of COVID-19 and to prevent infection by several indigenous groups of people from different parts of the globe (Benkhaira et al. 2021; Chhetri et al. 2021; Phumthum et al. 2021; Tegen et al. 2021; Chebaibi et al. 2022; De Meyer et al. 2022; Mlozi 2022; Shinde et al. 2022; Table 18.1). Although adequate scientific understanding of active phytochemicals, effectiveness, pharmacological characteristics, and mechanism of action of such

Table 18.1 Ethnobotanical investigation of the use of medicinal plants against COVID-19 infection

Survey population/area	Data collection method used	Sample size (<i>n</i>)	Major finding related to medicinal plant	References
Buddhabhumi Municipality, Kapilvastu, Nepal	Online and interpersonal interview	59 COVID-19 survivors and 111 inhabitants	They used 41 medicinal plants belonging to 24 families and 37 genera, of which <i>Curcuma longa</i> was the most frequently used species	Chhetri et al. (2021)
Cameroon	Published literature	119 papers were reviewed	Out of 230 Cameroonian medicinal plants, 90 species belonging to 53 botanical families have a potential role in managing at least three symptoms of COVID-19, and 30 species have anti-COVID-19 secondary metabolites	Fongnzossie Fedoung et al. (2021)
Congolese community in Belgium	16 in-depth semi-structured interviews	263 peoples	Around 15 forms of traditional, complementary, and alternative medicine were documented, of which 13 were plants	De Meyer et al. (2022)
Fez city, Northern Morocco	Semi-structured interviews	50 well-known traditional herbalists	49 medicinal plant species belonging to 28 botanical families were recorded, of which <i>Syzygium aromaticum</i> , <i>Thymus vulgaris</i> , <i>Eucalyptus globulus</i> , and <i>Artemisia vulgaris</i> are the most important species	Benkhaira et al. (2021)
Ethiopian population	Published literatures reviewed	46 literatures analyzed	Out of 111 traditionally used medicinal plant species 56 plant species have antiviral properties.	Tegen et al. (2021)
Hilly tribal communities of Nanded district, India	Semi-structured questionnaires	180 respondents	17 traditional herbal medicinal plant species belonging to 13 different families are being used extensively for the improved immune system against COVID-19	Shinde et al. (2022)
Hmong and Karen ethnic groups of Thailand	Field interviews and Literature reviewed	56 villages and 1230 reports	Among 496 plant species, 60 medicinal plants are used frequently to treat mild symptoms associated with COVID-19. The most frequently used plant species are <i>Psidium guajava</i> ,	Phumthum et al. (2021)

(continued)

Table 18.1 (continued)

Survey population/ area	Data collection method used	Sample size (n)	Major finding related to medicinal plant	References
			<i>Phyllanthus emblica</i> , <i>Houttuynia cordata</i> , <i>Plantago major</i> , <i>Strobilanthes cusia</i>	
Iringa region of Tanzania	Collection of materials	Traditional practitioners	<i>Eucalyptus</i> species, <i>Piper nigrum</i> , <i>Zingiber officinale</i> , <i>Allium cepa</i> , <i>Allium sativum</i> and <i>Citrus</i>	Mlozi (2022)
Moroccan population	Interview	1263 peoples	21 plants belonging to 12 families have been used to treat or prevent COVID-19. The most frequently used plants are <i>S. aromaticum</i> , <i>Citrus limon</i> , and <i>A. sativum</i>	Chebaibi et al. (2022)
Western Colombia	Google Drive tool and a general questionnaire	100 COVID-19 patients	The uses of 37 plant species belonging to 22 families and 32 genera, and 12 distinct medicinal formulas have been documented. The most commonly used plants are <i>Z. officinale</i> , <i>E. globulus</i> , <i>Citrus</i> , <i>Gliricidia sepium</i> , and <i>Matricaria recutita</i>	Cordoba-Tovar et al. (2022)

usage is unknown, these types of ethnobotanical studies may serve as practical application-based leads for finding successful COVID-19 therapy. Some authors recommended providing substantial consideration to ethnopharmacological research considering the enormous challenges in the current pandemic. Some of the ethnobotanical studies related to the use of medicinal plants to treat COVID-19 are given below.

In the context of treatment and prevention of COVID-19 through traditional medicine, the ethnobotanical and ethnopharmacological information on promising medicinal plants used in Fez city was collected via Semi-structured interviews of 50 well-known traditional herbalists over a period of 2 months (November and December 2020), which documented use of 49 medicinal plants species belonging to 28 botanical families (Benkhaira et al. 2021). Among these, *Syzygium aromaticum*, *Thymus vulgaris*, *Eucalyptus globulus*, and *A. vulgaris* are the most important species. Most remedies are prepared in infusion form that includes aerial parts of the plants and administered orally. According to herbalists, 47.36% of customers were very satisfied with using herbal remedies to combat COVID-19

(Benkhaira et al. 2021). Confirmation of the safety and efficacy of these plants by pharmacological, toxicological, and phytochemical studies should be done.

According to the online and interpersonal interviews with 59 COVID-19 survivors and 111 residents of Buddhabhumi Municipality, Kapilvastu, Nepal, 41 medicinal plants of 24 families and 37 genera were used and most of which were collected from their home gardens (Chhetri et al. 2021). *C. longa* was the most frequently used species. Interestingly, all 15 most frequently cited medicinal plants were reported to have both antiviral and anti-inflammatory properties, and 14 of them were proclaimed to be immunomodulatory (Chhetri et al. 2021).

In Thailand, Hmong and Karen ethnic groups used 269 and 297 plant species, respectively, to treat mild symptoms associated with COVID-19, of which 60 plant species are used frequently (Phumthum et al. 2021). The most frequently used plant species are *Psidium guajava*, *Phyllanthus emblica*, *H. cordata*, *Plantago major*, *Strobilanthes cusia*.

According to a survey on medicinal plants used to treat or cure symptoms of SARS-CoV-2 by the Moroccan population along with their sociodemographic parameters, 61.9% of the total (1263) interviewed individuals used medicinal plants for preventing or treating COVID-19 either once (30.6%) during the pandemic, frequently daily use until recovery (47.8%) or single daily use (17.4%) (Chebaibi et al. 2022). In total, 21 plants of 12 families have been used to treat or prevent COVID-19, which includes *Eucalyptus*, *S. aromaticum*, *A. sativum*, *Lavandula officinalis*, *Trigonella foenum-graecum*, *Mentha pulegium*. Some plants with potential toxicity, such as *Juniperus thurifera*, *Olea europaea*, *Pistacia lentiscus*, *Artemisia herba-alba*, *Nerium oleander*, are incorporated in diets by the Moroccans to manage SARS-CoV-2, and hence extra caution should be given to consume non-approved natural products (Chebaibi et al. 2022).

De Meyer et al. (2022) conducted an exploratory study with 16 in-depth semi-structured interviews with 1263 selected (using purposive sampling) people of Congolese descent currently residing in Belgium on the use of the medicinal plant in the context of COVID-19 and analyzed, depicted the use of 15 forms of traditional, complementary and alternative medicine by this group of people, of which 13 were plants. These medicinal plants include *Allium cepa*, *A. sativum*, *Allium schoenoprasum*, *Picralima nitida*, *A. annua*, *Ocimum gratissimum*, *Tetradenia riparia*, *Moringa oleifera*, *Eucalyptus* spp., *P. guajava*, *Morinda morindoides*, *Citrus* spp., *Z. officinale*. In addition, they appear to be abandoning their Congolese identity and traditional knowledge in search of coping mechanisms for the COVID-19 epidemic.

Traditional practitioners in Tanzania frequently use medicinal plants, such as *Eucalyptus* species, *Piper nigrum*, *Z. officinale*, *A. cepa*, *A. sativum*, and *Citrus* to combat COVID-19 (Mlozi 2022). Phytochemical screening reveals that these plants possess flavonoids, tannins, fatty acids, steroids, terpenoids, and saponins, which may play significant antiviral activity against SARS-CoV-2 (Mlozi 2022).

Shinde et al. (2022) performed a semi-structured questionnaire-based investigation in hilly tribal communities of the Nanded district, India, and they documented that 17 traditional herbal medicinal plant species from 13 families are widely utilized

for the improved immune system against COVID-19. It includes *A. indica*, *A. vera*, *Ocimum sanctum*, *Momordica charantia*, *Embllica officinalis*, *W. somnifera*, *Astragalus membranaceus*, *C. longa*, *Z. officinale*, *Tinospora cordifolia*, *T. foenum-graecum*, etc.

Based on 46 papers analyzed, Tegen et al. (2021) reported the involvement of 111 medicinal plant species and their phytochemicals in Ethiopian traditional knowledge to cure various viral illnesses, among which 56 plant species have antiviral phytochemicals that might be used to treat COVID-19. Some of these include lycorine, gingerol shogaol, resveratrol, rhoifolin, oleanolic acid, kaempferol, rosmarinic acid, almond oil, ursolic acid, hederagenin, nigellidine, α -hederin, apigenin, nobiletin, tangeretin, chalcone, hesperidin, epigallocatechin gallate, alliin, diallyl trisulfide, ajoene, aloenin, artemisinin, glucobrassicin, curcumin, piperine, flavonoids, anthraquinone, hydroxychloroquine, and jensenone.

18.6 In Silico Studies of Medicinal Plants, Traditional Herbal Medicine, and Their Phytochemicals

The lack of effective drugs for SARS-CoV-2 treatment is greatly intriguing in in-depth phytochemical screening with computational analysis due to its efficacy in virtually screening a large number of plant-derived chemicals in a short period. Previously well-known medicine or formulations are predominantly used to identify new clinical applications against COVID-19 via in silico studies during the current public health crisis because their instant use is possible if they show significant results. As they are already effectively used against other diseases in some populations, they are safe. It will accelerate the traditional drug discovery process and reduce the expenses associated with the development of novel drugs by reducing some preclinical research. Besides this, in silico studies of medicinal plants, traditional herbal medicine, and their phytoconstituents are able to predict possible novel lead candidate molecules with improved therapeutic index, which will aid in the development of an antiviral drug against COVID-19 in the future. Several in silico studies have been conducted using natural phytochemicals via computational approaches against several key proteins of SARS-CoV-2 (Balkrishna et al. 2020; Habibzadeh and Zohalinezhad 2021; Sahithya et al. 2021; Tripathi et al. 2021; Varshney et al. 2020; Khasamwala et al. 2022; Maurya and Sharma 2022). These studies include molecular docking, molecular dynamics (MD) simulation, ADMET profiling, binding free energy calculation, target accuracy, pharmacophysical properties, and toxicity predictions. Plant bioactive metabolites are docked to various targets and the binding energies of these natural substances are compared with reference drugs, such as nelfinavir and hydroxychloroquine in in silico assay.

Several studies predicted several phytochemicals, such as withanolide (Balkrishna et al. 2020; Maurya and Sharma 2022); apigenin (Habibzadeh and Zohalinezhad 2021; Maurya and Sharma 2022); luteolin (Habibzadeh and

Zohalinezhad 2021); withanoside V (Tripathi et al. 2021); ginsenoside, glycyrrhizic acid, hesperidin and tribulosin (Kumar et al. 2021); michellamine B, quercetin 3-*O*-(2'',6''-digalloyl)-beta-D-galactopyranoside, corilagin, hypericin, and 1,2,3,4,6-Penta-*O*-galloyl-beta-D-glucose (Murali et al. 2022); coumarin, coumapherine and bisdemethoxycurcumin (Khasamwala et al. 2022); somniferine A, tinosporide, tinocordioside, orientin, flavonol glucoside, cyclocurcumin, Withanolide B, Kaempferol, Withanone and Withaferin A (Maurya and Sharma 2022); dihydrodieuginol B and tulsinol A, B, C, D, E, F, G (Varshney et al. 2020); rutin, 4,5-di-*O*-caffeoylquinic acid, and schaftoside (Hasan et al. 2022); mukonicine, *o*-methylmurrayamine A, koenine, and girinimbine (Wadanambi et al. 2022); andrographolide, andrographic acid, neoandrographolide, 14-deoxyandrographolide, 14-deoxy 11, 12-didehydroandrographolide, onysilin, 14-deoxy 14, 15-dehydroandrographolide, 3, 4-dicaffeoylquinic acid and isoandrographolide (Sahithya et al. 2021), etc., which has great potential to be used against SARS-CoV-2 (Table 18.2).

The molecular-docking analysis also elucidated several medicinal plants, such as *Artemisia herba-alba* (Hasan et al. 2022), *A. paniculata* (Sahithya et al. 2021), *M. oleifera* (Siddiqui et al. 2022), *W. somnifera* (Balkrishna et al. 2020; Tripathi et al. 2021), *O. sanctum* (Varshney et al. 2020), *Murraya koenigii* (Wadanambi et al. 2022), *Isatis tinctoria*, *T. nucifera*, *P. corylifolia*, *Rheum palmatum* (Mandal et al. 2021), *Rhazya stricta* (Baeshen et al. 2022), etc., with potential beneficiary inhibitory actions against COVID-19.

Molecular docking investigation displayed that apigenin and luteolin the constituents of *Matricaria chamomilla*, could inhibit the function of the major protease protein of SARS-CoV-2 via a strong binding affinity with it (Habibzadeh and Zohalinezhad 2021). Apigenin and luteolin demonstrated -7.86 and -7.24 docking scores against the 6LU7 receptor, respectively, with a combination of hydrogen bonding, van der Waals, and other hydrophobic interactions in the docked complexes (Habibzadeh and Zohalinezhad 2021). These findings imply that *M. chamomilla* or its phytochemicals (apigenin and luteolin) might be used to prevent SARS-CoV-2 infection in humans. According to the determined inhibition constants (K_i), luteolin inhibits more effectively effect than apigenin (Habibzadeh and Zohalinezhad 2021).

The screening of 40 phytochemicals from the herbal medicinal plant through in silico molecular docking revealed that only 12 phytochemicals were able to block or stabilize the major functional sites of the main protease (M^{pro}) and structural spike protein of SARS-CoV-2 (Kumar et al. 2021). Among these, ginsenoside, glycyrrhizic acid, hesperidin and tribulosin have high binding energy with both main protease and spike protein, whereas etoposide and teniposide have high binding energy only with spike protein and main protease, respectively (Kumar et al. 2021).

Out of 40 potential phytoconstituents evaluated, four phytoconstituents of *W. somnifera* demonstrated the greatest docking energy: Withanoside II, Withanoside IV, Withanoside V, and Sitoindoside IX (Tripathi et al. 2021). Based on subsequent MD simulation studies and binding free energy score, withanoside V

Table 18.2 Potential anti-SARS-CoV-2 phytochemicals derived from medicinal plants with their mode of action determined by in silico analysis

Name of the proposed potential drug candidates	Chemical nature of potential phytotherapeutic agent	Biological source of phytotherapeutic agent	Total number of bioactive phytochemicals screened	Inhibitory site of COVID-19	References
Amritoside	Clerodane furano diterpene glucoside	<i>Tinospora cordifolia</i>	32	SARS-CoV-2 M ^{pro}	Murugesan et al. (2021)
Amentoflavone, Bilobetin, Ginkgetin	Biflavonoid	<i>Torreya nucifera</i>	12	SARS-CoV-2 M ^{pro}	Ghosh et al. (2022)
Andrographiside	Diterpenoid	<i>Andrographis paniculata</i>	1066	SARS-CoV-2 3CL ^{pro} and RdRp	Wu et al. (2020)
Andrographic acid, Neoandrographolide, Isoandrographolide, Onysilin; 14-deoxyandrographolide; 14-deoxy 11, 12-didehydroandrographolide; 14-deoxy 14, 15-dehydroandrographolide; 3, 4-dicaffeoylquinic acid; isoandrographolide	Favones, sterols, and diterpene	<i>A. paniculata</i>	17	Inhibited multiple SARS-CoV-2 receptor targets	Sahithya et al. (2021)
Andrographolide	Diterpenoid	<i>A. paniculata</i>	8	SARS-CoV-2 M ^{pro} or 3CL ^{pro}	Rajagopal et al. (2020)
			Direct study		Enmozhi et al. (2021)
			17		Sahithya et al. (2021)
Apigenin	Flavonoid	<i>Matricaria chamomilla</i>	Direct study	SARS-CoV-2 M ^{pro}	Habibzadeh and

Apigenin, Cyclocureumin, Somniferine A, Tinosporide, Tinocordioside, Orientin, Withanolide, Withanolide B, Kaempferol, Withanone and Withaferin A	Flavonoid, Steroidal saponin, Flavonol glucoside, etc.	Ayurvedic Kadha— <i>Ocimum sanctum</i> , <i>Curcuma longa</i> , <i>T. cordifolia</i> , <i>Zingiber officinale</i> , <i>Elettaria cardamomum</i> , <i>Withania somnifera</i> , <i>Syzgium aromaticum</i> , <i>Piper nigrum</i> , lemon	108	SARS-CoV-2 M ^{pro}	Zohalenezhad (2021)
Apigenin-6-C-glucosyl 7-O-glucoside	Flavone glucoside	<i>T. cordifolia</i>	32	SARS-CoV-2 M ^{pro}	Murugesan et al. (2021)
Ashwagandhanolide	Dimeric withanolide	<i>W. somnifera</i>	110 from six plants	SARS-CoV-2 Spike protein	Patel et al. (2021)
Asparoside-C, Asparoside-D, Asparoside-F	Steroidal saponin	<i>Asparagus racemosus</i>	32	NSP15 Endoribonuclease and spike receptor-binding domain	Chikhale et al. (2021b)
Astragaln, Pectolinarin	Flavonoid	<i>Phyllanthus niruri</i>	35	SARS-CoV-2 M ^{pro}	Murugesan et al. (2021)
Azadironic acid, Nimbionone, Nimbionol, Nimocinol	Diterpenoid	<i>Azadirachta indica</i>	47	SARS-COV-2 RdRp, 3CL ^{pro} , PL ^{pro}	Adegbola et al. (2021)
Baicalin	Flavonoid	<i>Scutellaria baicalensis</i>	1066	SARS-CoV-2 PL ^{pro}	Wu et al. (2020)
Berberine	Alkaloid	<i>T. cordifolia</i>	5	SARS-CoV-2 3CL ^{pro}	Chowdhury (2021)
Catechin	Phenolic compound	NM	17	SARS-CoV-2 M ^{pro}	Jamali et al. (2021)
Corilagin	Phenolic compound	<i>Chamaesyce hyssopifolia</i>	151	SARS-CoV-2 RdRp	Murali et al. (2022)
Coumarin, coumapherine, bisdemethoxycoucurmin	Phenolic compound		11	SARS-COV-2 RdRp	Khasamwala et al. (2022)

(continued)

Table 18.2 (continued)

Name of the proposed potential drug candidates	Chemical nature of potential phytotherapeutic agent	Biological source of phytotherapeutic agent	Total number of bioactive phytochemicals screened	Inhibitory site of COVID-19	References
Curcumin, Cyclocurcumin	Phenolic compound	Trikadu comprises of <i>Z. officinale</i> , <i>P. nigrum</i> , <i>P. longum</i>	7	SARS-CoV-2 M ^{pro}	Rajagopal et al. (2020)
Desacetylgedunin	Triterpenoid	<i>A. Indica</i>	19	SARS-CoV-2 PL ^{pro}	Baidya et al. (2021)
Dihydrodieuginol-B	–	<i>O. sanctum</i>	7	SARS-CoV-2 M ^{pro} and PL ^{pro}	Varshney et al. (2020)
Dihydroxy-dimethoxy flavone	Flavonoid	<i>A. paniculata</i>	8	SARS-CoV-2 M ^{pro}	Rajagopal et al. (2020)
Famesiferol B, Assafoetidinol A, Coniferol	Sesquiterpene coumarin	<i>Ferula asafoetida</i>	46 from 28 species	SARS-CoV-2 Spike protein and M ^{pro}	Natesh et al. (2021)
Glabridin, Fisetin	Flavonoid	NM	17	SARS-CoV-2 M ^{pro}	Jamali et al. (2021)
Gallocatechin-3-gallate, Epigallocatechingallate, Epigallocatechin gallate	Polyphenol	<i>Camellia sinensis</i>	8	SARS-CoV-2 M ^{pro}	Ghosh et al. (2021)
Ginsenoside	Triterpene saponin	NM	40	SARS-CoV-2 Spike protein and M ^{pro}	Kumar et al. (2021)
Glycyrrhizic acid	Terpenoid saponin	NM	40	SARS-CoV-2 spike protein and M ^{pro}	Kumar et al. (2021)
Glycyrrhizic acid, Isoliquiritin apioside	Terpenoid saponin	<i>Glycyrrhiza</i> species	20	SARS-CoV-2 M ^{pro}	Sinha et al. (2021)
Hesperidin	Flavonoid	NM	40	SARS-CoV-2 M ^{pro} and PL ^{pro}	Kumar et al. (2021)

Hesperidin, Neohesperidin	Hesperidin	<i>Citrus aurantium</i>	1066	SARS-CoV-2 3CL ^{pro}	Wu et al. (2020)
Hypericin	Quinone	<i>Hypericum perforatum</i>	151	SARS-CoV-2 RdRp	Murali et al. (2022)
I-Asarinin	Lignan	<i>P. longum</i>	32	SARS-CoV-2 PL ^{pro}	Lakhera et al. (2021)
Isopomiferin, Silydianin B, Silybin C, Silydianin	Flavonoid	LT	2750 from 463 plants	SARS-CoV-2 spike protein	Majeed et al. (2021)
Kouitchenside I	Flavonoid	<i>Sweritia kouitchensis</i>	1066	SARS-CoV-2 3CL ^{pro}	Wu et al. (2020)
Luteolin	Flavonoid	NM	12	SARS-CoV-2 Spike protein and M ^{pro}	Abd El-Mageed et al. (2021)
Luteolin	Flavonoid	<i>Matricaria chamomilla</i>	Direct study	SARS-CoV-2 M ^{pro}	Habibzadeh and Zohalmezhad (2021)
Luteolin-7-glucoside-3'-glucuronide	Flavone	<i>Melissa officinalis</i>	12	SARS-CoV-2 Spike protein and M ^{pro}	Prasanth et al. (2021)
Michellamine B	Alkaloid	<i>Ancistrocladus korupensis</i>	151	SARS-CoV-2 RdRp	Murali et al. (2022)
Lycopene	Carotenoid	LT	2750 from 463 plants	SARS-CoV-2 spike protein	Majeed et al. (2021)
Mukonicine, Koenine, O-methylmurrayamine A, Girinimbine	Alkaloid	<i>Murraya koenigii</i>	8	SARS-CoV-2 M ^{pro}	Wadanambi et al. (2022)
Mundulinol	Flavonoid	NM	12	SARS-CoV-2 Spike protein and M ^{pro}	Abd El-Mageed et al. (2021)

(continued)

Table 18.2 (continued)

Name of the proposed potential drug candidates	Chemical nature of potential phytotherapeutic agent	Biological source of phytotherapeutic agent	Total number of bioactive phytochemicals screened	Inhibitory site of COVID-19	References
Panduratin A (Pa-A) and its derivatives	Chalcone	<i>Boesenbergia rotunda</i>	28	SARS-CoV-2 M ^{pro}	Vergoten and Bailly (2022)
Platycodin D	Triterpenoid	<i>Platycodon grandiflorus</i>	1066	SARS-CoV-2 PL ^{pro}	Wu et al. (2020)
Racemoside A	Steroidal saponin	<i>A. racemosus</i>	110 from six plants	SARS-CoV-2 Spike protein	Patel et al. (2021)
Rutin, schaftoside, 4,5-di- <i>O</i> -affeoylquinic acid	Phenolic compounds	<i>Artemisia herba-alba</i>	24	SARS-CoV-2 M ^{pro}	Hasan et al. (2022)
Tulsinol A-G	–	<i>O. sanctum</i>	7	SARS-CoV-2 M ^{pro} and PL ^{pro}	Varshney et al. (2020)
Tribulosin	Steroid glycoside	NM	40	SARS-CoV-2 M ^{pro} and PL ^{pro}	Kumar et al. (2021)
Tinocordiside	Sesquiterpene glycoside	<i>T. cordifolia</i>	28	SARS-CoV-2 M ^{pro}	Shree et al. (2022)
Tinosponone	Clerodane diterpene	<i>T. cordifolia</i>	11	SARS-CoV-2 3CL ^{pro}	Krupanidhi et al. (2021)
Sesaminol, Sesamol, Sesami	Lignan	<i>Sesamum indicum</i>	46 from 28 species	SARS-CoV-2 spike protein and M ^{pro}	Natesh et al. (2021)
Shogaulonic acid A, Gingerenone-A, Isogingerenone-B, Zonarenebe, [6]-Paradol, [6]-Shogaol, Cedr-8-ene, Copaene	Phenolic compound, Sesquiterpene, etc.	<i>Z. officinale</i>	42	SARS-CoV-2 M ^{pro}	Prasanth et al. (2020)
Somniferine	Alkaloid	<i>W. somnifera</i>	28	SARS-CoV-2 M ^{pro}	Shree et al. (2022)

Speciophylline, Uncarine F, Uncaric acid	–	<i>Uncaria tomentosa</i>	26	dual strong interaction with interface of the RBD-ACE-2 and the ACE-2 binding site on SARS-CoV-2 RBD viral spike	Yepes-Pérez et al. (2022)
Quadranside-III	Triterpene glucoside	<i>Melissa officinalis</i>	12	SARS-CoV-2 Spike protein and M ^{pro}	Prasanth et al. (2021)
Quercetin	Flavonoid	<i>Embllica officinalis</i>	30	SARS-CoV-2 M ^{pro}	Murugesan et al. (2021)
Quercetin 3-O-(2'',6''-digalloyl)-β-D-galactopyranoside	Flavonoid	<i>Acer okamotoanum</i>	151	SARS-CoV-2 RdRp	Murali et al. (2022)
Ursolic acid	Triterpenoid	<i>O. sanctum</i>	46	SARS-CoV-2 M ^{pro}	Shree et al. (2022)
Vicenin, Isorientin 4'-O-glucoside 2''-O-p-hydroxybenzoate	Flavonoid glycoside	<i>O. sanctum</i>	46	SARS-CoV-2 M ^{pro}	Shree et al. (2022)
Withanone	Polyoxygenated steroids triterpenoid steroidal lactones	<i>W. somnifera</i>	9	Inhibit S-Protein RBD (receptor binding domain) and ACE2	Balkrishna et al. (2020)
Withanoside V	Steroidal saponin	<i>W. somnifera</i>	40	SARS-CoV-2 M ^{pro}	Tripathi et al. (2021)
			28	SARS-CoV-2 M ^{pro}	Shree et al. (2022)
Withanoside VI	Withanolide glycosides	<i>W. somnifera</i>	110 from six plants	SARS-CoV-2 Spike protein	Patel et al. (2021)
Withanoside X		<i>W. somnifera</i>	48	SARS-COV-2 (S) and (N) proteins inhibitor NSP15 endoribonuclease and prefusion spike RBD from SARS-CoV-2	Chikhale et al. (2021a)

(continued)

Table 18.2 (continued)

Name of the proposed potential drug candidates	Chemical nature of potential phytotherapeutic agent	Biological source of phytotherapeutic agent	Total number of bioactive phytochemicals screened	Inhibitory site of COVID-19	References
1,2,3,4,6-Penta- <i>O</i> -galloyl- β -D-glucose	Tannin	<i>Acer okamotoanum</i>	151	SARS-CoV-2 RdRp	Murali et al. (2022)
7-ketositosterol	Sterol	<i>E. officinalis</i>	30	SARS-CoV-2 M ^{pro}	Murugesan et al. (2021)
2,5 dimethyl 1-H Pyrrole; 2,3 diphenyl cyclopropyl methyl phenyl sulphoxide; Benzonitrile m phenethyl	–	<i>Bauhinia variegata</i>	57	SARS-CoV-2 M ^{pro}	More-Adate et al. (2022)
5,7,3',4'-tetrahydroxy-2'-(3,3-dimethylallyl) isoflavone, myricitrin, calceolarioside B, methyl rosmarinat, 3,5,7,3',4',5'-hexahydroxy flavanone-3- <i>O</i> -beta-D-glucopyranoside, (2S)-eriodictyol 7- <i>O</i> -(6'- <i>O</i> -galloyl)-beta-D-glucopyranoside, myricetin 3- <i>O</i> -beta-D-glucopyranoside, licoleafol, and amaranthin	Miscellaneous	Medicinal plant database	32,297	SARS-CoV-2 3CL ^{pro}	ul Qamar et al. (2020)

(1E,6E)-1,2,6,7-tetrahydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione	–	<i>C. longa</i>	267	SARS-CoV-2 M ^{pro}	Gupta et al. (2021)
(4Z,6E)-1,5-dihydroxy-1,7-bis(4-hydroxyphenyl)hepta-4,6-dien-3-one					
10 flavonoids such as Narcissi, Astragalin, Mauritianin, etc.	Flavonol glycosides	<i>Salvadora persica</i>	11	SARS-CoV-2-3CL ^{pro}	Owis et al. (2020)

SARS-CoV-2 main protease (SARS-CoV-2 M^{pro}), SARS-CoV-2 3-chymotrypsin-like protease (SARS-CoV-2 3CL^{pro}), SARS-CoV-2 RNA-dependent RNA polymerase (SARS-CoV-2 RdRp), and SARS-CoV-2 papain like protease (SARS-CoV-2 PLpro)

was recommended as the best potential inhibitor against Mpro of SARS-CoV-2 to combat COVID-19 (Tripathi et al. 2021).

Among 110 antiviral phytochemicals tested from *W. somnifera*, *A. racemosus*, *Zinziber officinalis*, *A. sativum*, *C. longa*, and *Adhatoda vasica*, three phytochemicals (racemoside A, ashwagandhanolide, and Withanoside VI) were recommended as the most effective lead compounds to inhibit the interaction of SARS-CoV-2 S-protein with ACE2 based on the molecular docking and molecular dynamics evaluations (Patel et al. 2021).

In another in silico investigation of 20 phytochemicals found in liquorice (*Glycyrrhiza* species), an essential medicinal plant in traditional Chinese medicine, six compounds (glycyrrhizic acid, isoliquiritin apioside, glyasperin A, liquiritin, 1-methoxyphaseollidin and hedysarimcoumestan B) with high affinity towards the Mpro protein target were identified (Sinha et al. 2021). Based on other parameters, glycyrrhizic acid and isoliquiritin apioside are recommended as the best alternative for developing new leads from liquorice plant against COVID-19 (Sinha et al. 2021).

In a similar in silico molecular docking analysis, andrographolide, andrographic acid, neoandrographolide, 14-deoxyandrographolide, 14-deoxy 11, 12-didehydroandrogra-pholide, onysilin, 14-deoxy 14, 15-dehydroandrographolide, 3, 4-dicaffeoylquinic acid and isoandrographolide derived from *A. paniculata* showed more effective inhibition activity against multiple SARS-CoV-2 receptor targets (Sahithya et al. 2021).

Mandal et al. (2021) enlisted medicinal plants, such as *I. tinctoria*, *T. nucifera*, *P. corylifolia*, *R. palmatum*, and phytochemicals such as bavachinin, psoralidin, betulinic acid, curcumin, hinokinin, etc., which have strong anti-3CL protease activity against coronavirus and their mode of inhibition via literature search.

A total of 89 phytoconstituents from *T. cordifolia* (10), *W. somnifera* (60), and *Punica granatum* (19) were selected for molecular docking analysis to identify potential hits against three structural targets—spike glycoprotein, main protease (M^{Pro}), and uridylate-specific endoribonuclease (EndoU) of novel coronavirus for the multi-target approach of drug design (Rai et al. 2021). Compounds that can target all three targets are likely to have a higher therapeutic index and effectiveness than a single target strategy (Rai et al. 2021). The 24 (six compounds from *T. cordifolia*, 12 compounds from *W. somnifera*, and six compounds from *P. granatum*) potential hits were identified that targeted all three selected proteins (Rai et al. 2021). Among these 24 compounds, 27-hydroxywithanolide B, 27-deoxy-14-hydroxywithaferin A, withanolide, cianidanol, and luteolin show a high potential for development as leads to efficacious drugs against coronavirus (Rai et al. 2021).

In another study, the promising activity of luteolin and mundulinol as inhibitors of COVID-19 via strong stable binding affinity towards the spike protein (Sp) and major protease (M^{Pro}) of SARS CoV-2 with safe and non-toxic effect to the host cell, among the various medicinal plant-derived flavonoids was reported via various computational approaches, such as molecular docking, MD stimulation, drug-likeness and ADMET studies (Abd El-Mageed et al. 2021).

Another molecular-docking analysis elucidated that 13 and 18 phytochemicals present in the ethanolic extract of *M. oleifera* fruits exhibited good binding affinity

towards SARS-CoV-2 spike glycoprotein and human ACE2 receptor, respectively (Siddiqui et al. 2022). Eleven phytoconstituents are recommended for potential use against the COVID-19 pandemic as the majority of them exhibited drug-like properties with no predicted toxicity, although further research is required to validate their effects and mode of action in vitro (Siddiqui et al. 2022).

In similar research, four carbazole alkaloids, namely, mukonicine, *o*-methylnurrayamine A, koenine, and girinimbine derived from the leaves of *Murraya koenigii* have been recommended as lead compound for drug development based on their potential role to restrict SARS-CoV-2 replication by inactivating the Mpro catalytic activity, high affinity, favorable physicochemical and ADMET properties, pharmacophoric features, stability, etc. (Wadanambi et al. 2022).

Among 57 compounds retrieved from the aqueous and methanol extract of *Bauhinia variegata*, three potential lead inhibitors (2,5 dimethyl 1-H Pyrrole, 2,3 diphenyl cyclopropyl methyl phenyl sulphoxide, and Benzonitrile *m* phenethyl) of SARS-CoV-2 M^{pro} were identified by in silico technique which has strong pharmacokinetic characteristics, drug-likeness properties high binding affinity (−5.719 to −5.580 kcal/mol), favorable confirmation and stability towards SARS-CoV-2 M^{pro} (More-Adate et al. 2022). However further in vitro and in vivo studies are required for the development of medication against SARS-CoV-2.

Thirty-four natural phytochemicals and three drugs were identified as potential SARS-CoV-19-RdRp inhibitors after the initial screening of 151 phytochemicals with known antiviral activity from medicinal plant literature and 18 anti-HIV drugs by computational studies (Murali et al. 2022). Further studies, predicted that Michellamine B, Quercetin 3-*O*-(2'',6''-digalloyl)-beta-D-galactopyranoside, Corilagin, Hypericin, and 1,2,3,4,6-Penta-*O*-galloyl-beta-D-glucose residues are the possible drug candidates for COVID-19 treatment that may be further studied in vitro and in vivo to aid in the development of efficient anti-COVID-19 drugs (Murali et al. 2022).

In another study, the molecular docking and molecular dynamic simulation study predicted withanoside V and somniferine from *W. somnifera*; Tinocordiside from *T. cordifolia*; and Vicenin, Ursolic acid, and Isorientin 4'-*O*-glucoside 2''-*O*-*p*-hydroxybenzoate from *O. sanctum* as potent phytochemicals with inhibitory effect on M^{pro} of SARS-CoV-2, among 102 different active phytoconstituents from above stated medicinal plants (Shree et al. 2022). Drug-likeness, ADMET profile, MD simulation of these phytoconstituents showed their safe, stable drug-like properties with no toxicity (Shree et al. 2022).

“Trikadu” is an Indian traditional medicinal formulation made up of *Z. officinale*, *P. nigrum*, and *Piper longum*, which is used as an immune boost in herbal remedies (Khasamwala et al. 2022). A recent in silico analysis on the potential therapeutic benefits of trikadu against COVID-19 showed that several phytochemicals, especially coumarin, coumapherine, and bisdemethoxycurcumin have shown significant bonding interactions with both RNA-dependent polymerase protein and enveloped protein of the SARS-CoV-2 virus (Khasamwala et al. 2022).

Somniferine A, Tinosporide, Tinocordiside, Orientin, Flavonol glucoside, Withanolide, Apigenin, Cyclocurcumin, Withanolide B, Kaempferol, Withanone,

and Withaferin A are the phytochemicals found in the Ayurvedic Kadha (commonly includes *O. sanctum*, *C. longa*, *T. cordifolia*, *Z. officinale*, *Elettaria cardamomum*, *W. somnifera*, *S. aromaticum*, *P. nigrum*, lemon) which have a significantly higher binding affinity with different SARS-CoV-2 proteins (M^{pro}, S, E, N), and significant anti-inflammatory property indicating their potential inhibitory role in viral infection, replication and transcription as well as reduction of the inflammatory response, and boosting the individual's immunity (Maurya and Sharma 2022). They also identified other phytochemicals that have a significant binding affinity with the host ACE2 protein and furin protein inhibiting viral contact with the host receptor and slowing or stopping viral genome entrance into the host (Maurya and Sharma 2022). Similar studies identified withanone from *W. somnifera* (Balkrishna et al. 2020); Dihydrodieuginol B and Tulsinol A, B, C, D, E, F, G from *O. sanctum* (Varshney et al. 2020); rutin, 4,5-di-*O*-caffeoylquinic acid, and schaftoside from *A. herba-alba* (Hasan et al. 2022).

18.7 In Vitro Studies of Medicinal Plant Extract, Traditional Herbal Medicine, and Formulation Against COVID-19

A traditional Chinese poly-herbal formulation (containing 11 herbal ingredients) known as Lianhuaqingwen, strongly suppresses SARS-CoV-2 replication, induces morphological abnormality in the virus, and significantly reduces pro-inflammatory cytokines (TNF- α , IL-6, CCL-2/MCP-1, and CXCL-10/IP-10) production at mRNA levels in a dose-dependent manner with an IC₅₀ of 411.2 μ g/mL when Vero E6 cells infected with 100 TCID₅₀ of SARS-CoV-2 and incubated with Lianhuaqingwen at various concentrations for 72 h (Runfeng et al. 2020). These findings might be intriguing because cytokine storm has been identified as one of the COVID-19 fatal consequences.

In vitro testing revealed that PHELA inhibited >90% of SARS-CoV-2 and SARS-CoV infection at concentrations ranging from 0.005 to 0.03 mg/mL and nearly 100% of MERS-CoV infection at concentrations ranging from 0.1 to 0.6 mg/mL, with average IC₅₀ values of 0.01 mg/mL for all the three viruses (Matsabisa et al. 2020). Furthermore, in silico docking investigations of phytoconstituents of PHELA revealed that Compound 5 had a strong affinity for the SARS-CoV-2 protein. All these findings suggested that PHELA, the herbal mixture of four African medicinal herbs, *Clerodendrum glabrum*, *Gladiolus dalenii*, *Rotheca myricoides*, and *Senna occidentalis*, could be considered a candidate for developed as a COVID-19 therapeutic (Matsabisa et al. 2020).

Shufeng Jiedu capsules (SFJDC), an approved and patented herbal drug composed of eight medicinal plants (*Polygonum cuspidatum*, *Forsythia suspensa*, *I. indigotica*, *Bupleurum chinense*, *Patrinia scabiosifolia*, *Verbena officinalis*, *Phragmites communis*, and *Glycyrrhiza uralensis*), is used to treat various viral

respiratory diseases (Lu et al. 2021). Lu et al. (2021) documented the antiviral and anti-inflammatory properties of SFJDC in the HCoV-229E mouse model based on parameters such as lung index, viral load in the lung, the release of cytokines, and on T- and B-lymphocytes. SFJDC considerably reduced the viral load and inflammatory factors IL-6, IL-10, TNF- α , and IFN- γ in the lung of HCoV-229E mice, and increased the amount of CD4+ and CD8+ cells in the blood compared to control via the influence of 11 inflammation and immunomodulation-related pathways by its bioactive components (Lu et al. 2021). They also identified quercetin, wogonin, and polydatin to be important SARS-CoV-2 Mpro inhibitors. Clinical data also revealed some encouraging indications that the use of SFJDC within the first eight days following the start of symptoms along with standard antiviral therapy, significantly shortens the clinical recovery time of COVID-19, fatigue, and cough compared to AVD alone (Lu et al. 2021).

In vitro investigation with the DCM: MeOH extract of *Gunnera perpensa*, an African traditional medicinal plant used for SARS-CoV-2 management, revealed effective disruption of RBD-ACE2 interactions with an IC₅₀ of <0.001 $\mu\text{g/mL}$ (Invernizzi et al. 2022). Further UPLC-IMS-HRMS analysis of this plant extracts able to identify two ellagitannins, punicalin (2.12% w/w) and punicalagin (1.51% w/w), which antagonized RBD-ACE2 binding with respective IC_{50s} of 9 and 29 nM (Invernizzi et al. 2022).

In vitro assay of hot-water (tea infusion) extract of *A. annua* showed IC₅₀ values of 0.3–8.4 μM (based on measured artemisinin content) and 11.0–67.7 $\mu\text{g DW}$ (based on dried leaf weight) without any significant cell toxicity (Nair et al. 2022). Oral consumption of hot-water (tea infusion) extract of *A. annua* was advised as a potential cost-effective treatment against wild SARS-CoV-2 and all five infectious variants to assist ward off the recent rapid transmission based on the extract inhibition of SARS-CoV-2 and cell viability assay (Nair et al. 2021, 2022). Such treatment offers two additional benefits: a decreased inflammatory response and blunting of fibrosis (Nair et al. 2021).

In vitro inhibition assays were performed to validate the docking simulation results that predicted seven potential inhibitors of SARS-CoV-2 Mpro with affinity scores ranging from -8.8 to -7.4 kcal/mol (Zhu et al. 2022). This study confirmed that (+)-dihydroquercetin, (+)-dihydromyricetin, kaempferol, quercetin, myricetin, isoquercitrin, and rutin are effective in vitro SARS-CoV-2 Mpro inhibitors with IC₅₀ values ranged from 0.125 to 12.9 μM (Zhu et al. 2022).

18.8 Clinical Trials and Observational Studies

The potential of traditional medicine/phytoextract of medicinal plants is being actively investigated through clinical trials in the form of a single drug, single drug formulation, multi-drug formulations, or multi-formulation regimen to uncover both preventative and therapeutic phytoagent. The overall approach to manage the outbreak of COVID-19 broadly comprises preventive and prophylactic, symptom

management, and add-on interventions to conventional care. Several clinical trials of different traditional medicinal plants, formulations, or medicine, especially traditional Chinese medicine and Indian Ayurvedic medicine, have been undertaken to evaluate their potential efficacy and safety for the treatment of COVID-19 patients. Some of these clinical trials are cited in Table 18.3, which covers both completed and ongoing trials as of August 1, 2022, registered in the Chinese Clinical Trial Registry (ChiCTR) (<http://www.chictr.org.cn>), ClinicalTrials.gov (<http://clinicaltrials.gov>), Clinical Trials Registry-India (<http://ctri.nic.in>), the United Kingdoms' ISRCTN registry (<http://www.isrctn.com>), and WHO International Clinical Trials Registry Platform (<https://www.who.int/ictrp/en/>).

18.8.1 Traditional Chinese Herbal Medicine and COVID-19

The previous experiences of developing and using more than 100 herbal traditional Chinese medicine formulations to save the life from epidemic diseases were capitalized widely in a few hospitals in China during the COVID-19 pandemic (Lee et al. 2021). In its COVID-19 treatment guidelines, the Chinese government encourages and recommends traditional Chinese medicine (TCM) as one of the approved therapeutic alternatives for treating COVID-19. Patients receiving TCM treatment in Wuhan and other traditional Chinese medicine hospitals had ten times fewer ICU admissions compared to western treatment methods, including the use of antibiotics and painkillers (Lee et al. 2021). However, scientific evidence supporting the effectiveness of TCM treatment is still inadequate. Thus, several clinical trials are being conducted to generate credible scientific information and validate the effectiveness of TCM treatment (Table 18.3).

Several recently published reviews examined and summarized the potential application of traditional Chinese medicines or formulations derived from medicinal plants (Yang et al. 2020, 2021; Chu et al. 2021; Lee et al. 2021; Luo et al. 2021; Ren et al. 2021a). According to Lee et al. (2021), more than 30 traditional Chinese medicine formulae were implemented in Wuhan, and 121 registered protocols of traditional Chinese medicine were identified from the Chinese Clinical Trial Registry to combat the various stages of the COVID-19 pandemic. *Glycyrrhiza*, *S. baicalensis*, *Z. officinale*, *P. cocos*, *Paeonia lactiflora*, *Ziziphus jujube*, *Pinelliae Rhizoma Praeparatum Cum Zingibere*, *Ephedra sinensis*, *Ramulus Cinnamomi*, *B. chinense*, *P. ginseng*, *Platycodon grandiflorus*, *Magnolia officinalis*, *Agastache rugosa*, *A. membranaceus*, *P. cuspidate*, *R. palmatum* is the most frequently used herbs among the total of 76 medicinal herbs used in more than 30 TCM formulae for treating COVID-19 in China (Lee et al. 2021). The composition and applications of a few forefront traditional Chinese medicines used against COVID-19 are mentioned here.

Currently, Jinhua Qinggan granule, a 12-herbal ingredient containing Chinese formulation originally developed to treat H₁N₁ influenza, is recommended in clinically suspected and asymptomatic COVID-19 patients to suppress disease

Table 18.3 Clinical trials of different traditional/herbal medicine for the treatment of SARS-CoV-2 infection

Traditional herbal medicine/formulation/medicinal plant extract	Name of plant species present as ingredients	Title	Registration number	Design type/Study design	Sample size	Phase
AEV01 (Kutki)	Root extract of <i>Picrorhiza kurroa</i>	A randomized, placebo-controlled, double-blind, parallel group clinical study to evaluate the efficacy and safety of AEV01 (Kutki) for mild COVID-19 in elderly patients at risk of complications	CTRI/2021/04/032804	Randomized, parallel	70	3
Arogya Kashayam-20 decoction	<i>Tinospora cordifolia</i> , <i>Glycyrrhiza glabra</i> , <i>P. niruri</i> , <i>Terminalia chebula</i> , <i>Zingiber officinale</i> , <i>Piper nigrum</i> , <i>Piper longum</i>	Evaluation of efficacy of Arogya Kashayam in asymptomatic and mild symptomatic positive cases of COVID-19—A randomized control study	CTRI/2020/06/026221	Randomized, parallel	100	2
Ashtadashang Ghana Vati	Not available	Effect of Malla Chandrodaya and Ashtadashang Ghana Vati in patients with mild to moderate Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infections	CTRI/2020/06/026229	Non-randomized	100	2
Ashwagandha	<i>Withania somnifera</i>	A prospective non-randomized open labeled controlled interventional study on the effect of Ashwagandha (<i>Withania somnifera</i>) as a prophylactic measure among high-risk population (Health Care Workers/Containment Zone Population) exposed to COVID-19	CTRI/2020/05/025429	Non-randomized	5000	2/3

(continued)

Table 18.3 (continued)

Traditional herbal medicine/formulation/medicinal plant extract	Name of plant species present as ingredients	Title	Registration number	Design type/ Study design	Sample size	Phase
Traditional herbal medicine/formulation/medicinal plant extract		A study of Ashwagandha administration in participants vaccinated against COVID-19 on safety, immunogenicity, and protection: A randomized, double-blind, placebo-controlled, multi-centric clinical trial	CTRI/2021/06/034496	Randomized, parallel	1200	3
Ashwagandha, Sudarshana Ghana Vati, Shanshamani Vati	Ashwagandha— <i>W. somnifera</i> ; Sudarshana Ghanavati— <i>Swerdia chirata</i> , <i>T. chebula</i> , <i>Embllica officinalis</i> , <i>Holarrhena antidiysenterica</i> , etc.; Shanshamani Vati— <i>T. cordifolia</i>	Impact of Ayurvedic interventions in prevention of COVID-19 infection in containment areas of Delhi—A community-based study	CTRI/2020/05/025069	Single arm study	9200	3/4
ASMO capsule	<i>Adathoda vasica</i> , <i>Tylophora ashamatica</i> , <i>Coelus forskohlii</i> , <i>Ocimum sanctum</i> , <i>Inula racemosa</i> , <i>Alpinia galangal</i>	A clinical study to assess the efficacy of cap ASMO in COVID-19 positive cases an open labelled single arm study	CTRI/2020/12/029714	Single arm study	100	2
AYUSH-64 tablet	<i>Alstonia scholaris</i> , <i>P. kurroa</i> , <i>S. chirata</i> , <i>Caesalpinia crista</i>	Efficacy of Ayurveda intervention (AYUSH 64) as add-on therapy in COVID-19 patients—An open label randomized controlled trial	CTRI/2020/06/025855	Randomized, parallel	200	2
		Evaluation of efficacy and safety of Ayurveda intervention (AYUSH-64) in the	CTRI/2020/05/025338	Single arm study	40	2/3

AYUSH-64 tablet, Yashtimadhu, Sanshamani Vati	AYUSH 64 tablet— <i>A. scholaris</i> , <i>P. kurroa</i> , <i>S. chirata</i> , <i>C. crista</i> ; Yashtimadhu—root extract <i>G. glabra</i> ; Sanshamani Vati— <i>T. cordifolia</i>	management of COVID-19 infection (asymptomatic and mild to moderate symptoms)—An open label single arm prospective clinical trial	CTRI/2020/06/025557	Randomized, parallel	420	2
AYUSH 64 tablet, Sanshamani Vati, Vataleshma Jwarhar decoction	AYUSH 64 tablet— <i>A. scholaris</i> , <i>P. kurroa</i> , <i>S. chirata</i> , <i>C. crista</i> ; Yashtimadhu—root extract <i>G. glabra</i> ; Sanshamani Vati— <i>T. cordifolia</i>	A randomized controlled clinical study on Ayurveda intervention (Ayush 64, Sanshamani Vati and Vataleshma Jwarhar Kwatha) along with standard care treatment in mild to moderate patients of COVID-19	CTRI/2021/05/033651	Randomized, parallel	30	NA
AYUSH Kwath	<i>O. sanctum</i> , <i>Cinnamomum zeylanicum</i> , <i>Zingiber officinale</i> , <i>P. nigrum</i>	Evaluation of the efficacy of Ayush Kwath in the prevention of COVID-19 pandemic among quarantine persons—an open label single arm prospective study	CTRI/2020/06/025779	Single arm study	50	NA
AYUSH medicine NOQ19 (NF2)	Polyherbal formulation	A randomized, double-blind, placebo-controlled trial to determine the therapeutic	CTRI/2021/10/037423	Randomized, parallel	100	3/4

(continued)

Table 18.3 (continued)

Traditional herbal medicine/formulation/medicinal plant extract	Name of plant species present as ingredients	Title	Registration number	Design type/Study design	Sample size	Phase
Bresol tablet	<i>Curcuma longa</i> , <i>Z. officinalis</i> , <i>P. longum</i> , <i>P. nigrum</i> , <i>T. chebula</i> , <i>Terminalia bellerica</i> , <i>Phyllanthus emblica</i> , <i>O. sanctum</i> , <i>A. vasica</i> , <i>Embelia ribes</i> , <i>Cyperus rotundus</i> , <i>C. zeylanicum</i> , <i>Elettaria cardamomum</i> , <i>Cinnamomum tamala</i> , <i>Mesua ferrea</i>	efficacy of Ayush medicine NOQ19 (NF2) in treatment of symptomatic COVID-19 patients along with standard allopathic treatment A clinical study to evaluate the role of herbal Immunomodulators (Tab Septilin and Tab Bresol) as add on treatment in asymptomatic and mildly symptomatic COVID-19 confirmed cases	CTRI/2020/06/025801	Randomized, parallel	40	2/3
Bufei Huoxue capsule	<i>Astragalus membranaceus</i> var. <i>mongholicus</i> , <i>Paeonia lactiflora</i> , <i>Psoralea corylifolia</i>	A randomized, double-blind, controlled trial for Bufei Huoxue capsule in the treatment of convalescent COVID-19 patients	ChiCTR2000032573	Parallel	120	0
CLEVIRA tablet	<i>Carica papaya</i> , <i>Melia azedarach</i> , <i>Andrographis paniculata</i> , <i>Vetiveria zizanioides</i> , <i>T. cordifolia</i> , <i>C. rotundus</i> , <i>Z. officinale</i> , <i>P. nigrum</i> , <i>Mollugo cerviana</i> , <i>Trichosanthes dioica</i>	A randomized, multicentric, open label, parallel group clinical study to evaluate the efficacy and safety of CLEVIRA in mild to moderate COVID-19 positive patients	CTRI/2020/05/025483	Randomized, Parallel Group Trial	100	3/4

Colchicine	<i>Colchicum autumnale</i> , <i>Gloriosa</i> spp. etc.	Colchicine Coronavirus SARS-CoV-2 trial (COLCORONA)	NCT04322682	Randomized, parallel	4506	3
Colchicine		Preemptive therapy with colchicine in patients older than 60 years with high risk of severe pneumoniae due to Coronavirus SARS-CoV-2 (COVID-19)	NCT04416334	Randomized, parallel	954	3
Colchicine		Randomized clinical trial for the treatment of moderate to severe cases of COVID-19 with Chloroquine and Colchicine	U1111-1255-6304	Randomized, parallel	-	-
Colchicine plus herbal phenolic monoterpene fractions Oral	<i>C. autumnale</i> , <i>Gloriosa</i> spp. etc.	Application of colchicine plus herbal phenolic monoterpene fractions to treat COVID-19	NCT04392141	Randomized	120	1/2
Curcumin tablet	<i>C. longa</i> , <i>P. nigrum</i>	Evaluation of effects of Tablet Curcumin and adjuvants in exposed individuals and carriers of corona virus, in terms of changes in acute phase reactants and clinical status over 15 days	CTRI/2020/05/025482	Randomized, parallel	50	NA
Danggui Shaoyao powder	<i>Angelica sinensis</i> , <i>P. lactiflora</i> , <i>Ligusticum chuansiong</i> , <i>Poria cocos</i> , <i>Atractylodes macrocephala</i> , <i>Alisma orientalis</i>	Clinical study for Danggui Shaoyao powder in the treatment of novel coronavirus (COVID-19) pneumonia	ChiCTR2000032098	Single arm study	300	NA
Dashamula Kwatha, Pathyadi Kwatha with Trikatu Churna, Sansamani	<i>Aegle marmelos</i> , <i>Premna optusifolia</i> , <i>Gmelina arborea</i> , <i>Oroxylum indicum</i> , <i>Stereospermum colais</i> ,	Evaluation of efficacy of an Ayurved Management as a supportive therapy in SARS-CoV-2 mild positive patients—	CTRI/2020/07/026433	Randomized, parallel	25	NA

(continued)

Table 18.3 (continued)

Traditional herbal medicine/formulation/medicinal plant extract	Name of plant species present as ingredients	Title	Registration number	Design type/Study design	Sample size	Phase
Vati, AYUSH 64, Yastimadhu Ghanavati	<i>Desmodium gangeticum</i> , <i>Solanum anguivi</i> , <i>S. virginianum</i> , <i>Tribulus terrestris</i> , <i>Uraria picta</i> , <i>T. chebula</i> , <i>E. officinalis</i> , <i>A. paniculata</i> , <i>C. longa</i> , <i>P. nigrum</i> , <i>P. longum</i> , <i>Z. officinalis</i> , <i>T. cordifolia</i> , <i>A. scholaris</i> , <i>P. kurroa</i> , <i>S. chirata</i> , <i>C. crista</i> , <i>Glycyrhiza glabra</i> , etc.	An open labeled randomized active control trial				
ES16001 Oral	<i>Elaeocarpus sylvestris</i> var. ellipticus extract	A prospective, randomized, double-blind, placebo-controlled clinical study to evaluate the efficacy and safety of ES16001 as an adjunct to standard of care treatment on mild to moderate COVID-19 positive subjects undergoing treatment for COVID-19 in Hospital Quarantine (Dedicated COVID Health Centre)	CTRI/2020/09/027775	Randomized, parallel	60	2
<i>Eucalyptus</i> oil	<i>Eucalyptus citriodora</i> , <i>E. globulus</i> , <i>Mentha piperita</i>	Eucalyptus oil as adjuvant therapy for Coronavirus disease (COVID-19), a randomized open-label clinical trial	NCT05398965	Randomized	54	2
Gorochanadi Vati	46 ingredients, such as <i>Z. officinale</i> , <i>Cinnamomum</i>	A study of Gorochanadi Vati as an add on efficacy in moderate	CTRI/2021/06/034114	Non-randomized	60	2

	<i>camphora</i> , <i>P. longum</i> , <i>Convolvulus prostratus</i> , <i>Elaeocarpus angustifolius</i> , etc.	and severe COVID-19 positive subjects with reduced oxygen saturation				
Guduchi Ghan Vati capsule/tablet	<i>T. cordifolia</i>	Evaluation of efficacy and safety of Ayurveda intervention (Guduchi Ghan Vati) in the management of COVID-19 infection (Asymptomatic and Mild symptoms)—An open label single arm prospective clinical trial	CTRI/2020/05/025370	Single arm study	40	NA
		A prospective randomized controlled clinical trial to evaluate the efficacy and safety of Guduchi Ghan Vati in the management of COVID-19 infection.	CTRI/2020/07/026840	Randomized, parallel	30	NA
Guduchi tablet	<i>T. cordifolia</i>	An open label, prospective, clinical study to evaluate the role of herbal immunomodulators (Tab Immusante and Tab Guduchi) in boosting the immunity among healthcare workers assigned to COVID-19 wards	CTRI/2020/07/026579	Randomized, parallel	100	3
Gu-Biao Jie-Du-Ling	Not available	Clinical study for Gu-Biao Jie-Du-Ling in preventing of novel coronavirus (COVID-19) pneumonia in children	ChiCTR2000029487	Case-control study	200	0
Han Shi Yi Decoction	Not available	The Efficacy of HanShiYi Decoction for COVID-19	ChiCTR2200058384	Parallel	300	0

(continued)

Table 18.3 (continued)

Traditional herbal medicine/formulation/ medicinal plant extract	Name of plant species present as ingredients	Title	Registration number	Design type/ Study design	Sample size	Phase
Haldi	<i>C. longa</i>	Patients: A Randomized, Controlled, Open-label Clinical trial Efficacy of Ayurvedic medicine Haldi 30 drops as an add-on medication to standard of care in adult patients of mild to moderate COVID-19. A phase II randomized, single-blind, parallel group, placebo-controlled trial	CTRI/2020/07/026841	Randomized, parallel	260	2
Homeopathic medicine	<i>Neolamarckia cadamba</i>	Drug proving and checking its effectiveness in treatment of COVID-19	CTRI/2020/04/024947	Randomized, parallel	100	3
Immunofree tablet	<i>A. paniculata</i> , <i>P. niruri</i> , <i>G. glabra</i> , <i>O. sanctum</i> , <i>T. cordifolia</i> , <i>L. racemose</i> , <i>A. galangal</i> , <i>Vitis Vinifera</i> , <i>C. longa</i> , <i>T. chebula</i> , <i>Aloe barbadensis</i> , etc.	An open label, multicenter, randomized, controlled, clinical study to evaluate the efficacy and safety of a combination of treatment of Regimmune capsule and Immunofree tablets compared with standard treatment protocol in the treatment of mild to moderate COVID-19 patients	CTRI/2020/08/026957	Randomized, parallel	100	3/4
Imusil tablet	<i>P. kurroa</i> , <i>T. cordifolia</i> , <i>E. officinalis</i>	A prospective, randomized, multi-center, open-label, interventional study to evaluate the	CTRI/2022/02/040687	Randomized, parallel	100	4

Immusante tablet	<i>Prosopis glandulosa</i> , <i>Symplocos racemosa</i>	safety and efficacy of Imusil (kutki extract 200 mg plus guduchi extract 60 mg plus amla extract 60 mg) tablet in treatment of adult subjects with mild COVID-19	CTRI/2020/07/026579	Randomized, parallel	100	3
Jing-Guan-Fang decoction	Not available	An open label, prospective, clinical study to evaluate the role of herbal immunomodulators (Tab Immusante and Tab Guduchi) in boosting the immunity among healthcare workers assigned to COVID-19 wards	NCT05236309	Retrospective case study	2000	NA
JieJiXuanFeiChuYi granule	Not available	Effect of JieJiXuanFeiChuYi granule on patients with COVID-19 (Omicron): A randomized, open-label, active drug parallel controlled trial	ChiCTR2200058245	Parallel	60	0
Jing-Yin granule	Not available	Efficacy and safety of Jing-Yin granule in the treatment of novel coronavirus pneumonia (COVID-19) wind-heat syndrome	ChiCTR2000030255	Parallel	300	4
Kang-Bing-Du granule	Not available	A multicenter, randomized, open and controlled trial for the efficacy and safety of Kang-Bing-Du granules in the	ChiCTR20000029781	Parallel	160	4

(continued)

Table 18.3 (continued)

Traditional herbal medicine/formulation/medicinal plant extract	Name of plant species present as ingredients	Title	Registration number	Design type/Study design	Sample size	Phase
Kan-Jang capsule	<i>A. paniculata</i> , <i>Eleutherococcus senticosus</i>	treatment of novel coronavirus (COVID-19) pneumonia Effect of Kan-Jang® supplementation in patients diagnosed with COVID-19: A randomized, quadruple-blind, placebo-controlled trial	NCT04847518	Randomized, parallel	140	2/3
Ke Su Ting syrup/Ke Qing capsule	Not available	A randomized, open-label, controlled trial for the safety and efficiency of Kesuting syrup and Keqing capsule in the treatment of mild and moderate novel coronavirus (COVID-19) pneumonia	ChiCTR2000029991	Parallel	72	4
Lian-hua Qing-wen	<i>Forsythia suspensa</i> , <i>Lonicera japonica</i> , <i>Ephedra sinica</i> , <i>Dryopteris crassirhizoma</i> , <i>Isatis indigotica</i> , <i>Pogostemon cablin</i> , <i>Rhodiola saera</i> , <i>Houttuynia cordata</i> , <i>Rheum palmatum</i> , <i>Prunus armeniaca</i> var. <i>ansu</i> , <i>Glycyrrhiza uralensis</i> , and menthol	Effective cluster randomized controlled study of Lianhua Qingwen in the treatment of asymptomatic infections and mild confirmed cases of novel coronavirus	ChiCTR2200058639	Parallel	30,000	4
Lian-hua Qing-wen		The efficacy of lianhuaqingwen in the treatment of patients infected with novel coronavirus variant Omicron	ChiCTR2200057841	Single arm	400	4
Lian-Hua Qing-Wen capsule		Real-world study on the prevention effect of Lian-Hua Qing-Wen capsules on close	ChiCTR2100043012	Parallel	2200	4

Lian-hua Qing-wen capsule	contacts of novel coronavirus (COVID-19) pneumonia A randomized, double-blind, international multi-center clinical study to investigate efficacy and safety of Lianhua Qingwen capsules compared to placebo and combined with standard of care (SOC) in adult patients with mild to moderate COVID-19	ChiCTR2200056727	Parallel	860	4
Lian-hua Qing-wen capsule	A randomized, open-label, blank-controlled trial for Lian-Hua Qing-Wen capsule in the treatment of novel coronavirus (COVID-19) pneumonia	ChiCTR2000029434	Parallel	284	4
Lian-Hua Qing-Wen capsule/granule	A randomized, open-label, blank-controlled, multi-center clinical study for Lian-Hua Qing-Wen capsule/granule in the treatment of asymptomatic patients with novel coronavirus (COVID-19) pneumonia	ChiCTR2100042066	Parallel	120	4
Medihope	Not available	CTRI/2020/09/027975	Other	880	3
MSL	Mahasudarshan Ghana vati, Samsamani vati, Lavangadi vati	CTRI/2020/08/027040	Randomized, parallel	200	3

(continued)

Table 18.3 (continued)

Traditional herbal medicine/formulation/ medicinal plant extract	Name of plant species present as ingredients	Title	Registration number	Design type/ Study design	Sample size	Phase
Pinak tablet	<i>Erythrina indica</i> , <i>Magnefera indica</i> , <i>Eugenia jambolana</i> , <i>Jusminum sambac</i>	progression of severity of the disease in asymptomatic and mild symptomatic cases of COVID-19: A randomized controlled trial				
	<i>Erythrina indica</i> , <i>Magnefera indica</i> , <i>Eugenia jambolana</i> , <i>Jusminum sambac</i>	Ayurvedic proprietary adjuvant oral intervention Tab PINAK for COVID patients towards early recovery.	CTRI/2020/05/025326	Randomized, parallel	30	2
Qingjin Yiqi granules	16 herbs: Renshen (<i>Ginseng Radix Et Rhizoma</i>), Maidong (<i>Ophiopogonis Radix</i>), Wuweizi (<i>Schisandrae Chinensis Fructus</i>), Fuling (<i>Poria</i>), Banxia (<i>Pinelliae Rhizoma</i>), Xuanshen (<i>Scrophulariae Radix</i>), Cangzhu (<i>Atractylodis Rhizoma</i>), Chenpi (<i>Citri Reticulatae Pericarpium</i>), Gancao (<i>Glycyrrhizae Radix Et Rhizoma</i>), Chaihu (<i>Bupleuri Radix</i>), Shengma (<i>Cimicifugae Rhizoma</i>), Yiyiren (<i>Coicis Semen</i>), Huangqin (<i>Scutellariae Radix</i>), Mabiancao (<i>Verbenae Herba</i>), Lugen (<i>Phragmitis Rhizoma</i>),	Qingjin Yiqi Granules for patients in pneumonia recovery phase (COVID-19, Omicron): A clinical study	ChiCTR2200056638	Parallel	312	0

	and Danzhuye (<i>Lophatheri Herba</i>) found in <i>Dimorphandra mollis</i> , <i>Allium cepa</i>						
Quercetin (Flavonoid)		The effectiveness of phytotherapy in SARS-CoV-2 (COVID-19)	NCT04851821	Randomized, double-blind	80	1	
Raj Nirwan Bati	<i>P. nigrum</i> , <i>Artemisia nilagirica</i> , <i>Carum copiticum</i>	To determine the efficacy of an Ayurvedic preparation Raj Nirwan Bati (RNB) on symptomatic COVID-19 patients: A double-blind, randomized, controlled trial	CTRI/2020/06/025998	Randomized, parallel	60	3	
		Therapeutic response of allo-vedic preparation Raj Nirwan Bati (RNB) on severe COVID-19 patients: A double-blind, randomized, placebo-controlled clinical trial	CTRI/2020/08/027467	Randomized, parallel	40	3	
ReDuNing injection	<i>Artemisia annua</i> , <i>L. japonica</i> , <i>Gardenia jasminoides</i>	An open, prospective, multicenter clinical study for the efficacy and safety of Reduning injection in the treatment of novel coronavirus (COVID-19) pneumonia	ChiCTR2000029589	Parallel	60	0	
Septilin tablet	<i>Commiphora wightii</i> , <i>T. cordifolia</i> , <i>E. officinalis</i> , <i>G. glabra</i> , <i>Rubia cordifolia</i> , <i>Moringa pterygosperma</i>	A clinical study to evaluate the role of herbal immunomodulators (Tab Septilin and Tab Bresol) as add on treatment in asymptomatic and mildly symptomatic COVID-19 confirmed cases	CTRI/2020/06/025801	Randomized, parallel	40	2/3	

(continued)

Table 18.3 (continued)

Traditional herbal medicine/formulation/ medicinal plant extract	Name of plant species present as ingredients	Title	Registration number	Design type/ Study design	Sample size	Phase
Shen Cao Gan Jiang Tang (Gan Cao Gan Jiang Tang with Ginseng)	<i>Glycyrrhiza</i> sp., <i>Z. officinale</i> , <i>Panax ginseng</i>	Efficacy of Shen Cao Gan Jiang Tang (Gan Cao Gan Jiang Tang with Ginseng) on mild and moderate COVID-19 patients: A multicenter, prospective, randomized controlled trial	NCT05055427	Randomized, parallel	300	2/3
Shen Fu injection	<i>P. ginseng</i> , <i>Aconitum carmichaeli</i>	Shen-Fu injection in the treatment of severe novel coronavirus (COVID-19) pneumonia: A multicenter, randomized, open-label, controlled trial	ChiCTR2000030043	Parallel	300	4
Shen-Qi Fu-Zheng injection	<i>P. ginseng</i> , <i>A. membranaceus</i>	A multicenter, randomized, open, controlled trial for the efficacy and safety of Shen-Qi Fu-Zheng injection in the treatment of novel coronavirus (COVID-19) pneumonia	ChiCTR2000029780	Parallel	160	4
Shirashadi Kasai	<i>Albizia lebbbeck</i> , <i>Solanum xanthocarpum</i> , <i>A. vasica</i> , <i>G. glabra</i> , <i>Cinnamum tamal</i>	A trial of Shirashadi Kasai in adults hospitalized with moderate to severe COVID-19	CTRI/2020/06/026190	Randomized, parallel	160	2
Shuang-Huang-Lian oral liquid	<i>Scutellariae baicalensis</i> , <i>L. japonica</i> , <i>F. suspense</i>	A randomized, open-label, blank-controlled, multicenter trial for Shuang-Huang-Lian oral solution in the treatment of novel coronavirus (COVID-19) pneumonia	ChiCTR2000029605	Parallel	400	4

Shugan Jieyu capsules	<i>E. senticosus</i> , <i>Hypericum perforatum</i>	A randomized, double-blind, placebo-controlled trial of Shugan Jieyu capsules for improving sleep and mood disorders during recovery from novel coronavirus (COVID-19) pneumonia	ChiCTR2100043108	Parallel	100	4
Swarna Amalaki	<i>E. officinalis</i>	Study of Swarna Amalaki as an immunomodulator in COVID-19 Patients	CTRI/2020/10/028414	Randomized, parallel	60	2
Tablets of purified aqueous extract of <i>Cocculus hirsutus</i>	<i>Cocculus hirsutus</i>	An open label, multicenter, randomized, controlled clinical trial to evaluate the efficacy and safety of Tablets of purified aqueous extract of <i>Cocculus hirsutus</i> in treatment of moderate COVID-19 disease	CTRI/2020/05/025397	Randomized, parallel	210	2
TADIOS	<i>Taraxacum officinale</i> , <i>Dioscorea batatas</i> and <i>Schizonepeta tenuifolia</i>	Multicenter, double-blind, placebo-controlled, maximum 10 days administration study to evaluate the efficacy and safety of TADIOS as an adjuvant therapy in patients diagnosed with mild to moderate COVID-19	NCT05552625	Randomized, parallel	100	2/3
Tanreqing capsule	Five water-soluble herbals extracts: <i>S. baicalensis</i> , bear bile powder, <i>Naemorhedus goral</i> , (7%), <i>L. japonica</i> , <i>F. suspense</i>	Tanreqing capsule: a randomized, double-blind, placebo-controlled, multicenter clinical trial of efficacy and safety in the treatment of COVID-19 (mild, general)	ChiCTR2200058676	Parallel	480	4

(continued)

Table 18.3 (continued)

Traditional herbal medicine/formulation/ medicinal plant extract	Name of plant species present as ingredients	Title	Registration number	Design type/ Study design	Sample size	Phase
Tanreqing injection		Clinical trial for Tanreqing capsules in the treatment of novel coronavirus (COVID-19) pneumonia	ChiCTR2000029813		72	0
		A real-world study for the efficacy and safety of large dose Tanreqing injection in the treatment of patients with novel coronavirus (COVID-19) pneumonia	ChiCTR2000029432	Sequential	72	4
	Vasa Ghana, Guduchi Ghana, Vasa-Guduchi Ghana	Whole aqueous extract of <i>A. vasica</i> , <i>T. cordifolia</i>	CTRI/2020/09/028043	Randomized, parallel	50	4

Vyaghryadi Kashay tablet	<i>S. xanthocarpum</i> , <i>Z. officinale</i> , <i>T. cordifolia</i> , <i>P. longum</i>	An interventional, single arm study to observe and assess the effectiveness of “Vyaghryadi Kashaya Tab” in preventing the progression of severity in asymptomatic, mild to moderate COVID-19 cases.	CTRI/2020/10/028570	Single arm study	30	2/3
Vyaghryadi Kashaya decoction		A comparative clinical study to involving Ayurvedic drug intervention in the management of COVID 19 patients.	CTRI/2020/06/025800	Randomized, parallel	120	2
Xinguan Fuzheng Zhuanyin decoction	Not available	A randomized, double-blind, placebo-controlled trial for Xinguan Fuzheng Zhuanyin decoction to reduce the viral load of the delta variant of COVID-19	ChiCTR2100047596	Parallel	100	0
Xi Yan Ping injection	<i>A. paniculate</i>	A multicenter, randomized, open, parallel controlled trial for the evaluation of the effectiveness and safety of Xiyanping injection in the treatment of common type novel coronavirus (COVID-19) pneumonia	ChiCTR2000030117	Parallel	348	4
YinHu QingWen decoction	Eleven traditional Chinese medicine such as <i>Polygonum cuspidatum</i> , <i>Lonicera</i> , <i>Nepeta</i> , <i>Ligustrum lucidum</i> , <i>Schizonepeta</i> , etc.	Adaptive, randomized, single-blind, three-arm parallel controlled clinical trial of Yinhu Qingwen decoction in the treatment of mild/common COVID-19	NCT04278963	Randomized, parallel	300	2/3

(continued)

Table 18.3 (continued)

Traditional herbal medicine/formulation/medicinal plant extract	Name of plant species present as ingredients	Title	Registration number	Design type/ Study design	Sample size	Phase
Yinhu Qingwen granula		An adaptive, randomized, double-blind, parallel-controlled clinical trial of Yinhu Qingwen granula for the treatment of severe COVID-19	NCT04310865	Randomized, parallel, double-blind	116	2/3
Yiqing oral liquid	Not available	Yiqing oral liquid and paxlovid in the treatment of novel coronavirus infection randomized controlled clinical studies	ChiCTR2200059502	Parallel	80	0
Yu-Ping-Feng formula, Xiang-Sha-Liu-Jun formula, Liu-Wei-Di-Huang formula	Not available	Based on the theory of “Body Constitution of Chinese Medicine” and “Combination of Prescription and Syndrome” to improve COVID-19 susceptible body constitution of residents in Hong Kong	NCT04668222	Randomized, parallel	480	1/2
	<i>Tricholoma matsutake</i> , <i>Cannabis sativa capsule</i> and <i>Dendrobium candidum</i>	Evaluation of the effect of taking <i>Tricholoma matsutake</i> , <i>Cannabis sativa capsule</i> and <i>Dendrobium candidum</i> to nutrition intervention of patients with novel coronavirus (COVID-19) pneumonia during convalescence	ChiCTR2000030920	Parallel	100	NA

TD0069 capsule	TD0069 capsule is a product based on the traditional medicine named “Ren shen bai du san”	A phase III, randomized, double-blind, placebo-controlled study to evaluate the safety and efficacy of TD0069 capsule for patients with mild to moderate COVID-19	NCT05249777	Randomized, parallel	573	3
Hesperidin and Diosmin mixture	Not available	Randomized, double-blind, controlled parallel study of (Hesperidin and Diosmin Mixture) for treatment of COVID-19 newly diagnosed patients in Egypt	NCT04452799	Randomized, parallel	100	1

development and prevent disease progression in individuals with mild to severe infection (Kageyama et al. 2022). It is made up of Jinyin hua (*Lonicerae Japonicae* Flos, honeysuckle), Shigao (Gypsum fibrosum moles, Gypsum fibrosum), *Ephedra herba*, Kuxing ren (Amygdalus Communis Vas, Armeniacae Semen Amarum), *S. baicalensis*, Lianqiao (Forsythiae Fructus, *Fructus forsythiae*), Zhebeimu (*Fritillariae thunbergii* Bulbus, Thunberg Fritillary Bulb), Zhimu (Anemarrhenae Rhizoma, Rhizoma Anemarrhenae), Niubangzi (Fructus Arctii, Arctii Fructus), Qinghao (*A. annua*, *Artemisiae annua herba*), Bohe (*Menthae Herba*, *Menthae haplocalycis* Herba) and Gancao (Licorice, Liquorice) (Kageyama et al. 2022). According to an open-label, single-arm study, Jinhua Qinggan granule may also be beneficial in reducing disease progression in a patient with moderate to severe infection due to its rapid immunomodulatory effects via rapidly decreasing of two critical COVID-19 exacerbating factors in blood (neutrophils and IL-6), and increasing blood levels of lymphocytes and IFN- γ (Kageyama et al. 2022). Another short retrospective course showed that the Jinhua Qinggan granule administered at the onset of COVID-19 was safe and effective in reducing hospitalization and mortality in elderly COVID-19 patients who are at risk of progression (Lin et al. 2022).

Based on the principle of collateral disease, the Lian-hua Qing-wen capsule was developed under the guidance of traditional Chinese medicine and has now become a representative proprietary Chinese medicine that has been approved in various countries such as Singapore, Philippines, Thailand, Kuwait, Romania, Kenya, etc. for drug registration (Sun et al. 2022). It is composed of 11 herbs, namely *F. suspensa* (forsythia fruit), *L. japonica* (honeysuckle flower), the fried stem of *Ephedra sinica*, the root of *Dryopteris crassirhizoma*, root of *I. indigotica*, *Pogostemon cablin* (patchouli), *Rhodiola saera* (integripetal rhodiola herb), *H. cordata* (houத்துynia), root and rhizome of *R. palmatum* (rhubarb), the fried seed of *Prunus armeniaca* var. ansu (apricot), the root of *Glycyrrhiza uralensis* (licorice), along with menthol and a traditional Chinese mineral medicine, *Crystalline gypsum* (Jia and Wu 2021). According to Sun et al. (2022), nine trials with 1152 participants with COVID-19 treated with Lian-hua Qing-wen capsule alone or in combination with chemical drugs showed significantly higher computed tomography recovery rate, increased disappearance rates of fever, cough, fatigue, shortness of breath, and muscle soreness compared to the control group.

Qingfei Paidu decoction that made up of 21 herbal components, namely, *Ephedra sinica*, *G. uralensis*, *Prunus armeniaca*, Raw Gypsum, *Cinnamomum cassia*, *Alisma plantago-aquatica*, *Polyporus umbellaru*, *Atractylodes macrocephala*, *P. cocos*, *B. chinensis*, *S. baicalensis*, *Iris domestica* *Pinellia Ternata*, *Z. officinale*, *Aster tataricus*, *Tussilago farfara*, *Asarum sieboldii*, *Dioscorea oppositifolia*, *Citrus aurantium*, *Citrus reticulate*, *Pogostemon cablin* (Ren et al. 2021a, b), is one of the first herbal medicine approved as a general prescription for treatment of COVID-19 (Zhang et al. 2022). An open-label, single-arm pilot study showed that Qingfei Paidu decoction elevates the blood levels of pro-inflammatory cytokines (Kageyama et al. 2021). According to network pharmacology study of Qingfei Paidu decoction, it contains 948 chemically diverse ingredients that influence virus invasion and viral

replication, along with the suppression of the cytokines activation via interaction with 790 potential target proteins (Zhao 2020).

Several clinical studies have been conducted with another Chinese herbal medicine, Qingjin Yiqi granules, against COVID-19 patients, especially during the post-COVID-19 recovery phase (Pang et al. 2022; ChiCTR2200056638). It comprises 16 herbals: Renshen (*Ginseng Radix Et Rhizoma*), Maidong (*Ophiopogonis Radix*), Wuweizi (*Schisandrae Chinensis Fructus*), Fuling (*Poria*), Banxia (*Pinelliae Rhizoma*), Xuanshen (*Scrophulariae Radix*), Cangzhu (*Atractylodis Rhizoma*), Chenpi (*Citri Reticulatae Pericarpium*), Gancao (*Glycyrrhizae Radix Et Rhizoma*), Chaihu (*Bupleuri Radix*), Shengma (*Cimicifugae Rhizoma*), Yiyiren (*Coicis Semen*), Huangqin (*Scutellariae Radix*), Mabiancao (*Verbenae Herba*), Lugen (*Phragmitis Rhizoma*), and Danzhuye (*Lophatheri Herba*) (Pang et al. 2022).

In TCM, ReDuNing injection is popular for its antibacterial, antiviral, antipyretic, and anti-inflammatory effects. Few preliminary studies showed that it helps to relieve clinical symptoms of COVID-19 and reduces SARS-CoV-2 infection by regulating inflammatory cytokine-related disorders without any adverse effect when applied to COVID-19 patients (Ma et al. 2021; Xu et al. 2021). As a result, the National Health Council and the National Administration of Chinese Medicine have both endorsed ReDuNing for the diagnosis and treatment of SARS-CoV-2-associated pneumonia. It is composed of three herbs—*A. annua*, *L. japonica*, and *Gardenia jasminoides* (Xu et al. 2021).

Shuang-Huang-Lian is a well-known Chinese patent medicine that comprises dried root of *Scutellariae baicalensis*, flowers or flower buds of *L. japonica*, and dry fruit of *F. suspense*. UHPLC-Q-TOF-MS analysis of Shuang-Huang-Lian has detected the presence of four flavonoids, six quinic acid derivatives, three saponins, and two phenylethanoid glycosides (Zhang et al. 2015). Ni et al. (2021) conducted a randomized, open-label, parallel-controlled, multicenter trial of the oral application of Shuang-Huang-Lian to 176 COVID-19 patients in addition to standard care, which showed its application in combination with standard care was safe and partially effective for the treatment of COVID-19.

Tan Re Qing injection, a traditional Chinese medicine formula, is composed of dried root of *S. baicalensis* (*Scutellariae Radix*), bear bile powder, *Caprae Hircus Cornu*, flowers, or flower buds of *L. japonica* (*Lonicerae japonicae Flos*) and dry fruit of *F. suspensa* (*Forsythiae Fructus*) used to treat bronchitis and upper respiratory infections (Qiao et al. 2018). Several clinical studies have evaluated its effectiveness against COVID-19 in capsule or injectable form (Table 18.3).

Xi Yan Ping injection, a Chinese herbal medicine primarily composed of 9-dehydro-17-hydro-andrographolide and sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate derived from *A. paniculate*, prescribed for the treatment of respiratory infection and pneumonia (Chong et al. 2013; Zhang et al. 2021). According to a prospective, multicenter, open-label, controlled clinical trial conducted among 130 COVID-19 patients with mild to moderate symptoms from five study sites, Xi Yan Ping injection in combination with standard therapy or standard supportive therapy alone is safe and effective in improving the recovery of patients with mild to moderate COVID-19 (Zhang et al. 2021). Significant reduction

in time to cough cessation, fever resolution, and viral clearance; and less frequency of severity was observed without significant adverse effects in Xi Yan Ping injected COVID-19 patients compared to control (Zhang et al. 2021).

A multicenter, double-blind, randomized, controlled trial consisting of 131 patients showed that the Bufei Huoxue capsule has the potential to produce significant rehabilitative effects on physiological activity in patients recovering from COVID-19, which may reduce tiredness symptoms and enhance exercise tolerance (Chen et al. 2022). Bufei Huoxue capsule is a widely known Chinese patent medicine that consists of three ingredients—the dried root of *A. membranaceus* var. *mongholicus* (*Astragali radix*), the dried root of either *P. lactiflora* or *Paeonia veitchii* (*Paeoniae radix rubra*), and dried matured fruit of *P. corylifolia* (*Psoraleae fructus*).

Besides these, the efficacy of several other Traditional Chinese herbal medicine, such as Shen-Qi Fu-Zheng injection, Shugan Jieyu capsule, Xinguan Fuzheng Zhuanyin decoction, Kangbingdu granules, Ke Su Ting Syrup/Ke Qing Capsule, Han Shi Yi Decoction, Gu Biao Jie Du Ling, etc., in the treatment of COVID-19 has been assessed in numerous clinical trials in China (Table 18.3). Shenfu injection is the Chinese State Food and Drug Administration-approved traditional Chinese herbal medicine, composed of the dried root or rootstalk of *P. ginseng* and the lateral root of *Aconitum carmichaeli* (Zhu et al. 2020). Shen-Qi Fu-Zheng injection consists of ginseng and dried root of *A. membranaceus* (*Radix Astragali*) (Chen et al. 2019), while Shugan Jieyu Capsule is a compound mixture composed of *Eleutherococcus senticosus* and *Hypericum perforatum* (Wu et al. 2022). Kangbingdu granules made up of *Isatidis Radix*, *Forsythiae Fructus*, *Gypsum Fibrosum*, *Anemarrhenae Rhizoma*, *Phragmitis Rhizoma*, *Rehmanniae Radix Praeparata*, *Pogostemon cablin*, *Acoritataninowii Rhizoma* and *Curcumae Radix*. The efficacy of Kangbingdu Granules has been assessed by molecular docking technology as well as by clinical trials (Yao 2020; ChiCTR2000029781).

Dayuan decoction is composed of *Areca catechu*, *M. officinalis*, *A. macrocephala*, *Citrus reticulata*, *Amomum tsao-ko* Crevost et Lemaire, *Ephedra sinica*, *Z. officinale*, *Citrus × aurantium*, *Pogostemon cablin*, *Hansenia weberbaueriana* in the mass ratio of 10:10:15:10:6:6:10:10:10 (Zhang et al. 2020a, b). According to network pharmacology and molecular docking study, it may treat COVID-19 through multiple targets, multiple channels, and multiple pathways and is worthy of clinical application and promotion (Ruan et al. 2020).

Ma xing shi gan decoction consists of four herbs including *Ephedra sinensis*, *Semen armeniacae amarum*, *Glycyrrhiza*, *Gypsum fibrosum*, and Gancao ganjiang decoction consists of two herbs including *Radix glycyrrhizae* and *Rhizoma zingiberis* are the two most popular TCM formulae used in the early phase of the COVID-19 infection (Lee et al. 2021). *Sheganmahuang* decoction consists of nine herbs, including *Rhizoma Belamcandae*, *Ephedra sinensis* *Rhizoma Zingiberis* Recens, *Asarum sieboldii*, *Radix Asteris*, *Flos Farfarae*, *Ziziphus jujube*, *Pinelliae Rhizoma Praeparatum Cum Zingibere*, *Schisandra chinensis* seeds, which relieves asthma and reduces airway restriction (Lee et al. 2021).

Luo et al. (2021) also reported the use of other Traditional Chinese herbal medicine such as Shufeng Jiedu Capsule, Qingfei Paidu decoction, Xuebijing Injection, Xingnaojing Injection, Shenmai Injection, Shengmai Injection, Shenfu Injection, Kangbingdu Granules, Reyaning Oral Liquid, Siji Kangbingdu Mixture, Huoxiang Zhengqi Capsule, Qingxuan Zhike Granules, Jinzhen Oral Liquid, Jinye Baidu Granules, Qingkailing Injection. The main herbs in Shufeng Jiedu formula are *Reynoutria japonica*, *F. suspensa*, *I. indigotica*, *Bupleurum abchasicum*, *Patrinia heterophylla*, *Verbena officinalis*, *G. glabra*, etc. (Luo et al. 2021). Huo Xiang Zhengqi dropping pills composed of *Pogostemon cablin*, *Atractylodes lancea*, *M. officinalis* Cortex, *Angelicae dahurica* Radix, *P. cocos*, *A. catechu*, *P. ternate*, *Glycyrrhizae* Radix et Rhizoma, *Perilla frutescens*, and *Citrus reticulata* (Xiao et al. 2020). Clinical trials were conducted to evaluate the clinical efficacy and prognosis of Huoxiang Zhengqi Oral Liquid in the combination of Jinhao Jiye Granules or Lianhua Qingwen granules in COVID-19 patients (Xiao et al. 2020; Yan et al. 2020). The use of Huoxiang Zhengqi dropping pills and Lianhua Qingwen granules combined with western medicine may have clinical advantages for COVID-19 patients in improving clinical symptoms, reducing utilization rate of anti-infective drugs, and improving patient prognosis, which could pave the way for the use of complementary medicine in treating this infection (Xiao et al. 2020).

18.8.2 Traditional Indian Medicine and COVID-19

WHO recognized and acknowledged the significant role of the centuries-old system of plant-based Indian traditional medicine, such as Ayurvedic, Homeopathy, Unani, and Siddha, in public health sectors. More than 5000 years of the ancient medical system of Ayurveda is an intrinsic part of Indian culture, which classified therapies into two types—Swasthasyorjaskara, which improves health strength (immunity), and Roganut, which treats diseases. In general, strong immune responses may be effective in minimizing the possibility of severity in COVID-19 patients by restricting viral propagation and spreading. However, it may help to reduce the inflammatory response or cytokine storm in the severe stage. Thus, medicinal plants with immune-boosting qualities may be used in the early, non-severe stage, whereas herbs with anti-inflammatory and anti-thrombotic activities may be used in the later, severe stage.

Indian researchers are also actively involved in numerous clinical trials of medicinal plant-based COVID-19 therapy. Several publications offer an extensive summary of COVID-19 research in India, emphasizing the clinical trials registered in the Clinical Trials Registry—India (Abinaya et al. 2021; Batta et al. 2021; Rao et al. 2021; Chaudhari and Somvanshi 2022). According to Rao et al. (2021), 122 registered trials on COVID-19 were collected from the CTRI database, which includes modern medicine ($n = 42$), traditional medicine (Ayurveda—45, homeopathy—14, Yoga and Naturopathy—3, Unani— n , and Siddha—3), and miscellaneous ($n = 13$) as of June 5, 2020. These clinical trials determine the safety and

efficacy of several Ayurvedic medicines, formulations, homeopathic drugs, or patented ayurvedic products as a new therapy being implemented in the management of COVID-19 patients alone or in combination with standard allopathic treatment by providing research evidence (Batta et al. 2021).

Several traditional herbal medicines and formulations with antiviral, antioxidant, anti-inflammatory, and immunomodulatory properties, such as “*Ayush Kwath*,” AYUSH-64, Arogya Kashayam 20 (AK-20), AEV01 (Kutki), Ashtadashang Ghana Vati, Sudarshana Ghana Vati, Shanshamani Vati, Vataleshma Jwarhar decoction, Dashamula Kwatha, Pathyadi Kwatha with Trikatu Churna, Gorochanadi Vati, Guduchi Ghan Vati, Guduchi tablet, Mahasudarshan Ghana vati, Lavangadi vati, Raj Nirwan Bati, Shirashadi Kasai, Vyaghryadi Kashaya decoction, Ashwagandha, Yashtimadhu, Colchicine, Curcumin, Danggui Shaoyao powder, ES16001, Immusante tablet, Immunofree tablet, Imusil tablet, Pinak tablet, etc., are now being used in several clinical trials to assist the prevention of virus proliferation, speedy recovery of patients with COVID-19, faster discharge from hospitals, and the prevention of further deterioration (Maideen et al. 2022). A few of these are described here (Table 18.3). *T. cordifolia*, *G. glabra*, *C. longa*, and *W. somnifera* are the most commonly used herbs in Ayurvedic research, whereas, *Arsenic album*, *Bryonia alba* is most frequently used in studies from Homeopathy system. It is not always possible to obtain the composition of all herbal formulations, particularly for diverse patent and proprietary pharmaceuticals.

AYUSH-64 is a multi-herbal formulation developed and patented by the Central Council for Research in Ayurvedic Sciences, Ministry of AYUSH, Government of India that contains four ingredients—*Alstonia scholaris*, *Picrorhiza kurroa*, *Swertia chirata*, and *Caesalpinia crista* (Panda et al. 2022; Singh et al. 2022). It has immunomodulatory, anti-inflammatory, antipyretic, antioxidant, and antiviral properties (Panda et al. 2022). Based on substantive evidence derived from several robust clinical and experimental studies on AYUSH-64, it has been included in the Government of India’s National COVID-19 management protocol. The Ministry of AYUSH has initiated mass distribution of AYUSH-64 in India to reduce the large-scale morbidity associated with COVID-19 and the burden on the hospital-based healthcare delivery system by effectively managing the home-isolated asymptomatic, mild to moderate COVID-19 patients as an adjunct to standard care (Reddy et al. 2020; Chopra et al. 2021; Panda et al. 2022; Singh et al. 2022). AYUSH-64, as an adjunct to standard conventional care, is safe and hastens clinical recovery in COVID-19 patients with mild to moderate disease by significantly reducing the levels of pro-inflammatory markers such as IL-6, D-dimer, CRP, LDH, serum ferritin, and HRCT chest score (Singh et al. 2022).

Arogya Kashayam 20 (AK-20) is an Ayurvedic herbal decoction composed of seven herbs, including *T. cordifolia*, *G. glabra*, *Phyllanthus niruri*, *Terminalia chebula*, *Z. officinale*, *P. nigrum*, and *P. longum* (Shukla et al. 2019). To assess the efficacy of AK-20 in the prevention and management of COVID-19, the Government of Madhya Pradesh, Department of AYUSH performed multi-centric research in 30 districts over 88 centers across Madhya Pradesh, which revealed

that the standalone intervention with AK-20 produced statistically equivalent effects when combined with HCQ therapy (Shukla et al. 2019).

Several studies demonstrated the clinical effectiveness and efficacy of colchicine in alleviating the health crisis and preventing the collapse of the health system in consecutive waves of the SARS-CoV-2 pandemic (Estebanez et al. 2021; Lopes et al. 2021; Tardif et al. 2021), whereas others disagreed with its benefit (Pascual-Figal et al. 2021; Absalón-Aguilar et al. 2022). Estebanez et al. (2021) found that lowering the consequences of COVID-19 in a highly sensitive population may be possible through early treatment of colchicine. Lopes et al. (2021) recommended that colchicine effectively reduces the length of supplemental oxygen therapy and hospitalization. Another clinical trial showed that colchicine administration reduces the rate of the composite of death or hospitalization in PCR-confirmed COVID-19 patients than placebo (Tardif et al. 2021). However, according to Absalón-Aguilar et al. (2022) colchicine is safe but ineffective in the treatment of severe SARS-CoV-2 infection based on a randomized clinical trial in which colchicine had no effect on the primary outcome and on the days of ICU and hospital stays among 65 in patients administrated with colchicines. Colchicine treatment did not enhance the clinical status or the inflammatory response in hospitalized COVID-19 patients (Pascual-Figal et al. 2021; Clinical Trial No. NCT04350320).

The proprietary herbal formulation “*Ayush Kwath*,” also known as “*Ayush Kudineer*” or “*Ayush Joshanda*,” comprises *O. sanctum*, *Cinnamomum zeylanicum*, *Z. officinale*, is well known for its immune-modulatory, antiviral, antioxidant, anti-inflammatory, anti-platelet, anti-atherosclerotic, hepato-protective, reno-protective properties (Gautam et al. 2020; Juliet and Meenakumari 2020). Considering the necessity of immunity-boosting measures in the present COVID-19-induced public health crisis, the Ministry of AYUSH, Government of India, has recommended *Ayush Kwath* as a preventive, curative, and restorative intervention to combat the infection of COVID-19 based on several preventive and curative studies of *Ayush Kwath* (Rao et al. 2021; Sharma et al. 2022).

A pilot study on a new Ayurvedic drug, Raj Nirvan Bati, which has *P. nigrum*, *Artemisia nilagirica*, *Carum copticum* as ingredients, effectively reduced viral shedding duration and symptoms of fever, cough, and fatigue when administrated twice daily with 5 ml of honey (Kumar et al. 2020).

18.9 Conclusion

The outbreak of SARS-CoV-2 has impacted millions of people globally in unimaginable ways and created an unprecedented global health crisis. SARS-CoV-2 causes pneumonia and ARDS, which may progress to multi-organ failure and ultimately causes death. Phytoconstituents, medicinal plant-based medicines and formulations are essential, potent contributors to disease treatment from prehistoric times. They frequently demonstrate pleiotropic effects, which may give a multimodal approach to COVID-19 therapy via antiviral, anti-inflammatory, and immunomodulatory

actions. Thus, the potential role of medicinal plants, traditional herbal medicines, and formulations against COVID-19 should be evaluated, along with vaccination programs. A major disadvantage of pooling data from published research is the qualitative and quantitative diversity in the same herbal extracts utilized in various investigations, as well as a lack of well-reported standardization data of the examined formulations.

Several *in silico*, *in vitro* researches and clinical trials, have been conducted with known as well as new medicinal plant-derived phytochemicals, traditional herbal medicines, and formulations. Some of them show promising results in the *in silico* or early phases of clinical trials. In COVID-19 treatment and management-based research, these preliminary claims of herbal medicine and formulations should be included, and further research and clinical trial-based validation of these potent medicines are recommended in the upcoming period. Moreover, the advancement and strengthening science of phytomedicine should be encouraged globally during the current pandemic. In addition to their direct effect on COVID-19 management, several other issues such as the identification of bioactive ingredients, safe dose specific to formulations, and bioavailability, should also be addressed.

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Chapter 19

Bioactive Compounds from Medicinal Plants and its Therapeutic Uses in the Traditional Healthcare System



Subir Chandra Dasgupta

Abstract The plant-based traditional medicine systems continue to play a crucial role in the healthcare system. Bioactive compounds derived from plants have been reported in large number of publications, and novel compounds are identified with technological developments in isolation and identification of phytochemicals from known or new species. Most common bioactive phytochemicals are polyphenols, saponins, terpenes, alkaloids, etc. Human health is largely dependent on gut microflora. Many phytochemicals possess antioxidant and anti-inflammatory properties that may positively affect the gut microflora, including polyphenols, carotenoids, phytosterols/phytostanols, lignans, alkaloids, glucosinolates and terpenes. Gut microbes influence host metabolism by altering the composition of bile acid pool through bile acid synthesis and reuptake. Traditional medicine refers to any ancient and culturally-based healthcare practice largely transmitted orally by communities of different cultures. African traditional medicine and its use in healthcare system is the oldest therapeutic systems. China has been successful in promoting its therapies with more research and science-based approach. In India, Ayurvedic herbs are time tested for their health and other benefits. The nutritive values that they pack are highly recommended for their healing powers. The Unani system of medicine is a great healing art as well as a science. Nearly 70% of homeopathic medicines are derived from herbs. The science of animal self-medication is called zoo pharmacognosy. The scientists researching zoo pharmacognosy are convinced that humans can learn from the animals, particularly in finding new medications.

Keywords Phytochemicals · Gut microflora · Traditional medicine · Healthcare · Zoo pharmacognosy

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19.1 Introduction

History of herbalism is closely related with history of medicine since prehistoric times. Evidence from archaeological standpoint reveals that human were using traditional medicinal plants since the Palaeolithic period. In Mesopotamia, the documents on the study of herbs are 5000 years old to Sumerians and hundreds of medicinal plants were documented (Sumner 2000). The herbs used by Egyptian healers were mostly indigenous only few were imported. They listed about 800 plants having medicinal importance (Sumner 2000). The use of chaulmoogra for leprosy, ephedra and hemp for anti-nociceptive has been mentioned in Chinese traditional medicine and Chinese pharmacopoeia. The book written by Pedanius Dioscorides was a good reference on herbalism in the seventeenth century containing around 1000 formulation of medicines from plants (Collins 2000). Ayurveda, which is the ancient healthcare system in India, is practiced 5000 years ago. As per the old literatures on Ayurveda, it was practiced during the Vedic period in India. About 700 plants were described and documented in Atharva Veda Charaka Samhita and Sushruta Samhita. This medical system is widely practiced in other parts of the world as a form of complementary medicine. The literal meaning of Ayurveda is the 'Science of Life'. Herbal treatments are the most popular form of Traditional Medical System (Nuan 2005). The plant-based traditional medicine systems continue to play a crucial role in the healthcare system. The demand for herbal-based medicine, health products, pharmaceuticals, food supplements, nutraceuticals and cosmetics are increasing worldwide (Shakhya 2016). In the twenty-first century, natural products represent more than 50% of all drugs in clinical use. Up to 50% of the approved herbal drugs during the last three decades are either directly or indirectly from natural products including plants, microorganisms, fungi and animals. As per the records of the National Medicinal Plant Board (NMPB), the Indian herbal industry may likely to increase by Rs. 80–90 billion by 2022. However, India is moving forward in popularising the Traditional Medical System of AYUSH (Ayurveda, Yoga, Unani, Siddha and Homeopathy) in the healthcare sector through global networks. The objective of this present review is to highlight the nature of bioactive compounds from herbs and the assessment of its therapeutic efficiency in the traditional healthcare system.

19.2 Prevalence of Use

Strategies formulated by WHO for 2014–2023 stressed the role and use of traditional medicine and utilisation of plant parts to improve the human healthcare system. The majority of global population (87.5%) use the traditional herbal medicine to combat different types of diseases (Devesa et al. 2004). Pharma industries show keen interest in the formulation of new herbal medicines from natural products which allure

consumers to procure plant-based health medicine as a second and alternative choice of drugs for health issues (McLay et al. 2016).

Food industries are more enthusiastic towards the development of new plant-based products such as soya extract (isoflavonoids), tomato extract (lycopene), spinach (lutein), oat (β glucan), green tea (epicatechin), garlic oil (Allicin), etc. (Bhanu et al. 2017). Plant-based bioactive compounds can also be used as food preservatives. Many plant products contain antimicrobial, antioxidant and contain essential oil which may be used as a safe alternative for synthetic preservatives. Nowadays, majority of good food contains angelica, basil, citrus peels, lemongrass, thyme, ylang-ylang, cinnamaldehyde, citral, *p*-cymene, eugenol, limonene, menthol, linalool, etc. which were considered as safe food preservatives.

19.3 Nature of Bioactive Compounds

Bioactive compounds derived from plants have been reported in large number of publications, and novel compounds are identified with technological developments in isolation and identification of phytochemicals from known or new species. The most common bioactive phytochemicals are polyphenols, saponins, terpenes, alkaloids, etc.

Polyphenols are widely distributed in the plant kingdom. Polyphenols have one or two hydroxyl group ($-\text{OH}$) attached directly to aromatic ring which is common in almost all secondary metabolites which are the simplest phenol. Simple phenolics are the substituted phenol (C_6C_n) possessing a single substitution of phenolic ring with aldehyde, alcohol or carboxylic acid (three categories are mentioned below).

Flavonoids comprise of 60% polyphenols including kaempferol, catechins, anthocyanin, etc. Polyphenolic amides consist of 20% polyphenols; for example, capsaicinoids in chillies. Phenolic acid constitutes 20% polyphenols, stilbenes and lignans found in fruits, whole grain, etc.

19.3.1 Saponins

Saponins are structurally complex phytochemicals compounds that are composed of glycone and aglycone moieties. Saponins are ubiquitously distributed in the plant kingdom. The complexity of saponin structure is due to the different glycan moieties that are attached to the triterpenoid or steroidal aglycones (water non-soluble part). The hydrolysis of saponin molecule produces two portions, aglycone and a sugar moiety. Like polyphenols, saponins are present in plant foods. Soya foods are one of the best sources of saponins.

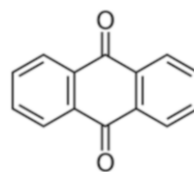
19.3.2 Terpenoids

Terpene, a class of hydrocarbons occurring widely in plants and animals and empirically regarded to be built up from isoprene, is a hydrocarbon consisting of five carbon atoms attached to eight hydrogen atoms (C_5H_8). The term is often extended to the terpenoids, which are oxygenated derivatives of these hydrocarbons. A terpene is a naturally occurring hydrocarbon based on combinations of the isoprene unit. Terpenoids are compounds related to terpenes, which may include some oxygen functionality or some rearrangement; however, the two terms are often used interchangeably. Terpenoid compounds give violets and roses their scents and are found in turpentine and rubber. Carotenoids or tetraterpenoids are yellow, orange and red organic pigments produced by plants, algae and several bacteria and fungi. Within the carotenoid group are more than 600 known natural structural variants. Terpenes are the straight chain structural group formed by the condensation of isoprene molecules and by the binding of 5-carbon-containing isoprene molecules. Due to their antioxidant behaviour, terpenes have been shown to provide relevant protection under oxidative stress conditions in different diseases such as liver, renal, neurodegenerative and cardiovascular diseases, cancer, diabetes as well as ageing processes. Steroids, which are derived from terpenoid building block isopentenyl pyrophosphate, are a subclass of terpenoids that contain a characteristic arrangement of four cycloalkane rings joined to each other. Carotenoids, also known as tetraterpenoids, are organic pigments found in plants and bacteria. Vegetable and animal body colourations are largely dependent on this carotenoid pigments. Carotenoids are two types: xanthophyll (contains oxygen) and carotenes (purely hydrocarbon without oxygen), but all are derived from tetraterpenes having 8 isoprene molecules and 40 carbon atoms. The absorption spectra of carotenoids are 400–550 nm which causes the compounds to be either yellow, violet or green colour. Some naturally occurring carotenoids are lycopene, phytofluene, α -carotene, β -carotene, rhodopsin, etc.

19.3.3 Anthraquinones

Anthraquinones, also known as anthraquinonoids, are a class of naturally occurring phenolic compounds based on the 9, 10-anthraquinone skeleton (Fig. 19.1). Anthraquinones (9, 10-dioxoanthracenes) constitute an important class of natural and synthetic compounds with a wide range of applications. They are widely used industrially and occur naturally. Its derivatives are widely used in different medical applications such as laxatives, as antimicrobial, as anti-inflammatory, etc. It has tremendous industrial application for the production of hydrogen peroxide and anthracene oxidation, naphthalene oxidation, condensation of 1, 4-naphthoquinone with butadiene. Anthraquinones has a potent anticancer activity. It is found naturally in some plants such as senna, buckthorn, yellow dock, etc. (Khan 2019).

Fig. 19.1 Basic structure of anthraquinones



Anthraquinones is a potent aromatic compound that besides being used commercially, it offers numerous therapeutic benefits as it inhibits cancer growth by inducing apoptosis, eases bowel movement, etc.

19.4 Phytochemicals and Human Gut Flora

Human health is largely dependent on gut microflora (GM). Many phytochemicals possess antioxidant and anti-inflammatory properties that may positively affect the GM, including polyphenols, carotenoids, phytosterols/phytostanols, lignans, alkaloids, glucosinolates and terpenes. The modest estrogenic-like activities (phytoestrogen) was obtained from soyabean, a situation similar to that of some isoflavonoids. Diet rich in lignans are also absorbed through intestine. These compounds show estrogen independent action with a strong activity of inhibition of angiogenesis, lowering diabetes and suppressing tumour growth. Gut microbes influence host metabolism by altering the composition of bile acid pool through bileacid synthesis and reuptake. Recent studies have shown that microbial habitat in the gut is altered due to phytochemicals such as phenols, carotenoids, butyrate through bacterial fermentation in colon. In chronic inflammatory diseases such as immune dysfunction, colon cancer, obesity, and diabetes, the entire profile of gut bacteria is changed. Dietary phytochemical-induced anti-inflammatory activities are also controlled by gut microflora. The advent of modern biology and its applications elucidate the exact mechanism of action of different phytochemical and microbiome interactions (Yin et al. 2019). Certain polyphenols have shown to influence bacterial communication, interacting with quorum sensing. Finally, phytochemicals can be metabolised in the gut into bioactive constituents, e.g. equol from daidzein and enterolactone from secoisolariciresinol, while bacteria can use glycosides for energy (Dingeo et al. 2020). Research on the interaction of 359 polyphenols with gut microbiota has mainly focussed on catechins, flavan-3-ols from green tea, including epigallocatechin gallate; ellagic acid and ellagitannins, non-flavonoids present, e.g. in pomegranate, raspberries, blackberries, strawberries and chestnuts (Henning et al. 2017).

19.5 Use of Traditional Healthcare Systems

Medicinal plants are an important element of indigenous traditional healthcare system used throughout the world. Traditional medicine refers to any ancient and culturally-based healthcare practice largely transmitted orally by communities of different cultures. It is the sum total knowledge of theories, skill, practices and belief of different tribes for betterment of human health (WHO 2013). Traditional medicinal plants are an important element of indigenous medical systems in China and the rest of the world. Traditional medicine adopted by other populations (outside its indigenous culture) is often termed complementary or alternative medicine.

African traditional medicine and its use in healthcare system is the oldest therapeutic system. Tribal people often use herbs which are easily accessible and affordable in the majority of rural Africa. Adequate scientific information on promising medicinal plants in Africa is still lacking. The medicinal uses of African plants and traditional systems are becoming a pressing need because of the rapid loss of the natural habitats of some of these plants due to anthropogenic activities and the erosion of valuable traditional knowledge (Mahomoodally 2013).

Some plants in use include *Acacia senegal* (Gum Arabic), widespread from Southern to Northern Africa and sub-Saharan arid zone, which is used to treat infections such as bleeding, bronchitis, diarrhoea, leprosy, gonorrhoea, typhoid fever and upper respiratory tract infections. Gum acacia is also used to bind pills and to stabilise emulsions. It is also used in aromatherapy for applying essential oils (Gurib-Fakim et al. 2010). *Aloe ferox*, which is native to South Africa, is considered to be the most common *Aloe* species in South Africa and used in cosmetic industries and as laxative agents (Brendler et al. 2010). *Artemisia herba-alba*, a perennial dwarf shrub native to the Northern Africa, Arabian Peninsula and Western Asia is used to treat arterial hypertension and diabetes, bronchitis, diarrhoea, hypertension and neuralgias (Segal et al. 1987). *Aspalathus linearis*, an endemic South African species, is a well-known herbal tea, also commonly known as rooibos. Its caffeine-free and comparatively low tannin status is combined with its potential health-promoting properties, most notably antioxidant activity. It has been used as a refreshment drink and as a healthy tea beverage (Brendler et al. 2010). *Centella asiatica* has a pan-tropical distribution used mainly in burns, ulcers, leprosy, tuberculosis, lupus, skin diseases, eye diseases, fever, inflammation, asthma, hypertension, rheumatism, syphilis, epilepsy, diarrhoea and mental illness (Kim et al. 2009). *Catharanthus roseus* is a well-known medicinal plant that has its root from the African continent, and it is the source of the anticancer alkaloids vincristine and vinblastine (Pereira et al. 2010). *Cyclopia genistoides* is an indigenous herbal tea to South Africa and considered as a health food and used as a restorative and as an expectorant in chronic catarrh and pulmonary tuberculosis (Wyk 2008). *Harpagophytum procumbens*, also known as Devil's cow, is native to the red sand areas in the Transvaal of South Africa, Botswana and Namibia. It has spread throughout the Kalahari and Savannah desert regions and is used in allergies, analgesia, anorexia, antidiabetic, antiarrhythmic, antiphlogistic, antipyretic, appetite

stimulant, arteriosclerosis and child birth difficulties (Mncwangi et al. 2012). *Pelargonium sidoides* is native to the coastal regions of South Africa and effective in alleviating symptoms of acute rhinosinusitis and the common cold, and also in relieving symptoms of acute bronchitis in adults and children and sinusitis in adults (Timmer et al. 2008). *Momordica charantia*, a tropical vegetable grown throughout Africa, is evaluated for its antilipolytic and lipogenic activities, and hypoglycaemic activity (Agbabiaka et al. 2008).

From approximately 8300 wild plants with known medicinal species in the North-western China, some 400 species are used in traditional Chinese medicines (TCM) or by drug manufacturing units in China. China has been successful in promoting its therapies with more research and science-based approach, while Ayurveda still needs more extensive scientific research and evidence. Traditional Chinese medicine aims to promote health and enhance the quality of life, with therapeutic strategies for treatment of specific diseases or symptoms in holistic fashion (Bhushan et al. 2005). Some traditionally used medicinal species in China are mentioned below.

Acorus tatarinowii is used for rheumatism, scabies; literature: Rheumatism (Alba and Brito 1996). *Angelica pubescens* is used as anti-rheumatic, kills cancer cell and fungus, and reduces anxiety (Chen et al. 1995). *Amaranthus spinosus* are used for pruritus, abscess bleeding, anti-diabetic, hemorrhoids bleeding, eczema, scabies (Xie et al. 2010). *Begonia cathayana* is used in eliminating stasis to activate blood circulation, detumescence, injuries from falls, rheumatism and numbness, carbuncle, skin ulcer (Tian et al. 2018). *Cucubalus baccifer* is used for amenorrhea, injuries from falls, rheumatism, dropsy, urinary system infection, fracture (Chen et al. 2001). *Euodia lepta* is used for rheumatoid arthritis, ache in waist and legs, eczema, injuries from falls, eczema, dermatitis (Croom 1983). *Gelsemium elegans* is used for eczema, scrofula, carbuncle, furuncle, injuries from falls, rheumatism; literature: eczema, ringworm, measles (Lin et al. 2021). *Glycyrrhiza uralensis* is used as anti-diabetic, for hypokalemia, sodium retention producing hypertension, edema, and headache (Liao et al. 2012). *Glechoma longituba* helps in eliminating stasis to activate blood circulation, menoxenia, profuse uterine bleeding, leucorrhea, rheumatoid arthritis, literature, ulcer, eczema (Lin et al. 2021). *Murraya tetramera* is extensively used in rheumatoid numbness, pain in bones and muscles, urticaria, eczema, bitten by viper; literature: pruritus, eczema (Etkin 1993). *Panax ginseng* has antioxidant and anti-inflammatory effects. It could also help regulate blood sugar levels and has benefits for some cancers (Zhang et al. 2020). *Ranunculus japonicus* is used in skin ulcer and ringworm, rheumatic arthritis, injuries from falls, fracture, uterine prolapse, menoxenia (Li et al. 2006). *Viola inconspicua* is extensively used to alleviate pain, itch, leucorrhea, uterine prolapse, and *Zippelia begoniaefolia* activates blood circulation (Li et al. 2006).

19.6 Relation with Unani and Ayurvedic Science

The science that comprises Unani healing can be traced back to Claudius Galenus of Pergamum, who lived in the second century of the common era. The basic knowledge of Unani medicine as a healing system was collected by Hakin Ibn Sina (known as Avicenna) in 980 CE in Persia. Subsequently, Ajmal Khan, who was born in India in 1864, is generally acknowledged to be the most significant twentieth century contributor to Unani medicine in India (Ansari 2013). Unani uses eight diagnosis methods for effective results which include pulse, stool, urine, tongue, speech, vision, touch and appearance. *Unani* means Greek and Unani medicine is an ancient Greek system of medicine based on the qualities of four temperaments and four humours. Unani medicine recognises the mental, emotional, spiritual and physical causes of illness or health and believes that each individual should take responsibility for their own well-being. The Hellenistic origin of Unani medicine is still visible in its being based on the classical four humours: phlegm (balgham), blood (dam), yellow bile (şafra) and black bile (saudā'), but it has also been influenced by Indian and Chinese traditional systems. The history of Unani system of medicine can be traced back to ancient Egypt and Babylon. Egyptians made use of medicinal plants as a remedy for ailments. They also initiated surgery as a method of treatment. Charaka is called 'Father of Ayurveda'. In his book 'Charaka Samhita', he had mentioned about 340 plant types. Ayurveda is the system of medicine that evolved in India with a rationale logical foundation, and it has survived as a distinct entity from remote antiquity to the present day. Ayurveda focuses on the imbalances of the dosha—Vata, Pitta and Kapha, and curates medicine that helps balance these doshas. The original conception of Ayurveda in its entirety is essentially linked to Dhanwantari who is considered as God of Hindu Medicine. Dhanwantari is considered as a mythical deity born with ambrosia in one hand and Ayurveda on the other at the end of the churning of milk ocean. Ayurveda believes that the entire universe is composed of five elements: Vayu (Air), Jala (Water), Aakash (Space or ether), Prithvi (Earth) and Teja (Fire). These five elements (referred to as Pancha Mahabhoota in Ayurveda) are believed to form the three basic humours of the human body in varying combinations. The body, which is the seat of the mind and soul, maintains its optimum health by regulating ahara (diet), svapna (sleep) and abrahmacharya (conjugal life). These are the three pillars of health according to Ayurveda which regulate the body by virtue of their actions in the body. The Unani physicians who settled in India have added new drugs to the system and therefore the Unani system practiced in India is somewhat different from the original Greek form. Ayurvedic herbs are time tested for their health and other benefits. The nutritive values that they pack are highly recommended for their healing powers. Known to induce no side effects, they have a unique aroma and flavour and when consumed regularly, they act as a perfect mechanism to bring about a balanced harmony between mind and body. They rejuvenate the whole system instead of focusing on one specific organ or body part. Ayurvedic herbs and spices such as black pepper, cinnamon, aloe, sandalwood, ginseng, red clover, burdock, bayberry and safflower

are used to heal wounds, sores and boils. To reduce fever and the production of heat caused by the condition, certain antipyretic herbs such as Chirayita is used.

Black pepper and sandalwood are recommended. Sandalwood and cinnamon are great astringents apart from being aromatic. Sandalwood is especially used in arresting the discharge of blood, mucus, etc. Ajwain, Amalaki, Aswatha, etc. serve as antacids and are recommended for healthy gastric acid flow and proper digestion. Herbs like cardamom and coriander are renowned for their appetising qualities. Other aromatic herbs such as peppermint, cloves and turmeric add a pleasant aroma to the food, thereby increasing the taste of the meal. Herbs such as aloe, sandalwood, turmeric, Sheetraj Hindi and Khare Khaskhas are commonly used as antiseptic and have very high medicinal values. Chamomile, basil, cardamom, ginger, peppermint and coriander are known to promote blood circulation in the body and keep the heart healthy. Right from the ancient times, Ayurvedic herbs offered a solution to all kinds of diseases, some of which were considered impossible to treat by other fields of medical sciences. They are derived from a plant source, where the leaves, flowers, fruits, seeds, roots, bark and resin are used to prepare medicines. These herbal medicines have the potential to maintain a balance between the mind and the spirit.

Brahmi, also called *Bacopa monnieri*, herb of grace, thyme-leaved gratiola, is one of the best Ayurvedic herbs that has been in use from ancient times to treat neurological disorders. It can be found in wet and tropical environments and can also be used as an Ayurvedic remedy for improving memory, reducing anxiety and treating epilepsy. Certain researches have pointed out that it may be effective in boosting brain function as well. The leaves of the plant are considered as highly sattvic or pure and were eaten by yogis to assist in meditation in the olden times.

By considering the importance of various traditional or complementary alternative medical systems (CAM), we have reviewed the basic concepts and role of the five major CAM systems used in India, i.e. AYUSH (Ayurveda, Yoga, Unani, Siddha and Homeopathy). Unani system of medicine is a great healing art as well as science. It treats a person as a whole not as a group of individual parts. It is aimed at treating the body, mind and soul. The World Health Organization (WHO) has recognised the Unani System of Medicine (USM) as an alternative system to cater to the healthcare needs of human population. Unani is one of the most well-known traditional medicine systems and draws on the ancient traditional systems of medicine of China, Egypt, India, Iraq, Persia and Syria (Qasmi 2013).

19.7 Relation with Homeopathic Medicine

The herbs used in homeopathy are mostly derived from angiosperms (flowering plants), although some conifers and ferns as well as fungi, including lichens, brown and red algae are also used. The medicines are prepared as per the standard prescribed in the official Pharmacopeia's. Homeopathic products come from plants (such as red onion, arnica [mountain herb], poison ivy, belladonna [deadly

nightshade] and stinging nettle), minerals (such as white arsenic) or animals (such as crushed whole bees). While it can scarcely compare in antiquity with Chinese or Indian medicine, homeopathy is the longest established CAM to have arisen in Europe. It was founded by Samuel Hahnemann (1755–1843), who grew up in Meissen in Germany, received his medical degree in Erlangen in 1779, and died a millionaire in Paris in 1843. The drug strength appears after the name with a numeral followed by an X or C. Since X is the Roman numeral for 10, a 6X potency states that the drug has been diluted at a ratio of 1–10 and succussed for a total of six times. In C terms, the concentration ratio is 1–100 (C is the Roman numeral for 100). It is well known that many homeopathic medicines are ultramolecular—that is, they are diluted to such a degree that not even a single molecule of the original solute is likely to be present. As drug actions are conventionally understood in biochemical terms, homeopathy presents an enormous intellectual challenge, if not a complete impasse. Many scientists have suggested that the clinical effects of homeopathic medicines are solely due to the placebo effect. However, there have been rigorous, replicated, double blind, randomised trials showing significant differences between homeopathic and placebo tablets.

Nearly 70% of homeopathic medicines are derived from herbs. These are prepared from any part of the plant such as fruit, seed, stem, bark, flower, leaf, stigma or root as well as a non-woody plant. The word ‘herb’ was derived from an old French word ‘*herbe*’ and from the Latin word, ‘*herba*’. Earlier, the term herb was only applied to non-woody plants, including those that come from trees and shrubs. Now, herb refers to any part of the plant. The World Health Organization (WHO) estimates that 80% of people worldwide rely on herbal medicines for the common healthcare needs. Homeopathic medicines are mainly categorised into classical, combinations and formulations. The classical medicines consist of single ingredient or source material and therefore, their standardisation at the manufacturing level is easy. In higher potencies, the original substance is difficult to detect through conventional drug assay. This is a big challenge in quality assurance as well in identification of homeopathic medicines. For the manufacturing of homeopathic medicines, only small quantity of the original drug material is sufficient. As such, it is eco-friendly and also called green medicine. Basic sources of homeopathic medicines are almost the same as the other drug systems. The difference lies mainly in the method of preparation and its use in treatment.

The curative effect of the homeopathic medicines is elicited through a process called drug proving or *homeopathic pathogenetic trials* (HPTs). In this, the effect of a medicine is studied on healthy volunteers. The medicine that produced a set of symptoms during the drug proving is given to cure similar symptom in a sick person. The theory behind this concept is that the substance that could induce artificial symptoms in a healthy person could cure those symptoms in natural disease. This principle is called ‘like cures like’, (Law of *Similia*).

19.8 Use of Plants by Animals

Plants are useful to animals as plants provide them food, medicines, timber, etc. Animals help plants by dispersing their seeds which further help in the planting of new plants. Animals also aid in the health and reproduction of trees. Many animals fertilise trees, and others work to disperse seeds and pollens from the plants into different areas, allowing more to grow. These animals eat whatever make them feel better, or prevent disease, or kill parasites such as flatworms, bacteria and viruses, or just to aid digestion. Even creatures with brains the size of pinheads somehow know to ingest certain plants or use them in unusual ways when they need them. Anyone who has seen a dog eat grass during a walk has witnessed self-medication. The dog probably has an upset stomach or a parasite. The grass helps them vomit up the problem or eliminate it with the faeces (Shrukin 2014). The science of animal self-medication is called zoo pharmacognosy. The scientists researching zoo pharmacognosy are convinced that humans can learn from the animals, particularly in finding new medications. Much of folk medicine, particularly in the undeveloped world, likely came from medicine men watching animals self-medicate. Animals use other plants that can combat schistosomiasis, plasmodium and leishmania—all terrible human afflictions and some medicines have been derived from them. The monarch's milkweed, for example, appears to have antiparasitic powers, and may be worth further study. Researchers have shown that the butterflies use milkweed chemicals called cardenolides to fend off a parasite called *Ophryocystis elektroscirrha* (Lefevre et al. 2010).

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Part III
Conservation of Medicinal Plants by
Biotechnology

Chapter 20

In Vitro Conservation and Propagation of Endangered Ethno-Medicinal Orchids from the Northeast Region of India



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Abstract Orchids are appreciated worldwide for their unique and beautiful flowers which hold a large commercial and floricultural value. Other than being ornamental, orchids are known to possess medicinal properties as well. Orchidaceae is one of the largest families of flowering plants with ~30,000 species reported worldwide. In India, there are over 1000 species of documented orchids and the Northeast region of India is the home to many such orchids. Many of them are rare and endangered species and belong to Appendix I of CITES (*Convention on International Trade in Endangered Species of Wild Fauna and Flora*). Their existence is highly threatened due to extensive exploitation and habitat destruction and as such their conservation is of utmost importance. One way to conserve such exotic wild species of orchids is through the intervention of biotechnological methods such as tissue culture. The chapter focuses on the up-to-date progress on the propagation and conservation of rare, endemic and endangered important orchids of the Northeast region of India which are of medicinal values. Phytochemicals that have been identified in many of the orchid species are responsible for their therapeutic properties.

Keywords Orchidaceae · Northeast region · Conservation · Ethnomedicine · Tissue culture · Phytochemicals

Abbreviations

AC	Activated charcoal
BAP	Benzyl Amino Purine
CW	Coconut water
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid

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NER	Northeast region
PLB	Protocorm-Like Bodies
TDZ	Thidiazuron

20.1 Introduction

Orchids are one of the most evolutionarily developed flowering plants with exquisite flowering patterns of various colours, shapes and sizes. They are one of the largest families of angiosperms with diverse species distributed worldwide, wherever favourable ecological conditions exist. It has nearly 850 genera with about ~30,000 species. The growth of orchid is climate specific with wide ecological range differing for each species. It requires sufficient indirect bright sunlight, high humidity and temperate temperatures for the plant to thrive (Biswas and Singh 2019). The Northeastern region (NER) of India exhibits a dynamic topography with varied altitudinal range and diverse bioclimatic conditions ranging from subtropical, near tropical to temperate and cool areas. Because of its favourable geoclimatic conditions, the NER is considered a mega biodiversity hotspot housing innumerable genetically diverse species. The NER of India lies between 22°N and 29°5'N latitude and 88°E and 97°30'E longitude with a land expansion of 262,379 km². NER of India serves as a repository of genetically rich and diverse flora and fauna and thus acts as one of the global biodiversity hotspots (Chakravarty et al. 2012). India's NER comprises of seven sister states (Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland and Tripura) and Sikkim. This region holds a rich array of orchid population contributing to some of the largest flora density and endemic species in this region. Orchids are considered to hold significant cultural importance in this region giving rise to many myths, folklore and symbolism based on the plants. Many of these states even have orchids as their state flowers. *Rhynchostylis retusa* is the state flowers of Assam and Arunachal Pradesh, *Paphiopedilum insigne* for Meghalaya, *Renanthera imschootiana* for Mizoram and *Dendrobium nobile* for Sikkim (Fig. 20.1). This geographical region accommodates numerous ethnic tribes that values ethnobotanical and traditional medicines since ancient period endowing people in the region with economical and natural healing remedies.

Owing to its rise in horticulture and medicinal values, overexploitation of orchids has become a perturbing trend. The population of orchids have drastically decreased due to anthropogenic activities, habitat destruction, climate changes, decline in pollinators' population and suitable symbiotic mycorrhiza along with poor harvesting techniques. In addition, the physiology of orchids drastically differs from other plants as well, resulting in a complicated and specialised life cycle. The flowers have structurally modified resupinate features and simulations to attract the pollinators. Unlike other monocots, orchid seeds lack endosperm for the developing embryo to independently germinate and mature on its own. It has been reported that

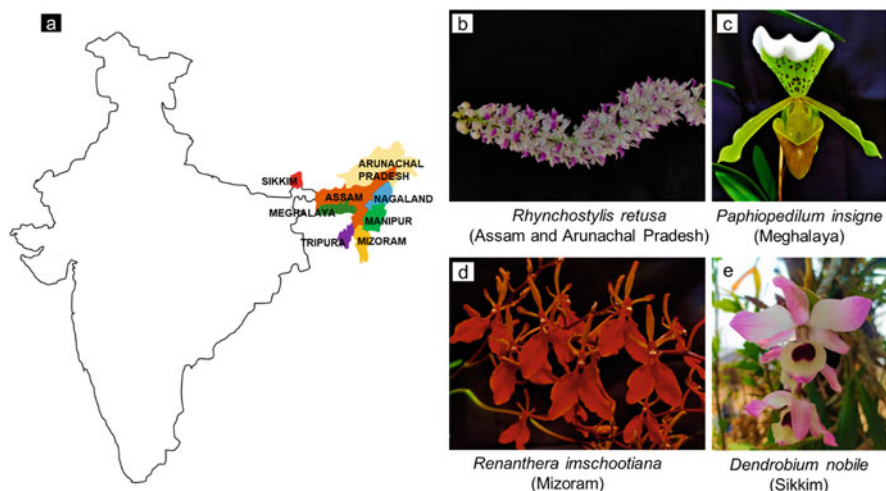


Fig. 20.1 Four orchid species as state flowers of five Northeast Indian states. (a) an outline map of India showing the eight Northeast states (map not to scale), (b) *Rhynchosstylis retusa* (state flower of Assam and Arunachal Pradesh), (c) *Paphiopedilum insigne* (state flower of Meghalaya) (d) *Renanthera imschootiana* for Mizoram and (e) *Dendrobium nobile* for Sikkim

the absence of fully developed endosperms is due to failure in development of polar chalazal complex into endosperm. This makes them rely on mycorrhizal association for their seed germination and embryo maturation (Arditti 1980; Chen and Chen 2007). To lessen the impacts of the mentioned activities and to conserve the declining numbers of threatened orchid species solid measures must be taken up. There are Legislative measures validated by IUCN to protect and avoid illegal trade of the threatened species. Under this legal protection measures, all orchids are included under the Appendix I and II of CITES. The government of India has also enacted Wild life Protection Act to help preserve and conserve the declining population of orchids. Conservation approach can be done at the species level or at a population level; for sympatrically occurring species. The later approach is considered to be an effective mode of conservation strategy (De and Singh 2015; De 2020). Modes of Orchid conservation are shown as a model in Fig. 20.2. Conservation of orchids will be successful with a wholistic approach wherein biodiversity maintenance will be a must. Knowing the root causes for the decline in the population of orchids and many rushing towards extinction, will prove beneficial in combating the process of their conservation. Habitat destruction (natural or man-made), over exploitation of the plants for various purposes, climatic changes etc. are a threat to the survival of orchids. This necessitates the conservation of the species. An integrated approach for conservation would be required which needs to be under the scrutiny of a consortium especially designated for orchid conservation. The consortium can be classified into various sections as per requirement, as research on different aspects of orchid, horticulture dealing with its modes of propagation and education as in the form of awareness, workshops or training

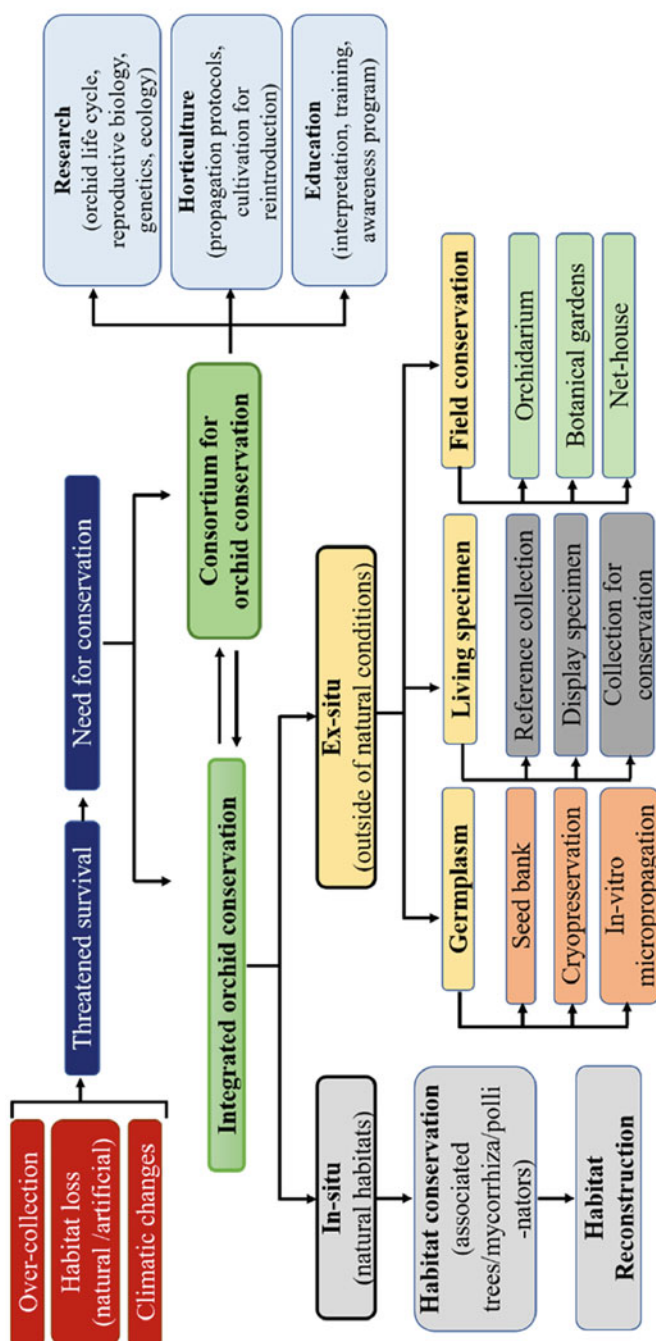


Fig. 20.2 Model for orchid conservation. (Adapted and modified from Botanic Gardens Conservation International, <https://www.bgci.org/about/about-plant-conservation/integrated-plant-conservation/>)

programs. The modes of integrated approach for conservation can be done in situ and ex situ. In situ conservation includes conservation of the species under its natural habitat. This includes conservation of the associated trees for epiphytes, mycorrhizal fungi and the pollinators as well. Re-establishment of a destroyed habitat would also aid in in situ conservation. The ex situ conservation can be in the form of germplasm conservation (seed bank, cryopreservation and in vitro propagation), living specimen collection for use as a reference or display specimen, collection for conservation purpose and field conservation in the form of orchidarium, botanical gardens or net-houses.

The NER of India has many tribes that hold belief in animism. The local communities conserve patches of forest rich in biodiversity as sacred grooves, limiting admittance within the certain region if not for socio-cultural and religious practices. They believe in the existence of a symbiotic relationship between the people and the nature in a holistic approach and deem importance in the protection of that certain area. There are quite a few numbers of sacred groves in each of the states of NER. A great deal of orchid species is well protected within this area of sacred groves.

There are 17 orchid species enlisted by BSI (<https://bsi.gov.in/uploads/documents/research-program/Threatened-plants-of%20India.pdf>) as threatened species of India. According to red list category, three are critically endangered (*Paphiopedilum druryi* (Bedd.) Stein, *Paphiopedilum fairrieanum* (Lindl.) Stein and *Zeuxine rolfiana* King & Pantl.), eight are endangered (*Paphiopedilum charlesworthii* (Rolfe) Pfitzer, *Paphiopedilum spicerianum* (Rchb.f.) Pfitzer, *Paphiopedilum venustum* (Wall. ex Sims) Pfitzer, *Habenaria dichopetala* Thwaites, *Cypripedium elegans* Rchb.f., *Cypripedium himalaicum* Rolfe ex Hemsl. *Paphiopedilum appletonianum* (Gower) Rolfe, *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer) and remaining six are vulnerable (*Paphiopedilum villosum* (Lindl.) Stein, *Luisia volucris* Lindl., *Vanda spathulata* Spreng., *Cypripedium cordigerum* D.Don, *Paphiopedilum hirsutissimum* (Lindl. ex Hook.f.) Stein and *Malaxis muscifera* (Lindl.) Kuntze).

The diminishing population of certain species of the family at unprecedented rate has resulted in orchids being the subject of aggressive conservation efforts. Effective conservation techniques involve applied modern biotechnological approaches through in vitro cultures, tissue culture and clonal propagation of endangered plants at minimal time period. Through this mode of conservation, the decline in orchid population for expansion of herb trades can be compromised without exhausting the bioresources and its genetic population. Mass multiplication of thousands of plants can be achieved from few explants, provided the right conditions for the plant to grow. It requires sterile controlled conditions with regulated temperature, humidity and light/dark cycle depending on the type of species.

20.2 History of Medicinal Orchids

Orchids are mostly famed for its extravagant and unique blooms holding high horticultural value as ornamental plants. Assessments on its nutritional and medicinal properties are comparatively less compared to its ornamental aspect (Marjoka et al. 2016). Orchids display various roles, be it ornamental because of its beautiful flowers that adds to its aesthetic values. They have cultural relevance as well, for example *R. retusa* is adorned by women in Assam during festivals. Not only do they have horticultural aspects, but also have medicinal properties and are used by local healers in different regions of the Northeast states. The use of medicinal plants and their extracts as the major source of treating and healing ailments has been acknowledged since ancient human civilisation even before the enlightenment of scientific revolution. It has also been adjudged that nearly 80% of the populace of the developing countries and emergent nations depend on plant based traditional treatments (Chauhan and Sharma 2017; Mukherjee and Wahile 2006; Palhares et al. 2015). A number of orchid species are known to possess medicinal properties. The evident use of orchids as medicine was initially popularised in the Ayurveda and traditional Chinese medicine (Singh and Duggal 2009). Indian system of traditional medicine has a long history of orchid use. Ashtavarga known as the ‘herb of immortality’ is an important ingredient of various classical Ayurvedic formulations such as Chyawanprash, which consists drugs of orchid’s origin such as *Crepidium acuminatum* (syn. *Malaxis acuminata*, *Microstylis wallichii*), *Malaxis muscifera*, *Habenaria intermedia* and *Platanthera edgeworthii* (*Habenariaedgeworthii*) (Jalal et al. 2009; Singh and Duggal 2009). *Dendrobium macraei* is used as a source of an important Ayurveda drug called ‘Jivanti’ (Kasera and Shukla 2003). To name a few, *Dendrobium fimbriatum*, *D. nobile*, *Papilionanthe teres*, *Eria musicucola*, *Eulophia compestris*, *Laparis odorata*, *Orchis latifolia*, *Vanda cristata*, *V. tessalata*, *V. coerulea*, etc. are some of the important medicinal orchids used by traditional healers in India (Ming et al. 2003; Hossain 2011; Medhi and Chakrabarti 2009). *Vanda* has a long history of use for its anti-inflammatory properties (Dash et al. 2008; Ho and Chen 2003).

They are also used as edibles and their usage in cosmetics are also known. These multifaceted roles of orchids are depicted in Fig. 20.3. Their therapeutic properties include antipyretic, antioxidative, anti-platelet aggregation activity, antibacterial, antiulcer, analgesic and aphrodisiacs. Their medicinal properties are attributed by the phytochemicals present. Presence of secondary metabolites such as phytoalexins, hydrocarbons, terpenoids, triterpenes, steroids stilbenoids, bibenzyls, phenanthrenes, alkaloids, phenols, flavonoids, polysaccharides etc. attribute to their phytochemicals responsible for their therapeutic nature. Further analysis on phytochemical screening of orchids reported the presence of flavonoids, glycosides, antioxidants, bibenzyl derivatives and various other secondary metabolites of potential medicament (Singh and Duggal 2009; Marjoka et al. 2016). The most commonly reported orchids that have medicinal significance include *Acampe*, *Aerides*, *Bletillia*, *Bulbophyllum*, *Calanthe*, *Coelogyne*, *Cypripedium*, *Cymbidium*, *Dendrobium*, *Eria*,

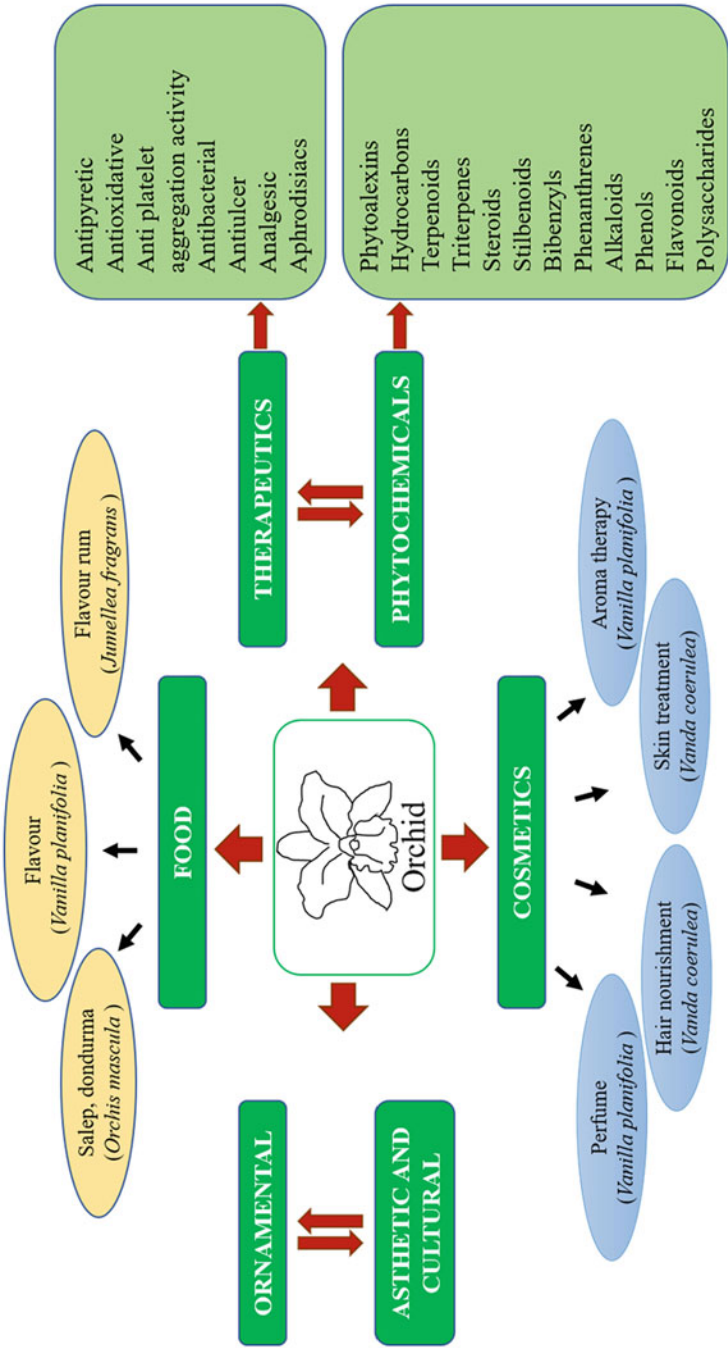


Fig. 20.3 Multifaceted roles of orchids. Scope of orchids and its potential in the field of industry, horticulture and pharmaceuticals is displayed

Eulophia, *Gastrodia*, *Habenaria*, *Malaxis*, *Nervilia*, *Pholidota* and *Vanda* (Yonzon et al. 2011; Singh et al. 2012; Behera et al. 2013; Teoh 2016, 2019).

Dendrobium chrysotoxum and *Dendrobium densiflorum* are two threatened ethno-medicinal orchids of NER India. The flowers, their medicinal uses and their phytochemicals identified are shown in Fig. 20.4. The 2-D structures of the phytochemicals are obtained from PUBCHEM, NCBI (<https://pubchem.ncbi.nlm.nih.gov/>).

The species that have potential bioactive compounds can be micro-propagated at large scale through in vitro propagation. Biotechnological methods and techniques can be employed to genetically manipulate the genes for upregulating the desired secondary products and tweaking the regulation of gene expression. Addition of elicitors in the growth medium such as chitosan has been indicated to enhance the production of phytochemicals in *Vanda coerulea* (Nag and Kumaria 2018). Increase in yield of secondary metabolites in *Dendrobium* hybrids has also been reported by action of light emitting diodes (LEDs) in PLB cultures by Yeow et al. (2020). The phytochemicals and bioactive compounds obtained from the cultured plants can be further identified and processed for qualitative and quantitative analysis. The extracted bioactive secondary metabolites obtained that are known to have pharmaceutical values can be exerted in the field of medicines. Once quality and ethical requirements are met, they could undergo clinical trials and have a better scope in providing framework of economical health care system. A typical flowchart for micropropagation steps via tissue culture technique and biotechnological interventions for isolation, identification and quantification of bioactive compounds is shown in Fig. 20.5.

20.3 Biotechnological Intervention for Conservation of Orchids

Orchids in general propagate through sexual reproduction but, they also reproduce at a slightly faster rate through vegetative propagation from shoot tips, nodal division, back bulbs, keikis and other possible meristematic regions. The growth rate of orchids is quite steady compared to other plants which is one of the many reasons leading to its threatened status. Propagation of the species for conservation and to aid in lifting up its threatened status undoubtedly requires understanding the biological mechanism of its reproduction, physiology of the seed and its interaction with environmental factors (Behera et al. 2013; Ramawat and Arora 2021). The integration of traditional knowledge of cultivation with modern biotechnological approaches with the help of scientific tools and new breeding techniques can optimise efficient propagating methods of orchids at a large scale.

Understanding the physiology and functional requirements of plants is a pre-requisite knowledge to propagate the plants in vitro. Orchid seeds have a complex physiology. Unlike other monocotyledonous angiosperm, cotyledons in

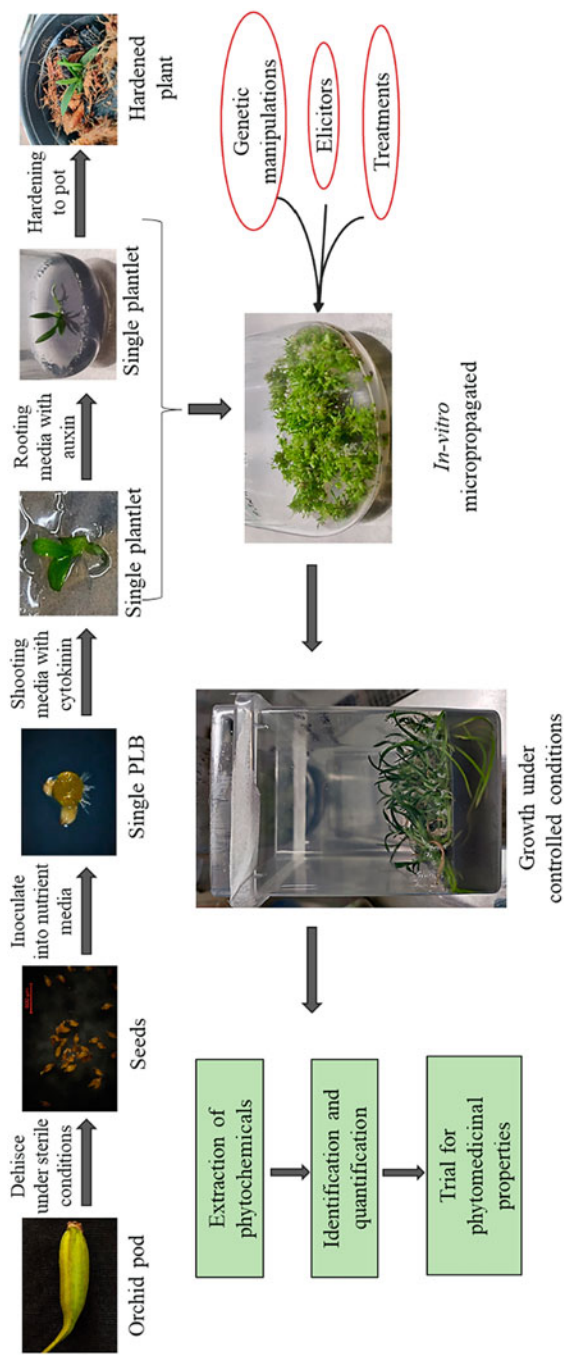


Fig. 20.5 A typical flowchart for micropropagation steps via tissue culture technique and biotechnological interventions for isolation, identification and quantification of bioactive compounds

orchids are not developed, leading to poorly developed embryo. The lack of sufficient functional endosperms results in immature embryo that fails to germinate independently. To balance the inadequacy of its lack of endosperms, the seeds have acquired modifications in its suspensors. The suspensors are temporary organs which are formed during the embryonic stage to secure the embryo proper with the seed coat, and help obtain nutrients for the developing embryo. To prevent the seed from being dehydrated, cuticular and secondary walls are formed and layered up in the seed coat as an adaptation (Yeung and Clutter 1979; Arditti 1980; Yeung 2017). Analysing the limitations and differences of the seed development could help us design and implement necessary procedures to grow a mature plantlet. John Lindley, the father of orchidology, was among the pioneers who laid down the concepts on orchid pollination and fertilisation. Reviewing earlier studies and experimentation, Knudson (1922) successfully achieved asymbiotic germination of orchid seeds by providing external nutrient source. With this as a trailblazer, many studies have been reported where in vitro propagation of orchids from seeds and other meristematic explants have been carried through. Cognizance of functional and physiological requirements of the explants during germination, regeneration, differentiation and development would be a constructive approach towards designing and formulation of efficacious culture media. Various standard protocols for micropropagation have been established using different kinds of media, supplemented with additional growth regulators and phytohormones.

Designing an artificial culture media requires in depth understanding of the roles and functions of each and every composition that goes into formulating the medium. The growth and development requirement for different explants of different species varies but the elemental components required for basic growth of the plants is well established in all the media.

Macronutrients such as nitrogen, magnesium, potassium, sulphur, phosphorous and calcium are added in the form of salts as balanced nutrient. Micronutrients are relatively required in trace quantity and include iron, cobalt, chlorine, manganese, copper, molybdenum, zinc, boron and nickel. They are both required by the plant cells to perform basic cellular metabolism and synthesis of biomolecules required for cell division and growth. In vitro cultured plants are partially heterotrophic in its early stage. Therefore, to obtain optimum growth they are supplemented with organic nutrients such as vitamins, amino acids, sugars, phytohormones and additional organic extracts. Generally used plant hormones are auxins such as IAA, NAA, IBA and 2,4-D for callus formation, vascular differentiation, shoot and root morphogenesis (George et al. 2008a) and cytokinins such as 6-BAP, Kinetin, Zeatin and TDZ. They initiate cell division by regulating mitotic activity, helps regulate seed germination, stimulate shooting and axillary shoot multiplication while decreasing rooting. The ratio of auxin to cytokinin influences the nature of organogenesis and callus induction in plant tissue cultures. Gibberellins induce seed germination, callus growth and cell elongation. Absciscic acid promotes seed dormancy and inhibit development of embryo into later stage but enhances shoot proliferation. Lowering ABA level can help progress asymbiotic seed germination. Additional organic supplements such as coconut water, banana powder, chitosan,

casein hydrolysate, yeast extract etc, promote cell division and development of plantlets. Addition of activated charcoal in the media enhances the growth of cultured cells by irreversible absorption of inhibitory compounds in the media (Saad and Elshahed 2012; Miransari and Smith 2014; Yeung 2017).

Besides the above mentioned supplements, the main inevitable components that furnish energy for the proliferating and developing cells are the sugar or carbohydrate source. They function as source of energy and helps in regulating osmotic potential of plant cells (de Paiva Neto and Otoni 2003). Sucrose serves as the main choice of carbon source since it is the form of carbohydrate found in phloem sap. On autoclaving, sucrose hydrolyses into simpler forms of monosaccharide glucose and fructose which can be easily utilised by the cultured cells. Koch (1996) articulated that abundant availability of carbohydrate source in the media suppresses the functionality of photosynthesis of the growing plantlets. Therefore, a balanced addition of necessary nutrients is a requisite. The chemical stability and efficacy of the media also depends on the pH of the medium. It is necessary to maintain an appropriate pH based on the composition and requirement of the explant taken. Orchid cultures showed optimum growth between a pH range of 5.5–5.8 for solid media, while liquid suspension cultures prefer a slightly acidic condition of pH 4.9 (Gamborg et al. 1976; Yam and Arditti 2017; Kumar 2018). The appropriate light intensity for incubation of orchid cultures is reported to be 20–50 $\mu\text{mol}/\text{m}^2/\text{s}$ photon flux density with a photoperiod 16/8 (light/dark) condition at a temperature of 22–26 °C. Different species have specific preferences of growing conditions. Terrestrial orchids such as *Paphiopedilum* sps. prefer 24 h dark condition for seed germination and 16–18 h photoperiod at 23–25 °C for its plantlet to grow depending on the type of explant taken. *Vanda* sps. shows optimum growth at a slightly warmer temperature. It shows preference of growth at 25 ± 2 °C with 12–16 h photoperiod (Yam and Arditti 2017; Setiaji et al. 2021).

In vitro propagation in suspension of well formulated nutrient media is practised for culture of protoplast, PLBs and root explants. Many studies reported convincing results for propagation in liquid suspension medium. However, this type of culture medium is restricted for certain explants and production of specific desired end products. Limitations of liquid nutrient medium include deceleration of growth of cultured cells and tissues due to excessive hydration and hypoxia. Water potential becomes higher for submerged cells that results in decrease in respiratory rate of the cells and causes decrease in metabolic function of the cells. To increase the availability of oxygen content in the medium, cultures can be placed in shaker incubators. Addition of solidifying agents in the medium results in better aeration provided through the porous gelling agents such as agar, gellan gum, alginates, starch, carrageenan and other porous supports (George et al. 2008b). The fully developed micropropagated plantlets are than established under controlled conditions provided with required humidity, temperature and light/dark cycle before acclimatisation and establishment in the open field.

Various explants have been used for the propagation of different orchid species. Seeds from capsules (Baby et al. 2019), leaves (Islam and Bhattacharjee 2015), leaf tips (Goswami et al. 2016), roots (Islam and Bhattacharjee 2015), rhizome tips

(Bhadra and Hossain 2003), stems (Kaur and Bhutani 2010), node (Bhowmik et al. 2020), pseudobulb (Bhandari et al. 2011), etc. have been documented. Symbiotic germination of orchid seeds in nature and its propagation is an extremely time-consuming process. To control the steep decline in the population of this family and threatened status of certain species, micropropagation through callus culture is an alternative solution. Callus is an undifferentiated parenchymatous mass of cells that dedifferentiate from the tissue of explant taken to undergo re-differentiation and give rise to a new plantlet (Bhatia 2015). In orchids, callus culture is difficult to obtain as callus formation often accompanied with necrosis. Nevertheless, Giri et al. (2012) studied callus induction in *H. edgeworthii* from seeds grown in ½ MS supplemented with 1.0 µM NAA within 8 weeks. Leaf of *Laelia speciosa* was cultured in MS media with 2.5 mg/L of BA for callus induction that developed into PLBs and mature plantlets (Sarabia-Ochoa et al. 2010).

20.4 Medicinal Orchids of Northeast Region

Some of the representative medicinal orchids of NER of India are enlisted below with their ethno-medicinal usage, phytochemicals and tissue culture studies.

20.4.1 Acampe

20.4.1.1 Ethno-Medicinal

Roots of *Acampe praemorsa* (syn. *Acampe papillosa*) are used in treating rheumatism (Deb et al. 2009) and arthritis (Dash et al. 2008). Its root decoction is used as Indian herbal *Rasna*. It is also used to promote healing of fractured bones (Teoh 2016).

20.4.1.2 Phytochemical

Acampe praemorsa contains flavidin and praemorsin (Teoh 2016; Anuradha and Prakasa Rao 1993, 1994). *Acampe papillosa* showed the presence of saponins, tannins, glycosides, flavonoids and steroids (Akter et al. 2018).

20.4.1.3 Tissue Culture

Tissue culture by direct somatic embryogenesis from leaves of *A. papillosa* has been reported by Jena et al. (2013).

20.4.2 *Aerides*

20.4.2.1 Ethno-Medicinal

Species of *Aerides* are used for treating local infections such as wounds, boils, other skin disorders and earache (Teoh 2016). *Aerides multiflorum* is shown to possess anti-bacterial activity (Singh and Duggal 2009). While roots and leaves paste of *A. odoratum* mixed with leaf paste of *Azadirachta indica* when taken orally is said to reduce joint pain and swellings. The fresh leaf juice is taken orally to cure tuberculosis (Dash et al. 2008).

20.4.2.2 Phytochemical

Study on α -Glucosidase inhibitory effect of compounds Aerimultin A, B, C, Dihydrosinapyl dihydroferulate, 6-Methoxy coelonin, Gigantol isolated from *A. multiflorum* indicated that the biphenathrene aerimultin C is the most potent inhibitor, with potency much higher than the drug acarbose (Thant et al. 2021).

20.4.2.3 Tissue Culture

In vitro generated plantlets have been developed for *A. multiflorum* by Bhowmik et al. (2020) from leaves and nodes. In vitro propagation of leaf segment cultures and cryopreservation has been carried out for *A. odorata* (Hongthongkham and Bunnag 2014).

20.4.3 *Anoectochilus*

20.4.3.1 Ethno-Medicinal

Anoectochilus roxburghii is used for clearing heat and cooling blood, eliminating dampness, and detoxification (Zhang et al. 2015). It is also used to treat hepatitis, splenic disorders, hypertension, cancer, tuberculosis, impotence, fever, snake bites and even slow development in children (Lin et al. 2000; Teoh 2016). Along with *A. formosanus*, it is also used for numerous ailments that include pleurodynia and other pulmonary conditions, liver disease, hypertension, paediatric malnutrition and snake bites (Teoh 2016).

20.4.3.2 Phytochemical

The pharmacological activities such as antidiabetic, antilipemic, anti-inflammatory, antiviral, liver protective, renal protective, immunomodulatory, abirritant, sedative, and antineoplastic effects of *A. roxburghii* is attributed by the phytochemicals such as polysaccharides, flavonoids, glycosides, organic acids, volatile compounds, steroids, triterpenes, alkaloids, and nucleosides (Ye et al. 2017). Other compounds isolated from this plant include beta-D-glucopyranosyl-3R-hydroxybutanolide, stearic acid, palmitic acid, beta-sitosterol, succinic acid, *p*-hydroxybenzylaldehyde, daucosterol, methyl 4-beta-D-glucopyranosyl-hutanoate, *p*-hydroxycinnamic acid, *o*-hydroxy phenol, Ferulic acid, quercetin, daucosterol and cirsilineol (Teoh 2016).

20.4.3.3 Tissue Culture

Nodal segments has been used as explants for generating tissue culture plantlets of *A. roxburghii* in half strength MS media (Zhang et al. 2015).

20.4.4 Arundina

20.4.4.1 Ethno-Medicinal

Rhizome paste of *Arundina graminifolia* (bamboo orchid) is used to cure bacterial infected wounds (Singh and Duggal 2009).

20.4.4.2 Phytochemical

Stilbenoids, phenanthrenes, quinones, ketones, phenolic acids, glycosides, and essential oils have been isolated from *A. graminifolia* (Zhang et al. 2021). Benzyldihydrophenanthrene: arundinaol, stilbenoid: arundinan and phenanthrene constituents are also present in *A. graminifolia* (Singh and Duggal 2009).

20.4.4.3 Tissue Culture

Micropropagation of the species through protocorm-like-bodies using node explants has been studied (Kottackal 2007).

20.4.5 *Bulbophyllum*

20.4.5.1 Ethno-Medicinal

According to Dash et al. (2008), dried root powder of *Bulbophyllum cariniflorum* and powder of black pepper mixed with milk is helpful to induce abortion within 2–3 months of pregnancy. Pseudobulbs of *Bulbophyllum sterile* are used as tonic for good health (Teoh 2016). Roots decoction of *Bulbophyllum umbellatum* (syn. *Cirrhopetalum maculosum*) is used as revitalise *swarna jiwanti* in Ayurvedic preparations (Duggal 1971).

20.4.5.2 Phytochemical

Glycosides, flavonoids, saponins, tannins, steroids, quinine and coumarin are isolated from *B. cariniflorum* (Aswandi and Kholibrina 2021). Cirrhopetalanthrin is a dimertic phenanthrene derivative isolated from *Bulbophyllum umbellatum* (syn. *Cirrhopetalum maculosum*) (Majumder et al. 1990). Petroleum fraction of *Bulbophyllum sterile* bulbs (PFB) and roots (PFR) was found to be most active in HCT-116 cell lines with IC₅₀ value of 94.2 ± 6.0 and 75.7 ± 9.8 , respectively. The active fractions of bulbs and roots seem to possess anticancer activity likely by inducing apoptosis through phospho-p53 dependent pathway (Biswas et al. 2016).

20.4.5.3 Tissue Culture

Data unavailable.

20.4.6 *Calanthe*

20.4.6.1 Ethno-Medicinal

Root extract of *Calanthe triplicata* is effective in diarrhoea and teeth cavities. Whole plant parts used for ailments of stomach and intestine. Flower poultice is used to reduce ulcer pain; roots decoction used to cure diarrhoea (Deb et al. 2009).

20.4.6.2 Phytochemical

Extraction of the compound 4H-Pyran-4one, 2,3-dihydro-3,5 dihydroxy-6-methyl from *Calanthe triplicata* suggests its anti-inflammatory and anti-arthritic activities (Mythili et al. 2013).

20.4.6.3 Tissue Culture

In vitro seed germination and rhizome based micropropagation protocol was established in *Calanthe densiflora* (Bhowmik and Rahman 2017).

20.4.7 Coelogyne

20.4.7.1 Ethno-Medicinal

Dried pseudobulbs of *Coelogyne punctulata* are pounded into powder which is applied to spots of burn injuries. It relieves pain immediately and helps in healing of the wound (Deb et al. 2009). Fresh pseudobulbs of *Coelogyne stricta* is used to cure headache and fever (Yonzon et al. 2011). *C. cristata* is used in dysentery and diarrhoea (Naing et al. 2011).

20.4.7.2 Phytochemical

Presence of phenolic compound coelonin was reported in *C. stricta* (Majumder et al. 1982b). Plant extracts of *C. cristata* also shows presence of phenanthrin derivative coelogin, coeloginin, coeloginanthrin and coeloginanthridin (Majumder et al. 1982a, 2001).

20.4.7.3 Tissue Culture

Mass propagation of *Coelogyne cristata* through PLB was cultured on MS media containing TDZ and CP (Naing et al. 2011). In vitro germinated seed from immature seed capsules of *C. stricta* was developed into mature plantlets in MS media supplemented with NAA and BAP (Parmar and Pant 2016).

20.4.8 Cremastra

20.4.8.1 Ethno-Medicinal

Fresh tubers of *Cremastra wallichiana* is used to cure acute toothache. *Cremastra appendiculata* is used in treatment of liver cancer, lung cancer, breast cancer and other cancers and has hypoglycemic properties (Yang et al. 2021).

20.4.8.2 Phytochemical

Pseudobulbs of *Cremastra appendiculata* contain colchicine, quercetin, rutin, adenosine, oleanolic acid, myristate, rhein and emodin (Yang et al. 2021).

20.4.8.3 Tissue Culture

Micropropagation protocol of *Cremastra appendiculata* in $\frac{1}{4}$ MS has been developed (Yang et al. 2021).

20.4.9 Cymbidium

20.4.9.1 Ethno-Medicinal

Fresh leaves of *Cymbidium aloifolium* are heated and the juice is dropped in the ear for the cure of otitis and other inflammatory conditions. Seeds are used for healing wounds and used as haemostatic. Root powder is helpful to reduce paralysis. Entire plant is used as purgative, emetic, tonic and in treating earache. Mature seeds are used for healing wounds (Dash et al. 2008; Medhi and Chakrabarti 2009). Another species *Cymbidium ensifolium*'s fresh flowers decoction is used in eye sores and rhizome decoction is used to cure gonorrhea (Yonzon et al. 2011). Fresh leaf juice of *Cymbidium iridoides* is used for clotting of blood in wound (Medhi and Chakrabarti 2009) while fresh roots decoction of *Cymbidium longifolium* is effectively used for nervous disorders, madness, epilepsy, hysteria, rheumatism, spasms (Yonzon et al. 2011).

20.4.9.2 Phytochemical

Leaf of *Cymbidium aloifolium* showed the presence of phytochemicals such as alkaloids, flavonoids, phenols, quinine, coumarin, saponins, carbohydrates, tannins (Akter et al. 2018).

20.4.9.3 Tissue Culture

In vitro seed germination of *Cymbidium aloifolium* was assessed on 0.8% (w/v) agar solidified KC, MS, PM, VW media, out of which MS medium proved to be best (93.34%) for early germination and greenish PLBs formation followed by PM (86.87%), VW (73.34%) and KC (73.34%) media (Bhowmik et al. 2020).

20.4.10 *Dactylorhiza*

20.4.10.1 Ethno-Medicinal

Tuberous roots of *Dactylorhiza hatagirea* are used to cure diabetes, dysentery, diarrhoea, anthelmintic, wounds, urinary trouble, fever, paralysis, convalescence and malnutrition. It is aphrodisiac, astringent, expectorant and nervine tonic. Salep prepared from the tuber is very nutritive and demulcent (Singh and Duggal 2009).

20.4.10.2 Phytochemical

Dactylorhiza hatageria, a source of high quality ‘salep’ used as nervine tonic showed the presence of alkaloids, flavonoids, saponins, and steroids (Kawra et al. 2020).

20.4.10.3 Tissue Culture

Dactylorhiza hatagirea pod culture was observed to grow best in MS medium supplemented with peptone (P) (1.0 g/L), morphinoethane sulphonic acid (MES) (1.0 g/L) and activated charcoal (AC) (0.1%) (Giri and Tamta 2012).

20.4.11 *Dendrobium*

20.4.11.1 Ethno-Medicinal

Whole plant parts of *Dendrobium aggregatum* is used to treat nerves, cholera, pimples and boils and that of *Dendrobium alpestre* as poultice in curing pimples, boils and skin diseases. Fresh shoots of *Dendrobium amoenum* extraction serves as antibiotic and inhibits the growth of bacteria in wounds and ulcers. Dried powdery seeds of *Dendrobium chrysanthum* is used as haemostatic (Deb et al. 2009).

Whole plant extract of *Dendrobium chrysotoxum* serves as anti-tumorous and anti-cancerous; powdery seeds dried and used as haemostatic (Deb et al. 2009).

Fresh leaf poultice of *Dendrobium densiflorum* is recommended for bone fracture (Yonzon et al. 2011); powdery seeds dried and used as haemostatic (Deb et al. 2009).

Decoction of flowers and leaves of *Dendrobium fimbriatum* is used for liver upsets and nervous debility. Poultice of fresh leaves is used to cure boils and pimples. Fresh leaves and flowers used to cure cholera (Medhi and Chakrabarti 2009).

Tuber of *Dendrobium macraei* is used as tonic and helpful to cure asthma, bronchitis and throat trouble. Roots paste is externally applied in skin allergy and affected part to cure eczema (Singh and Duggal 2009).

Fresh leaf juice of *Dendrobium moschatum* is used in Meghalaya for earache. Powdery seeds dried and used as haemostatic (Medhi and Chakrabarti 2009; Deb et al. 2009).

Whole plant parts of *Dendrobium nobile* is used in the treatment of pulmonary tuberculosis, flatulence, general debility, cut and wounds healing, dyspepsia, night sweats, fever and anorexia. It is antiphlogistic, pectoral, stomachic and tonic (Medhi and Chakrabarti 2009; Singh and Duggal 2009); powdery seeds and root-powder used to heal wounds; also used in nervous disorder (Deb et al. 2009).

20.4.11.2 Phytochemical

The medicinal and pharmaceutical activities of *Dendrobium* species are widely explored with its compounds isolated that are shown to exhibit antipyretic, anti-tumour, antioxidative and antimicrobial activities. Some of the compounds isolated are Shihunidine, Shihunine (*Dendrobium loddigesii*), dendrobine (*D. moniliforme*, *D. nobile*), Moscatilin, Moscatilin diacetate (*D. loddigesii*), Homoeriodictyol (*D. densiflorum*), Dendroside A; Dendroside D; Dendroside E; Dendroside F; Dendroside G and Dendronobiloside A (*D. nobile*), Isoamoenylin (*D. amoneum*), Dendromoniliside A; Dendromoniliside B and Dendromoniliside C (*D. moniliforme*), Alkyl ferulates (*D. moniliforme*), Erianin (*D. chrysotoxum*), Nobilin D, Nobilin E (*D. nobile*), moscatin (*D. loddigesii*), 4,7-Dihydroxy-2-methoxy-9,10-dihydrophenanthrene; Denbinobin (*D. nobile*), Dendrochrysanene (*D. chrysanthum*), Moniliformin; 2,6-Dimethoxy-1,4,5,8-Phenanthradiquinone; 7-Hydroxy-5,6-dimethoxy-1,4-phenanthrenequinone (*D. moniliforme*).

20.4.11.3 Tissue Culture

The genus is one of the most widely studied orchids for their propagation. In vitro germination and micropropagation studies have been studied in many species of these genus, for example *D. nobile* (Malabadi et al. 2005), *D. chrysotoxum* (Roy et al. 2007), *D. moschatum* (Kanjilal et al. 1999), *D. densiflorum* (Luo et al. 2008), *D. fimbriatum* (Roy and Banerjee 2003), *D. chrysanthum* (Mohanty et al. 2013), *D. aggregatum* (Hossain 2013), etc.

20.4.12 Eria

20.4.12.1 Ethno-Medicinal

Mixture prepared from whole plant parts of *Eria bambusifolia* and ripe fruits of *Aegle marmelos* are used to cure hyper acidity and stomach disorder (Dash et al. 2008). Whole plant parts of *Eria muscicola* are used to cure diseases of heart, lungs, eyes, ears and nervous system (Yonzon et al. 2011; Medhi and Chakrabarti 2009). Decoction prepared from fresh roots and leaves of *Eria pannea* is useful in bone ache. Whole plant is used in bath for treating ague (Medhi and Chakrabarti 2009).

20.4.12.2 Phytochemical

Nudol is a phenanthrene found as a minor constituent of *Eria carinata* and *E. stricta*. The compound is also present in the tubers of *Eulophia nuda* (Bhandari et al. 1985). Zhan et al. (2016) also reported the presence of a chemical compound bambusifolol from *E. bambusifolia*.

20.4.12.3 Tissue Culture

Asymbiotic seed germination of *E. bambusifolia* and development into plantlets was obtained in MS media along with additional growth regulators and NAA as rooting hormone (Basker and Bai 2010).

20.4.13 Eulophia

20.4.13.1 Ethno-Medicinal

Tubers of *Eulophia campestris* are used to treat worm infestation and scrofula. Tuber decoction serves as tonic and helpful to treat stomach problems, cough, paralysis, heart ailments and also used as aphrodisiac. It is demulcent and anthelmintic (Medhi and Chakrabarti 2009; Singh and Duggal 2009).

Powdered prepared from rhizomatous tuber of *Eulophia dabia* is used to treat spermatorrhoea, blood purifier, heart problems and stomach ache (Yonzon et al. 2011).

Poultice prepared from the pseudobulbs and whole plant parts of *Eulophia explanata* are used to heal wounds and spermatorrhoea (Yonzon et al. 2011).

Tuber pieces of *Eulophia herbacea* fried with ghee and used to cure leucorrhoea and used as tonic (Yonzon et al. 2011).

Fresh tubers of *Eulophia nuda* are used to treat worm infestation, bronchitis, tumours and scrofula (Singh and Duggal 2009).

Tubers of *Eulophia spectabilis* are used against aphrodisiac and leaf decoction is used against vermifuge (Dash et al. [2008](#)).

20.4.13.2 Phytochemical

Eulophiol and nudol are compounds present in the tubers of *E. nuda* (Bhandari et al. [1985](#)).

20.4.13.3 Tissue Culture

An in vitro propagation and tuberisation method was successfully established for *Eulophia nuda* on Murashige and Skoog (MS) medium containing 44.4 M 6-benzyladenine (BA) and additives (283.8 M ascorbic acid, 67.8 M adenine sulphate, 143.5 M arginine and 118.9 M citric acid) (Panwar et al. [2012](#)).

20.4.14 Geodorum

20.4.14.1 Ethno-Medicinal

Fresh root paste of *Geodorum densiflorum* mixed with honey is taken orally to regularise menstrual cycle in women (Dash et al. [2008](#)).

20.4.14.2 Phytochemical

The pseudobulbs showed alkaloids, glycosides, phytosterols, tannins and proteins and antimicrobial activity against *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aurigenosa* and *Salmonella typhi* (Borkar and Masirkar [2015](#)).

20.4.14.3 Tissue Culture

Seeds of *Geodorum densiflorum* were aseptically cultured on 0.8% (w/v) agar solidified MS and Phytamax (PM) media (Bhadra and Hossain [2003](#)).

20.4.15 **Goodyera**

20.4.15.1 **Ethno-Medicinal**

Whole plant decoction of *Goodyera repens* is used to treat common cold, snake bites, gastro-intestinal, toothache, kidney disorders, obesity, eye urinary and female diseases (Yonzon et al. 2011). *Goodyera schlectendaliana* is used for fever, pain, snake-bites and lung disease (Du et al. 2000).

20.4.15.2 **Phytochemical**

Goodyerin is isolated from *Goodyera schlectendaliana* (Du et al. 2000).

20.4.15.3 **Tissue Culture**

Symbiotic culture of *G. schlechtendaliana* seeds with a binucleate *Rhizoctonia* isolate was effective in promoting seed germination and protocorm development compared with non-symbiotic culture (Tomita 1995).

20.4.16 **Habenaria**

20.4.16.1 **Ethno-Medicinal**

Dried roots of *Habenaria commelinifolia* are used to cure spermatorrhea, urinary trouble. Roots and tubers are used as 'Salep'.

Infusion of the whole plant powdered is used for curing nasal bleeding and snake bite (Yonzon et al. 2011; Dash et al. 2008).

Whole plant of *Habenaria dentata* is useful as an analgesic, disinfectant, aphrodisiac, antirheumatic, urinary trouble and orthopaedic ailments (Yonzon et al. 2011).

Fresh leaves and roots of *Habenaria edgeworthii* are used to treat diseases of the blood. It is cooling and spermopiotic. Tubers used for cough, asthma, insanity, fever and rejuvenation (Singh and Duggal 2009).

Fresh rhizome of *Habenaria intermedia* is used as an aphrodisiac and heart tonic (Yonzon et al. 2011; Singh and Duggal 2009).

Fresh tuber decoction of *Habenaria marginata* mixed with honey is taken daily on an empty stomach for treatment of malignant ulcer (Dash et al. 2008).

Fresh leaves of *Habenaria pectinata* are crushed and applied in snake bites. Tubers mixed with condiments are used in arthritis rheumatism. Roots are used as blood purifier and revitaliser (Singh and Duggal 2009).

20.4.16.2 Phytochemical

Giri et al. (2012) reported the presence of certain phenolic compounds such as gallic acid, catechin and hydroxybenzoic from extracts of in vitro cultured callus and wild stem of *Habenaria edgeworthii* that showed antioxidant property.

20.4.16.3 Tissue Culture

An efficient in vitro propagation protocol for *H. edgeworthii* using seed-derived callus was established. The maximum seed germination was observed in MS medium supplemented with 1.0 μM α -naphthalene acetic acid (NAA). Induction of callus was achieved on full and $\frac{1}{2}$ -strength MS medium supplemented with 1.0 μM NAA (Giri et al. 2012).

20.4.17 Liparis

20.4.17.1 Ethno-Medicinal

The genus *Liparis* is known for its importance in traditional medicine. *Liparis odorata* has been reported to be used in treatment of elephantiasis in Malabar (Teoh 2016). Fresh leaf juice is used in burns, cancerous ulcers and gangrene (Yonzon et al. 2011). The entire plant has been reported to be used in treating cough, clearing phlegm, dampness, heat and detoxification of blood, hemostasis, swollen sores, lumbago, hemoptysis, and arresting leucorrhoea (Liang et al. 2019).

20.4.17.2 Phytochemical

Li et al. (2014) and Xie et al. (2015) studied and reported the presence of phenolic glycosides, Liparisglycoside, in *Liparis odorata* which might have potential hypolipidaemic effect and anti-inflammatory action. According to Li et al. (2014), three phenolic glycosides viz. liparisglycoside A, B and C, along with unnamed compound, succinic acid, phloic acid, batatasin III and luteolin, derivative from enzymatic hydrolysis of the compound anodendrosin A, were isolated from *L. odorata*. Compounds liparisglycoside C and derivative of anodendrosin A had potential anti-inflammatory activities, and compounds liparisglycoside A, liparisglycoside C and derivative of anodendrosin A expressed potential hypolipidaemic effects.

20.4.17.3 Tissue Culture

In vitro propagation of various species of the genus *Liparis* through stem sections and leaf tips has been reported but micropropagation of *Liparis odorata* could not be found.

20.4.18 Luisia

20.4.18.1 Ethno-Medicinal

Luisia teretifolia (*L. tritis*) has long been known to be utilised by herbal practitioners. Whole plant decoction is used to treat boils, abscesses tumours and inflorescence used for healing of wounds, to treat fractures, leaf juice is used for treating chronic wounds and for deworming. *Luisia trichorhiza* is used to treat liver dysfunction and diabetes mellitus (Yonzon et al. 2011; Teoh 2016). Its root paste is mixed with turmeric and ginger and consumed to cure jaundice and root extract reduces muscle pain (Yonzon et al. 2011; Dash et al. 2008).

20.4.18.2 Phytochemical

A trace amount of alkaloid has been reported to be present in *L. teretifolia* and *L. trichorhiza* as mentioned by Teoh (2016).

20.4.18.3 Tissue Culture

Micropropagation has been accomplished for *L. teretifolia* and *L. trichorhiza* from leaf tissues. Apical and basal explants were cultured in MS media with 1 mg/L kinetin, 1 mg/L BA and 1 mg/L NAA. Addition of activated charcoal and banana homogenate enhances the multiplication and development of PLBs to grow into plantlets in *Luisia trichorhiza* (Yam and Arditti 2017).

20.4.19 Malaxis

20.4.19.1 Ethno-Medicinal

This genus consists of some medicinally important species such as *Malaxis acuminata* (*Crepidium acuminatum*) and *Malaxis muscifera*. They are included in the ‘Ashtvarga’ of Indian Ayurveda and were believed to have antioxidant properties and impart vitality (Arora et al. 2017). The stem and leaves of *M. acuminata* are used

as blood purifier, aphrodisiac, spermoprotic and for curing burning sensation and arthritis. The pseudobulbs of *M. muscifera* are reported to be used as aphrodisiac, styptic, and febrifuge and for curing dysentery, tonic, burns, debility and sterility (Sharma et al. 2015a).

20.4.19.2 Phytochemical

Compounds reported from pseudobulbs of *M. acuminata* are β -Sitosterol, ceryl alcohol, glucose, rhamnose and choline (Bhatnagar et al. 1971).

20.4.19.3 Tissue Culture

Micropropagation of *M. acuminata* from pseudobulbs and shoot inductions from stem segments has been reported (Kaur and Bhutani 2010). Addition of 5 mg/L of BA, 0.5 mg/L of IAA and 0.5 mg/L of GA₃ in MS media showed successful plantlet development from the pseudobulb explant (Bhandari et al. 2011).

20.4.20 Nervilia

20.4.20.1 Ethno-Medicinal

Fresh leaves decoction of *Nervilia aragoana* is used in Ayurveda as a protective medicine after childbirth in Malaysia, whole plant acts as antipyretic, stops bleeding, helps in diuresis, decreases swellings and to treat discharges, menorrhagia and weak kidneys. Whole stem decoction of *Nervilia plicata* is used for treatment against cough, urinary trouble, diarrhoea, vomiting, jaundice, asthma. Its leaf and stems and leaf are used as antidiabetic. Whole plant of *Nervilia prainiana* helps in treating asthma, colic and urinary ailments and to cure epilepsy. *N. aragoana* and *N. plicata* are reported to show presence of alkaloids and triterpenes (Yonzon et al. 2011; Sharma et al. 2015a; Teoh 2016). Haridas et al. (2014) observed the antibacterial and antifungal properties of *N. plicata* that could have medical significance.

20.4.20.2 Phytochemical

Compounds isolated from *N. aragoana* by GCMS analysis include 5-hydroxy-2-(hydroxyl methyl)-4H-pyran-4-one (antibacterial and antifungal), 2-octenoic acid, pentadecanoic acid (fragrance and flavouring agents), hexadecanoic acid (anti-inflammatory), 2-chloroethyl linoleate, isoamyl laureate (flavouring and skin conditioning), phthalic acid, vitamin C, 2-propyl thiophene (Thomas et al. 2013).

20.4.20.3 Tissue Culture

The tissue culture of *N. aragoana* has been successfully achieved by using apical stem segments as explants cultured in Woody plant medium (WPM) with 0.7% agar and 0.5 mg/L BA. Subculturing in VW, KC or WPM medium supplemented with banana homogenate or activated charcoal helps in plantlet maturation (Yam and Arditti 2017).

20.4.21 Oberonia

20.4.21.1 Ethno-Medicinal

Oberonia genus has quite some species that have medicinal significance. *Oberonia caulescens* tubers are useful to treat liver diseases (Yonzon et al. 2011). Whole plant is used to stop bleeding, remove bruises and to treat fractures (Teoh 2016).

20.4.21.2 Phytochemical

Data unavailable.

20.4.21.3 Tissue Culture

Micropropagation of *Oberonia caulescens* is not yet popularly found but micropropagation of some species of the genus is being performed following the methods of *Acampe praemorsa* from leaf explants (Yam and Arditti 2017).

20.4.22 Papilionanthe

20.4.22.1 Ethno-Medicinal

Papilionanthe teres (*Vanda teres*) leaf paste is applied during high fever. Roots decoction used as an anti-fertility medicine and leaves decoction used to cure typhoid fever (Yonzon et al. 2011; Medhi and Chakrabarti 2009).

20.4.22.2 Phytochemical

Plant extracts of *P. teres* contain alkaloid, flavonoid, tannin, saponin, etc. (Mazumder et al. 2010).

20.4.22.3 Tissue Culture

Ex situ symbiotic germination of *P. teres* seeds were performed by leaving seed packets in its host plant bark to allow ecological interaction of the symbiotic associating fungi and enhancing the seed germination (Zhou and Gao 2016). In vitro propagation was also achieved from seed and shoot tip culture. Asymbiotic seed germination of *P. teres* was successfully observed in MS media supplemented with BAP and NAA. Pandey et al. (2020) reported the significant need of GA₃ instead of auxins in seed germination of *P. teres*.

20.4.23 Pecteilis

20.4.23.1 Ethno-Medicinal

Roots of *Pecteilis susannae* is reported to strengthen kidney and spleen. It is used in treating back pain, chronic nephritis, impotence, orchitis, to treat blebs, hernia and indigestion, and also to make salep (Teoh 2016). The fresh tubers are used to cure boils (Yonzon et al. 2011).

20.4.23.2 Phytochemical

Data unavailable.

20.4.23.3 Tissue Culture

The genus shows similarity with *Habenaria* (Teoh 2016) and therefore similar culturing methods can be employed as done by Chutima et al. (2011) following the protocols of Stewart and Kane (2006) in *Habenaria macroceratitis*. Endophytic fungal isolates obtained from root of explants introduced into Oat meal agar media along with seeds inoculum of *P. susannae* showed efficient seed germination and protocorm development.

20.4.24 Phaius

20.4.24.1 Ethno-Medicinal

The genus includes medicinal plant *Phaius tankervilleae*. Whole plant part in addition to wild ginger is utilised to treat dysentery and healing of fractured bones. Pulverised pseudobulbs, leaves and roots are used to treat boils, infected wounds,

abscesses, reduce pain and treating swollen hands and legs (Hossain 2011; Yonzon et al. 2011).

20.4.24.2 Phytochemical

Plant extracts of *P. tankervilleae* contains alkaloids (Lawler and Slaytor 1969).

20.4.24.3 Tissue Culture

In vitro propagation of *P. tankervilleae* has been conducted through asymbiotic seed germination and shoot tip culture (Thokchom et al. 2017; Vimal et al. 2018). Tissue culture from shoot tips on MS in addition with BAP and NAA has been successfully conducted (Pant and Shrestha 2011). Introduction of additional supplements such as coconut water and potato extract to the culture media in varying concentration of 150ml/L and 50g/L respectively, also aids in plantlet development (Punjansing et al. 2019).

20.4.25 Pholidota

20.4.25.1 Ethno-Medicinal

Pholidota articulata is used as medicinal plant. It is crushed and applied on fractured bones for healing (Jalal et al. 2009). The entire plant is also used in making tonic (Yonzon et al. 2011). The powder of its root is used in treatment of cancer and the juice of the plant is applied to treat skin disorders. Pseudobulb of *Pholidota pallida* is pulverised and applied to treat stomach ache, wounds, to relieve rheumatic pains while powder of pseudobulb helps to induce sleep (Teoh 2016; Pant et al. 2018). Nagananda et al. (2013) reported the presence of alkaloids, flavonoids, phenols and phytosterols in *P. pallida* which might have pharmaceutical significance. Antibacterial and antifungal activity of *P. articulata* and *P. pallida* has been confirmed and reported by Marasini and Joshi (2012).

20.4.25.2 Phytochemical

Three phenanthrenes (flaccidin, flavidin and oxoflavidin) were isolated from ethyl acetate fraction of *P. articulata* (Sharma et al. 2015b). The study indicated the osteogenic effects of oxoflavidin and *P. articulata* extract in mice by modulating key pathways such as BMP signalling pathway. Eleven compounds have been isolated from *P. pallida* (coelonin, lusianthridin, flavanthrin, batatasin-III, 3',5-dihydroxy-2-(4-hydroxybenzyl)-3-methoxybibenzyl, gigantol,

3-[2-(3-hydroxyphenyl) ethyl]-2,4-bis[(4-hydroxyphenyl) methyl]-5-methoxyphenol, hydroxytyrosyl butyrate, (24R)-ethylcholest-5-en-3-ol-7-one, taraxerone, friedelin). The presence of hydroxytyrosyl butyrate in natural resource can be used as a potential chemotaxonomic marker for *P. pallida* (Yu et al. 2021).

20.4.25.3 Tissue Culture

In vitro culture of *P. articulata* through seed germination is accomplished in $\frac{1}{2}$ MS media (Prasad et al. 2021). Asymbiotic seed germination of *P. pallida* can be achieved in Lindemann orchid medium and MS medium supplemented with 3 mg/L IBA and 1 mg/L KN (Prasathkumar and Ramesh 2016). Seed germination in KC is also found to be effective as reported by Mary and Divakar (2015).

20.4.26 Pleione

20.4.26.1 Ethno-Medicinal

Fresh rhizome of *Pleione maculata* is used in treating liver and stomach ailments (Pant 2013; Teoh 2016).

20.4.26.2 Phytochemical

The phenolics and flavonoids of *P. maculata* showed potent antioxidant and antibiofilm properties (Sympli et al. 2021).

20.4.26.3 Tissue Culture

Young shoot explants of *P. maculata* are used for micropropagation as reported by Apang (2015). Additional supplements such as peptone, casein hydrolysate and yeast extract also help in multiple shoot buds and callus formation (Chauhan and Sharma 2017).

20.4.27 Rhynchosstylis

20.4.27.1 Ethno-Medicinal

Rhynchosstylis retusa leaves are used in the treatment of rheumatism. Its roots are crushed and paste made to apply on wounds and cuts, and to cure dysentery (Yonzon et al. 2011; Pant et al. 2018).

20.4.27.2 Phytochemical

R. retusa showed the presence of saponins, tannins, glycosides, flavonoids, steroids, terpenoids and phlobatannins (Akter et al. 2018).

20.4.27.3 Tissue Culture

Low-cost substitutes of sucrose and agar in the medium were experimented and shown to give plantlets without any compromise (Kurniasari et al. 2019; Lal et al. 2020). Symbiotic seed germination in artificial medium was successfully initiated with fungal isolate *Ceratobasidium* sp. isolated from its root segments (Bhatti et al. 2016). Germination of seeds via in vitro condition was reported by Fonseka 2020. Naing et al. (2010) and Sharma (2012) studied and reported the effective role of cytokinin in growth of plantlets.

20.4.28 Satyrium

20.4.28.1 Ethno-Medicinal

Tubers of *Satyrium nepalense* are utilised in treatment of malaria and diarrhoea and in making tonic (Pant et al. 2018).

20.4.28.2 Phytochemical

S. nepalense showed the presence of alkaloids, flavonoids, saponins, and steroids (Kawra et al. 2020).

20.4.28.3 Tissue Culture

In vitro propagation and asymbiotic seed germination of *S. nepalense* was achieved in Mitra's basal media (Babbar and Singh 2016). Mahendran and Bai (2009) also successfully obtained seed culture of *S. nepalense*.

20.4.29 Vanda

20.4.29.1 Ethno-Medicinal

The juice of fresh leaves of *Vanda coerulea* is used in diarrhoea, dysentery, eye diseases and external skin diseases (Medhi and Chakrabarti 2009); decoction of fresh flowers taken as appetiser as well as tonic (Deb et al. 2009). Fresh leaves of *Vanda cristata* are used as tonic and expectorant (Medhi and Chakrabarti 2009). Roots of *Vanda tessellata* (*Vanda roxburghii*) are alexiteric and antipyretic in nature. It is used to treat dyspepsia, bronchitis, inflammation, piles and hiccup.

20.4.29.2 Phytochemical

Stilbenoids including imbricatin, methoxycoelonin, gigantol, flavidin, and coelonin were isolated from *V. coerulea*, of which imbricatin and methoxycoelonin were able to inhibit COX-2 activity in a concentration-dependent manner thereby reducing PGE-2 production from irradiated HaCaT cells. This could be potentially used for skin protection against the damage caused by UVB exposure (Simmler et al. 2010). Chowdhury et al. (2014) evaluated the antinociceptive and cytotoxic effect of methanol and aqueous extracts of *V. tessellata* leaf.

20.4.29.3 Tissue Culture

Regeneration of PLBs from thin shoot tip sections of *Vanda coerulea* was observed when cultured on Vacin and Went's (VW) (1949) basal medium supplemented with 11.35 μ M thidiazuron (Malabadi et al. 2004). Rahman et al. (2009) reported the in vitro micropropagation of *V. tessellata* from shoot tip explant.

20.5 Conclusions and Future Prospects

Plants and plant-based products have a long history of eminence in diverse usage to mankind. The economical and potential applications of plants in treatment of ailments have created a significant futuristic scope in the field of medicine. Around

25,000 plant-based ethno-medicinal products have been utilised in India alone (Mukherjee and Wahile 2006). Many such traditional and folk medicinal formulation exists around the world which may or may not be documented. The methodological integration of traditional knowledge along with modern techniques can assert an effective and economical solutions in treatment of diseases. At the same time, over-collection of the herbal plants results in the population decline of many species and sometimes at the extend of extinction. Orchids are one of the prominent plants that have medicinal importance. They have been exploited for their ornamental and medicinal values which caused a major decline in their population and have been included under threatened and endangered species. With intervention of biotechnological methods such as plant tissue culture, plants that are at the verge of extinction and endangerment can be propagated at large scale to secure their declining population. Moreover, through genetic manipulations, the production of secondary metabolites that have pharmaceutical values can also be enhanced. Seed germination of endosperm deficient plants like that of orchidaceae family can be initiated under controlled conditions and mass culture of their derived PLBs for synthetic seed production. This would allow micropropagation at commercial scale to obtain the desired products. Tissue culture of not all potential medicinal plants is under process. There is dire need to focus on those endangered orchids with high medicinal values. This does not indicate the preferential negligence of other endangered or rare species. We put forward for the formation of a functional consortium where the genera or species for orchid conservation could be divided among the group members and their studies conducted to document the possible bioactive compounds that could be of pharmaceutical importance. Fear and trepidation of over exploitation of endangered medicinal plants could be an obstacle for extraction of phytochemical compounds that may have medicinal usage. For example, the chemical characteristics of the *H. edgeworthii* and compound(s) with medicinal value have not been reported in depth. Many such elite phytochemically active plant sources could be mass propagated in a pace to meet the required demands and studies conducted in the direction of conservation, extraction and its utilisation in pharmaceutical and clinical point of view. To assure the therapeutic efficacy of the herbal drugs, their standardisation is very crucial. Evaluation of the pharmacognostical as well as physico-chemical standards of these orchids with the help of various techniques need to be conducted at large so as to be able to bring up to the level of clinical trial. At the same time, it is important to check for dose dependency, side effects and toxicity of the compounds extracted from the orchids. This chapter summarises the some of the important medicinal orchids of the NER of India, their related bioactive compounds if studied and the studies on their propagation via tissue culture. The other areas of biotechnology like its cryopreservation, transgenic approach for genetic modification, secondary metabolite production in bioreactors, will open avenues for research in untapped potentials of the already available resources of nature. The scientific community needs to realise the potentials of these plants as medicines. All the while, we must focus on the conservation of these exotic and already endangered species.

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Chapter 21

Artificial Seed Production and Cryopreservation Technology for Conservation of Plant Germplasm with Special Reference to Medicinal Plants



Monika Sharma, Prasann Kumar, and Padmanabh Dwivedi

Abstract Medicinal plants are the most important group of plant genetic resources. These are used for the manufacturing of various herbal products, but these plants are decreasing day by day at a high speed. The use of biotechnology can be considered a great tool to meet the increasing demands due to overexploitation. Cryopreservation is a safe, easy, and cost-effective method for the preservation of germplasm and to manage the in vitro products for biotechnological tasks because the cultured plant cells in vitro are prone to different alterations. Artificial seed production is another important technique for the mass propagation of different plant genotypes and for propagating various crop types, which are commercially important. Artificial seeds are effectively created from encapsulated plant propagules. Artificial seeds are generally encapsulated somatic embryos or another vegetative part of the plant which is converted into seed for the generation of the whole plant under in vitro conditions. The vegetative parts used for the production of artificial seeds include shoot bud, cell cluster, micropropagules, etc. which can be sown like seeds and converted into the whole plant. Likewise, cryopreservation is the technique to preserve the medicinal plant species or germplasm in very low-temperature conditions. Different biotechnological techniques are used to conserve the plant germplasm for a longer period. The main purpose of this chapter is to provide a brief overview of artificial seed production and cryopreservation techniques and how these are used to conserve germplasm, especially the medicinal plants.

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Keywords Artificial seed production · Biotechnology · Cryopreservation · Conservation · Encapsulation · Germplasm · Medicinal plants · Synthetic seed · Poverty · Hunger · Sustainability

Abbreviations

DMSO	Dimethylsulfoxide
LN	Liquid nitrogen
M	Molar
MS	Murashige and Skoog medium
PVS	Plant vitrification solution

21.1 Introduction

Medicinal plants are the backbone of different herbal products and medicines. Therefore, there is a need to conserve them. However, it is distressing to know that 70% of the world's plants are presently on the Red List of endangered species, especially medical plants (Paunescu 2009). The primary causes of biodiversity loss and dispersion include over-collection, inadequate agricultural and forestry operations, industrialization, pollution, habitat destruction, degradation, and climate change (Pitman and Jørgensen 2002). Given the importance of these plants in health care, food, and the photo industry, it is critical to raise global awareness about the loss of such valuable herbs and to develop new conservation programs. For effective protection of these valuable genetic resources, this should be combined with strict legal measures (Sharma et al. 2010).

In biotechnology, new gene modification techniques are reported that are site-specific targeted or capable of designing new unique medicinal plants with diverse secondary metabolite profiles. For the selection, multiplication, and conservation of medicinal plants with important genotypes, many biotechnology methods are applied. For the production and conservation of medicinal plants, several technologies (such as micropropagation, synthetic seed technology, molecular marker-based approaches, and tissue culture) are applied. Artificial seed production and cryopreservation techniques have a great role in the conservation of medicinal plant species.

As the production of a seed is the result of sexual activity, naturally formed seeds in cross-pollinating species are genetically distinct from individual parent seeds. Seeds can maintain dormancy by undergoing extensive self-drying and allowing them to be stored for an extended long time. Various plant species which have pharmacological importance have different characteristics; however, they are tropical or subtropical. These plants make their conservation difficult through traditional techniques. Desiccation-sensitive or refractory seeds are found in many medicinal plant species, limiting storage time to a few weeks or months. Besides, strong

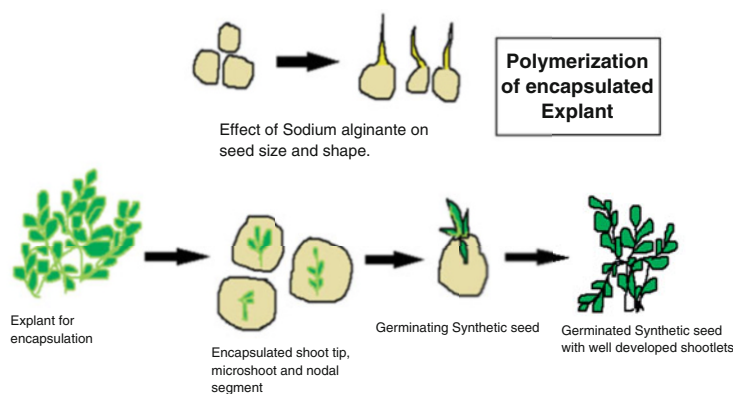


Fig. 21.1 Different stages of synthetic seed development

metabolic activity and a high prevalence of pathogenic infestation also contribute to a shorter storage period. Moreover, while collecting germplasm from field gene banks, the risk of germplasm attack rises due to pest and disease invasions as well as natural disasters. Because of the vegetative nature of germplasm, dissemination and exchange become difficult, and a significant risk of disease transmission exists (Chaudhury and Malik 2003). Recently, there has been a rise in interest in the use of synthetic seeds which are created by encapsulation technology (Fig. 21.1), which is a great way to ensure the safe conservation and interchange of different medicinal plant species. Synthetic seed technology has opened a new direction with huge opportunity for the preservation and large-scale micropropagation of exclusive rare hybrids, choice genotypes, and sterile unsteady genotypes, as well as genetically modified plants with non-availability of seeds or that requires a mycorrhizal-fungal liaison for their germination.

Nowadays, researchers' attention has been drawn to encapsulating technology for the transmission of germplasm and other analytical investigations. Although Kitto and Janick (1982) provided the primary account of the formation of artificial seed, Murashige defined the concept of artificial seed in 1977. Kitto and Janick reported on the development of dehydrated synthetic seeds using a water-soluble resin covering and polyoxyethylene glycol on carrot somatic embryos (SEs). Redenbaugh et al. (1993) later succeeded in producing synthetic alfalfa seeds by encapsulating SEs in alginate hydrogel. Since then, several research organizations have claimed to be working on synthetic seeds for a variety of plants, including grains, fruits, ornamentals, medicinal plants, vegetables, trees, and orchids (Rai et al. 2009).

Cryopreservation of medicinal plant in vitro is a valuable approach. Cryopreservation is a long-term storage procedure in liquid nitrogen (-196°C) that stops cell division, metabolic, and biochemical activities. Cryopreservation allows endangered medicinal plants to be preserved since complete plants may regenerate from frozen culture, for example, *Rauvolfia serpentina*, *D. lanalta*, and *A. belladonna*. Liquid nitrogen can hold a large number of cultivated materials.

21.2 Artificial Seed Technology

Many medicinal plants like recalcitrant or desiccation-sensitive seeds limit their storage to months or years. The potential use of synthetic seed synthesis is for the conservation of high-value medicinal species which is achieving a great importance in recent days. Synthetic seed technology is a recent advancement in plant biotechnology that has the potential to save a variety of medicinal plant species. A synthetic seed is described as an artificially encapsulated plant propagule with the potential to germinate into full plantlets under *in vitro* or *in vivo* circumstances. Plant propagules may include somatic embryos, shoot tips, protocorm-like bodies, axillary buds, hairy roots, calli, or any other tissue. This approach provides simple handling, large-scale plant production, storage transportation, and cost-effective germplasm interchange between laboratories and pharmaceutical companies without compromising plant viability (Wang et al. 2007). Several researchers have looked into synthetic seed production for a variety of plant species, including medicinal plants, vegetables, fruits, grains, orchids, ornamental plants, and various forest trees (Rihan et al. 2011).

Because of its wide variety of biotechnological applications in plant germplasm conservation, synthetic seed technology has shown tremendous potential in recent decades. As a result, synthetic seed synthesis for medicinal plant species would make biotechnological applications by end users more affordable. The cost-effective production of high-quality viable micropropagules remains a major challenge (Wang et al. 2007; Sujatha and Kumari 2008). Reduced shoot, root, and matrix development during bulk alginate encapsulation due to excessive sodium alginate concentration is also of major concern (Reddy et al. 2012). These difficulties will need to be addressed in future studies to ensure long-term conservation practices and the correct use of this biotechnological method.

Artificial seed development based on alginate encapsulation has recently emerged as a viable method for mass multiplication and storage of various medicinal plants of industrial value. This method produces a low-cost, large-scale plant manufacturing system. Artificial seeds can provide easy management of plant germplasm during transport or storage without compromising viability (Sharma et al. 2010). The alginate coating on plant components protects tissues from mechanical damage and avoids undesirable drying during storage (in a controlled environment) and germplasm exchange (Ara et al. 2000; Reddy et al. 2012).

21.2.1 Artificial Seed Concept

The artificial seed structure is similar to that of a natural seed. It consists of both explant material (gel agent and additional materials such as nutrients, growth regulators, anti-pathogens, bio-controllers, and biofertilizers), which mimics the zygotic embryo in the conventional seed, and the capsule (gel agent and additional materials such as nutrients, growth regulators, anti-pathogens, bio-controllers, and

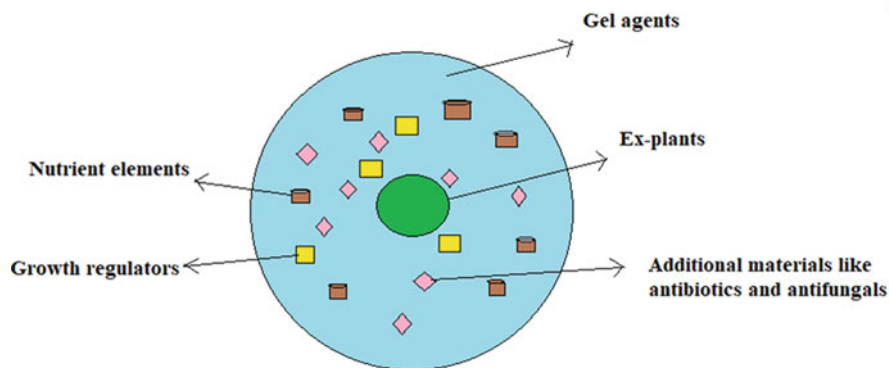


Fig. 21.2 Artificial seed concept

biofertilizers), which mimics the endosperm in the conventional seed (Cartes et al. 2009) (Fig. 21.2).

21.2.2 Requirements for Artificial Seed Production

21.2.2.1 Explant Materials

Explant materials are the artificial seed's core generative component. It may be somatic embryos, because their structures may form the radical and plumule axis, which can advance into the root and shoot in a single step; somatic embryos are the most common micropropagule employed for artificial seed generation. Artificial seeds created from somatic embryos can also generate a lot of offspring. Plant lines derived from somatic embryos can maintain their regeneration potential for a long period, resulting in uniform plant production (Leroy et al. 2000). The use of somatic embryos for artificial seed generation has grown in popularity, and the number of species that appear to be capable of propagation via this method is increasing.

Two types of artificial seeds are commonly produced: desiccated and hydrated (Bapat and Mhatre 2005).

Artificially Desiccated Seeds

Desiccation of somatic embryos, either naked or encapsulated in polyoxyethylene glycol, results in desiccated artificial seeds. Desiccation can be done quickly by putting synthetic seeds in open Petri dishes on the bench overnight to dry or slowly by lowering relative humidity over a longer length of time. These artificial seeds can only be created in plants with desiccation-tolerant somatic embryos. A high osmotic

potential of the maturation medium can be used to develop desiccation tolerance in somatic embryos. The osmotic potential can be raised by utilizing high gel strength or adding osmoticants such as mannitol, sucrose, and other sugars (Sundararaj et al. 2010). Desiccation tolerance is produced by sublethal stimuli such as food restriction or a low temperature (Pond and Cameron 2003).

Artificially Hydrated Seeds

Encapsulating somatic embryos in hydrogel capsules produce hydrated artificial seeds. These are formed in recalcitrant and desiccation-sensitive plant species. Encapsulation has been predicted to be the most effective method for providing protection and converting in vitro micropropagules into “artificial seeds” or “synseeds” (Redenbaugh 1993), and it is an important application of micropropagation for improving the success of in vitro-derived plant delivery to the field. Somatic embryos, on the other hand, are enclosed in a germination-promoting substance (Latif et al. 2007). In different plant species, apical shoot tips have been encapsulated to create artificial seeds since they lack a root meristem. Even though these explants required particular treatment to induce root reconstruction before encapsulation, several investigations observed the conversion of encapsulated banana and mulberry (Bapat and Rao 1990) buds into plantlets without any special induction therapy.

Additional Explant Materials

Several other explant materials, such as embryogenic masses and protocorm-like bodies, have been studied to see if these can produce artificial seeds. Despite this, Onay et al. (1996) were able to create artificial seeds using an embryogenic mass. They found that after 2 months of storage, the encapsulated embryogenic mass fractions restored their primary reproductive capacity. Datta et al. (1999) studied the manufacture of artificial seeds using encapsulated protocorm-like structures of orchid (*Geodorum densiflorum*). According to them, the encapsulated protocorm-like bodies remained alive after 3 months at 4 °C, but non-encapsulated protocorm-like entities looked non-viable after 1 month at 4 °C. However, there are a number of explants that may be utilized to make synthetic seeds, and this is mostly dependent on the plant type.

21.2.2.2 Adjuvant Materials and Artificial Seed Gelling Agents

The primary drawback of using somatic embryos as micropropagules for plant propagation is that these are delicate structures that lack a quiescent resting period. As a result, these require vital supplemental tissues that give nutritious elements as well as a protective shell that makes them easier to handle and store. The primary

goal of artificial seed research is to create an artificial seed structure that mimics the features of traditional seeds which include handling, storage, viability, and germination level (Ara et al. 2000). Polyoxyethylene was chosen as a good encapsulation medium for embryos at the start of artificial seed investigations because of its beneficial qualities, like prolonged embryo development, non-toxicity to explants, and water solubility (Kitto and Janick 1982). Redenbaugh et al. (1993) presented a novel approach involving hydrogel encapsulation. Since then, hydrogel polymers have become the primary framework for encapsulating somatic embryos, even though many gel materials such as agar, alginate, carrageenan, guar gum, and sodium pectate were investigated for artificial seed production. Alginate matrix was discovered to be the best encapsulation for artificial seed production due to its sensible thickness, weak spinnability of a solution, low micro-organism toxicity, low cost, bio-suitability characteristics, and fast gelation. This material increases capsule structure and bead stiffness, providing improved mechanical protection for covered explants (Ara et al. 2000; Saiprasad 2001). The addition of nutrients and growth regulators and nutrients to the capsule is a critical component of a successful artificial seed production approach, enhancing the seeds' germination and viability. These components are referred to as artificial endosperm, and they serve a critical function in artificial seed storage. However, many other compounds, such as pesticides, antibiotics, and fungicides, have beneficial impacts on capsule characteristics.

21.3 Cryopreservation

Plant germplasm preservation is important to avoid genetic loss and to maintain biodiversity. Cryopreservation is a significant biotechnological method for the long-term preservation and conservation of plant germplasm, with minimal risk of material loss and low cost and labour (Reed 2008; Keller et al. 2008). Cryopreservation is known as the most dependable method for storing plant germplasm for long periods (Sakai and Engelmann 2007). In terms of cost-effectiveness, long-term storage, and space savings, plant germplasm cryopreservation (in liquid nitrogen, -196°C) offers evident advantages over *in vitro* (Wang et al. 2018). Freezing, thawing, recovery, culture initiation and embryo rescue, osmotic pretreatments, and regeneration are examples of *in vitro* cryopreservation (Berjak et al. 2011). Cryopreservation is the storage of plant material at extremely low temperatures (-196°C) in a cryogenic environment, such as liquid nitrogen (Wen and Wang 2010). All cellular divisions and metabolic activities of plant cells halt at this temperature, and plant material is preserved unmodified for an unlimited period (Gonzalez-Arno et al. 2008; Shatnawi et al. 2011). Cryopreservation is a process that involves removing all freezable water from tissues by physical or osmotic dehydration, followed by superfast freezing. As a result, water drainage is essential for avoiding freezing damage and maintaining post-thaw viability (Gonzalez-Arno and Engelmann 2006). Crystallization after freezing is thought to be the most important element determining cryopreservation cell survival. When ice crystals develop

during the warming process, it compromises cellular integrity. Slow warming increases the likelihood of big ice crystals growing; thus, thawing should be done quickly by immersing the cryovials in a water bath at 37–40 °C for a few minutes. Furthermore, cryogenic storage is done in a limited volume, which eliminates contamination hazards and reduces maintenance costs. Cryopreservation is often used in most basic storage systems since it is both safe and cost-effective for long-term preservation. The capacity to regenerate into full plantlets and the survival rate of the seed are used to determine the success of cryopreservation. The use of cryopreservation has been described in a wide variety of medicinal plant species (Dwivedi 2004; Naidoo et al. 2016).

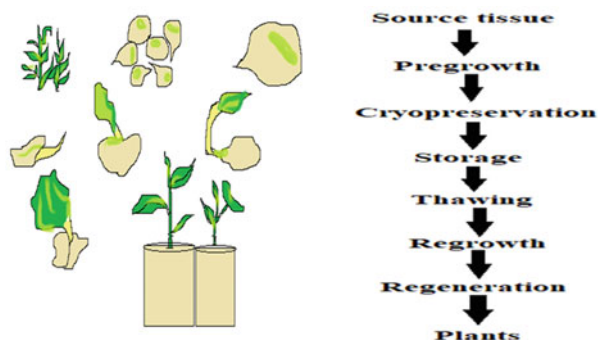
21.3.1 Techniques for Cryopreservation

Cryopreservation research has recently focused on the development of simple and easy procedures that may be applied to a variety of plant materials, including cultured cells, shoot tips, and somatic embryos (Sakai and Engelmann 2007). Cryopreservation processes include encapsulation-dehydration, vitrification, encapsulation vitrification, and droplet vitrification (Engelmann 2004) (Fig. 21.3).

21.3.2 Encapsulation-Dehydration

This method was developed by Fabre and Dereuddre (1990) in which shoot tips, somatic embryos, or callus cells are encapsulated in alginate beads and then cultured for 1–3 days in a medium containing elevated concentrations (0.7–1.5 M) of sucrose (Gonzalez-Arno and Engelmann 2006; Shatnawi et al. 2011). Before being submerged in LN, the beads are allowed to dry using silica gel or air under laminar airflow until the moisture content reduces to 20–30% (Fabre and Dereuddre 1990). As it is easy and affordable and provides a high level of genetic stability,

Fig. 21.3 Cryopreservation technique



encapsulation-dehydration is used on a broad scale (Shibli et al. 2006; Shatnawi et al. 2011).

Encapsulation and dehydration procedure steps include isolation and preculturing of shoot tips; encapsulation of precultured shoot tips followed by incubation in MS liquid medium enriched with sucrose; air dehydration in a laminar flow; placing the dehydrated shoot tips in cryovials and freezing them; thawing quickly; recovery media to subculture; and incubation in the dark for many days before transferring to normal light.

21.3.3 *Vitrification*

Vitrification is the process of supercooling a highly concentrated cryoprotectant to such a low temperature that it solidifies into a metastable glass state without crystallization (Sakai and Engelmann 2007). As a result, vitrification is a useful freeze-avoidance technique. Furthermore, once a glass state is reached, all chemical processes that need molecular mobility will cease, resulting in metabolic inactivity and stability over time (Sakai and Engelmann 2007). Vitrification is one of the most common and commonly used plant cryopreservation methods. The loading phase, dehydration with highly concentrated vitrification solutions, and unloading phase are the three key steps of vitrification (Ashmore 1997). Samples are subjected to cryoprotectants or diluted vitrification solutions during the loading phase (Withers 1991) and then dehydrated by a highly concentrated vitrification solution before being plugged in LN (Sakai et al. 1991). After quick thawing, the vitrification solution is drained from the cryovials and replaced with 1.2 M sucrose for 10–20 min during the unloading phase (Shibli et al. 2006; Shatnawi et al. 2011). Plant vitrification solution is an aqueous cryoprotectant solution that allows living materials to be chilled without forming significant ice, either intra- or extracellularly (Fahy et al. 1987). The capacity of this solution to raise the osmotic potential of the external medium confers protection (Reed 1990). This will enable water to flow out of the cells, resulting in tissue dehydration (Ashmore 1997). Cryoprotectants must be tuned at the right concentration to avoid toxicity, be water-miscible, and have a high capacity to infiltrate cells quickly (Shibli et al. 2006). Most vitrification techniques employ Plant Vitrification Solution 2 (PVS2) (Reed 2008). It contains 30% w/w glycerol, 15% w/w ethylene glycol, and 15% w/w dimethylsulfoxide DMSO in an MS medium supplemented with 0.4 M sucrose (Sakai and Engelmann 2007).

Vitrification technique steps includes isolation and preculturing of shoot tips; loading solution and PVS2 solutions treatment; placing the treated shoot tips in cryovials and submerging them in LN (liquid nitrogen); rapid freezing; unloading; and subculturing to recovery media, followed by incubation in the dark for several days before transferring to normal light.

21.3.4 Encapsulation-Vitrification

Explants should be enclosed in alginate beads with air or silica gel instead of dehydrating; encapsulation-vitrification dehydrates them with vitrification solutions before freezing (Younes 2012). Explants are allowed to freeze in vitrification for a confined time, which is difficult to apply when dealing with a large number of samples. Due to the relatively large size of the alginate beads, the encapsulation-dehydration approach takes a significantly longer time to apply, and handling the encapsulated explants is quite straightforward. So it is a novel technology that combines the benefits of vitrification with encapsulation-dehydration (Sakai and Engelmann 2007), making it suitable for large-scale cryopreservation (Hirai and Sakai 1999; Sharaf et al. 2012).

Encapsulation and vitrification steps are as follows:

1. Isolation and preculturing of shoot tips.
2. Precultured shoot tips are encapsulated and subsequently treated with loading solution and PVS2 solutions.
3. Place the treated shoot tips in cryovials, and submerge them in LN (liquid nitrogen).
4. Rapid freezing, unloading, and subculturing to recovery media, followed by incubation in the dark for several days before transferring to normal light.

21.3.5 Droplet Vitrification

Kartha et al. (1982) invented this procedure, in the shoot tips of cassava. In this method, shoot tips are immersed in cryoprotective medium droplets before ultra-cooling (Sakai and Engelmann 2007). Shoot tips are placed on aluminium foils and dehydrated using cryoprotectants like PVS2 in this procedure. The use of aluminium foils makes it easier to move shoot tips into and out of LN (Kaczmarczyk et al. 2011). Shoot tips are then treated individually with five to ten droplets of PVS2 on a piece of aluminium foil, which is subsequently submerged in LN. After warming, the aluminium foils are unloaded for 20 min in a liquid medium containing 1.2 M sucrose before being placed on recovery media. It is a relatively new technique that has only been applied to a small number of plant species (Sakai and Engelmann 2007).

Steps of droplet vitrification procedure include the following steps:

1. Isolation and preculturing of shoot tips.
2. Loading solution and PVS2 solutions treatment.
3. Place the treated shoot tips on aluminium strips in cryovials, and submerge them in LN (liquid nitrogen).
4. Rapid freezing, unloading, and subculturing to recovery media, followed by incubation in the dark for several days before transferring to normal light.

21.4 Conclusion

Medicinal and aromatic plants are the primary ingredients in pharmaceuticals, food, and other industries. These plants are currently threatened due to their widespread usage in medical, food, and beverage production. Therefore, there is a great need to protect these natural resources. Artificial seeds are effectively created from encapsulated plant propagules. This technology offers several advantages, including a low-cost delivery system, reduced plantlet costs, a straightforward methodology with high mass-production potential, a viable strategy for the *in vivo* usage of artificial seedlings, and a large storage capacity. This technique's progress is determined by the plant species used in the initial phase. Despite the benefits of artificial seeds, further study is needed to increase non-embryogenic artificial seed root production. More research is needed to increase artificial seed culture capability in commercial substrates and non-sterile circumstances. Furthermore, cryopreservation is a group of basic conservation strategies (encapsulation-dehydration, vitrification, encapsulation-vitrification, and droplet vitrification) that can retain the genetic integrity of stored plant material. This might be enhanced by using the right anti-diseases and antibiotics in the right quantities, and more extensive study is needed to increase the artificial seed cryopreservation ability in particular plant species.

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Chapter 22

Biotechnological Studies on *Nasturtium officinale* (Watercress): an Endangered Species of Significant Relevance in Medicine, Cosmetic, and Food Industries



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Abstract The chapter discusses the importance of plant biotechnology research on *Nasturtium officinale* (watercress), an endangered species of valuable pharmaceutical, cosmetic, and nutritional properties.

This is a comprehensive study focusing on the development of *N. officinale* in vitro cultivation methods with the aim of stimulating the production of glucosinolates (GSLs) and polyphenols—compounds that are important from the perspective of plant utility.

In addition, optimization of *N. officinale* in vitro culture conditions is discussed in detail, e.g., influence of plant growth regulators (PGRs) (cytokinins and auxins), LED light wavelength spectrum, and duration of growth periods on the production of bioactive metabolites in agar and agitated *N. officinale* microshoot cultures.

Furthermore, the antioxidant profile of the tested in vitro culture extracts is evaluated using the following assays: cupric reducing antioxidant capacity (CUPRAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and ferric reducing ability of plasma (FRAP).

The highest total GSL content was achieved in the agar-grown microshoots over 20 days on the Murashige and Skoog (MS) medium containing 1 mg/L 6-benzyladenine (BA) and 1 mg/L 1-naphthylacetic acid (NAA) under a combination of 50% green, 35% red, and 15% blue lights (RBG). The highest total

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polyphenol content was detected in agar cultures cultivated over 10 days on the MS medium with 1 mg/L zeatin (Zea) and 1 mg/L NAA. The experimental cultures showed a high antioxidant potential. The optimal antioxidant activity was found for the extracts of *N. officinale* agitated microshoot cultures grown for 20 days on the MS medium containing 1 mg/L kinetin (KIN) and 1 mg/L 3-indolylacetic acid (IAA), as determined using the abovementioned methods.

Keywords Watercress · Microshoot cultures · Biotechnology of endangered plants · In vitro cultures · Micropropagation · Secondary metabolite production · Glucosinolates · Phenolic compounds · Antioxidant potential

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	6-(γ,γ -Dimethylallylamino)purine
Ala	Alanine
B	Blue light ($\lambda = 430$ nm)
BA	6-Benzyladenine
CUPRAC	Cupric reducing antioxidant capacity
D	Darkness (no light)
DM	Dry mass
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DW	Dry weight
EFSA	European Food Safety Authority
F-C	Folin-Ciocalteu
FRAP	Ferric reducing ability of plasma
FW	Fresh weight
GAL	Gallic acid equivalent
GSLs	Glucosinolates
IAA	3-Indolylacetic acid
IBA	3-Indolylbutyric acid
Ile	Isoleucine
IPA	3-Indolylpyruvic acid
IUCN	International Union for Conservation of Nature
KIN	Kinetin
LED	Light-emitting diodes
Leu	Leucine
Met	Methionine
MS	Murashige and Skoog
NAA	1-Naphthylacetic acid
PGRs	Plant growth regulators
Phe	Phenylalanine
PPFD	Photosynthetic photon flux density

R	Red light ($\lambda = 670$ nm)
RB	A combination of 70% red light and 30% blue light
RBfR	A combination of 50% far red ($\lambda = 730$ nm), 35% red, and 15% blue
RBG	A combination of 50% green ($\lambda = 528$ nm), 35% red, and 15% blue light
RBUV	A combination of 50% UV-A ($\lambda = 400$ nm), 35% red, and 15% blue light
RBV	A combination of 50% yellow ($\lambda = 600, 630$ nm), 35% red, and 15% blue light
SIN	Sinigrin equivalent
TDZ	Thidiazuron
TE	Trolox equivalent
TIS	Temporary immersion system
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
WF	White fluorescent light ($\lambda = 390$ – 760 nm)
WLED	White LED light (33% warm color, 2700 K; 33% neutral color, 4500 K; 33% cold color, 5700 K)
Zea	Zeatin

22.1 Biotechnology of Endangered Plants

Species that are endangered or facing extinction can be protected using plant biotechnology approaches. The common in situ conservation strategies for the protection of threatened plants are management of wild populations and protection of natural habitats. However, ex situ strategies such as in vitro techniques, cryopreservation, and storage of germplasm are adapted during critical situations (Ayuso et al. 2019). One such approach is the use of plant in vitro culture methods, which have advantages such as high rates of propagation, production of disease-free plants, and germplasm storage. In vitro culture methods are also beneficial for the establishment of plant reintroduction programs and help obtain in vitro cultured plants for research purposes, with no ecological impact on their limited natural populations. Moreover, plant biomass with good biotechnological parameters (e.g., growth rate, biomass doubling time, cell weight yield) can be used in phytochemical and biological research, without depleting natural environments (González-Benito and Martín 2011; Kikowska et al. 2020).

Micropropagation is one of the major directions in plant biotechnology and has been used as an ex situ conservation strategy for endangered plant species. In vitro crops improve the growth rate and production of secondary metabolites compared with conventional breeding due to the possibility of implementing strict conditions (Karuppusamy 2009; Ayuso et al. 2019). Tree micropropagation processes are of

higher importance compared with the conventional methods of reproduction, especially in endangered species and species with poor germination (Ahmad et al. 2021). In pharmaceutically important and endangered plants, alternative methods of the production of compounds of therapeutic importance can be achieved using plant biotechnology approaches. Plant cell culture is an effective tool for the production of these compounds from whole-plant extracts (natural habitat) or via chemical synthesis (Javid et al. 2021).

In vitro culture techniques provide opportunities to stimulate the production of valuable secondary metabolites, e.g., selection of highly productive cell lines, optimization of culture conditions, selection of the basic composition of the culture medium, and testing of various plant growth regulators (PGRs). Other important parameters are the degree of biomass differentiation (callus, shoot, or root cultures) and the type of cultures conducted (agar, stationary liquid, agitated, or bioreactor cultures). Moreover, light conditions (e.g., illuminance, photoperiod, lack of light), temperature, and the use of elicitors and/or secondary metabolite precursors also influence metabolite production in in vitro cultures (Verpoorte et al. 2002; Szpitter and Królicka 2005; Pietrosiuk and Furmanowa 2006; Karuppusamy 2009). Large-scale in vitro cultivation of plant cultures is also possible, from which the production of compounds with a limited natural presence and compounds that are present in plants at risk of overexploitation can be achieved (Yancheva et al. 2019; Krol et al. 2020). In order to increase the production of biomass and metabolites in in vitro plant cultures, special bioreactors are used (Steingroewer et al. 2013; Krol et al. 2020). Many research groups are currently working on the development of protocols for the micropropagation of not only endangered species but also those whose population is drastically decreasing and may be considered close to threat in a few years. In addition, attempts are made to obtain pharmaceutically and cosmetically valuable active ingredients from in vitro cultures of protected species, such as *Aloe peglerae*, *Araucaria araucana*, *Arnica montana*, *Cullen corylifolium*, *Cytisus aeolicus*, *Eryngium alpinum*, *Ginkgo biloba*, *Picrorhiza kurroa*, and so on (Baskaran and Jayabalan 2009; Shinde et al. 2009; Lucchesini et al. 2010; Baskaran et al. 2011; Mishra et al. 2011; Petrova et al. 2012; Satdive et al. 2015; Hlatshwayo et al. 2020; Singh et al. 2020; Gallia et al. 2021; Nieto-Trujillo et al. 2021; Qaderi et al. 2021; Szewczyk et al. 2021). Some examples of species of endangered plants according to the International Union for Conservation of Nature (IUCN) or the State Books of Plant Protection are presented in Table 22.1. To obtain active substances, micropropagation of these plants was carried out.

According to the IUCN, *Nasturtium officinale* is classified as the “species of least concern” (IUCN). However, its classification varies between European countries, e.g., as an extinct or probably extinct species in Estonia, as an endangered species in Austria and Sweden, and as a partially endangered species in Poland (IUCN; Klimek-Szczykutowicz et al. 2018a, b). The species *N. officinale* is highly important due to its valuable chemical composition and scientifically proven therapeutic effects, which will be discussed in the following subsections.

Table 22.1 Examples of endangered species on which biotechnological research was conducted

Species	IUCN status	The National Red Lists/local threats to the species	Type of in vitro cultures	Production of compounds	References
<i>Aconitum violaceum</i>	Vulnerable	nd ^a	Shoot cultures	nd	Rawat et al. (2013)
<i>Aloe peglerae</i>	Endangered	nd	Shoot cultures	Total content of polyphenols and flavonoids	Hlatshwayo et al. (2020)
<i>Alternanthera sessilis</i>	Least concern	nd	Shoot cultures	nd	Sowmya et al. (2020)
<i>Araucaria araucana</i>	Endangered	Chile (natural monument)	Callus cultures	Total content of polyphenols and flavonoids	Gallia et al. (2021)
<i>Arnica montana</i>	Least concern	Bosnia-Herzegovina (vulnerable), Croatia (vulnerable), Czech Republic (protected), France (protected in several regions), Germany (vulnerable), Hungary (extinct), Lithuania (vulnerable), Luxembourg (critically endangered), Sweden (near-threatened), Switzerland (least concern)	Callus and hairy root cultures	Sesquiterpene lactones	Petrova et al. (2012) and Nieto-Trujillo et al. (2021)
<i>Chlorophytum borivilianum</i>	Critically endangered	nd	Shoot cultures	nd	Debnath et al. (2011)
<i>Crinum malabaricum</i>	Critically endangered	nd	Shoot cultures	Galanthamine	Priyadharshini et al. (2020)
<i>Cullen corylifolium</i>	Least concern	Sri Lanka (endangered), India (possibly threatened)	Callus and cell suspension cultures	Daidzein, genistein, and psoralen	Baskaran and Jayabalan (2009), Shinde et al. (2009) Baskaran et al. (2011), Satdive et al. (2015) and Singh et al. (2020)
<i>Cytisus acolicus</i>	Endangered	Sicily	Callus and shoot cultures	Genistein	Lucchesini et al. (2010)

(continued)

Table 22.1 (continued)

Species	IUCN status	The National Red Lists/local threats to the species	Type of in vitro cultures	Production of compounds	References
<i>Dyosma versipellis</i>	Vulnerable	nd	Callus and shoot cultures	Podophyllotoxin	Jiang et al. (2012)
<i>Eryngium alpinum</i>	Near-threatened	Austria (vulnerable), France (protected), Italy (vulnerable), Slovenia (protected), Switzerland (vulnerable)	Shoot cultures	Flavonoids and phenolic acids	Kikowska et al. (2020)
<i>Eryngium maritimum</i>	Least concern	Estonia (threatened), Latvia (threatened), Lithuania (threatened), Norway (threatened), Slovenia (extinct in a small coastal area)	Shoot and root cultures	Flavonoids and phenolic acids	Kikowska et al. (2022)
<i>Eryngium planum</i>	Least concern	Austria (critically endangered), Croatia (extinct)	Shoot and root cultures	Flavonoids and phenolic acids	Kikowska et al. (2022)
<i>Eryngium viviparum</i>	Endangered	France, Spain	Shoot cultures	Total content of poly-phenols and flavonoids	Ayuso et al. (2019)
<i>Gentiana cruciata</i>	Least concern	Bialorus (vulnerable), Czech Republic (endangered), Estonia (near-threatened), Germany (vulnerable), Hungary (near-threatened), Lithuania (vulnerable), Luxembourg (regionally extinct), Netherlands (near-threatened), Switzerland (vulnerable)	Shoot cultures	Iridoid glycosides	Hayta et al. (2011)
<i>Ginkgo biloba</i>	Endangered	nd	Callus and shoot cultures	Phenolic acids and quercetin	Qaderi et al. (2021) and Szewczyk et al. (2021)
<i>Glycyrrhiza glabra</i>	Least concern	Bulgaria (endangered), China (least concern)	Hairy root cultures	Glycyrrhizin	Pandey and Ayangla (2018)

<i>Hylocereus costaricensis</i>	Least concern	Costa Rica, Nicaragua	Callus and cell suspension cultures	Total content of polyphenols and flavonoids, betalain pigments	Winson et al. (2020)
<i>Lilium candidum</i>	Near-threatened	Greece, Israel	Shoot cultures	nd	Daneshvar Royandazagh (2019)
<i>Magnolia dealbata</i>	Near-threatened	Mexico	Shoot cultures	Honokiol, magnolol	González-Molina et al. (2010)
<i>Nilgiranthus ciliatus</i>	Vulnerable	nd	Callus cultures	Squalene	Rameshkumar et al. (2018)
<i>Picrorhiza kurroa</i>	Endangered	nd	Hairy root cultures	Picrotin and picrotoxinin	Mishra et al. (2011)
<i>Pterocarpus marsupium</i>	Near-threatened	Sri Lanka	Shoot cultures	nd	Ahmad et al. (2021)
<i>Rhodiola rosea</i>	Least concern	Bosnia and Herzegovina, Bulgaria, Czech Republic, Russia, Slovakia	Shoot cultures	Rosarin, rosavin, rosin	Javid et al. (2021)
<i>Rhododendron tomentosum</i>	Least concern	Czech Republic (vulnerable), Denmark (critically endangered), Norway (least concern)	Microshoot cultures	Compounds of essential oil	Jesionek et al. (2017, 2018)
<i>Silybum marianum</i>	Least concern	Croatia (near-threatened), United Kingdom (least concern)	Callus cultures	Silymarin	Shehzad et al. (2021)
<i>Salvia sclarea</i>	Least concern	Croatia, Cyprus, Serbia, Switzerland	Shoot cultures	Diterpenes	Erişen et al. (2020)

^and no data

22.2 *N. officinale*: Botanical and Chemical Characteristics and Relevance in Phytotherapy, Cosmetology, and Food Industry

Commonly known as watercress, *N. officinale* R. Br. (Robert Brown) (Fig. 22.1) is an aquatic or semiaquatic species belonging to the Brassicaceae family. It grows often in the wild, found colonizing in gently flowing and shallow streams, ditches, pond fringes, and marshes (IUCN, 2021).

N. officinale has numerous names, the most popular Latin names being *Nasturtium aquaticum* Wahlenb., *Rorippa nasturtium-aquaticum* (L.) Hayek, and *Rorippa officinalis* (W.T. Aiton) P. Royen. Its names in foreign languages are as follows: watercress (English); Brunnenkresse, Wasserkresse, and Brunnkresse (German); cresson de fontaine (French); berro (Spanish); and dou ban cai and 豆瓣菜 (Chinese).

N. officinale was first described by Linnaeus as *Sisymbrium nasturtium-aquaticum* in 1753. For three centuries, this species was mentioned in botanical descriptions as *N. aquaticum* Wahlenb. or *Sisymbrium aquaticum* Garsault. Linnaeus rejected both names and assigned a new three-component name: *Sisymbrium nasturtium-aquaticum* (Linnaeli 1753). In 1810, two British botanists reverted to the dual naming system: first, John Hill renamed it *N. aquaticum*; and later another botanist, Robert Brown, gave it a new name—*N. officinale*—which is now considered correct. The word “nasturtium” is probably derived from the Latin phrase “*nasus tortus*,” which means “twisted nose,” referring to the human reflex caused

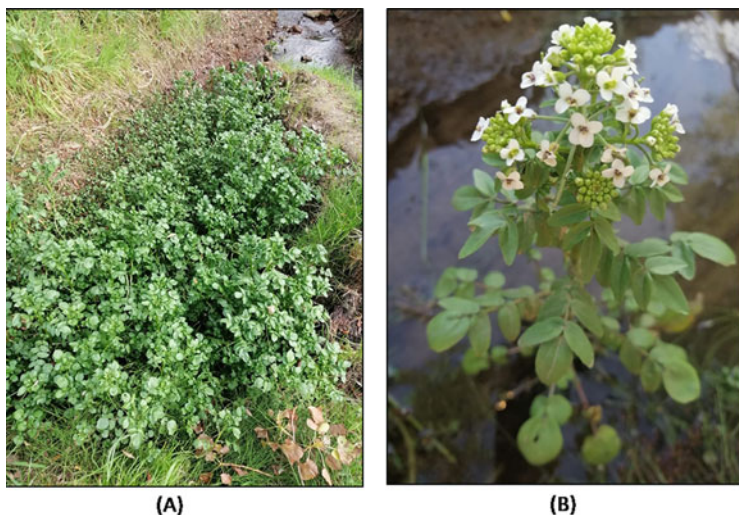


Fig. 22.1 Morphological appearance of *Nasturtium officinale*; (a) plants in a natural environment; (b) apical shoot fragments with flowers (photos by A. Szopa)

by the sharp, peppery smell of the plant (Greene 1896). The word “officinale” denotes a plant with recognized medicinal use (Greene 1896).

N. officinale is perennial, aquatic, or semiaquatic species with creeping or floating stems. The stems of *N. officinale* are 10–70 cm long, hollow, and irregularly shaped. The pinnate leaves of this plant are dark green, and inflorescence is clustered, with small white flowers with four petals. Its fruit is a shell with small reddish brown seeds (Manton 1935). Natural habitats of *N. officinale* are found in Europe, North Africa, and Asia. It is also found in North America, South America, and Australia. The first commercial cultivation of *N. officinale* was carried out in southern England in 1808 (Klimek-Szczykutowicz et al. 2018a, b). Currently, artificial breeding is the main source of production of this plant because the number of natural habitats is decreasing.

The raw material obtained from *N. officinale* is a herb (*Nasturtii herba*), which is a known phytomedicine not only in Europe but also worldwide (Suroowan and Mahomoodally 2016; Teixidor-Toneu et al. 2016; Klimek-Szczykutowicz et al. 2018a, b). Contemporary medicinal applications of *N. officinale* herb extracts are rooted in ancient Persian medicine and traditional medicine of Iran, Azerbaijan, Morocco, and Mauritius, in which *N. officinale* is used in the form of extracts from fresh or dried herb. In traditional medicine of Iran and Azerbaijan, *N. officinale* is especially recommended in the treatment of hypertension, hyperglycemia, and renal colic (Asadi-Samani et al. 2017). The leaves of *N. officinale* are used in traditional Moroccan medicine to improve the overall health in pediatric population (Teixidor-Toneu et al. 2016). In traditional medicine of Mauritius, *N. officinale* is used in the treatment of asthma, cough, and bronchial problems (Suroowan and Mahomoodally 2016).

In Germany, the herb *N. officinale* is an officially approved plant material for phytotherapy. According to the German Commission Monograph E (Blumenthal et al. 1999), *N. herba* can be used in the form of fresh or dried aerial parts of the plant in the treatment of rhinitis. In addition, *N. officinale* has an important position in the German Commission Monograph D (German Commission D 1989) on homeopathic medicines. It is used in the treatment of urinary tract inflammation.

Phytochemical tests of *N. officinale* showed qualitative and quantitative differences in the chemical composition depending on the plant organ analyzed. The major groups of secondary metabolites characteristic of *N. officinale* are as follows: glucosinolates (GSLs), isothiocyanates, and polyphenolic compounds (Afsharypuor and Salehi 2008; Jeon et al. 2017; Klimek-Szczykutowicz et al. 2018a, b).

GSLs are the most important metabolites responsible for the biological activity of *N. officinale*. GSLs are thioglycosides containing a glucose molecule, a sulfur atom, and an amino acid chain. GSLs are a special group of compounds found primarily in the species of the Brassicaceae family. These compounds are named after the glucose present in the sugar part of these compounds. Most of the GSLs are thermally and chemically stable. Plants with GSLs produce an enzyme called myrosinase (β -thioglucosidase), which hydrolyzes these glucoside molecules. GSLs and myrosinase are found in plants in distinct cells. When the fresh raw material is crushed and the tissue structure is destroyed, enzymatic decomposition

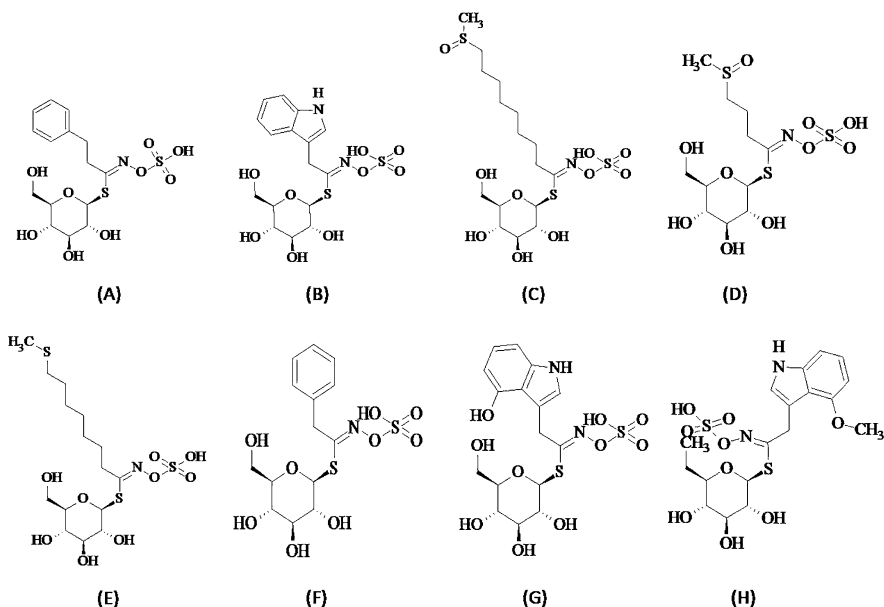


Fig. 22.2 Chemical structures of glucosinolates characteristic of *Nasturtium officinale*; (a) gluconasturtiin, (b) glucobrassicin, (c) glucohirsutin, (d) glucoiberin, (e) glucosiberin, (f) glucotropaeolin, (g) 4-hydroxy-glucobrassicin, and (h) 4-methoxy-glucobrassicin

of GSLs takes place, leading to the formation of products such as isothiocyanates, nitriles, elemental sulfur, thiocyanates, epithionitriles, oxazolidine-2-thions, and/or indole compounds (Holst and Williamson 2004).

GSLs are classified into three groups due to the amino acid side chain present in them: aliphatic GSLs, aromatic GSLs, and indole GSLs. Aliphatic GSLs are derived from alanine (Ala), leucine (Leu), isoleucine (Ile), methionine (Met), or valine (Val); aromatic GSLs are derivatives of phenylalanine (Phe) or tyrosine (Tyr); and indole GSLs are derivatives of tryptophan (Trp) (Holst and Williamson 2004; Blažević et al. 2020).

Eight GSLs are reported in *N. officinale*: Phe derivatives (gluconasturtiin and glucotropaeolin); Trp derivatives (glucobrassicin, 4-hydroxyglucobrassicin, and 4-methoxyglucobrassicin); and Met derivatives (glucohirsutin, glucoiberin, and glucosiberin) (Fig. 22.2). These compounds are present in extracts from *N. officinale* leaves, stems, roots, flowers, and seeds. The highest content of GSLs was observed in flowers and in the whole herb. Gluconasturtiin (2-phenylethylglucosinolate) is the dominant compound found quantitatively in all parts of the plant, and it hydrolyzes yielding 2-phenylethyl isothiocyanate (nasturtiin) (Fig. 22.3) (Afsharypuor and Salehi 2008; Jeon et al. 2017). Other isothiocyanates found in the *N. officinale* herb are 4-phenylbutyl isothiocyanate, heptyl isothiocyanate, isopropyl isothiocyanate, 4-methylsulfinylbutyl

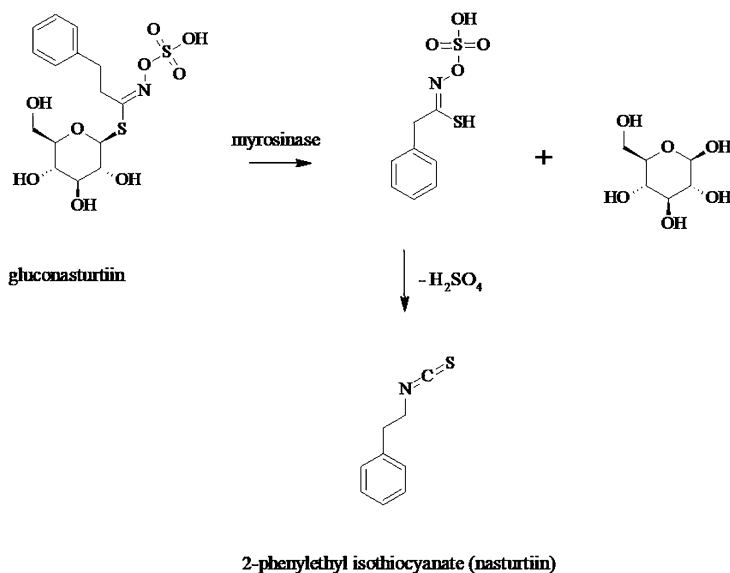


Fig. 22.3 Gluconasturtiin breakdown reaction by myrosinase to 2-phenylethyl isothiocyanate (nasturtiin) and glucose

isothiocyanate (sulforaphane), 7-methylsulfinylheptyl isothiocyanate, octyl isothiocyanate, and sec-butyl isothiocyanate (Afsharypour and Salehi 2008; Jeon et al. 2017).

Polyphenols such as flavonoids, phenolic acids, and proanthocyanidins are another important group of secondary metabolites found in *N. officinale*. Besides the glycoside combinations of quercetin and kaempferol, the presence of apigenin and rutoside was confirmed in leaf and herb extracts. Among phenolic acids, the presence of chlorogenic acid, ferulic acid derivatives, gallic acid, caffeic acid, caffeoylmalonic acid, caftaric acid, *p*-coumaric acid, and sinapic acid, among others, has been documented (Martínez-Sánchez et al. 2008; Boligon et al. 2013; Zeb 2015). Proanthocyanidin B₁ is found in *N. officinale* herb (Zeb 2015). In addition, monoterpenoids (carvacrol, 1,8-cineole, limonene, neoiso(iso)pulegyl acetate, pulegone, and *trans-p*-menth-2-en-1-ol), diterpenoids (isophytol, *E*-phytol, and *Z*-phytol), tetraterpenoids (β -carotene, lutein, and zeaxanthin), and sesquiterpenoids (α -bisabolene A oxide) are also present in *N. officinale* herb (Afsharypour and Salehi 2008; Martínez-Sánchez et al. 2008; Quezada-Lázaro et al. 2016). Analysis of *N. officinale* herb extracts also showed the presence of water-soluble vitamins (e.g., ascorbic acid, niacin, pyridoxine, riboflavin, thiamine), fat-soluble vitamins (e.g., α -tocopherol), and palmitic acid (Martínez-Sánchez et al. 2008). Furthermore, *N. officinale* is a source of essential oil, which can be obtained from the herb, flowers, stems, or leaves. A number of compounds have been reported in essential oil from flowers, limonene being the most important (Amiri 2012).

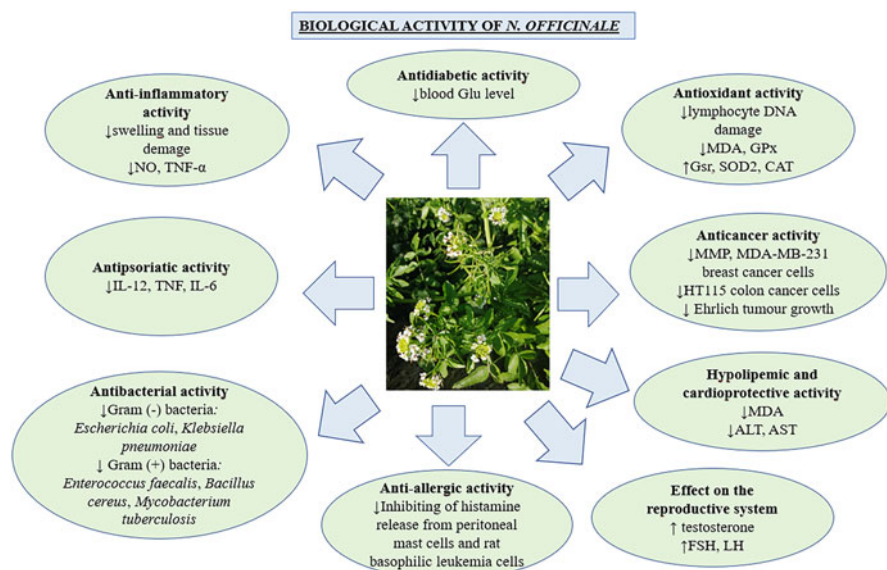


Fig. 22.4 Biological activities of *Nasturtium officinale* herb extracts

The biological activity of *N. officinale* extracts (Fig. 22.4) depends primarily on the presence of GSLs and isothiocyanates. Currently, an increasing number of scientific researches on the pharmacological action of *N. officinale* herb extracts, as well as compounds isolated from them, are conducted. Strong anticancer and antioxidant properties have been proven in *N. officinale* extracts and isothiocyanates (Martínez-Sánchez et al. 2008; Amiri 2012; Casanova et al. 2013; De Souza et al. 2016; Li et al. 2016; Yuan et al. 2016; Shahani et al. 2017; Giallourou et al. 2019). Recent studies have reported the anticancer effect of *N. officinale* extracts in the treatment of lung, colon, breast, liver, and bile duct cancer (Boyd et al. 2006; De Souza et al. 2016; Li et al. 2016; Yuan et al. 2016; Schuchardt et al. 2019; Arumugam et al. 2020). The anti-inflammatory and antipsoriatic effects of sulforaphane—an isothiocyanate isolated from the herb *N. officinale*—have been demonstrated (Yehuda et al. 2012; Camponogara et al. 2019). In vitro and animal studies have confirmed the antiallergic, antibacterial, and hypolipemic effects of *N. officinale* herb extracts (Basch et al. 2006; Bahramikia and Yazdanparast 2008; Yazdanparast et al. 2008; Sadeghi et al. 2014; Quezada-Lázaro et al. 2016; Zafar et al. 2017; Mahdavi et al. 2019; Schuchardt et al. 2019). The ability of these extracts to inhibit the angiotensin-converting enzyme has also been reported, which in turn affects the functioning of the circulatory system (Yaricsha et al. 2017). Moreover, *N. officinale* herb extracts lower the blood glucose levels and positively affect the functioning of the reproductive system (Hoseini et al. 2009; Mohammadi et al. 2017).

Unfortunately, *N. officinale* herb extracts have not yet been recognized as a popular ingredient in legalized medicinal preparations. However, they are used in the production of antioxidant dietary supplements, e.g., Yango (Poland) and Nutrilite (USA). Extracts of *N. officinale* herb are used in cosmetic preparations and patented cosmetic compositions. The first references to the cosmetic use of *N. officinale* are found in the textbook of the Italian doctor and writer Trota from Salerno, entitled *De Ornatu Mulierum* from the twelfth century (Głusiuk 2018). Nowadays, *N. officinale* herb extracts have been scientifically documented for their valuable cosmetological, antioxidant, antiaging, skin-brightening, anti-acne, anti-inflammatory, antibacterial, and tyrosinase-inhibiting properties (Klimek-Szczykutowicz et al. 2018a, b; Thibane et al. 2019).

Extracts of *N. officinale* herb can be found in cosmetics produced by many companies, including Polish (e.g., FLOSLEK-Pharma, Farmona, Synoptis Pharma), English (Skin Chemists), Japanese (Kanebo, Sensai), and Canadian (Attitude). These cosmetics are mainly used in the care of oily and mature skin.

The species *N. officinale* has long been used as a culinary plant. Due to its valuable prohealth properties, low calorific value, rich chemical composition, and taste, it is used as an ingredient in salads and added to other dishes. On Gran Canaria—one of the Canary Islands, the soup of *N. officinale* (“potaje de berros”) is a well-known regional dish. In Mexican cuisine, the plant is also served as a local dish in the form of a cream soup (“crema de berros”). Currently, *N. officinale* is frequently used in modern European cuisine (Klimek-Szczykutowicz et al. 2018a, b). It has also been classified as a safe vegetable by the European Food Safety Authority (<http://www.efsa.europa.eu/>. Accessed 25 Jan 2022). Every year, the importance and popularity of *N. officinale* as a culinary plant is increasing. This is evidenced by the fact that *N. officinale* is one of the so-called healthy foods. Moreover, *N. officinale* is listed in the USDA National Nutrient Database (US Department of Agriculture), with a caloric value of 100 g dry mass (DM) = 11 kcal (USDA (<https://ndb.nal.usda.gov/ndb/>)). Additionally, it has recently been recognized as the healthiest vegetable in the world by the Centers for Disease Control and Prevention (<https://www.cdc.gov/>. Accessed 31 Mar 2022).

22.3 Micropropagation as the Tool for *N. officinale* Protection

In plant biotechnology, research on in vitro cultures of *N. officinale* is scarce. Biotechnological studies on *N. officinale* have mainly focused on the development of micropropagation techniques. Micropropagation is considered important because of the partial protection of this species. It can contribute to the conservation of endangered species in a controlled manner (Wainwright and Marsh 1986; Gilby and Wainwright 1989; Wielanek et al. 2009; Rubin et al. 2018a, b).

In 1985, Wainwright and Marsh began the first biotechnological research with the aim of developing a micropropagation protocol for *N. officinale* (Wainwright and

Marsh 1986). Studies reported a high efficiency of multiplication for cultures grown on a 1/4 Murashige and Skoog (MS) medium containing 2% sucrose. The multiplication index was dependent on the type of explant used (shoot apex, single node, and two nodes) and its location (distal and proximal) (Wainwright and Marsh 1986). In 1989, using somatic embryogenesis, the same team of researchers developed a protocol for the regeneration of *N. officinale* from callus tissue. The study included testing the MS medium and the addition of various auxins, such as 1-naphthylacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), and cytokinins, such as 6-benzyladenine (BA) and thidiazuron (TDZ). The highest shoot multiplication index was obtained for the media containing 2,4-D (Gilby and Wainwright 1989). In another study, the best coefficient of regeneration was obtained when callus was induced on a medium containing 0.25 mg/L TDZ and 0.05 mg/L 2,4-D, followed by regeneration on 0.25 mg/L TDZ (Arnold et al. 1995). Claxton et al. (Claxton et al. 1998) tested different concentrations of TDZ and 2,4-D in the MS medium to regenerate adventitious shoots from the callus formed. The highest microshoot regeneration rate of 18 shoots per responding explant was reported for the stem callus formed on a medium containing 5 μ M TDZ and 0.05 μ M 2,4-D (Claxton et al. 1998).

Recently, Ogita et al. have studied *N. officinale* micropropagation (Ogita et al. 2009). Internodes of ca. 1 cm excised from the in vitro shoot culture were placed on a 1/2 MS medium containing 3 μ M 2,4-D. Then, to obtain multiple shoots, the explants were transferred to an MS medium containing 1 μ M TDZ and maintained for 3 weeks (Ogita et al. 2009).

22.4 Biotechnological Studies on *N. officinale* Agar Cultures

22.4.1 Initiation of Microshoot Cultures

In the biotechnological studies performed in the Department of Pharmaceutical Botany, Faculty of Pharmacy, Jagiellonian University Medical College, in vitro cultures of *N. officinale* were initiated and optimized for the production of bioactive metabolites.

The cultures were initiated from seeds obtained from the Garden of Medicinal Plants of the Faculty of Pharmacy, Jagiellonian University Medical College, Kraków, Poland. The seeds were then sterilized for 5 min with 0.1% HgCl₂ (mercury chloride), washed thrice with sterile redistilled water, and transferred to the MS (Murashige and Skoog 1962) medium with 0.72% agar (w/v), 3% sucrose (w/v), 1 mg/L BA, and 1 mg/L NAA. After about 2 weeks, green, viable, well-grown microshoot cultures were obtained (Fig. 22.5). The in vitro cultures were maintained at a temperature of 25 ± 2 °C under continuous artificial white LED lighting of 2.75 W/m² (photosynthetic photon flux density (PPFD) 40 μ mol m⁻² s⁻¹) and passaged at 2-week intervals.

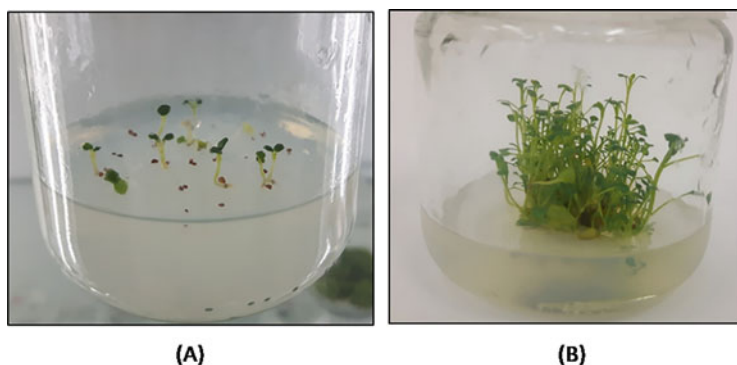


Fig. 22.5 Initiation of *Nasturtium officinale* microshoot cultures; (a) germination of seeds and (b) microshoot cultures after 3 weeks of stable sowing

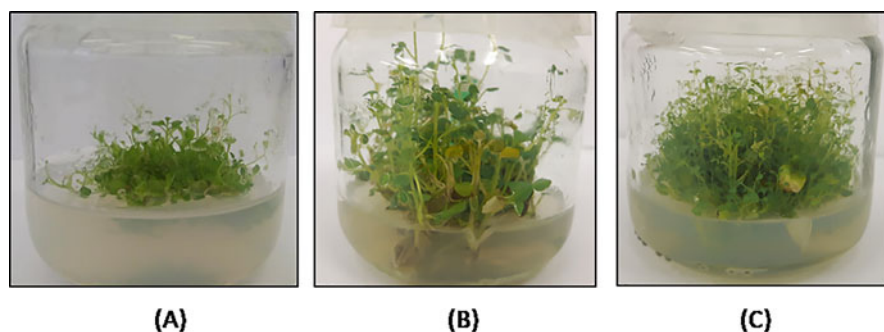


Fig. 22.6 *Nasturtium officinale* microshoot agar cultures; MS medium containing 1 mg/L BA and 1 mg/L NAA; (a) 10 days of cultures; (b) 20 days of culture; (c) 30 days of cultures (Klimek-Szczykutowicz et al. 2020)

22.4.2 Biomass Increases and Survival of Cultures

Studies on the optimization of the agar MS media (Fig. 22.6) involved testing of various cytokinins at a concentration of 1 mg/L, such as BA, 6-(γ , γ -dimethylallylamino)purine (2iP), kinetin (KIN), and zeatin (Zea). The culture growing on the MS medium without the addition of PGRs was treated as the control (Klimek-Szczykutowicz et al. 2020). Good results were obtained regarding the growth of biomass and the accumulation of phenolic compounds in *N. officinale* cultures grown on the MS medium containing 1 mg/L BA, which prompted us to test this cytokinin together with the auxin NAA at concentrations of 2 and 1; 1 and 2; and 1 and 1 (mg/L), respectively. The results showed that a cytokinin/auxin ratio of 1:1 was the best for the growth of biomass of the cultures and the accumulation of phenolic compounds. Subsequently, studies were carried out on the combination of four cytokinins (BA, 2iP, KIN, and Zea) and five auxins (3-indolylacetic acid (IAA),

Table 22.2 Tested variants of MS medium with different combinations of with 1 mg/L cytokinin and 1 mg/L auxin in *Nasturtium officinale* agar microshoot cultures

Cytokinin	Auxin
6-Benzyladenine (BA)	3-Indolylacetic acid (IAA)
	3-Indolylbutyric acid (IBA)
	3-Indolylpyruvic acid (IPA)
	2,4-Dichlorophenoxyacetic acid (2,4-D)
	1-Naphthylacetic acid (NAA)
6-(γ,γ -Dimethylallylamino)purine (2iP)	3-Indolylacetic acid (IAA)
	3-Indolylbutyric acid (IBA)
	3-Indolylpyruvic acid (IPA)
	2,4-Dichlorophenoxyacetic acid (2,4-D)
	1-Naphthylacetic acid (NAA)
Kinetin (KIN)	3-Indolylacetic acid (IAA)
	3-Indolylbutyric acid (IBA)
	3-Indolylpyruvic acid (IPA)
	2,4-Dichlorophenoxyacetic acid (2,4-D)
	1-Naphthylacetic acid (NAA)
Zeatin (Zea)	3-Indolylacetic acid (IAA)
	3-Indolylbutyric acid (IBA)
	3-Indolylpyruvic acid (IPA)
	2,4-Dichlorophenoxyacetic acid (2,4-D)
	1-Naphthylacetic acid (NAA)

3-indolylbutyric acid (IBA), 3-indolylpyruvic acid (IPA), 2,4-D, and NAA) at a concentration of 1 mg/L each, resulting in 20 MS medium variants differing in the combination of PGRs (Table 22.2). A total of 27 variants of MS medium containing different combinations of PGRs were tested. Cultures were grown in cycles of 10, 20, and 30 days (three series, $n = 10$) under continuous white LED illumination (2.75 W/m^2 ; PPFD $40 \mu\text{mol m}^{-2} \text{ s}^{-1}$) at a temperature of $25 \pm 2 \text{ }^\circ\text{C}$ (Klimek-Szczykutowicz et al. 2020).

Depending on the applied PGRs, the biomass of the cultures, the length of the microshoots, and the ability to create branches differed from each other. In all tested variants with 2,4-D, dying off of biomass was observed after 10 days of cultivation; therefore, these variants were studied further. After 10-, 20-, and 30-day growth cycles, fresh biomass of agar cultures was weighed, and after lyophilization, their dry weight (DW) was also determined to calculate the biomass growth index (G_i) for fresh and dry biomass. Growth rates of fresh and dry biomass (G_i) were calculated according to the formula $G_i = \frac{(DW_1 - DW_0)}{DW_0}$, where DW_1 represents the mass of the sample after the end of the growth cycle and DW_0 represents the mass of the sample at the time of establishing the culture (inoculum) (Grzegorzczak and Wysokińska 2008).

The increase in the fresh and dry biomass depended on the concentration of PGRs used and the duration of the growth cycles. The G_i index ranged from 0.05 to 5.05

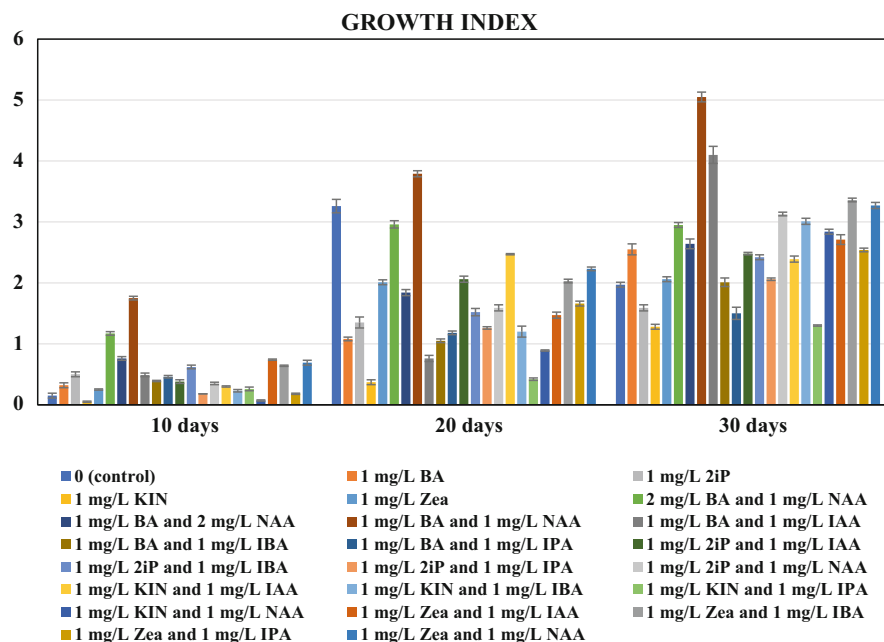


Fig. 22.7 $\text{Gi} \pm \text{SD}$ values of *Nasturtium officinale* microshoot agar cultures depending on growth cycle periods and MS medium variants of different PGR combinations. Data are expressed as mean value $\pm \text{SD}$ ($n = 3$)

(Fig. 22.7). Good results were obtained with the use of BA as a cytokinin and NAA as an auxin in different concentrations as follows: 2 and 1; 1 and 2; and 1 and 1 (mg/L), respectively. Microshoots growing on all these variants were characterized by a large number of microshoots and the dark green color of the leaves. The highest dry biomass increment index (Gi) of 5.05 was found for microshoots grown on the MS medium containing 1 mg/L BA and 1 mg/L NAA for 30 days (Fig. 22.7) (Klimek-Szczykutowicz et al. 2020).

22.4.3 Production of GSLs

The total GSL content was analyzed using a spectrophotometric method (Gallaher et al. 2012). Extracts from agar cultures containing 1 mg/L each of cytokinin (BA, 2iP, KIN, and Zea) and auxin (IAA, IBA, and NAA) in the MS medium (6 medium variants) were tested. In the biomass extracts grown on the tested MS medium variants, an obvious effect of the applied PGRs on the accumulation of secondary metabolites was observed. In the agar cultures, the total GSL content ranged from 100.23 to 194.77 mg sinigrin equivalent (SIN)/100 g DW. The highest GSL content was observed for the microshoot cultures grown on the medium containing 1 mg/L

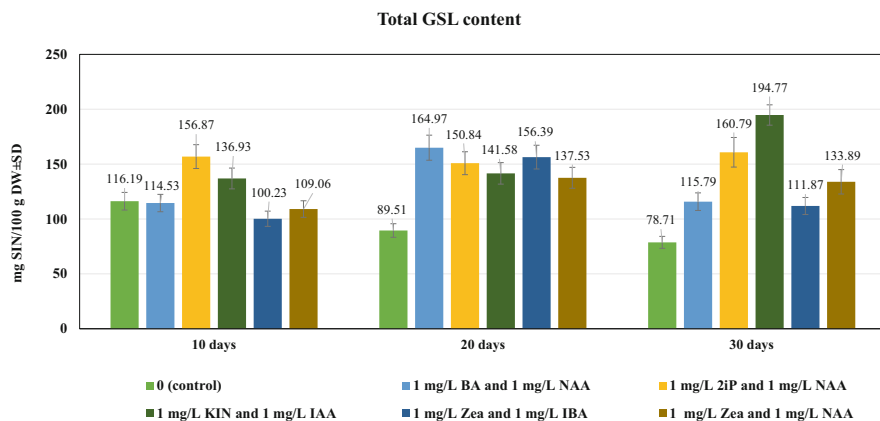


Fig. 22.8 Maximum total GSL contents (mg SIN/100 g DW $\pm$ SD) in the extracts of *Nasturtium officinale* microshoot agar cultures depending on growth cycle periods and MS medium variants of different PGR combinations. Data are expressed as mean value $\pm$ SD ($n = 3$)

KIN and 1 mg/L IAA after 30 days of the growth cycle, which was about 1.68 times higher than that of control cultures grown on the MS medium without PGRs (Fig. 22.8).

22.4.4 Production of Polyphenol Compounds

The total polyphenol content in microshoot cultures was determined using the spectrophotometric Folin-Ciocalteu method (F-C) (Singleton et al. 1999; Bach et al. 2015). In the biomass extracts from agar cultures, the total polyphenol content ranged from 455.27 to 1634.85 mg gallic acid equivalent (GAL)/100 g DW. The highest polyphenol content was observed in the extracts of cultures grown on the MS medium variant containing 1 mg/L Zea and 1 mg/L NAA harvested after 10 days of cultivation. The lowest total polyphenol content was observed in the extracts of agar cultures grown on the MS medium containing 2 mg/L BA and 1 mg/L NAA after 30 days of cultivation (Fig. 22.9) (Klimek-Szczykutowicz et al. 2020).

22.4.5 Antioxidant Potential

The antioxidant potential of in vitro cultures biomass extracts was determined using the cupric reducing antioxidant capacity (CUPRAC) (Özyürek et al. 2007), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Blis 1958), and ferric reducing ability of plasma (FRAP) (Benzie and Strain 1996) tests. The antioxidant activity of the extracts from in vitro cultures ranged from 1.50 to 4.13 mmol Trolox equivalent

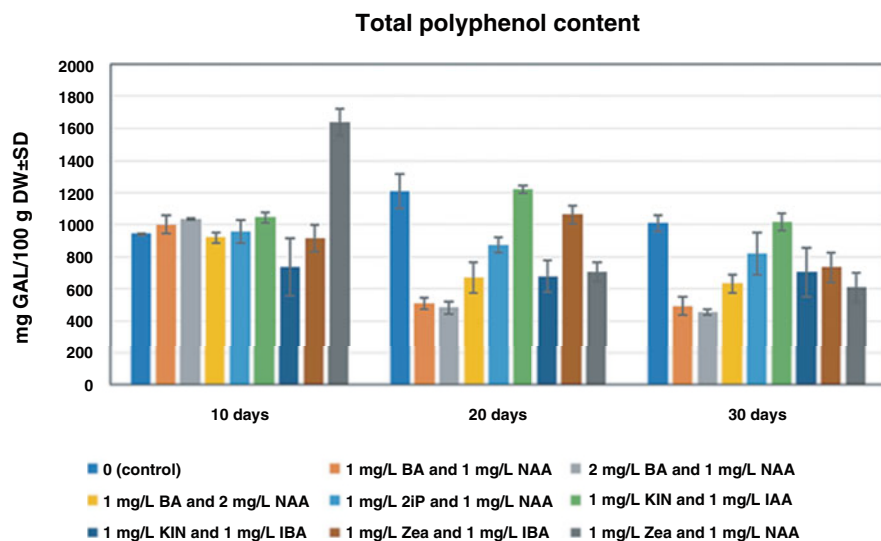


Fig. 22.9 Total polyphenol contents determined using the F-C method (expressed in mg GAL/100 g DW $\pm$ SD) of *Nasturtium officinale* microshoot agar cultures depending on growth cycle periods and the MS medium variants of different PGR combinations. Data are expressed as mean value $\pm$ SD ($n = 3$)

(TE)/100 g DW via CUPRAC, from 0.49 to 30.89 via DPPH, and from 0.51 to 1.42 mmol TE/100 g DW via FRAP. The highest antioxidant potential measured by the CUPRAC and DPPH methods was observed for the cultures grown on the medium containing 1 mg/L KIN and 1 mg/L IAA collected after 30 days of culture duration, whereas the highest antioxidant potential measured by the FRAP method was observed for the cultures grown on the medium containing 2 mg/L BA and 1 mg/L NAA harvested after 10 days of culture (Fig. 22.10) (Klimek-Szczykutowicz et al. 2020).

22.5 Biotechnological Studies on *N. officinale* Agitated Cultures

22.5.1 Biomass Increases and Survival of Cultures

The conditions for agitated microshoot cultures (Fig. 22.11) were optimized on the MS medium variants selected based on studies carried out with agar cultures. The four cytokinins (BA, 2iP, KIN, and Zea) and three auxins (IAA, IBA, and NAA) (six variants) were tested at the concentration of 1 mg/L (Table 22.3) and with different durations of culture growth periods: 10 and 20 days. The 30-day cultivation cycles were not tested due to the dying off of the biomass. The dynamics of biomass

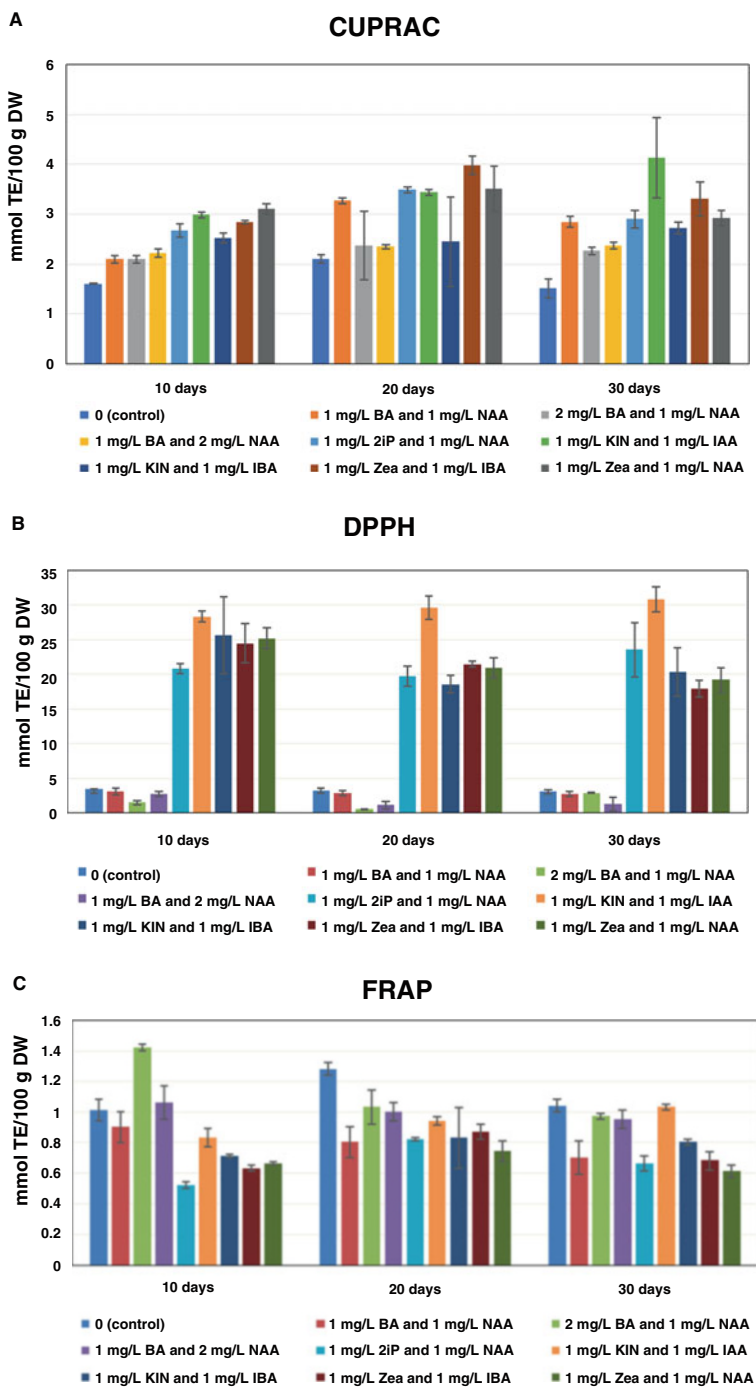


Fig. 22.10 The highest antioxidant potential (expressed in mmol TE/100 g DW $\pm$ SD) of *Nasturtium officinale* microshoot agar cultures depending on growth cycle periods and MS medium

increases and the accumulation of secondary metabolites were investigated (Klimek-Szczykutowicz et al. 2020).

Depending on the applied PGRs, the cultures differed from each other in the appearance of biomass, the length of the microshoots, and the ability to create branches. The highest number of green microshoots was achieved after 20 days of growth on the medium containing 1 mg/L Zea and 1 mg/L NAA. The cultures growing over 20-day growth periods in the medium containing 1 mg/L BA and 1 mg/L NAA were characterized by a large number of microshoots. The lowest number of microshoots was obtained after 10 and 20 days of growth on the medium containing 1 mg/L KIN and 1 mg/L IAA (Klimek-Szczykutowicz et al. 2020).

The Gi values ranged from 1.47 to 10.48 and were dependent on the medium variants and duration of culture growth periods. The highest biomass increases were obtained for the cultures cultivated over 20 days on the variant containing 1 mg/L Zea and 1 mg/L NAA. The lowest Gi values were obtained after 10 days on the MS medium containing 1 mg/L KIN and 1 mg/L IAA (Fig. 22.12) (Klimek-Szczykutowicz et al. 2020).

22.5.2 Production of GSLs

Extracts from the agitated cultures containing 1 mg/L each of cytokinin (BA, 2iP, KIN, and Zea) and auxin (IAA, IBA, and NAA) in the MS medium (six medium variants) for the total GSL contents were tested using the spectrophotometric method (Gallaher et al. 2012). In the agitated cultures, the total GSL content ranged from 78.09 to 182.80 mg SIN/100 g DM. The highest content was observed in the microshoot cultures grown on the medium containing 1 mg/L BA and 1 mg/L NAA over 10-day periods (Fig. 22.13). The lowest GSL content was observed after 20 days for the cultures grown on the MS medium containing 1 mg/L Zea and 1 mg/L IBA (Klimek-Szczykutowicz et al. 2020).

22.5.3 Production of Polyphenol Compounds

The analysis using the F-C method (Singleton et al. 1999; Bach et al. 2015) showed that in the biomass extracts from agitated cultures, the total polyphenol content ranged from 468.44 to 985.80 mg GAL/100 g DW. The highest polyphenol content was observed in the extracts of the cultures grown on the MS medium variant containing 1 mg/L KIN and 1 mg/L IAA harvested after 20 days of cultivation.



Fig. 22.10 (continued) variants of different PGR combinations, estimated by (a) CUPRAC, (b) DPPH, and (c) FRAP tests. Data are expressed as mean value $\pm$ SD ($n = 3$)

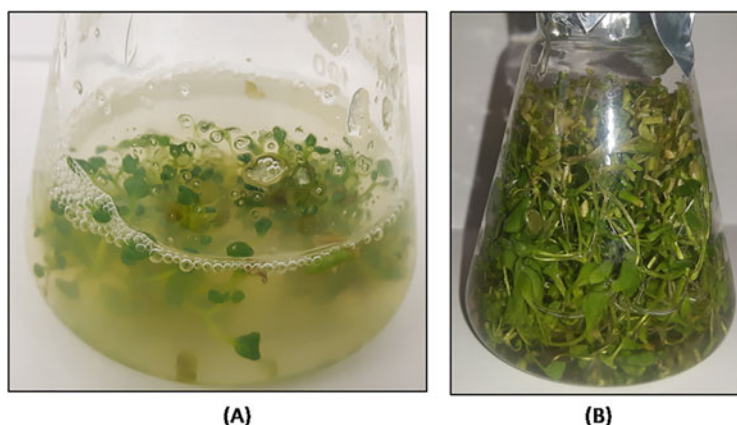


Fig. 22.11 *Nasturtium officinale* microshoot agitated cultures on MS medium containing 1 mg/L Zea and 1 mg/L NAA after the tested growth periods: (a) 10 days and (b) 20 days

Table 22.3 Tested variants of MS medium for *Nasturtium officinale* agitated microshoot cultures with 1 mg/L cytokinin and 1 mg/L auxin

Cytokinin	Auxin
6-Benzyladenine (BA)	1-Naphthylacetic acid (NAA)
6-(γ,γ -Dimethylallylamino)purine (2iP)	1-Naphthylacetic acid (NAA)
Kinetin (KIN)	3-Indolylacetic acid (IAA)
	3-Indolylbutyric acid (IBA)
Zeatin (Zea)	1-Naphthylacetic acid (NAA)
	3-Indolylbutyric acid (IBA)

The lowest total polyphenol content was observed for the extracts of agar cultures grown on the MS medium containing 1 mg/L 2iP and 1 mg/L NAA after 20 days of cultivation (Fig. 22.14) (Klimek-Szczykutowicz et al. 2020).

22.5.4 Antioxidant Potential

The antioxidant potential of the biomass extracts of agitated microshoot culture was determined using the CUPRAC (Özyürek et al. 2007), DPPH (Blis 1958), and FRAP (Benzie and Strain 1996) tests, which ranged from 2.52 to 5.26, 14.55 to 25.95, and 0.51 to 1.26 mmol TE/100 g DW, respectively. The highest antioxidant potential measured by the CUPRAC method was found for the agitated cultures grown on the medium containing 1 mg/L KIN and 1 mg/L IAA collected after 20 days of culture duration, whereas the same measured by the DPPH and FRAP methods was observed for cultures grown on medium containing 1 mg/L KIN and

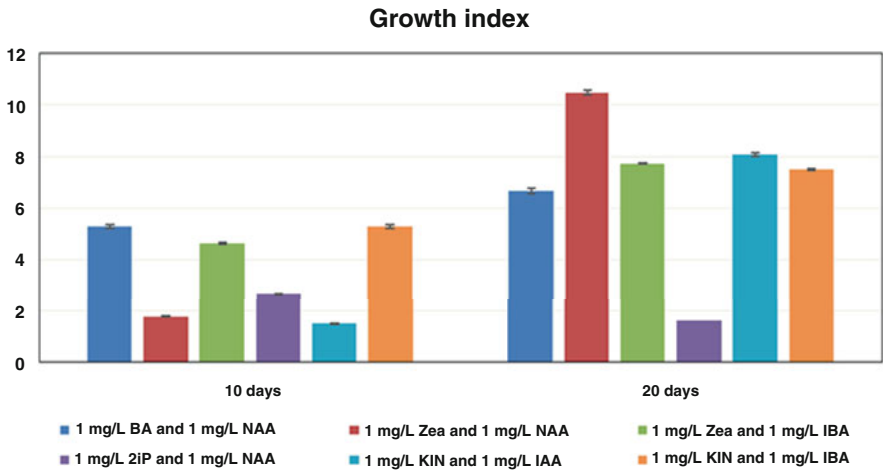


Fig. 22.12 $G_i \pm SD$ values of *Nasturtium officinale* microshoot agitated cultures depending on the duration of growth periods and MS medium variants of different PGR combinations. Data are expressed as mean value $\pm SD$ ($n = 3$)

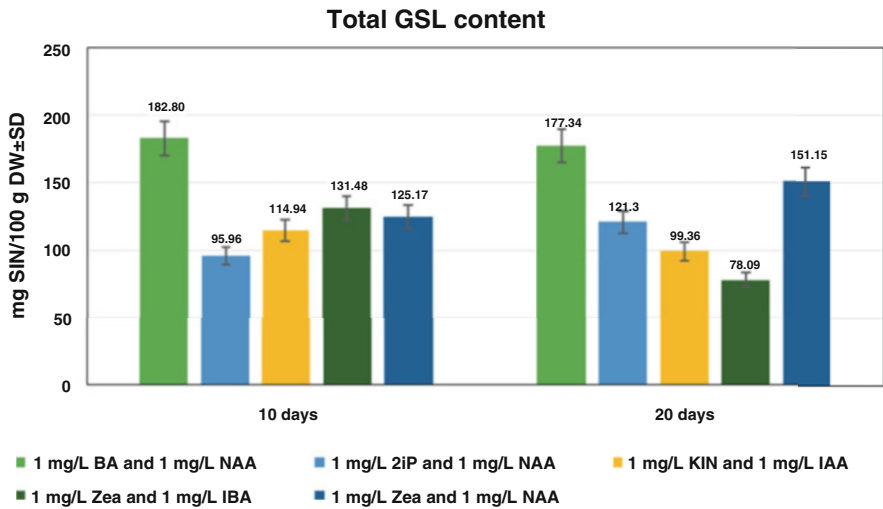


Fig. 22.13 The highest total GSL contents (mg SIN/100 g DW $\pm SD$) in the extracts of *Nasturtium officinale* microshoot agitated cultures depending on growth cycle periods and MS medium variants of different PGR combinations. Data are expressed as mean value $\pm SD$ ($n = 3$)

1 mg/L IAA harvested after 20 days of culture (Fig. 22.15) (Klimek-Szczykutowicz et al. 2020).

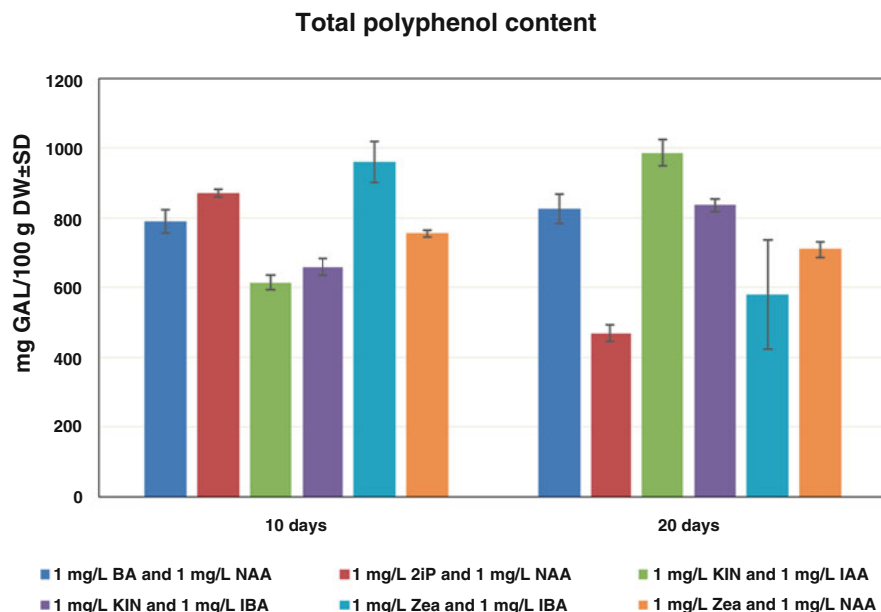


Fig. 22.14 Total polyphenolic content determined using the F-C method (expressed in mg GAL/100 g DW $\pm$ SD) of *Nasturtium officinale* microshoot agitated cultures depending on growth cycle periods and MS medium variants of different PGR combinations. Data are expressed as mean value $\pm$ SD ($n = 3$)

22.6 Influence of Light Conditions on Secondary Metabolite Production in *N. officinale* Agar Cultures

22.6.1 Biomass Increases and Survival of Cultures

No studies have discussed the effect of different wavelengths of LED light on the production of metabolites such as GSLs and polyphenolic compounds and the antioxidant activities of *N. officinale* microshoot cultures.

Based on previous studies, an optimal medium was chosen for the best “growth” and “production” in terms of accumulation of GSLs, polyphenols and antioxidant activity—the MS variant containing 1 mg/L BA and 1 mg/L NAA (Klimek-Szczykutowicz et al. 2020). The tests were carried out on agar microshoot cultures over 20-day growth periods. Cultures of *N. officinale* were carried out in a phytotron under photoperiod conditions of 16/8 h (day/night), at a temperature of $25/23 \pm 1$ °C, and a relative humidity of 70%. Eight variants of LED lighting were in the experiments (combinations of different wavelengths): blue light, B ($\lambda = 430$ nm); red light, R ($\lambda = 670$ nm); a combination of 70% red light and 30% blue light, RB; a combination of 50% UV-A ($\lambda = 400$ nm), 35% red, and 15% blue light, RBUV; a combination of 50% far red ($\lambda = 730$ nm), 35% red, and 15% blue, RBfR; a

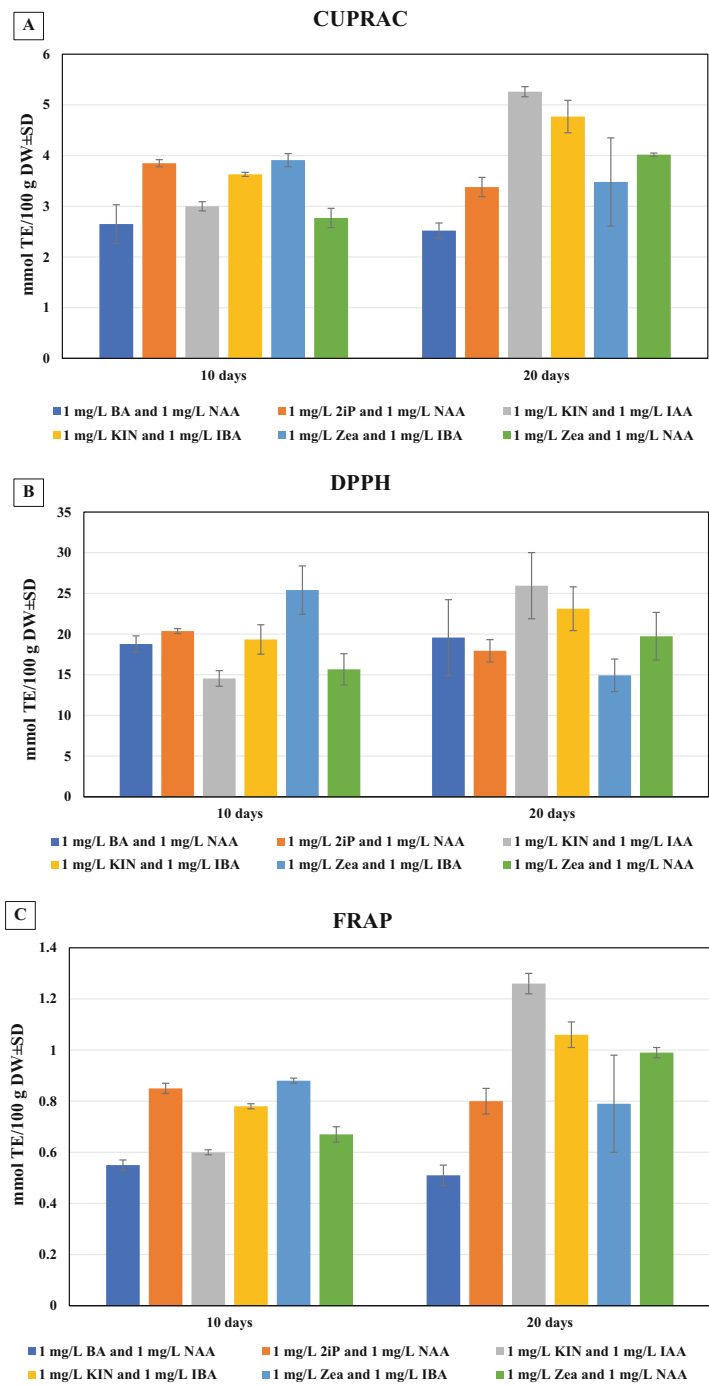


Fig. 22.15 The highest antioxidant potential (expressed in mmol TE/100 g DW ± SD) of *Nasturtium officinale* microshoot agitated cultures depending on growth cycle periods and MS

combination of 50% green light ($\lambda = 528$ nm), 35% red, and 15% blue, RBG; a combination of 50% yellow light ($\lambda = 600, 630$ nm), 35% red, and 15% blue, RBY; and white LED light, WLED (33% warm color, 2700 K; 33% neutral color, 4500 K; 33% cold color, 5700 K). The microshoot cultures grown under white fluorescent light—WF ($\lambda = 390\text{--}760$ nm)—and in darkness (D, no light) were used as control cultures. In total, ten different lighting conditions were tested. PPFD was set at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ in all lighting variants (Klimek-Szczykutowicz et al. 2021).

Depending on the applied LED lighting conditions, different appearance and growth rates of *N. officinale* microshoot cultures were observed (Fig. 22.16). A high number of microshoots and a dark green color were observed in in vitro cultures grown in B, RB, RBY, RBUV, and RBG lighting conditions. Microshoots grown under R, RBfR, and D light conditions showed a light green color, and their growth was the lowest. The Gi values ranged from 2.52 to 13.38. The highest Gi values were obtained for microshoots grown under RBY, RBG, and RB conditions—13.38, 11.81, and 8.25, respectively. A clear, stimulating effect of all the tested variants of LED light on the increase in biomass in comparison with the control conditions was observed in the studied cultures (Klimek-Szczykutowicz et al. 2021).

22.6.2 Production of GSLs

The experiments showed a significant influence of the studied combinations of LED light on the total GSL content, which ranged from 40.86 to 237.92 mg SIN/100 g DW. The highest content was observed in the microshoot cultures grown under RBG light conditions, which was 5.8 and 1.8 times higher than that under WF (40.86 mg SIN/100 g DW) and WLED (135.61 mg SIN/100 g DW) light conditions, respectively. High GSL contents were also observed under D (165.52 mg SIN/100 g DW) and RBfR (150.09 mg SIN/100 g DW) light conditions. The lowest GSL content (40.86 mg SIN/100 g DW) was observed under WF light conditions, which proves that LED light favored the production of GSLs (Fig. 22.17) (Klimek-Szczykutowicz et al. 2021).

22.6.3 Production of Polyphenols

The total polyphenol content ranged from 190.84 to 229.18 mg GAL/100 g of DW (Fig. 22.18). The highest content was observed in the extracts from microshoot cultures grown under WLED light. Similar results were also obtained for WF

Fig. 22.15 (continued) medium variants of different PGR combinations as estimated using (a) CUPRAC, (b) DPPH, and (c) FRAP tests. Data are expressed as mean value $\pm$ SD ($n = 3$)



Fig. 22.16 Appearance of *Nasturtium officinale* microshoot cultures depending on the LED light variants used. From left: B, R, RB, WF, RBUV, RBfR, RBY, RBG, WLED, and D

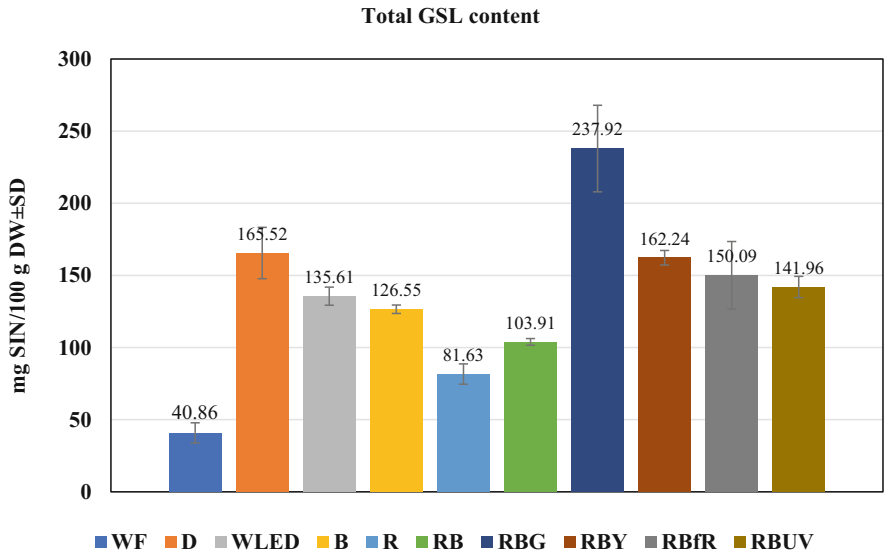


Fig. 22.17 Total GSL (mg SIN/100 g DW ± SD) contents in *Nasturtium officinale* microshoots cultivated under different LED light conditions. Data are expressed as mean value ± SD ($n = 3$)

(226.20 mg/100 g DW), RBUV (224.84 mg/100 g DW), and D (224.63 mg/100 g DW). This shows that there is no significant influence of LED light variants on the production of polyphenolic compounds (Klimek-Szczykutowicz et al. 2021).

22.6.4 Antioxidant Potential

In vitro analysis of the antioxidant potential of extracts from the tested cultures showed that the highest antioxidant potential was achieved using the CUPRAC method (2.39 mmol TE/100 g DW) in the cultures grown under RBY conditions and that the lowest (1.65 mmol TE/100 g DW) was obtained under D conditions. Using the DPPH method, the highest antioxidant potential was achieved under WLED conditions (0.71 mmol TE/100 g DW), and the lowest was achieved under RBY conditions (0.37 mmol TE/100 g DW). Using the FRAP method, the highest

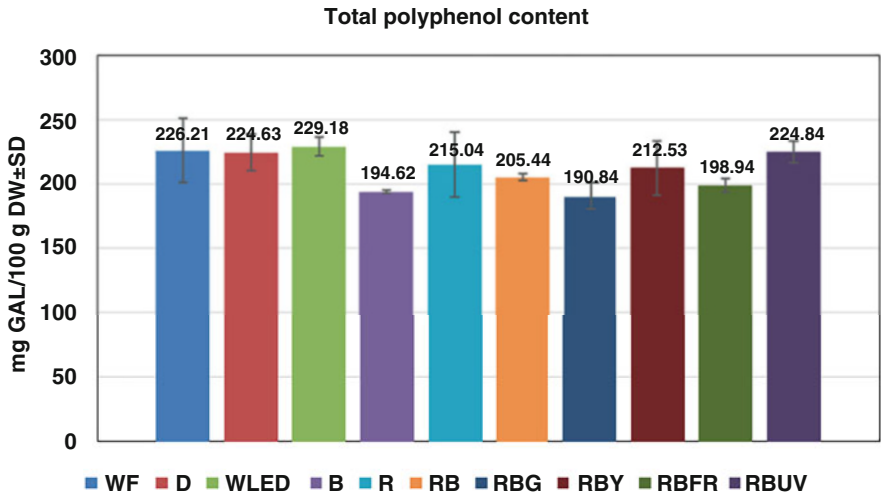


Fig. 22.18 Total polyphenol content (mg GAL/100 g DW ± SD) of *Nasturtium officinale* microshoots cultivated under different LED light conditions. Data are expressed as mean value ±SD (*n* = 3)

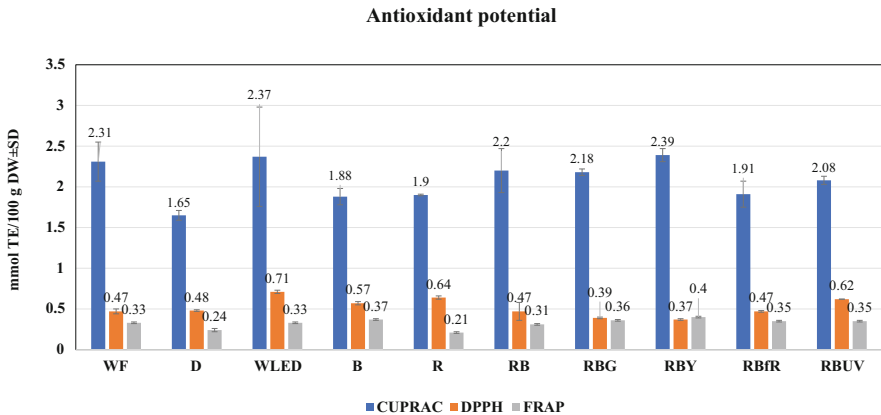


Fig. 22.19 Antioxidant potential estimated using CUPRAC, DPPH, and FRAP assays (mmol TE/100 g DW ± SD) for *Nasturtium officinale* microshoots cultivated under different LED light conditions. Data are expressed as mean value ±SD (*n* = 3)

antioxidant potential was achieved under B conditions (0.37 mmol TE/100 g DW) and the lowest under R conditions (0.21 mmol TE/100 g DW) (Fig. 22.19). These results demonstrated a slight influence of the applied LED lighting conditions on the antioxidant potential. This may be attributable to the slight influence of the tested light conditions on the production of the polyphenolic compounds in *N. officinale* microshoot cultures (Klimek-Szczykutowicz et al. 2021).

22.7 Evaluation of Results with the Parent Plant Material and Conclusions

Biotechnological studies focusing on endangered plants are of high significance. In particular, for the *ex situ* conservation of endangered plant species, micropropagation protocols are of higher importance compared with conventional methods of reproduction. Plant biotechnology studies also provide possibilities for obtaining biologically active metabolites from *in vitro* propagated biomass of rare species.

The species *N. officinale* is worth paying attention to due to its declining natural population in various European countries. In addition, it has valuable health-promoting and cosmetic properties. The present study focused on the development of *in vitro* cultivation protocols for this species. The methods developed aimed at stimulating the production of compounds that are important from the perspective of plant utility, such as GSLs and polyphenolic compounds.

The effects of the PGR contents in the medium and the duration of culture growth periods in two types of *in vitro* cultures—agar and agitated cultures—were investigated. Furthermore, research on agar cultures was complemented with studies on the influence of light conditions on the production of bioactive compounds.

The highest total GSL content was obtained after 20 days of growth period for agar cultures maintained on the MS medium containing 1 mg/L BA and 1 mg/L NAA grown under RBG light conditions, which was 2.1 times lower than that found in the herb of the parent plant (Table 22.4). The highest total polyphenol content was observed in the agar cultures cultivated over 10-day growth periods on the MS medium containing 1 mg/L Zea and 1 mg/L NAA, which was 3.6 times higher than that found in the herb of the parent plant (Table 22.4).

The antioxidant potential of the *in vitro*-propagated microshoots was analyzed using three methods—CUPRAC, DPPH, and FRAP. Using the CUPRAC method, the highest antioxidant potential was achieved for the agitated cultures grown over 20 days on the MS medium containing 1 mg/L KIN and 1 mg/L IAA. Using the DPPH method, the highest antioxidant potential was obtained for the agar cultures grown over 30 days on the MS medium containing 1 mg/L KIN and 1 mg/L IAA. On the other hand, using the FRAP method, the highest antioxidant potential was achieved by the agar cultures grown for 10 days on the MS medium containing 2 mg/L BA and 1 mg/L NAA (Table 22.4).

The present study has proved the usefulness of *N. officinale* *in vitro* cultures as an alternative to the parent plant material. The results obtained were compared with those of the parent plant material, which is presented in Table 22.4.

Table 22.4 Comparison of the maximum contents of secondary metabolites and the antioxidant potential of *Nasturtium officinale* microshoot culture and herb of the parent plant extracts

Analyzed parameter		In vitro cultures					Parent plant material (herb)
		Maximum value ±SD	Type of cultures	Variant of MS medium	Growth cycles (days)	Gi ± SD	
Total content	Total content of GSLs (mg SIN/100 g DW ± SD)	237.92 ± 30.00	Agar cultures	1 mg/L BA and 1 mg/L NAA, RBG light	20	11.81 ± 0.31	499.89 ± 34.59
	Total content of polyphenols (mg GAL/100 g DW ± SD)	1634.85 ± 82.78	Agar cultures	1 mg/L Zea and 1 mg/L NAA	10	2.47 ± 0.09	459.32 ± 15.18
Antioxidant potential (mmolTE/100 g DW ± SD)	CUPRAC	5.26 ± 0.01	Agitated cultures	1 mg/L KIN and 1 mg/L IAA	20	8.08 ± 0.06	4.45 ± 0.02
	DPPH	30.89 ± 1.82	Agar cultures	1 mg/L KIN and 1 mg/L IAA	30	2.39 ± 0.05	26.32 ± 8.23
	FRAP	1.42 ± 0.02	Agar cultures	2 mg/L BA and 1 mg/L NAA	10	1.17 ± 0.03	0.76 ± 0.08

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Chapter 23

Isatis tinctoria L. (Woad): Cultivation, Phytochemistry, Pharmacology, Biotechnology, and Utilization



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Abstract *Isatis tinctoria* L. (*Brassicaceae*) is an herbaceous species with an ancient and well-documented history as an indigo dye source in temperate climates and as a medicinal plant. After centuries of intensive cultivation in Europe, the crop was completely abandoned in the late nineteenth century, when the production of synthetic indigo dye replaced that of natural indigo. Currently, the rising demand for naturally sourced dyes by the dyestuff industry, together with the use for medicinal purposes and the production of cosmetics, has prompted a renewed interest in this species. Herein, an overview of the research focused on the economically sustainable cultivation of *I. tinctoria* is given, primarily aimed at achieving maximum yields of indigo dye. Additionally, a detailed account is reported of all attempts in plant biotechnology studies for the enhanced production of bioactive

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compounds from *I. tinctoria* hairy root and shoot cultures as an alternative to plant raw materials. Furthermore, the main uses of this valuable species are described.

Keywords *Isatis tinctoria* L. · Field cultivation · In vitro culture · Indigo dye · Medicinal plant · Cosmetic use

23.1 Introduction

Isatis tinctoria L. (*Brassicaceae*) has been long utilized as an ornamental crop and for animal feeding, with an ancient and well-documented history as an indigo dye source in temperate climates and as a medicinal plant.

From the twelfth up to the seventeenth century, *I. tinctoria* has been widely cultivated in Europe for indigo dye production and at the same time utilized for medicinal purposes (Hamburger 2002). In the late seventeenth century, the woad industry in Europe started to decrease rapidly as a consequence of the importation of the cheapest indigo blue from *Indigofera tinctoria* cultivated in Asia and later from other tropical *Indigofera* species. Subsequently, the production of synthetic indigo totally replaced the natural one; as a consequence, the crop was definitively abandoned in the late nineteenth century, and the use of *I. tinctoria* as a medicinal plant has also fallen into disuse (Danz et al. 2001; Hamburger 2002; Meijer et al. 2007; Spataro and Negri 2008; Nguyen et al. 2019).

The use of *I. tinctoria* for medicinal purposes is widespread in Traditional Chinese Medicine (TCM) (Hamburger 2002; Recio et al. 2006a; Condurso et al. 2006). Several in vitro and in vivo investigations have provided reasonable support for various traditional uses of *I. tinctoria*, demonstrating that the extracts or compounds isolated from different organs of this species have a wide range of biological activities. The phytochemical composition of *I. tinctoria* has been thoroughly characterized, showing that the plant represents a valuable source of bioactive compounds, including several alkaloids, among which tryptanthrin, indirubin, and indolinone are the most relevant, phenolic compounds, polysaccharides, glucosinolates, carotenoids, volatile constituents, and fatty acids (Speranza et al. 2020).

Currently, the rising demand for naturally sourced dyes by the dyestuff industry, together with the use for medicinal purposes and for the production of cosmetics, has prompted a renewed interest in *I. tinctoria*, encouraging the re-establishment of *I. tinctoria* crops in the European agricultural system (Sales et al. 2006; Spataro and Negri 2008). Its heat and water stress tolerance characteristics, long flowering period, and attractive inflorescences have also increased the use of *I. tinctoria* by horticulturists as an ornamental plant (Branca 2015). In addition, in recent years plant in vitro culture technology has emerged as a valuable alternative approach to field cultivation for the enhanced production of bioactive compounds from *I. tinctoria* (Speranza et al. 2020).

Herein, an up-to-date overview of the research focused on the economically sustainable cultivation of *I. tinctoria* is given, primarily aimed at achieving

maximum yields of indigo dye. Additionally, a detailed account is reported of all attempts in plant biotechnology studies for the enhanced production of bioactive compounds from *I. tinctoria* hairy root and shoot cultures as an alternative to plant raw materials. Furthermore, the main uses of this valuable species are described, i.e. as a source of indigo dye, as a medicinal plant, and as a natural ingredient for cosmetic purposes.

23.2 Morphology

Isatis tinctoria is a herbaceous biennial or short-lived perennial species (Fig. 23.1). The root system consists of a cylindrical, slightly tortuous tap root, 20 cm to 1.5 m long, brownish-yellow or greyish in colour, and numerous lateral roots up to 40 cm long. The plant begins as a rosette with long-petioled basal leaves 4–10 cm long and 0.8–4 cm wide. They are oblong-lanceolate, whole or toothed, blue-green, and usually pubescent. The stems are erect, branched above, ranging from 35 to 150 cm tall. Cauline leaves are sessile, straight, or sagittate, with an entire edge, narrower than basal leaves. Their size and length are gradually reduced upward of the stem. Typical of the *Brassicaceae* family, flowers are gathered in a racemose inflorescence on the top of the stems. They have yellow petals about 2.5–4 cm long, almost twice as long as the sepals, and androecium tetradynamous, consisting of



Fig. 23.1 *Isatis tinctoria* L.: flowering plants (a), flowers (b), fruits (c). (I. Kwiecień)

6 stamens with 2 filaments shorter than the others. Fruits are pendulous siliques, 13–22 mm long and 4–8 mm wide, usually 3–5 times as long as wide. The silique is oblong-obovate or elliptic-obovate with a single seed and characteristic purple to black colour at maturity. The aboveground parts can be both glabrous and hirsute. The authors note that there exist substantial morphological variations in the species globally observed (Ball 1964; Pignatti 1982; Guarino et al. 2000; Akeroid 2002; Kizil 2006; Zouhar 2009).

23.3 Natural Habitats and Ecology

According to some authors, *I. tinctoria* is native to the grasslands of South-eastern Russia to South-West Asia and perhaps to some parts of South-eastern Europe (Akeroid 2002; Hamburger 2002), while other studies indicate that this species is native to Central Asia (Spataro et al. 2007; Spataro and Negri 2008). It is spread widely across Europe and Eastern Asia, including China and Japan, by cultivation. By contrast, it is considered a noxious weed in most of the western United States (Akeroid 2002; Hamburger 2002; Monograph 2002).

The US National Plant Germplasm System states that the natural habitats of *I. tinctoria* are the following: Albania, Bulgaria, Bosnia and Herzegovina, Croatia, the Czech Republic, Denmark, Estonia, Finland, France, Greece, Germany, Hungary, Italy, Latvia, Montenegro, the Netherlands, North Macedonia, Romania, Russia, Serbia, Slovakia, Slovenia, Spain, Sweden, and Ukraine across Europe; Algeria and Morocco in Africa; and Lebanon, Turkey, Armenia, Ciscaucasia, Dagestan (Russian Federation), Kazakhstan, Tajikistan, Uzbekistan, China (Zhejiang Sheng, Fujian Sheng, Henan Sheng, Hebei Sheng, Hubei Sheng, Gansu Sheng, Jiangxi Sheng, Guizhou Sheng, Liaoning Sheng, Shanxi Sheng, Shandong Sheng, Shaanxi Sheng, Sichuan Sheng, Yunnan Sheng, Nei Mongol Zizhiqu, Xinjiang Uygur Zizhiqu, Xizang Zizhiqu), Korea, Japan (Hokkaidô, Honshu), India (Himachal Pradesh, Jammu, and Kashmir), and Pakistan in Asia. The species was naturalized at different times in Belarus, the Czech Republic, France, Lithuania, Norway, Russia, and the United Kingdom but also in North and South America: Canada (British Columbia, Québec, Ontario, Newfoundland and Labrador) and the United States (California, Idaho, Illinois, Missouri, Montana, New Mexico, Nevada, New York, Oregon, Utah, Virginia, Washington, West Virginia, Wyoming) and across Chile (USDA 2022).

The differences are due to the taxonomic conflict of two species, *I. tinctoria* (European woad) and *I. indigotica* (Chinese woad). Some researchers highlight the morphological, genetic, and physiological differences between *I. tinctoria* and *I. indigotica* (Angelini et al. 2007). Despite this, *I. indigotica* is currently often considered a synonym of *I. tinctoria* and not a separate species by the consulted taxonomic databases known as The Plant List, the International Plant Names Index (IPNI), Tropicos, and the Global Biodiversity Information Facility database. The GBIF (2022) provides numerous synonymic names for *I. tinctoria*, including

subspecies, forms, and varieties (GBIF Secretariat 2021). At the same time, the Euro + Med PlantBase does not mention *I. indigotica* in the list of synonyms.

Isatis tinctoria grows spontaneously in dry and sunny locations, in the rocky substrates, abandoned crops, open woods, clearings, and along the roads, from sea level up to the maximum altitude of 1900 m. It is associated with loose, alkaline, or neutral soils and tolerates an infertile environment. The optimal climate for the growth of this species is either a warm temperate climate with dry summers or a continental climate with dry summers (Aichele 1987; Poli Marchese 1991; CABI 2022).

23.4 Studies on Field Cultivation

Currently, the recent interest in alternative crops and the growing demand for natural products have prompted to reconsider *I. tinctoria* as a dye crop for the production of natural indigo. Within the perspective of expanding the cultivation of this crop, improving efficient and sustainable cultivation methods, and producing great amounts of high-quality plant material at low costs, across Europe various studies have been carried out to achieve scientific information regarding the agronomic aspects of *I. tinctoria* cultivation. Ecological variations such as altitude, location, light, and temperature could affect agronomical observations.

Kizil (2006) evaluated the adaptation ability and the morphological and agronomical characteristics of *I. tinctoria* plants obtained from seeds provided by the University of Ankara (Turkey). The field trial was designed as a collection garden in plots of 1.8×4 m. The sowing of 2–3 seeds in each hole was done in February in rows, at a depth of 1–2 cm. After emergence, one plant at the fourth to fifth leaf stage in each hole was left. The plots were weeded when needed and irrigated in an orderly manner. Harvesting was done in June. Among the data analysed, there were some phenological characteristics, such as the number of days from seeding to emergence, number of days to form stalks, number of days to 50% flowering, number of days from stalk formation to 50% flowering, number of days from sowing to flowering, number of days from 50% flowering to harvest, and number of days from sowing to harvest. Plant height, stem diameter, the number of branches per plant, the number of seeds per plant, seed yield per plant, seed width, seed length, and thousand seed weight were also evaluated. The results of the study showed that the number of days from seeding to emergence was 36, to form stalks 412, and to flowering 429, and the harvesting of *I. tinctoria* was done after 485 days. Plant height, an important parameter for mechanical harvesting, was 91.0 cm; it contributes to the number of branches, which was found to be 16.8 pieces/plant, which, in turn, influences the number of seeds per plant (17,918 seeds/plant), an important agronomic characteristic. Another important agronomic parameter is represented by the stem diameter (7.80 cm), which provides resistance against lodging due to wind or rains; fast blowing winds or rains could result in the breaking of seed-bearing branches or stems, negatively affecting the seed yield.

In the same period, Sales et al. (2006) studied agronomic conditions for optimizing *I. tinctoria* crops in the south-east of Spain (Valencia), with the aim of increasing indigo production. Field studies were conducted on seeds from the United Kingdom cultivated in two localities in Valencia, Bolbaite and Paiporta, with different paedological characteristics such as medium texture, pH, and rate of N, K, P, and organic carbon. Individual plots consisted of 10 m² areas (15 rows, 5 m long, row spacing of approximately 30 cm); the seeding rates were 0.5 g of siliquea/m²; assessed plant densities were 5, 10, and 20 plants/m²; and soil watering was done by flooding. The following agronomic factors were evaluated: sowing date, plant density, nitrogen fertilization, irrigation rate, and seedling transplanting. The results of the study demonstrated that early winter sowing dates (December and January) gave higher biomass productions when compared to the spring sowing dates (February and March); furthermore, it was observed that sowing date had no effect on seed production of *I. tinctoria* plots. The optimal plant density was in the range of 10–20 plants/m², depending on soil fertility and water availability, and these plant density values did not influence leaf indigo content. The results showed also that indigo production was maintained when nitrogen and water supplies were significantly restricted. Indeed, no significant variation of biomass production or leaf indigo content was observed with high-nitrogen fertilization (200 kg N/ha), as well as over a wide range of irrigation rates (70% ETo, ETo, 130% ETo). Finally, from the comparison of indigo production in plots established by seeding and by transplanting greenhouse-germinated seedlings, it was highlighted that transplanting produced significantly more biomass and indigo yield than the corresponding sowing experiment.

The behaviour of *I. tinctoria* cultivated under different climatic conditions was assessed by Spataro and Negri (2008). To assess crop adaptability to diverse altitudes, three accessions were cultivated in 4 experimental sites in central Italy located at the same geographical coordinates but at different altitudes (from 380 to 1700 m a.s.l.). For each accession, a total of 72 plants were arranged in a randomized complete block design with 2 replications, each plot consisting of 2 rows of 6 spaced (80 × 80 cm) plants. The following morphological and physiological characteristics were recorded for the single plants over a 2-year period: rosette vigour, plant growth habit, and survival percentage, which are indicators of yield capacity, suitability to mechanical harvesting, and standing ability, respectively. Other traits were recorded as indicators of seed production capacity (number of branches per scape, flowering and ripening dates, and silique dimensions) and of differences caused by diverse altitudes (height of the scape, branching type, and type of cauline leaves).

The obtained results demonstrated that *I. tinctoria* survived well even at the highest altitude, where the temperatures were below zero for most of the winter; the best values for rosette vigour, plant growth habit, and survival percentage were observed at an altitude of 925 m a.s.l.

Water represents an important factor in leaf production and indigo yield. The study of Sales et al. (2006), described above, indicated that *I. tinctoria* cultivated in a Mediterranean region of Spain (Valencia) was not a high water-demanding crop; this is an essential point since water is a limiting resource in that region. For efficient use

of water resources, the knowledge of crop coefficient (Kc) in the different plant growth stages is of vital importance to estimate the crop water requirements (CWR). Indeed, in a later study carried out by Angelini and Bertolacci (2008) to assess the seasonal crop water requirements, the crop coefficient (Kc) values for *I. tinctoria* cultivated in Pisa countryside (central Italy), the crop water requirements, and the effects of irrigation on both production quantity and quality were determined. The investigation was conducted during 2 growing seasons (2002 and 2003). *Isatis tinctoria* seeds were sown in paper pots in March and incubated in germination cabinets under controlled air temperature; at the fourth to sixth true leaf stage, the plants were transplanted in soil, whose physical and chemical characteristics were measured, with 30 cm inter-row and 10 cm intra-row distances and a crop density of 330,000 plants/ha, and maintained under identical fertilization conditions (100/100/100 kg/ha of N/P₂O₅/K₂O applied at pre-planting and 50 kg N/ha supplied after the first and the second harvest of the leaves). A randomized block design experiment with four replications was performed to compare six irrigation levels (T100, T80, T60, T40, T20, T0); the crop evapotranspiration (ETc) during the growing season was determined. Plants grown in the same area were harvested four times in each season, and fresh and dry leaf production and indigo yield were estimated. From the results differences in the daily maximum ETc and in the seasonal CWR were highlighted between the 2 years; the Kc values ranged from 0.30 to 0.47 in relation to plant development; leaf dry production and indigo yield were unaffected by the level of irrigation. An increment of 16% in dry leaf and indigo yield has been observed in the driest growing season going from T0 to T40, indicating that a seasonal irrigation volume not over 40% ETc should be provided when severe drought occurs.

23.5 Chemical Composition

Isatis tinctoria is characterized by a broad spectrum of secondary metabolites. Among them are alkaloids, flavonoids, phenolic acids, mono- and oligolignols, glucosinolates, *volatile constituents*, and other compounds (Fig. 23.2). Their source is the leaves, root, and seeds of the plant.

23.5.1 Alkaloids

I. tinctoria alkaloid metabolites can be divided into two groups. One of them includes metabolites produced and stored in plant cells. The products of postharvesting processes belong to the second one. Indolic alkaloids of this species are commonly known as an indigo dye and are used to dye textiles, cosmetics, food, and pharmaceutical preparations as well. The plant synthesizes several precursors of these molecules in the leaves. After harvesting, during fermentation, precursors are

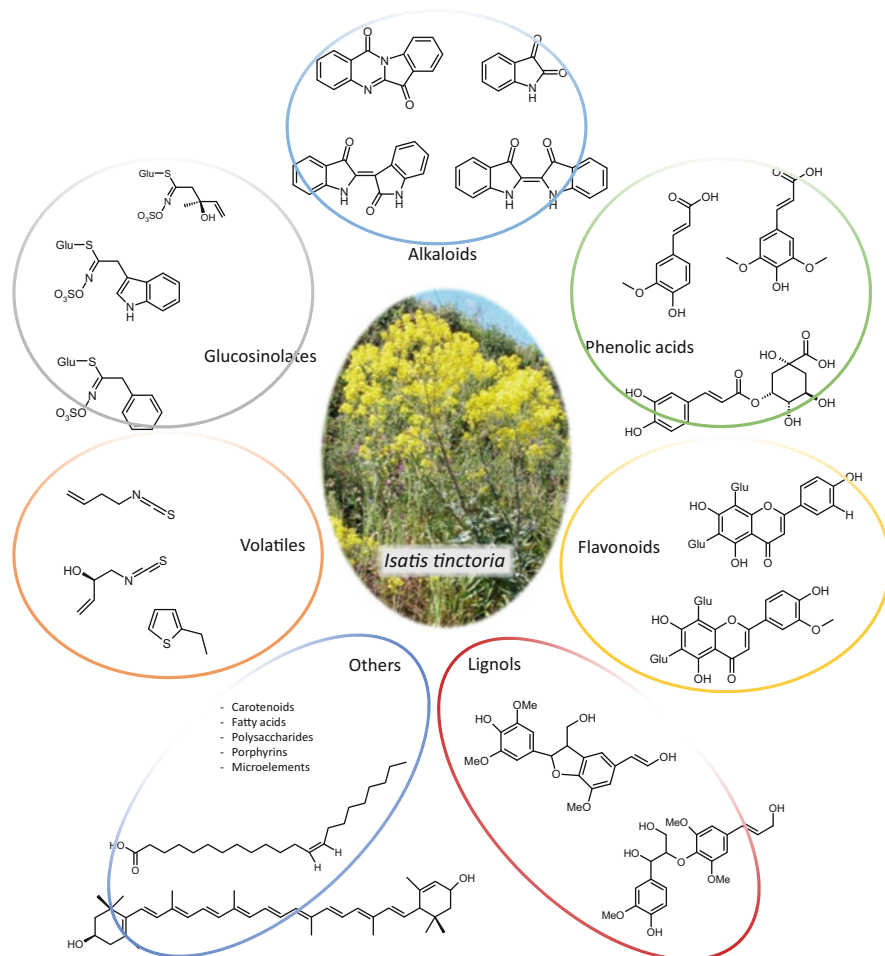


Fig. 23.2 Chemical constituents of *Isatis tinctoria*

hydrolysed and oxidized by exposure to *air*, finally converted to indigo dyes (Song et al. 2010). Besides blue indigo (*cis*-indigo), blue *trans*-indigo (indigotin), red dyes *trans*-indirubin and *cis*-indirubin (*isoindirubin*), or *isoindigo* (brown) can be formed (Maugard et al. 2001).

The most important alkaloid metabolite of *I. tinctoria* is tryptanthrin. The tryptanthrin biosynthetic pathway is not known. But it is one of the products of the postharvest process. Unlike indigo dyes, it is not formed during leaf fermentation. The drying process, especially at elevated temperatures, favoured tryptanthrin synthesis (Danz et al. 2002b; Oberthür and Hamburger 2004).

23.5.2 Flavonoids

The main class of secondary metabolites in polar extracts of *I. tinctoria* is represented by phenolic compounds such as flavonoids (Taviano et al. 2018). The identified flavonoids are derivatives of flavones and of flavonols (Miceli et al. 2017). Flavonoids detected in hydroalcoholic leaf and flower extracts of *I. tinctoria* are vicenin-2, stellarin-2, isovitexin, luteolin glucuronide, quercetin, buddleoside, isoscoparin, and apigenin glucosides (Taviano et al. 2018). Vicenin-2 turned out to be the most abundant flavonoid detected in the cauline leaf extract, while luteolin glucuronide and stellarin-2 were the main compounds for the basal leaves and flowers. Researchers from France also found in leaf extract flavonoid compounds esterified with *p*-coumaric, ferulic, or sinapic acid, for example, 4'-*O*-feruloyl isoscoparin-3''-*O*-glucoside-7-*O*-glucoside, 2''-*O*-feruloyl isoscoparin-3''-*O*-glucoside-7-*O*-glucoside, isoscoparin-3''-*O*-glucoside-7-*O*-feruoylglucoside, isoscoparin-3'-*O*-feruoylglucoside, isoscoparin-3'-*O*-sinapoylglucoside, and isoscoparin-3'-*O*-*p*-coumaroyl glucoside (Nguyen et al. 2017).

23.5.3 Phenolic Acids

Among phenolic acids, the sinapic and ferulic acids are dominant in leaf extract (Mohn et al. 2009). Additionally, other researchers also reported the presence of neochlorogenic, chlorogenic, caffeic, and coumaroylquinic acids in leaves and flowers extracts (Miceli et al. 2017; Taviano et al. 2018). In addition to free phenolic acids, six flavone glucosides conjugated with hydroxycinnamic acid, many conjugates between hydroxycinnamic acids and a hexose or dihexose, glycerate, malate, or glucaric acid were detected in leaf extracts. These compounds showed one or two phenylpropanoic acid ester moieties with *p*-coumaric, ferulic, or sinapic acid (Nguyen et al. 2017).

23.5.4 Mono- and Oligolignols

From the group of phenolic compounds in extracts of *I. tinctoria* rosette leaves, mono- and oligolignols were also found. They are derivatives of coniferyl and sinapyl alcohols, such as the 5-hydroxy-coniferylalcohol hexose ferulic acid adduct, the isodihydrodehydrodiconiferyl alcohol hexose formic acid adducts, and the pinoresinol dihexose formic acid adduct. Furthermore, several compounds containing units of guaiacyl, syringyl, formic acid, and hexose were detected (Nguyen et al. 2017).

23.5.5 *Glucosinolates*

I. tinctoria is a source of glucosinolates, characteristic metabolites for the *Brassicaceae* family, which are synthesis and storage products of isothiocyanates. They are classified into three groups: aliphatic, aromatic, and indole glucosinolates. *I. tinctoria* seeds are characterized by aliphatic glucosinolates such as gluconapin, progoitrin, and epiprogoitrin. In addition, some indolic glucosinolates such as glucoisatisin, epiglucoisatisin, glucobrassicin, and neoglucobrassicin were identified (Mohn and Hamburger 2008). The glucosinolate pattern of the leaves is slightly different. This group of metabolites in leaves is represented by indolic glucosinolates that include glucobrassicin, neoglucobrassicin, sulfoglucobrassicin, and aromatic glucotropaeolin (Mohn et al. 2007). Newer reports also show the presence of glucoiberin, glucobrassicin, and 4-methoxyglucobrassicin (Taviano et al. 2018) and gluconapoleiferin as well (Nguyen et al. 2019).

23.5.6 *Volatile Components*

During plant tissue damage, glucosinolates are released and converted by myrosinase (Condurso et al. 2006). The enzyme hydrolyses the glucose moiety by giving isothiocyanates and other types of products such as thiocyanates, indoles, epithionitriles, and nitriles (MacLeod et al. 1981). These compounds are one of the major volatile constituents of *I. tinctoria*.

The presence of volatile components of this species was confirmed in leaves and roots. The analysis of leaves resulted in the identification of several compounds such as acids, alcohols, aldehydes, esters, ethers, furans, hydrocarbons, isothiocyanates, thiocyanates, ketones, nitriles, sulphurated compounds, terpenes, and sesquiterpenes (Table 23.1) (Condurso et al. 2006). The dominant compound was 3-butenylisothiocyanate, a product of gluconapin decomposition. 2-Hydroxy-3-butenyl isothiocyanate, which is the aglycone of progoitrin and epiprogoitrin, was also identified. After isothiocyanates, aldehydes (especially (*Z*)-2-esenal), sulphurated compounds (e.g. 2-ethylthiophene), saturated and unsaturated alcohols with tetradecanol as the main component, monoterpenes, and sesquiterpenes (e.g. limonene) were identified. Verzera et al. (2010) carried out the extraction of volatile constituents from the root of *I. tinctoria* (Table 23.2). The dominant metabolite was also 3-butenyl isothiocyanate which represents 82% of the total volatiles. In addition to isothiocyanates, alcohols and nitriles were the most abundant groups, constituted 4.9% and 3.6%, respectively.

Table 23.1 Volatile compounds of *Isatis tinctoria* leaves (Condurso et al. 2006)

Group of compounds	Metabolites
Isothiocyanates and thiocyanates	3-Butenyl isothiocyanate, benzyl isothiocyanate, 2-hydroxy-3-butenyl isothiocyanate, hexyl isothiocyanate, 3-methylthiopropyl isothiocyanate, allyl isothiocyanate, pentyl isothiocyanate, methyl thiocyanate
Sulphurated compounds	2-Ethylthiophene, thiophene, cyclopentanethiol, carbon disulphide, carbonyl sulphide
Alcohols	Nonadecan-1-ol, 2-methylhexadecan-1-ol, heptadecan-1-ol, pentadecan-1-ol, tetradecan-1-ol, <i>trans</i> -hex-3-en-1-ol, <i>cis</i> -pent-2-en-1-ol, 2-butyloctan-1-ol, butan-1-ol
Aldehydes	Tetradecanal, 4-ethylbenzenecarbaldehyde, <i>cis,trans</i> -nona-2,6-dienal, benzenecarbaldehyde, nonanal, hexanal, but-2-enal, <i>trans</i> -oct-2-enal, <i>trans,trans</i> -hexa-2,4-dienal, <i>trans,trans</i> -hepta-2,4-dienal, <i>trans</i> -pent-2-enal, <i>trans</i> -hex-2-enal, 3-methylbutanal
Terpenes and sesquiterpenes	Geranyl acetone, δ -cadinene, valencene, β -cyclocitral, <i>p</i> -cymene, myrtenal, terpinolene, γ -terpinene, limonene, <i>p</i> -cymene, sabinene, δ -3-carene, eucalyptol
Nitriles	2-Phenylacetonitrile, octanenitrile, heptanenitrile, 4-pentenitrile, 3-hydroxy-4-pentenitrile
Ketones	<i>Trans</i> - β -ionone, octane-2,5-dione, 1-penten-3-one
Acids	Octanoic acid, acetic acid
Esters	Butyl tetradecanoate, methyl 2-hydroxybenzoate
Ethers	Diphenyl ether, 1-methoxy-4-prop-2-enylbenzene
Furans	2-Ethylfuran
Hydrocarbons	Tetracosene, heneicosene, eicosene, nonadecene, heptadecane, <i>trans</i> -1,5-heptadiene

Table 23.2 Volatile compounds of *Isatis tinctoria* dried roots (Verzera et al. 2010)

Group of compounds	Metabolites
Isothiocyanates and thiocyanates	3-Butenyl isothiocyanate, cyclopentyl isothiocyanate, methyl thiocyanate
Alcohols	Heptanol, hexanol, furfuryl alcohol, 2-phenylethyl alcohol, 1-octen-3-ol, phenol
Aldehydes	Benzaldehyde, furfural
Terpenes	6-Methyl-5-hepten-2-one, guaiacol, camphor, geranyl acetone
Nitriles	2-Pentenitrile, 4-pentenitrile, 2,4-pentadiene nitrile
Ketones	2-Nonanone, (E,E)-3,5-octadien-2-one, 1-phenyl-1-propanone, 2-heptanone
Acids	Butyric acid, octanoic acid
Esters	Methyl butyrate
Hydrocarbons	Pentadecane, decane, heptadecane, tridecane

23.5.7 Other Compounds

Among other popular plant metabolites, carotenoids were extracted from *I. tinctoria* leaves. Dichloromethane and hexane/acetone extracts showed the presence of

(all-*E*)- β -, (15*Z*)- β -, and (9*Z*)- β -carotene isomers and (13*Z*)-, (13'*Z*)- (9*Z*)-, (9'*Z*)-, (all-*Z*)-lutein isomers. Among them, less-known carotenoids like (all-*E*)- and (*Z*)-neochrome and (15*Z*)- and (di-*Z*)-violaxanthin were detected (Mohn et al. 2009). Studies also show chlorophyll degradation products such as phaeophorbide a, a' and pyropheophorbide, and 10-hydroxy phaeophorbide (Mohn et al. 2009).

The root of *I. tinctoria* besides other specific compounds is rich in polysaccharide fractions with a maximum yield of over 11% (Han et al. 2011).

The seeds of *I. tinctoria*, like those of other plants belonging to the *Brassicaceae* family, show an interesting fatty acid composition. The dominant was erucic acid followed by oleic, linoleic, linolenic, palmitic, stearic, arachidic, and tetracosanoic acids (Kizil et al. 2009). Furthermore, the presence of ursolic, palmitoleic, and α -lysolecithin was found in leaf extract (Mohn et al. 2009). Seeds are a rich source of microelements, too (Kizil et al. 2009).

23.6 Pharmacology: Medicinal and Health-Promoting Properties

The utilization of *I. tinctoria* as a medicinal plant in both Eastern and Western cultures has an ancient and well-documented history. *I. tinctoria* has a 1000-year history of use in Traditional Chinese Medicine (TCM) for the prevention and treatment of several diseases. Currently, the Chinese Pharmacopoeia reports for *I. tinctoria* three monographs relating to different herbal preparations with diverse medicinal properties (Monograph 2002; Mohn et al. 2009):

- *DàQīngYè* (*Isatidis folium*) (Fig. 23.3), consisting of the dried leaves of *I. tinctoria*, is used to treat the virus of B-encephalitis, mumps, influenza, and leptospirosis (Huang 1993; Zhu 1998).



Fig. 23.3 *Isatidis folium* (left) and *Isatidis radix* (right)

- *Qing Dai* (*Indigo naturalis*) is a dark blue powder prepared from the leaves of various plants including *I. tinctoria*, which is utilized in the treatment of bacterial and viral infections and as a haemostatic, antipyretic, and anti-inflammatory remedy (Tang and Eisenbrand 1992; Kim et al. 2012). This TCM is used to treat various inflammatory diseases, including inflammatory bowel diseases (IBD), such as ulcerative colitis, and psoriasis (Sugimoto et al. 2016; Naganuma 2019).
- *BǎnLánGēn* (*Isatidis radix*) (Fig. 23.3) is the dried root of *I. tinctoria*. It is most commonly processed into granules and consumed dissolved in hot water or tea and is indicated to treat a variety of ailments such as eruptive epidemic diseases, pharyngitis, laryngitis, scarlet fever, erysipelas, carbuncles, hepatitis, mumps, flu, mononucleosis, viral skin diseases, epidemic cerebrospinal meningitis, and diphtheria (Duke and Ayensu 1985; Zhu 1998; Han et al. 2011; Zhang et al. 2019). This preparation has been one of the eight major therapies endorsed by the Chinese government for the prevention and control of severe acute respiratory syndrome (SARS) (Ke et al. 2012). Currently, it has been favoured by Chinese health officials as an effective remedy for fighting SARS-Cov-2/Coronavirus-19 (COVID-19). Indeed, it is one of the TCM plant-based formulations used in clinical trials to treat patients with COVID-19 infection (Akalin et al. 2020).

Another TCM preparation is *Dang Gui Long Hui Wan*, consisting of 11 herbal medicines, including *I. tinctoria*, indicated for the treatment of various chronic diseases, among which chronic myelocytic leukaemia (Meijer et al. 2007).

In Europe, the ethnobotanical use of *I. tinctoria* for the treatment of wounds, ulcers, and haemorrhoids has been documented by Hippocrates (460 B.C.), Galen (129–216 B.C.), and Pliny (23–79 B.C.) (Hamburger 2002; Buhner 2013). The same medicinal properties have been widely described in a number of Renaissance and Baroque herbal texts, which recommended *I. tinctoria* also as an anti-tumour remedy (Hamburger 2002). From the twelfth century until the seventeenth century, *I. tinctoria* was extensively cultivated in Europe for the production of indigo for dyeing and at the same time used as a medicinal plant to treat snake bites, wounds, and other inflammatory ailments (Hamburger 2002; Oberthür et al. 2005). The loss of importance of *I. tinctoria* in Europe as the source of indigo made it fall into disuse also as a medicinal plant.

In addition to those mentioned above, other medicinal properties have been reported for *I. tinctoria*. The leaves have been utilized as a remedy for typhoid, measles, and flu, against the skin inflammation due to the St. Anthony's fire (Perry 1980; Garland 1988), and also for the treatment of iposideremicanemic diseases, scurvy, and hypovitaminosis (Palma 1964). Moreover, the leaf extracts have been used to treat acrodermatitis, haemorrhagic diathesis, eczema from scrofulous, furunculosis, intoxications, hypercholia, jaundice, gastric neurosis, hives, torpid sores, scrofulous, heartburn, and seborrhoea (Palma 1964). The roots have been utilized to reduce the fever in scarlet fever and for the treatment of solid tumours and leukaemia (Monograph 2002).



Fig. 23.4 Biological activities of *Isatis tinctoria*

In recent years, the consolidated utilization of *I. tinctoria* root in TCM, mostly as an antiviral remedy, has become more and more popular also in Europe, significantly increasing its spread (Buhner 2013). As a consequence, a monograph for *I. tinctoria* root (*Isatidis radix*) (Fig. 23.3) has been included in the European Pharmacopoeia starting from 2011 and up to the latest edition, for the usage in official European phytotherapy (European Directorate for the Quality of Medicines 2011, 2020).

The above-reported information clearly indicates that *I. tinctoria* is utilized as a medicinal plant in the treatment of a wide variety of diseases, but the most relevant ethnopharmacological uses are linked to the antiviral, anti-inflammatory, and anti-cancer properties of this species. The use in traditional medicine has, therefore, opened a vast field of research aimed at confirming these activities, as well as at identifying the bioactive components present in *I. tinctoria* (Fig. 23.4).

23.6.1 Anti-inflammatory Activity

I. tinctoria has been appreciated for its anti-inflammatory properties which have been proven by numerous in vivo and in vitro studies. In vivo models, e.g. carrageenan-induced rat paw oedema, 12-*O*-tetradecanoilforbol-13-acetate (TPA) ear oedema, *Pseudomonas aeruginosa* lung infection mimicking cystic fibrosis, adjuvant-induced arthritis and ovalbumin (OVA)-induced mouse asthma, rabbit platelet-activating factor (PAF) receptors, and delayed-type hypersensitivity (DHT) induced by dinitrofluorobenzene (DNFB), were studied. Moreover, in vitro assays on cyclooxygenase 1 and 2 (COX-1 and COX-2), lipoxygenase 5 and 15 (5-LOX and 15-LOX), leukotriene B₄ (LTB₄), thromboxane A₂/prostaglandin H₂ (TXA₂/PGH₂), phospholipase A₂ (PLA₂), phospholipase C (PLC), and human leucocytic elastase were performed. Inhibition of adhesion factor expression was assayed for intercellular adhesion molecule 1 (ICAM-1), endothelial adhesion molecule (ELAM-1), and vascular adhesion molecule 1 (VCAM-1). Cell-based assays were carried out for COX-2 (in HUV-EC cells) and iNOS activity (in RAW 264.7 cells) (Speranza et al. 2020).

The in vivo effects of SFE and dichloromethane (DCM) extracts of *I. tinctoria* rosette leaves were assessed in two experimental models of acute inflammation—carrageenan-induced mouse paw oedema and TPA-induced mouse ear oedema. The SFE (75–125 mg/kg per os) and DCM (125–175 mg/kg per os) extracts showed dose-dependent anti-inflammatory activity in the carrageenan-induced mouse paw oedema; particularly, the potency of SFE extract gave better results than DCM extract. In the in vivo model of acute inflammation, the topical application of the DCM and SFE extracts (0.5 mg/ear) significantly inhibited the TPA-induced ear oedema. The reduction of oedema was smaller after oral administration (Recio et al. 2006b).

The anti-inflammatory properties of *I. tinctoria* rosette leaf lipophilic extracts were proved also in models of acute and sub-chronic inflammation. Under this study the inhibition of COX-2, 5-LOX, iNOS, histamine, and serotonin release and of leucocytic elastase was indicated as the mode of action (Hamburger 2002). According to Danz et al.'s (2001, 2002a) studies, the anti-oedematous effect of *I. tinctoria* lipophilic extracts could be related mainly to tryptanthrin, a potent inhibitor of leukotriene and prostaglandin synthesis, in particular being highly selective towards the COX-2 isoenzyme. It was supposed that the synergistic effects of various mechanisms play a role in the anti-inflammatory activity of *I. tinctoria* lipophilic extracts such as α -linolenic acid and indolin-2-one, which inhibited 5-LOX and histamine release, respectively (Rüster et al. 2004).

The effect of aqueous extract of *I. tinctoria* plant raw material was studied in a model of chronic *Pseudomonas aeruginosa* lung infection mimicking cystic fibrosis in rat. *I. tinctoria* (400 mg/kg b.w., s.c. once a day for 10 days) reduced the frequency of lung abscess and the severity of the macroscopic pathological modifications in the lungs (Song et al. 1996).

In the model of adjuvant-induced arthritis in rats, the effect of a DCM *I. tinctoria* extract on chronic inflammatory diseases like rheumatoid arthritis was proved (Recio et al. 2006a). In this model, the DMC extract (150 mg/mL once/day per os, on days 17–23 by injection of the adjuvant) led to a significant reduction of paw oedema induced by injection of *Mycobacterium butyricum*; this effect was not dose-dependent. A decreased joint damage, a reduction of tissular and articular inflammatory markers (oedema, articular damage, cell infiltration, cysts, pannus, etc.), and bone and cartilage erosion was observed. The treatment didn't cause significant difference in tissue levels of COX-1 and COX-2.

The anti-inflammatory effects of *I. tinctoria* leaf supercritical CO₂ extract in a murine experimental model of allergic airway disease (asthma) were studied. The extract (10–100 µg/mouse per nose) was administered to ovalbumin-immunized BALB/c mice and determined a dose-dependent inhibition of the allergic reaction, decreasing the methacholine-induced airway hyperresponsiveness (AHR) and eosinophil recruitment, as determined both in the bronchoalveolar lavage (BAL) fluid and in the in-lung homogenates. The mechanism of this action was based on inhibition of the production of the typical mediators of Th2 immune response IL-4, IL-5, and RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted). The authors considered alkaloid indolin-2-one as the main active compound responsible of the antiallergic properties (Brattström et al. 2010).

The anti-inflammatory activity of *I. tinctoria* was also evaluated in a clinical pilot research on healthy human volunteers using two experimental models, namely, sodium lauryl sulphate-induced irritant contact dermatitis and UVB-induced erythema. The efficacy of the topical administration of different lipophilic extracts of *I. tinctoria* leaves showed that they were effective when administered during the induction phase of dermatitis; however, they did not show any anti-inflammatory activity in the UVB-induced erythema model (Heinemann et al. 2004).

The studies on anti-inflammatory activities of *I. tinctoria* extracts have indicated that the compounds responsible for this action are indolinone, alpha-linolenic acid, and indirubin (Speranza et al. 2020).

Lotts et al. (2020) screened for the anti-inflammatory properties of different extracts from the leaves of *I. tinctoria* using COX-2 activity assays and in the mouse model. The petroleum ether extract resulted in significantly reduced ear swelling, oedema, and inflammatory cell density. In mouse skin and human keratinocyte cultures, petroleum ether extract inhibited pro-inflammatory cytokine expression. Furthermore, human mast cell degranulation was significantly inhibited in LAD2 cell cultures.

Lee and Cho (2019) described the anti-inflammatory effect of the *I. tinctoria* root extract ("Antifect"; 200 mg/kg) on lipopolysaccharide-induced periodontitis in rats. This treatment, compared to the control group, significantly reduced the inflammation, decreasing the levels of IL-1β, IL-6, and TNF-α, reducing the gingival tissue lesions, and preserving bone alveolar resorption.

23.6.2 Analgesic Activity

The lipophilic extract of *I. tinctoria* rosette leaves (DCM extract) showed the analgesic potential under in vivo acetic acid-induced abdominal writhing test. The oral administration of the extract (200 mg/kg), before the intraperitoneal injection of acetic acid, reduced the number of writhing and stretching movements in mice, while tryptanthrin (40 mg/kg) was not effective (Recio et al. 2006b).

23.6.3 Antioxidant Activity

During the last few years, research has highlighted the significant antioxidant properties of *I. tinctoria* ascribed to bioactive compounds contained in its phytochemical profile. Evidence confirmed polyphenols as the main class of natural antioxidants acting in various ways. In fact, polyphenols are considered radical scavengers due to their phenolic groups, excellent nucleophiles able to inhibit lipid peroxidation. Also, polyphenols act as breakers of the oxidation reaction by binding with free radicals generated through lipid peroxidation. Their activity as chelators of metal ions causing oxidation has also been proved.

Dating back to 2009, Fialova et al. reported the first data regarding the antioxidant activities of hydroalcoholic extract of *I. tinctoria* leaves. Then, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) test (SC50 = 103.9 g/mL) detected a good radical scavenging activity (Fialova et al. 2009). However, a study subsequently published by Yang et al. (2013) on ethanol extract from Chinese *I. tinctoria* showed a weak activity in DPPH, compared to the previous study, and also in the Trolox Equivalent Antioxidant Capacity (TEAC) together with reducing power assays.

Recent studies (Miceli et al. 2017; Taviano et al. 2018) have highlighted the in vitro antioxidant properties of polar phytocomplexes extracted from flowers, rosette, and cauline leaves of *I. tinctoria*. Taviano et al. (2018) established a correlation between the phenolic content and the observed biological effects through the characterization of the phenolic profile by HPLC-PDA-ESI-MS analysis. Results have identified flavonoids, among the others vicianin-2 and isovitexin, as the most involved compounds in such activity. The polar extracts (MeOH 70%) showed a good radical scavenging (DPPH test) and ferrous ions' chelating activity as well as moderate reducing power. Further in vitro assays on cauline leaves confirmed good radical scavenging properties due to flavonols of the phenolic-rich fraction (Miceli et al. 2017).

Recently, Zhao et al. (2017) evaluated the antioxidant effects of the indole alkaloids. Indigo and indirubin exhibited good scavenging activities on a DPPH-free radical and appeared more effective for superoxide anion scavenging due to the presence of some electrophilic groups that contribute to the superoxide anion. Despite differences in their chemical composition, not only leaves but also roots from this plant were found to be a source of natural antioxidants capable of exerting

protective effects against oxidative stress in the biological system (Nicosia et al. 2022).

A study revealed (Han et al. 2011) that in vitro, the polysaccharides exhibited an appreciable radical scavenging activity in the 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay reaching, at the maximum tested concentration (0.3 mg/mL), a percentage of inhibition similar to the reference compound vitamin C.

23.6.4 Antiviral, Antibacterial, and Antifungal Activities

In recent years, the antiviral effect of the polysaccharides contained in *I. tinctoria* roots has attracted increasing attention. These compounds have been demonstrated to display anti-influenza virus activity. Polysaccharide compounds have shown powerful anti-influenza A virus activity against human seasonal influenza viruses (H1N1 and H3N2) and avian influenza viruses (H6N2 and H9N2). The effect was speculated to be related to the reduction of the upregulated expression of pro-inflammatory cytokines/chemokines induced by the virus, through the inhibition of Toll-like receptor (TLR)-3 (Li et al. 2017). A polysaccharide isolated from *I. tinctoria* roots has been reported to display protective effect on red blood cells by inhibiting the attachment of influenza virus while also promoting the production of anti-influenza virus IgG antibodies, thus indicating its possible use as adjuvant for antiviral vaccines. Moreover under this study, the authors indicated also that polysaccharide compounds are effective against type II herpes simplex virus (HSV-2) in vitro (Wang et al. 2018).

The antiviral properties of *I. tinctoria* root polysaccharides against influenza virus have also been demonstrated in vivo. The intraperitoneal injection of a polysaccharide solution in mice infected with influenza virus can stimulate non-specific immunological function, humoral immunity, and cellular immunity, thereby giving mice enhanced resistance to virus infection (Zhao et al. 2008).

Indirubin has been shown to act as anti-influenza virus, too; the effect, observed on A/NWS/33- and B/Lee/40-infected bronchial epithelial cells H292, was related to the inhibition of RANTES expression (Mak et al. 2004).

Recently Cai et al. (2021) screened the extracts from *I. tinctoria* plant material (roots, leaves, herb) and commercial products and typed the dominant components, searching in silico their potential mechanism of anti-influenza virus 1 action. A total of 269 components were screened by drug-likeness and ADME (absorption, distribution, metabolism, and excretion) evaluation. Through experimental evaluation, six compounds, eupatorin, dinatin, linarin, tryptanthrin, indirubin, and acacetin, exhibited good inhibitory activity against wild-type H1N1 and H3N2. The authors indicated that the mechanism of anti-influenza virus 1 action of these compounds is multidirectional, which is also admitted by other authors of similar works.

Recent studies claim that some isolated compounds from *I. tinctoria* hydroalcoholic leaf extracts can be effective in the treatment of dengue fever

based on docking study using a crystal structure of dengue viral protein (Adawara et al. 2020).

The *I. tinctoria* leaf hydroalcoholic (75% ethanol) extract has shown the enhancing antimicrobial effect of four antibiotics (penicillin G, gentamicin, ciprofloxacin, and ceftriaxone) against *Staphylococcus aureus* strains (methicillin-resistant isolated from hospital patients and standard ATCC25923) (Yang et al. 2005).

The methanolic and ethanolic extracts of *I. tinctoria* leaf, stem, and flower are able to inhibit the growth of *Aspergillus niger* and *Candida albicans* strains. The water extracts are not effective (Odabaş-Serin et al. 2020).

23.6.5 Anticancer Activity

The use of *I. tinctoria* in the treatment of solid tumours dates back to ancient times. However, in the last decades a considerable number of in vitro and in vivo studies have shown the anticancer efficiency against a broad range of tumour types mainly due to the alkaloids indirubin and tryptanthrin (Taviano et al. 2018). Indirubins arrest cancer cell proliferation, induce cancer cell apoptosis, and inhibit tumour angiogenesis and cancer cell migration and invasion (Yang et al. 2022). Several trials published in China about 40 years ago already emphasized the effectiveness of the lipophilic compound indirubin in the treatment of human leukaemia (Cooperative Group of Clinical Therapy of Indirubin 1980). Indirubin orally administered at a dose of 150–200 mg/day led to complete remission in 26% and partial remission in 33% of 314 patients affected by chronic myelocytic and chronic granulocytic leukaemia. Moreover, the low toxicity and limited adverse effects related to this bisindole compound were detected. Later, an in vitro study identified a weak binding of indirubin to DNA behind its efficacy in treating chronic granulocytic leukaemia. The use of an isotope labelling method together with thermal denaturation measurements revealed the presence of a hydrogen bond instead of the usual ionic bond (Wu et al. 1982). In addition, Sethi et al. (2006) observed the ability of indirubin to downregulate the expression of the nuclear factor (NF)- κ B-dependent protein cyclin D1, induced by tumour necrosis factor (TNF), and suppressed the proliferation of KBM-5 human chronic myeloid leukaemia cells.

According to another experiment, indirubin blocked prostate tumour growth by suppressing tumour angiogenesis in xenograft mouse models (BALB/c nude mice) after 25 days of treatment (10 mg/kg per day). The inhibitory effect was confirmed through a chick chorioallantoic membrane (CAM) assay and mouse corneal model. Further Western blot analysis showed that the anti-tumour angiogenesis occurs via blocking vascular endothelial growth factor receptor 2 (VEGFR2)-mediated Janus kinase (JAK)/STAT3 signalling in endothelial cells. In the same experiment, indirubin has proven to be useful in inhibiting proliferation and inducing apoptosis in human umbilical vein endothelial cells (HUVEC) (Zhang et al. 2011). Other in vitro research brought out an antimitotic potential of indirubin derivatives. Indeed, indirubins are considered cyclin-dependent kinase 1 (CDK1) potent inhibitors in

human tumour cells with a high selectivity for the ATP-binding site on CDKs. It was found out that these alkaloids inhibit the proliferation of human large-cell lung carcinoma LXFL529L and human mammary carcinoma MCF-7 cells. Marko et al. (2001) showed that the more soluble analogue indirubin-3-monoxime arrested the cancer cell proliferation by inhibiting CDK1/cyclin B binding and inducing cell cycle arrest in G1/M and G2/S phases (Eisenbrand et al. 2004; Marko et al. 2001; Yang et al. 2022). In Damiens et al.'s (2001) study, indirubin-3'-monoxime displayed efficacy towards human breast epithelial HBL-100 cells. After a transient treatment with indirubin, cells underwent an endoreplication resulting in death after necrosis. Therefore, the endoreplication may be considered a mechanism behind the anti-tumour properties of indirubins.

Likewise, the indole tryptanthrin quinazolinone displayed a relevant cytotoxic activity by interacting with targets involved in tumour-associated cell processes such as cell growth and cell cycle progression and survival. In this regard, studies demonstrated that tryptanthrin was able to arrest the growth of human gastric cancer (HGC) as well as of lung cancer (HLC) (Kimoto et al. 1999; Speranza et al. 2020). The cytotoxic efficacy of tryptanthrin against non-small cell lung cancer NCI-H460 together with human glioblastoma SF-268 and human breast cancer MCF-7 cell lines was also detected. In detail, it seems that tryptanthrin produces a multidrug resistance (MDR)-reversing effect in doxorubicin-resistant breast cancer MCF-7 cells, through downregulation of MDR1 gene expression and, in part, modulating the glutathione *S*-transferase Pi (GST-Pi)-related pathway (Yu et al. 2007, 2009). New in vitro and in vivo experiment on human breast adenocarcinoma MCF-7 revealed that in vitro tryptanthrin inhibited the proliferation and migration as well as the invasion of MCF-7 cells through the upregulation of E-cadherin protein levels and the downregulation of MMP-2 and Snail. In vivo, three doses of tryptanthrin (25, 50, and 100 mg kg⁻¹) on the 4T1 murine breast cancer model were assayed to examine its anti-tumour activity. Tryptanthrin suppressed tumour growth presumably through the downregulation of NOS1, COX-2, and NF- κ B expression, the upregulation of IL-2 and TNF- α , and the recovery of the serum IL-10 level (Zeng et al. 2021). However, according to Liao and Leung (2013), tryptanthrin inhibited the growth of the *N-myc*-amplified human neuroblastoma LA-N-1 cells together with human neuroblastoma cell lines SH-SY5Y and SK-N-DZ. Western blot analysis revealed a marked mechanism of downregulation of cyclin D1, D3, CDK4, and CDK6 underlying the cell cycle arrest at G0/G1 phase. Also, a significant reduction of the *N-myc* proto-oncogene expression has been identified after a treatment with the alkaloid. The same authors indicated both in vitro and in vivo anti-angiogenic effects of tryptanthrin as potential anticancer mechanisms. In fact, tryptanthrin inhibited the in vitro proliferation, migration, and tube formation of the human microvascular endothelial cells (HMEC-1) in a concentration-dependent manner and significantly suppressed angiogenesis in Matrigel plugs in mice. In addition, it seems that tryptanthrin acts by reducing the expression of several pro-angiogenic factors (Ang-1, PDGFB, and MMP-2) as well as by suppressing the VEGFR2-mediated ERK1/2 signalling pathway in HMEC-1 cells (Liao et al. 2013). Furthermore, Chan et al. (2009) analysed the anti-proliferative activity of tryptanthrin

towards myelomonocytic leukaemia induced in BALB/c mice by WEHI-3B JCS cells. The research showed that tryptanthrin exerted its anti-tumour effects by causing cell cycle arrest in G0/G1 phase in a dose-dependent manner and by triggering cell differentiation, as supported by morphological and functional studies. Kimoto et al. (2001) evaluated the antileukaemic activity of tryptanthrin both in human monocytic U-937 and promyelocytic leukaemia HL-60 cells. The study explained that low concentrations of tryptanthrin were able to enhance the expression of cell differentiation markers, whereas high concentrations inhibited cell proliferation and damaged the mitochondria causing, through a caspase-3/Fas antigen pathway, an apoptotic cascade followed by cell death. Later on, an in vitro experiment led by Miao et al. (2011) has proven that tryptanthrin significantly inhibited human chronic myeloid leukaemia K562 cell proliferation by blocking the cell cycle progression at the G0/G1 phase. The resulting apoptotic process is due to the damage of the mitochondrial membrane as well as to the cyt-c-caspase-3-dependent mechanism. A recent study conducted by Shankar et al. (2020) attributed to tryptanthrin a new suppressor effect against non-melanoma skin cancer (NMSC). A DMBA/PMA-induced skin carcinogenesis model was set up by using Swiss albino mice. It turned out that tryptanthrin attenuated inflammation, impeded the proliferation of hair follicle (HF) cells, and suppressed the activation of β -catenin, a major driver of HF cell proliferation. Also, chemo-preventive efficacy in intestinal tumour formation was noticed in F344 rats affected by azoxymethane-induced intestinal tumour (Koya-Miyata et al. 2001).

Besides alkaloids, further investigations have also attributed to the phenolic profile of *I. tinctoria* a good anticancer potential. In Taviano et al.'s (2018) study, the polar extracts, obtained from both *I. tinctoria* rosette and cauline leaves, exhibited anti-proliferative effects against three different human anaplastic thyroid carcinoma (ATC) cell lines: CAL-62, 8505C, and C-643. After 48 h of treatment, the basal leaf extract expressed the strongest activity through a remarkable growth inhibition in CAL-62 cells. The data explained a significant (85%) reduction of viability at the highest dose (1 mg/mL). Also, the characterization of extracts through HPLC-PDA/ESI-MS indicates flavonoids and phenolic acids as the major constituents of polar extracts involved in anticancer activity of *I. tinctoria* (Miceli et al. 2017). Another study conducted by the same authors revealed that *I. tinctoria* cauline leaf extract at the highest tested dose (0.1 mg/mL) caused 80% growth inhibition in CAL-62 cells and 65% in 8505C cells after 48 h of exposure (Taviano et al. 2018). Recently, by using a fluorescently tagged plant cell line, Guan et al. (2021) demonstrated that extracts of both leaves and roots from this plant exert anti-microtubular activity. Through bioactivity-guided HPLC fractionation, glucobrassicin was selected, among the others, as a candidate compound accounting for such activity.

23.6.6 *Neuroprotective Property*

Lately, research has gained deep interest in the neuroprotective potential of *I. tinctoria* mainly ascribed to the high concentration of flavonoids and other phenolic compounds in its leaves and closely related to its anti-inflammatory, nitric oxide (NO) inhibiting, and antioxidant properties against free radicals (Taviano et al. 2018). Oxidative stress and inflammation play a crucial role in the development of prevalent neurodegenerative diseases along with psychiatric disorders such as anxiety and major depressive disorder. In fact, the brain tissue is particularly susceptible to the oxidative damage caused by the abnormal expression of reactive oxygen species (ROS) and overproduction of NO (Nicosia et al. 2022). Therefore, neuroprotection may be worthwhile to prevent brain tissue damage and preserve neuron integrity and function. In this regard, a recent in vitro study evaluated the neuroprotective activities of isolated alkaloids from *I. tinctoria* leaves against human neuroblastoma SH-SY5Y cells damaged by H₂O₂ using Trolox as the positive control. The cell viability determined by MTT assay indicated the constituents 5a/5b and 6a/6b improved in the neuroprotective activity at 50 µM compared with the responsible for neuroprotective activity (Atukuri and Mujavar 2021).

The most recent preliminary study conducted by Nicosia et al. (2022) demonstrated that *I. tinctoria* cauline leaf extract reduces stress-induced behavioural and cellular disorders in mice. According to behavioural tests (locomotor activity, social interaction, dark/light box, and the splash tests), stressed mice showed a decreased locomotor activity and anxiety-like behaviour as well as reduced interaction with other individuals. The study showed that a single administration of extract was able to reverse this behavioural response to the stress induced in the mouse model of acute restraint stress (ARS) by modulating oxidative stress, neuroinflammation, and NO reduction. Significant effects in reducing stress symptoms were reported by the behavioural studies at the dose of 500 and 50 mg/kg: the lowest (50 mg/kg) significantly reduced the spontaneous mobility compared to the control group suggesting its sedative effect. Contrarily, the locomotor activity increased at the highest dose (500 mg/kg) of extract. Moreover, in vitro assays displayed significantly increased levels of catalase (CAT), reduced levels of NO in the brain, and C-reactive protein (CRP) in plasma after a single administration assuming that *I. tinctoria* may be valuable in treating stress-related disorders such as depression and anxiety by improving resilience to stress.

23.6.7 *NO Inhibiting Activity*

Numerous studies highlighted the nitric oxide (NO) inhibiting activity of *I. tinctoria*. NO is a messenger molecule widely distributed in cells which plays an important modulatory role in a wide range of physiological and pathological processes. Increased NO production causes tissue damage generating various inflammatory

diseases. Zhang et al. (2020) identified four undescribed sulphur-containing **indole alkaloids** from *I. tinctoria* roots showing inhibitory effects on NO production in RAW 264.7 cells induced by LPS, indicating a good potential anti-inflammatory activity.

23.7 Significance in the Industry

23.7.1 *Isatis tinctoria* for Indigo Dye Production

Until the beginning of the century, natural dyes have been widely used and traded, representing the only available source of colour. Unfortunately, nowadays most of the colours utilized by the industries for modern textile dyeing are synthetic, and natural dyes are rarely used, except for a few specialist companies and craft dyers (Gilbert and Cooke 2001).

Currently, there has been a growing interest in natural dyes in fabric colouring, due to the rigorous environmental standards imposed by many countries in response to the toxic and allergic reactions associated with synthetic dyes (Comlekcioglu et al. 2015). The increasing interest in obtaining raw materials from renewable sources, the negative impact on the environment due to the release of polluting wastewaters by the synthetic dyeing industry, and the classification of many synthetic dyes as toxic when in contact with the skin are the main reasons for the renewed search for natural dyes by the dyestuff industry (Rocha et al. 2011; Osimani et al. 2012).

As a consequence of this demand, most of the work on dye plants that could be reintroduced into the marketplace has been concentrated on the production of indigo. Indeed, there is an immediate commercial potential, in comparison with other naturally produced colours, due to the already existing demand for this product in the denim fabric manufacturing process (Gilbert and Cooke 2001; Bhattacharjee et al. 2019). This use reevaluated indigo as a dye in the mid-nineteenth century, when Levi Strauss, a Bavarian tailor, marketed indigo-dyed cotton drill trousers for use by gold miners in California for heavy-duty jobs (Gilbert 2003).

The indigo blue pigment is one of the oldest natural dyes known to man. Indigo has been an important dyestuff from prehistoric times; historians claim that it was used as early as the Neolithic age. There are archaeological finds from over 4000 years ago in Egypt, India, and China. It has been found on some of the oldest textile fragments ever discovered. It is known that indigo was imported (probably from India) to dye hemp covering mummies in ancient Egypt, from about 2500 BC (Gilbert and Cooke 2001; de Melo et al. 2006). It was used to dye the garments of the high priests of Jerusalem in Biblical times (Wood 1986). For centuries, indigo has been obtained from a wide variety of plant sources from different tropical, sub-tropical, and temperate zones (Gilbert and Cooke 2001; Sales et al. 2006; Osimani et al. 2012).

One of the earliest known sources of indigo is *I. tinctoria*, the most commonly utilized species in temperate climates (Gilbert and Cooke 2001; Angelini et al.

2007). The discovery of blue-dyed textile remains preserved from the Neolithic, Bronze, and Iron Age indicates that this species was introduced to Europe in the late prehistory and protohistory (Zech-Matterne and Leconte 2010). Historical sources which date back to Roman times report that Celtic and Germanic people used woad to paint their body and hair for prophylactic or religious purposes. The use of woad indigo by Celts to colour themselves in order to obtain a fearsome appearance has been reported by Julius Caesar in the book *De Bello Gallico* (Guarino et al. 2000; Meijer et al. 2007). *Isatis tinctoria* was the only source of blue dye in Europe from the Middle Ages until the end of the seventeenth century, and the renowned trade centres were Toulouse in France and Erfurt in Germany, which currently still have relevance in the woad commerce. Its cultivation decreased rapidly as the dye market was invaded by the importation of the cheapest pigment obtained in greater quantities from other *Indigofera* species (Meijer et al. 2007; Spataro and Negri 2008; Osimani et al. 2012; Nguyen et al. 2019).

The crop was definitively abandoned in Europe in the late nineteenth century when the production of synthetic indigo dye and chemical reduction agents replaced that of natural indigo. Synthetic indigo destroyed almost completely the production of tropical indigo as well. Consequently, starting from some years after the synthesis of indigo (1897) onwards, the industrial production of natural indigo had almost ceased to exist (Gilbert and Cooke 2001). Recently, the rising demand for naturally sourced dyes has driven a renewed interest in *I. tinctoria*.

The indole alkaloid indigo is not found as a native compound in the plant, which instead produces its precursors. In most cases, the indigo precursor, indoxyl, is present in most of the indigo-producing plants as the glucoside indican (indoxyl- β -D-glucoside); however, as described by Epstein et al. (1967), the major indigo precursor in *I. tinctoria* is the ester isatan B (indoxyl-5-ketogluconate) (Stoker et al. 1998; Gilbert and Cooke 2001; Maugard et al. 2001). In nature, when the leaves are damaged and exposed to air, indigo precursors are submitted to enzymatic hydrolysis by β -D-glucosidase and β -glucuronidase. After cleavage of the *O*-glycosidic linkage, the indoxyl is released and subsequently oxidized to form indigo in contact with air (Maugard et al. 2001; Song et al. 2010) (Fig. 23.5). Once formed, indigo is a very stable molecule and remains so for many centuries, as evidenced by the textile fragments such as those found in the tombs of the ancient Egyptians (Gilbert and Cooke 2001).

Quantitative analysis showed that isatan B, indicant, and indigo amounts vary significantly throughout the year, being higher in spring and summer, and that the precursors are present at higher concentrations in the younger leaves (Maugard et al. 2001).

Natural indigo is produced by fermenting the leaves of indigo-bearing plants. The traditional method for the production of indigo dye from *I. tinctoria* was based on the formation of woad balls (Hurry 1930). The freshly harvested leaves were crushed by a horse-drawn millstone “woad mill” into a pulpy paste. The crushed leaves were then kneaded into large woad balls (about 10–12 cm diameter), which were allowed to dry over a period of 4–6 weeks until reduction to between a half and a third of their early size. This procedure led to the formation of indigo at the cut surfaces of the

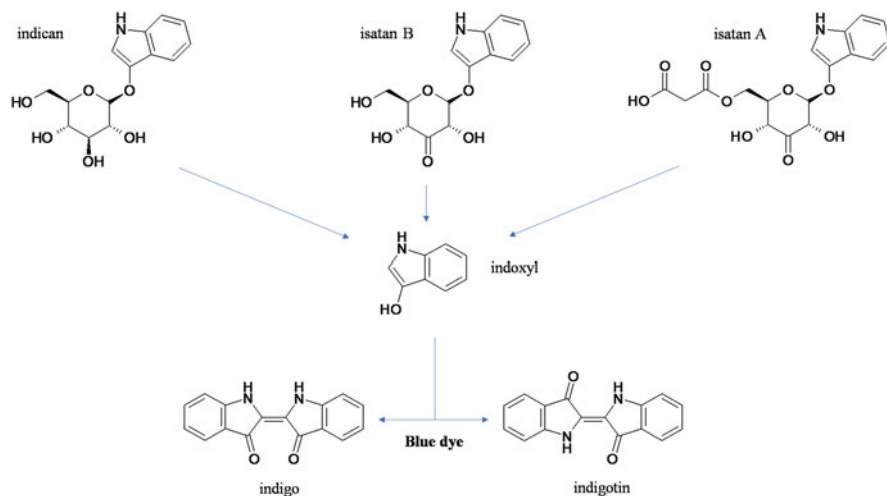


Fig. 23.5 The formation of a blue dye during postharvest processes. (According to Maugard et al. 2001 and Oberthür et al. 2004)

leaves by oxidation. The balls were then crushed, heaped into piles, wet with water, and allowed to ferment aerobically for some weeks, to produce “couched woad” containing indigo (Stoker et al. 1998; Gilbert and Cooke 2001).

During the fermentation of *I. tinctoria* leaves, water-soluble isatan B is hydrolysed to produce indoxyl, which further reacts with another indoxyl molecule, producing indigo (Gilbert et al. 2004).

However, the traditional fermentation method is now unacceptable to modern dyers, as it relies on “dirty” processing, produces a putrid odour, and is inefficient, usually giving a low dye yield (Stoker et al. 1998; Comlekcioglu et al. 2015).

Although indigo could be produced from the plants in large quantities, the decrease in trade of natural indigo has been mainly due to the lack of knowledge of simple procedures for its production from the plant material and the high production cost (Gilbert and Cooke 2001). For this reason, much research has focused on developing more effective extraction systems which do not rely on the old fermentation procedure. Indeed, several reports on the extraction of indigo from *I. tinctoria* leaves can be found in the existing literature (Stoker et al. 1998; Gilbert et al. 2004; Tozzi et al. 2005; Sales et al. 2006; Angelini et al. 2007; Rocha et al. 2011; Comlekcioglu et al. 2015).

The modern extraction methods are based on the water solubility of the indigo precursors in steeping the leaves in hot water. Several protocols based on this principle have been described, such as that developed by Hill (1992), in which indigo is extracted by boiling the leaves in water for a few minutes and adding an ammonia solution to the cooled water extract, followed by aeration. This method produces a “clean” indigo product, but it cannot be used for large-scale extraction because it is too expensive (Gilbert and Cooke 2001).

In a work carried out by Stoker et al. (1998), an alternative method has been developed for the extraction of natural indigo from *I. tinctoria* based on a technique used to extract indigo from *Indigofera* spp. The method is cheap, clean, and efficient, which opens the way for the large-scale production of woad indigo on a commercial basis. To improve the process, intact leaves must be harvested to avoid the spontaneous formation of indigo; then, the greatest amounts of the soluble precursors are extracted and subsequently hydrolysed and oxidized to form the final product. After harvesting, the leaves are immediately transferred into mesh bags and then put into a tank fitted with a pump-driven circulation system. To facilitate precursor extraction, the leaves are covered with hot water (60 °C), which damages the wax surface; then, after reduction of the temperature to 38 °C, to attain the optimal extraction of isatan B, the procedure involves steeping the leaves, covered with a piece of wire mesh and weighted down with stone blocks to maintain anaerobic conditions, in warm water at low pH (3.5) overnight, oxidation of the precursors at alkaline pH (9–10) in a settling tank aerated for 2–4 h using a compressor, ultrafiltration after 24 h, and air-drying of the produced indigo.

In more recent work, an efficient and eco-friendly method for colouring textiles by using natural indigo and determining the amount of indigo potentials in different species of *Isatis* has been developed by Comlekcioglu et al. (2015). In the experimental protocol, the fresh rosette leaves from different *Isatis* spp. washed and cut into small pieces were used as raw materials for the extraction. Two different procedures have been utilized and compared, namely, fermentation (24 h) at room temperature and hot water extraction (60, 70, or 80 °C for 10 min) with subsequent rapid cooling to 50 °C. For extracting the indigo precursors, two different pH were tested (9.0 and 11.0), followed by aeration to enable their oxidation. The efficacy of the extraction procedures was established by evaluating the dyeing potential of indigo from the *Isatis* spp. on cotton yarns after the addition of the reducing agent sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$). Results indicated that the dye from hot water application produced the desired dyeing quality at pH 11 and that the temperature of 70 °C could be favourable for *I. tinctoria*.

After extraction, the quantitative determination of indigo is usually attained by spectrophotometry of solutions using a variety of organic solvents, among which ethyl-acetate is the most utilized (Stoker et al. 1998; Gilbert et al. 2004; Sales et al. 2006; Comlekcioglu et al. 2015).

Some studies have been conducted to obtain rapid analysis methods to predict the indigo content of the leaves of *I. tinctoria* through the quantification of its precursors. This could be useful to implement a breeding program aimed at the optimization of the production of indigo, in which the identification of high indigo-yielding genotypes is needed.

The ratio of indican to isatan B in woad has been routinely analysed by TLC (Gilbert et al. 2004). In a study aimed at investigating the seasonal variation of isatans A and B, and of indican in defined woad accessions, as well as the effect of different postharvest treatments, Oberthür et al. (2004) have developed and validated a densitometric assay for the quantitative determination of these precursors in *I. tinctoria* leaves through HPTLC separation on silica gel. For the chromatographic

assay, a mixture of chloroform/methanol/water (60:35:5) was used as the developing eluent. After development, and removal of the eluent by drying in a stream of cold air, the HPTLC were immersed for 4 s into 5% hydrochloric acid for the detection of indican and into 5% sodium hydroxide for the detection of isatans. Then, the plates were dried under a stream of warm air, heated at 120 °C for 2 min, and analysed with a CD 60 densitometer. The amount of indican, isatan A, and isatan B in the extracts was calculated with the aid of calibration curves of reference solutions.

Several protocols based on the detection of the indigo precursors through HPLC analysis have been developed (Gilbert et al. 2000, 2004; Maugard et al. 2001; Angelini et al. 2007; Rocha et al. 2011; Nguyen et al. 2019). The work of Gilbert et al. (2000) showed the first analytical procedure for the identification of indican and isatan B from *I. tinctoria* leaf extracts using HPLC coupled to an evaporative light-scattering detector and confirmed using HPLC coupled, on line, to a particle beam mass spectrometer (MS). For the analysis, leaf discs were taken from middle-sized leaves and extracted with boiling water for 5 min. After filtration through a 0.22 mm Millipore membrane filter or centrifugation, the resulting extract, opportunistically diluted, was analysed by HPLC-ELSD or by HPLC-MS. Maugard et al. (2001) have described for the first time a quali-quantitative HPLC method for the identification and the separation of indigoid pigments and precursors contained in *I. tinctoria* fresh leaves. For the analysis, the leaves were ground in a mortar, suspended in acetone/acetic acid 1% v/v, and stirred at 24 °C for 4 h. After filtration, the solvents were evaporated, and the residue was dissolved in methanol and analysed. The study demonstrated that *I. tinctoria* produces three indigo precursors, isatan B as a major precursor, indican as a minor precursor, and a new precursor found in the young leaves of *I. tinctoria* harvested from June, which has been named “isatan C”. Subsequently, Gilbert et al. (2004) have described a method for the quantitative determination of indigo precursors in the leaves of *Isatis* spp. by HPLC-ELSD analysis. For the extraction, the leaf discs mixed with a solution of water/acetonitrile/formic acid (75%/25%/0.1%) were heated at 90 °C in a water bath for 2 min. After removing the leaf material, the remaining mixture was cooled to 25 °C, and an aliquot was mixed with ethyl-acetate/formic acid 0.1%. The obtained upper fractions were added with formic acid (0.1%) in methanol and analysed by HPLC-ELSD.

In a later study aimed at identifying high-yielding genotypes of *Isatis* spp. to be included in a further selection and breeding program for indigo production in Italy, to determine the indigo potential of the genotypes screened in the study, extraction and quantification of indigo precursors were accomplished by the method of Gilbert et al. (2004) with some modifications (Angelini et al. 2007). In particular, the leaf discs were extracted with a mixture of water/acetonitrile/formic acid (75%/25%/0.1%) at 80 °C for 2 min; after removal of the leaf discs and rapid cooling, an aliquot of each extract was mixed with ethyl-acetate/formic acid 0.1%, and then the ethyl-acetate solutions were directly analysed by HPLC-ELSD, at a difference with Gilbert's method.

In more recent work, Nguyen et al. (2019) have proposed simple statistical models, based on a small number of predictor metabolites, that predict moderately

well the postharvest accumulation of indole alkaloids in *I. tinctoria* leaves, including indigo. For this purpose, the dichloromethane extracts of dried leaves and the methanol extracts of fresh leaves from the same plants were analysed by HPLC-Hybrid Ion Trap-Orbitrap Mass Spectrometry. The determination of the polar metabolites showing the strongest predictive power for the lipophilic indole alkaloids was achieved by a stepwise selection procedure starting from the integration of 122 compounds detected in fresh leaves and of 5 selected lipophilic indole alkaloids in dried leaves, generating 5 predictive linear models. The simplest and at the same time one of the most accurate models was obtained for indigo; it was found that isatan B and dioxindole malonylglucoside contributed positively to the amounts of indigo in dried leaves. Certainly, the predictive power of a limited set of metabolite predictors in the fresh leaves for the postharvest formation of the lipophilic indole alkaloids could represent a simple and valuable procedure to be used in *I. tinctoria* cultivation program for a high indigo yielding.

23.7.2 *Isatis tinctoria* in the Cosmetic Industry

In the early years of the cosmetic industry, most of the ingredients used were natural, mineral resources and products of plant or animal origin. With the development of synthetic chemistry, a broad range of natural compounds was replaced with synthetic derivatives, which were utilized in cosmetics as “nature-like” ingredients (Boeriu 2015).

In modern times, there has been an ever-increasing interest in the use of natural ingredients from botanical sources for cosmetic purposes as an alternative to synthetic products, to formulate new, safe, and more effective cosmetics, which are easily accepted by consumers.

Currently, different organs of *I. tinctoria* are utilized in the cosmetic industry as ingredients for the production of a wide range of cosmetics, including perfumes (e.g. eau de toilette), skin care (e.g. face and body creams), hair care (e.g. colouring), and toiletry (e.g. soaps) products. The roots (powder/extract) have astringent and skin-protecting properties. The seed oil and the leaves (powder/extract) are cosmetic ingredients for skin and hair conditioning due to their emollient and moisturizing properties (Spataro and Negri 2008). These raw materials are listed in the CosIng database elaborated by the European Commission to produce cosmetics in Europe (<https://ec.europa.eu/growth/sectors/cosmetics/cosing>).

One of the applications of *I. tinctoria* in cosmetics is as a source of the blue dye indigo for hair and nail colouring (Boeriu 2015). A problem related to the use of the indigo dye for hair colouring is that the uptake of the colour into the keratin fibres is very poor. This has driven research to overcome the problem by improving the stability of indigo, as well as of other natural plant-based colorants such as henna, which are unstable to light, temperature, and pH due to their chemical structure. Thus, some studies have been carried out to provide keratin fibre dyeing processes using natural dyes, which can produce less aggressive colorations that are to the hair

and resistant to external agents (light, bad weather, or shampooing), and that are fast and homogeneous, while at the same time remaining powerful, intensive, and/or chromatic. This is the case of the invention patented by Rharbi and Peron (2018), which relates to a composition comprising henna and/or indigo, oil, and a saccharide, and a process for dyeing keratin fibres, especially human keratin fibres such as the hair, using the formulated composition.

The oil obtained from the seeds of *I. tinctoria* is highly appreciated for manufacturing cosmetics due to its valuable moisturizing properties (Jeanvoine et al. 2009). *I. tinctoria* seed oil contains a high percentage of unsaturated fatty acids and sterols, which are well known to be of particular value in cosmetics (Kizil et al. 2009; Michalak and Kiełtyka-Dadasiewicz 2019). In a recent work published by Roche et al. (2016), higher levels of polyunsaturated acids, mainly linolenic acid, and phytosterols, mostly β -sitosterol, were found in the oil of seeds collected between 25 and 45 days after flowering.

In addition to lipids, proteins also contribute to the cosmetic value of *I. tinctoria* seeds. A research team of the Toulouse company Cocagne & Compagnie in association with the Institut National Polytechnique de Toulouse (INPT) has filed a patent related to the use of the protein extract of *I. tinctoria* seeds or a composition comprising such an extract preferably for cosmetic purposes. Indeed, seed proteins are supposed to have the ability to stimulate the production of collagen in fibroblasts, thus improving the appearance, flexibility, firmness, elasticity, density, texture, smooth appearance, and/or brightness of the skin; to prevent, limit, or reduce the formation of wrinkles; or to act as an anti-ageing agent (Juin et al. 2013).

With the use of natural plant extracts as cosmetic ingredients, a new generation of products has been introduced in the market of which a category has been claimed to contain biologically active ingredients with medicinal or drug-like effects. Indeed, the search for plants with “cosmeceutical” properties has become of huge interest to many cosmetic industries globally (Ribeiro et al. 2015; Sagbo and Mbeng 2019). Plants traditionally used for their medicinal properties have been rediscovered for the extraction and utilization of the bioactive principles, which satisfy the needs of both beauty and health.

Starting from the traditional use of *I. tinctoria* for the treatment of skin inflammations, which has been confirmed by acute and sub-chronic murine experimental models and by a clinical pilot study using sodium lauryl sulphate-induced irritant contact dermatitis and UVB-induced erythema models (Speranza et al. 2020), recently Forner et al. (2019) have investigated the skin caring and soothing effects, the sensorial characteristics, and the tolerability of a body cream (oil-in-water emulsion) containing *I. tinctoria* extract, in subjects with dry and pruritic skin. After twice daily topical application over 2 weeks, the treated subjects positively evaluated the cosmetic and sensorial characteristics of the product; tolerability and a lack of negative skin sensations reached the highest scores underlining the favourable safety profile of the product. The suitability of the product for dry and sensitive skin reached high scores too, as did also the soothing, smoothing, moisturizing, and skin feeling variables. The results of the study indicated that the

I. tinctoria-containing cream is an effective and very well tolerated option for the treatment of dry, irritated, and pruritic skin.

23.8 Biotechnological Studies

Although *I. tinctoria* can be grown in the field, the number of bioactive compounds is often fluctuating due to unfavourable environmental conditions (e.g. seasonal changes, infestations, diseases, and other biotic and abiotic stresses); for this reason several in vitro culture techniques have been used to produce secondary metabolites in vitro. In addition, this strategy offers several advantages: firstly, the uniform quality of products collected everywhere under strictly controlled conditions; secondly, the production system that is ecologically sustainable regardless of climatic/ecological and agrochemical problems; and thirdly, the production of large-scale products for commercial purposes with a bioreactor.

As shown by the analysis of the latest scientific literature, biotechnological studies on *I. tinctoria* are limited and the first ones regarding the establishment of shoot culture that was carried out by Leal et al. (2009). In the study, the best culture medium to establish and proliferate cultures of woad in vitro, to be used for metabolites extraction, was evaluated. Nodal segments of young plants from two different populations (the Botanical Garden of the University of Coimbra, Portugal, and from the University of Bristol, UK) were cultured in Murashige and Skoog (MS) and Gamborg (B5) media supplemented with BAP or KIN, with or without NAA. In the Bristol population, higher values for all the parameters such as the number and length of shoots and the number of internodes were observed as compared with the Coimbra population. Considering the two basal media, B5, supplemented with 1.0 mg/L BAP, was more effective to induce multiplication; moreover, the addition of auxin to the culture media did not increase multiplication. On the contrary, the addition of activated charcoal showed a positive effect in the length of shoots and in the number of internodes, reaching the highest values when MS medium was supplemented with 1.0 mg/L KIN.

One year later, Saglam and Ciftci (2010) carried out a study on shoot regeneration of *I. tinctoria*. It was planned to develop an efficient and cheap in vitro regeneration methodology for a higher shoot regeneration of *I. tinctoria* by comparing the gelling ability of the agar and isubgol gelling agents. The results showed that the medium supplemented with isubgol was equally suitable for inducing shoot regeneration, and in addition it induced a greater number of shoots per explant compared to agar providing to the plant tissues a better diffusion of media components and increased availability of plant growth.

The most important studies on in vitro culture about this species were carried out by the team of two researchers: Q.-Y. Gai and J. Jiao of Northeast Forestry University in Harbin in China. *I. tinctoria* hairy root cultures were successfully initiated by explants of 3-week-old petioles co-cultivated in vitro with *Agrobacterium rhizogenes* in a half-strength Murashige and Skoog (MS/2)-based

solid medium. The hairy roots emerging from the explants were isolated and sub-grown on hormone-free MS/2 medium supplemented with acetosyringone and arginine. T-DNA integration was confirmed by PCR analysis of the *rolB*, *rolC*, and *aux1* genes. Eight different hairy root culture lines were obtained, and line III resulted to be the best both in terms of biomass production and for the accumulation of alkaloids. The optimization of the culture conditions can improve the biomass yield and the content of active ingredients. For this reason, the effects on the production of biomass and on the accumulation of active compounds of different parameters such as the composition of the medium, the pH, and nature of the carbohydrates, used as an organic carbon source, were optimized by Box-Behnken design. The best results were obtained by using a 0.75% inoculum and a half-strength MS (MS/2)-based solid medium with pH 5.8 and containing 3.14% sucrose while maintaining the culture at a temperature of 24.7 °C and harvest time of 23 days. By using LC-MS/MS analysis carried out on the extract obtained from *I. tinctoria* hairy root cultures, six alkaloids were determined such as epigoitrin, isatin, indole-3-carboxaldehyde, tryptanthrin, indigo, and indirubin. The total alkaloid content (521.77 g/g DW) was 1.12 times higher than that of the roots of 2-year-old plants grown in the field (464.69 g/g DW) (Gai et al. 2015a).

The same authors (Gai et al. 2015b) have shown that *I. tinctoria* hairy root cultures are able to accumulate also flavonoid compounds. Eight different hairy root culture lines (I–VIII) were identified and evaluated by comparing their performance in terms of biomass production and accumulation of flavonoid compounds. Lines III, V, and VII have proved to be highly productive in terms of biomass dry weight (DW) and flavonoid content; moreover, the V line showed the best biosynthetic capacity. Optimization of culture conditions has been shown to improve biomass yield and flavonoid content. The best results were obtained using 0.75% inoculum, an MS/2 liquid culture medium with pH 5.8, and 3.06% sucrose, keeping the culture at a temperature of 24.7 °C. Flavonoids such as rutin, neohesperidin, buddleoside, liquiritigenin, quercetin, isorhamnetin, kaempferol, and isoliquiritigenin have been determined by LC-MS/MS analysis performed on the extract obtained from the *I. tinctoria* hairy root cultures. The total flavonoid content (438.10 g/g DW) was 1.28 times higher than that of the roots of 2-year-old plants grown in the field (341.73 g/g DW) (Gai et al. 2015b).

In order to increase the production of bioactive metabolites, several elicitation experiments have been carried out on *I. tinctoria* hairy root cultures using abiotic and biotic elicitors such as chitosan, ultraviolet radiation, salicylic acid, acetylsalicylic acid, methyl jasmonate, and *Aspergillus* spp. Chitosan is a natural, low-cost, and non-toxic elicitor that can trigger defense responses of plants with the concomitant improvement of phytochemical biosynthesis. The study showed that exposure to moderate concentrations of chitosan (150 and 200 mg/L) resulted in an increase in both biomass and flavonoids of 7.08 and 7.22 times, respectively, in comparison with the control. On the contrary, prolonged exposure (72–96 h) to a high concentration of chitosan (400 mg/L) did not cause an increase in the concentration of flavonoids. This increase seems to be related to the upregulation of some

transcription of associated genes involved in the flavonoid biosynthesis pathway underlying chitosan elicitation (Jiao et al. 2018a).

The same authors have also shown that the increase in the concentration of flavonoids can be obtained by exposing *I. tinctoria* hairy root cultures to ultraviolet radiation (UV-A, UV-B, and UV-C). The UV light lamps (UV-A: 40 W, $\lambda_{\text{max}} = 365$ nm; UV-B: 40 W, $\lambda_{\text{max}} = 313$ nm; UV-C: 40 W, $\lambda_{\text{max}} = 254$ nm) were mounted on luminaires and placed on the upper part of the hairy roots, adjusting the distance from the crops to obtain a constant radiation intensity (3 W/m^2) measured with a photometer. During the elicitation experiments, three groups of cultures were individually subjected to UV-A, UV-B, and UV-C radiation with successive time points equivalent to different UV radiation doses expressed as kJ/m^2 . The control group was kept in the same conditions in the dark. The results showed that the maximum accumulation of flavonoids ($7259.12 \text{ } \mu\text{g/g DW}$) was achieved in hairy root cultures exposed to UV-B radiation (108 kJ/m^2). The total flavonoid content was 16.51 times higher in the elicited crops than in the control ones; the concentrations of rutin, neohesperidin, buddleoside, liquiritigenin, quercetin, isorhamnetin, kaempferol, and isoliquiritigenin were increased by 15.33, 2.76, 1.96, 5.67, 29.38, 21.91, 15.54, and 6.87 times, respectively, compared to control. Moreover, the expression of chalcone synthase gene was tremendously upregulated (up to 405.84-fold) in *I. tinctoria* hairy root cultures following ultraviolet-B radiation, which suggested chalcone synthase gene might play a crucial role in flavonoid increase (Jiao et al. 2018b).

The same research team further demonstrated the increase in the production of flavonoids also through co-cultivation of *I. tinctoria* hairy roots with two immobilized live GRAS (Generally Recognized as Safe) fungi such as *Aspergillus niger* and *Aspergillus oryzae*. The experiments were carried out on the fifth hairline of *I. tinctoria* for the high production capacity of flavonoids. The cultures of both fungi were grown on a culture medium containing potato dextrose agar (PDA) and incubated at 30 ± 1 °C until sporulation; the fungal spores were collected and counted for immobilization. With the aim to achieve the highest flavonoid production, several key parameters including spore concentration (10–104 spores/mL), temperature (25–32 °C), initial pH value of media (4.0–8.0), and time (0–96 h) were systematically optimized. The highest flavonoid production ($3018.31 \pm 48.66 \text{ } \mu\text{g/g DW}$) was achieved in *A. niger* treated culture under the optimal conditions of spore concentration ca. 104 spores/mL, temperature 30 °C, initial pH value of media 7.0, and time 72 h, which remarkably increased 6.83-fold in comparison with non-treated control ($441.91 \pm 7.35 \text{ } \mu\text{g/g DW}$). This study revealed that *A. niger* elicitation could trigger the sequentially transient accumulation of signal molecules and intensify the oxidative stress in the elicited cultures, which both contributed to the upregulated expression of associated genes involved in the flavonoid biosynthetic pathway (Jiao et al. 2018c).

Recently, the effects of exogenous signal molecules such as salicylic acid, acetylsalicylic acid, and methyl jasmonate, utilized as abiotic elicitors, on the accumulation of alkaloids and flavonoids in two high-productivity *I. tinctoria* hairy root cultures were demonstrated by the same research group. Lines III and V

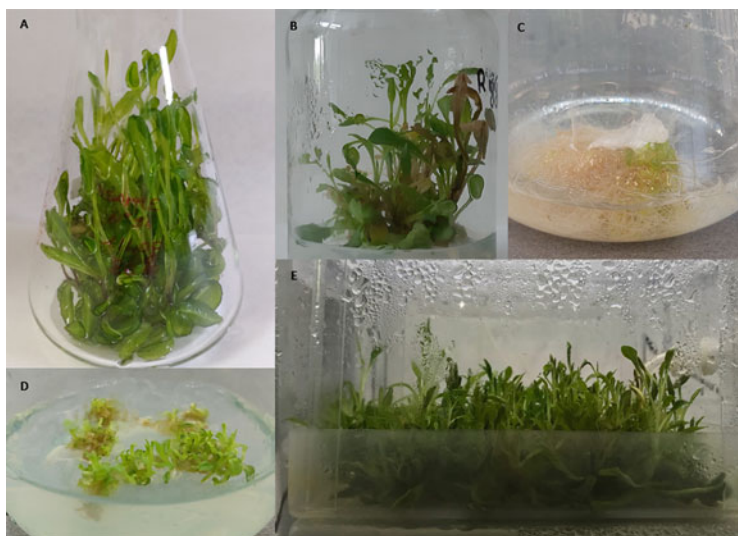


Fig. 23.6 In vitro cultures of *Isatis tinctoria*: agitated shoot culture (a), shoot culture on agar medium (b), hairy root culture (c), regenerated shoot culture (d), shoot culture in Plantform bioreactor (e). (I. Kwiecień)

have undergone elicitation, and the maximum accumulation of alkaloids has been obtained in *I. tinctoria* hairy root cultures of line III elicited by 142.61 μM salicylic acid for 28.18 h and for the flavonoids in those of line V elicited by 179.54 μM methyl jasmonate for 41.87 h. A 5.89-fold increase for the alkaloids epigoitrin, isatin, indole-3-carboxaldehyde, tryptanthrin, indigo, and indirubin and 11.21-fold for the flavonoids rutin, neohesperidin, buddleoside, liquiritigenin, quercetin, isorhamnetin, kaempferol, and isoliquiritigenin, as compared with controls, were observed. The increase in the accumulation of alkaloids and flavonoids in elicited *I. tinctoria* hairy root cultures can be attributed to the activation of genes related to the biosynthesis of alkaloids and flavonoids (Gai et al. 2019). Moreover, the transcription levels of the 11 investigated genes, encoding enzymes involved in alkaloid and flavonoid biosynthetic pathways, were significantly upregulated in *I. tinctoria* hairy root cultures undergoing salicylic acid and methyl jasmonate elicitation. Among the genes determined, *YUCCA monooxygenase*, *chalcone isomerase*, and *flavonoid 3'-hydroxylase* might play a crucial role in the target phytochemical augmentation.

The biotechnological studies reported above on *I. tinctoria* hairy root cultures describe the crucial role played by the optimization culture conditions and the use of elicitors aimed at increasing the production of secondary metabolites. Guided by the broad spectrum of biological activity of *I. tinctoria* leaf extracts and their metabolites, our team also undertook research on shoot cultures of this species (Fig. 23.6).

23.9 Conclusion

The high utility value of *Isatis tinctoria* has been long known in Asian and European countries. This species except to be indigo natural dye source has many medical indications. The herb, leaf and root extracts have been recommended especially as strong anti-inflammatory agents. The latest studies underline particularly antiviral and anticancer properties, too. Moreover, extracts show e.g. antioxidant, antimicrobial, analgesic and neuroprotective activities.

This raw material, currently, is of high use also in cosmetic industry, where is used not only as a natural dye, but also in production of e.g. perfumes, creams and soaps. Nevertheless, the indigo receiving is still the most popular utility aspect of *I. tinctoria* plant. Taking in account all usability attitudes of *I. tinctoria*, it is worth emphasizing especially that it is undemanding and easy to cultivate plant even in marginal lands of Europe and Asia. Moreover, plant in vitro culture technology has been emerged as an attractive alternative approach to the field cultivation of *I. tinctoria*.

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Chapter 24

Tissue Culture Techniques to Conserve Endangered Medicinal Plants with Antimicrobial and Antiviral Activities



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Abstract Approximately four-fifths of the world's population has long relied on medicinal plants in the form of traditional formulations for their primary healthcare systems and the treatment of some ailments. According to the IUCN Red List of Threatened Species, 4976 plant species are critically endangered, 9400 endangered, and 8959 are vulnerable. Numerous medicinal plants also had problems due to overharvesting and habitat destruction. Population growth, increased demand, and unlimited collection of medicinal plants combined with illegal trade have reduced endangered medicinal plants in their natural habitat. Managing the resources of medicinal plants, thus, is essential. Conventional propagation approaches take a long time for proliferation, and nonuniform cloning and the growing demand for herbal-based drugs ensure their mass propagation through plant tissue culture. Plant tissue culture technology is widely employed for large-scale plant propagation. Apart from being utilized as a study tool, plant tissue culture techniques have gained major industrial importance in the contemporary era in the field of plant propagation, disease elimination, plant breeding, production of secondary metabolites, and protection of endangered species. Tissue culture protocols have been developed for a wide range of medicinal plants, including rare and endangered plant species. This chapter will study various plant tissue culture systems, including the culture of organized structures and unorganized cells that are widely utilized to maintain, improve, and increase the production of phytochemicals in endangered medicinal

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plants with antimicrobial and antiviral properties, such as *Withania somnifera*, *Zhumeria majdae*, *Picrorhiza kurroa*, *Ginkgo biloba*, *Swertia chirayita*, and *Gymnema sylvestre*.

Keywords Antimicrobial activities · Conservation · Endangered medicinal plants · Tissue culture

24.1 Introduction

Indeed, medicinal plants have been attractive to humans since the ancient past and have been widely utilized in many fields such as food, color, cosmetics, perfume, and medicine. Medicines are usually made using various plant species that grow in other parts of the world. In the Neolithic period, for example, they were frequently used in the prevention and treatment of diseases and are still the main types of drugs used on the planet. Despite the decline in interest in medicinal plants due to synthetic drug production in the early twentieth century, herbal medicines became important again in the late twentieth century. This is mainly because they have lower side effects. Herbal medicine usage in developed nations is growing dramatically, and the rapid population growth in recent years shows that individuals prefer herbal medicines or their products to improve their health. Approximately 40% of the compounds used in the pharmaceutical industry are obtained directly or indirectly from plants, and the global market value of herbal medicinal products exceeds \$100 billion annually (Kin 2020; Ugboko et al. 2020). Medicinal plants are valuable plant resources. Some are acquired via cultivation, but most are obtained through harvesting from nature. Many plant species with medicinal properties, thus, are in danger of extinction due to excessive collection. A very conservative estimate reveals that the current losses of plant species are 100 times higher than the expected natural extinction rate, and the earth loses at least 1 significant medicinal plant every 2 years. According to the International Union for Conservation of Nature and the World Wildlife Fund, 50,000–80,000 flowering plant species are employed for medicinal purposes worldwide. Meanwhile, roughly 15,000 species are at risk of extinction due to overharvesting and habitat destruction, and one-fifth of their wild resources are now almost depleted by the boost in human population and plant consumption (Chen et al. 2016).

Currently, there is a steady decline in newly registered antimicrobials on the market. On the other hand, many of the available antimicrobial agents have not been able to inhibit microorganisms. In addition, new and emerging diseases caused by viruses, including SARS-CoV-2, threaten human existence (Rai and Kosalec 2022). Persistent infectious diseases are one of the major causes of global health threats. The World Health Organization (WHO) reported in 2013 that infectious diseases accounted for 61.7% (5.9 million) of the 9.6 million deaths in sub-Saharan Africa (Ugboko et al. 2020). Medicinal plants contain bioactive organic chemical compounds, often referred to as secondary metabolites that play a protective role against chronic, genetic, and infectious diseases. These compounds are found in grains,

vegetables, fruits, and other plant products. Phytochemical screening of medicinal plants is usually performed against a wide range of microorganisms to determine their antimicrobial activity based on the active compounds of plants, which are mainly secondary metabolites. The occurrence of antimicrobial drug resistance by most bacteria has created a crisis and the need for ongoing research for better and safer therapeutic agents. The antimicrobial activity of beneficial plants varies. The majority of them acts synergistically and reduces the side effects of synthetic drugs (Oladosu et al. 2015). Despite the success of antibiotic detection, infectious diseases have always been the second leading cause of death worldwide (Ugboko et al. 2020). As mentioned, microbial resistance to antibiotics is one of the major problems of the twenty-first century and has necessitated the constant quest for more robust and safer therapeutic agents.

Three main strategies are utilized in the biotechnology of medicinal plants. The first is a molecular modification to improve the product, the second is the artificial production of secondary metabolites with therapeutic value, and the last is micropropagation for mass production. The plant tissue culture technique is one of the promising tools to ensure the sustainable use of plant resources. The science of plant tissue culture originates from cell discovery and subsequent cell theory. In 1838, Schleiden and Schwann proposed that the cell is the basic structural unit of all living things. They imagined that the cell is an autonomous unit. It, thus, should be possible for each cell to become a complete plant again under suitable conditions. Based on this hypothesis, Gottlieb Haberlandt, a German physiologist, attempted to culture cells isolated from leaves in a salt-knop solution enriched with sucrose. The cells survived for up to a month, their size increased, and starch accumulated but could not divide. Although he was unsuccessful, he laid the foundation for tissue culture technology, for which he is considered the father of plant tissue culture (Hussain et al. 2012). Plant tissue culture is the *in vitro* culture of cells, tissues, organs, or the whole plant under controlled nutritional, sterile, and environmental conditions with a suitable formula to produce a complete plant (Thorpe 2012). Controlled conditions provide a favorable environment for growth and multiplication. These conditions include an appropriate and adequate supply of nutrients, pH, temperature, and liquid environment. Apart from being used as research tools, plant tissue culture techniques have gained industrial importance in recent years in plant propagation, disease elimination, and production of secondary metabolites. Endangered, threatened, and rare species have been successfully grown and maintained by micropropagation. The reason why this is the case is their high reproduction rate and low requirement for the number of primary plants and space (Hussain et al. 2012). These are rapid propagation processes that can result in the production of virus-free plants (García-González et al. 2010). Although this method has disadvantages, such as the need for expensive and specialized techniques and skills, plants produced, unlike farm plants, are not dependent on food resources in a short time. Disadvantages of this can be noted as follows: sometimes calluses do not appear for months, or even they do not appear at all, seedlings are smallish at first and have undesirable characteristics, plants do not act autotrophically in cultivation before they can grow independently, they have to go through a transition phase, the chances of producing genetically abnormal plants

increase, and eventually the seedlings are more prone to contamination and water loss in the external environment, but overall the benefits are more lavish. These advantages include the following: it can be accomplished from the molecular level to large scale; there is no tedious work such as weeding, spraying, and irrigation; plant production in this method is season-independent; plant tissue culture can produce many clones; disease-free plants, valuable plants, and plants with nutritional value and high quality are producible. This process provides uniform biomass that is always available, ultimately enabling the production of medicinal compounds that are challenging to synthesize chemically (Bhatia 2015). Tissue culture mainly refers to the ability of a cell to express a complete genome by cell division. In addition to the full potential of plant cells, the capacity of cells to alter their metabolism, growth, and development is equally essential to regenerate the whole plant. The culture medium contains the necessary nutrients for plant growth and development. It is mainly composed of macro- and micronutrients, vitamins, other organic components, plant growth regulators, and carbon sources (Murashige and Skoog 1962). Murashige and Skoog (MS) culture media are the most common media for the propagation of many plant species. In general, the pH of the environment is also vital, which affects the growth of plants and the activity of the regulators. It is set between 5.4 and 5.8. Both solid and liquid media can be used for culture. The composition of the environment, especially plant hormones and the nitrogen source, has profound impacts on the initial explant response (Bhatia 2015).

In this chapter, plant threatening factors, types of tissue culture methods, culture medium, stress factors in tissue culture process, and the study of six endangered plant species with antimicrobial and antiviral properties, including *Withania somnifera*, *Zhumeria majdae*, *Picorrhiza kurroa*, *Ginkgo biloba*, *Swertia chirata*, *Gymnema sylvestre*, and *Tinospora cordifolia*, will be discussed in general.

24.2 Factors Affecting and Threatening Plant Populations

Biodiversity loss contributes to a reduction in the number of species, genes, individual organisms, and ecosystems in a given area. It is caused by five primary drivers, including habitat loss, invasive species, pollution, overexploitation, and climate change (Fig. 24.1).

24.2.1 *Habitat Loss*

Disorders caused by natural disasters such as fire, flood, and hurricane in habitats improve with gradual regeneration by species that spread from nearby areas. Nevertheless, the scale of human activity disturbances, especially in cases that possess several threatening processes in a zone, may be considered sufficient to prevent regenerating habitats, leading to the decline of some species and ecological

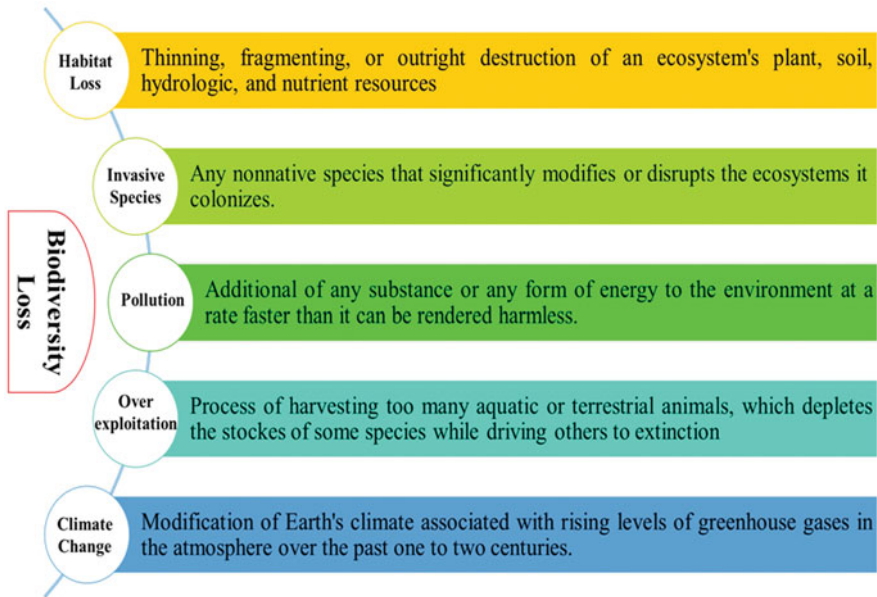


Fig. 24.1 Summary of the five primary drivers of biodiversity loss

communities. Remnants of small habitats cannot support populations for long periods and are more prone to threats and loss of biodiversity, especially if there are no suitable habitats nearby from which plants can regenerate. Conservation reserves separated by large areas often cannot guarantee the long-term survival of species and ecological communities and may need to be interconnected by native plant corridors. Also, freshwater habitats are sensitive to changes in water flow, temperature, and sediment that can be caused by drainage, dredging, dewatering, and roads (Young et al. 2017).

24.2.2 *Invasive Species*

Biological invasions are seen as a significant driver of biodiversity loss on the planet and cause damage through parasites, competition with native species, physical changes in the environment, and pathogen transmission. The globalization of trade and travel increases the spread of non-native species. A large number of species have been introduced to the land for agriculture, sports, and pets. Some of the introduced species have already flourished in native ecosystems and have a significant impact (Pötzelsberger et al. 2021). Animals and plants introduced for agricultural activities contribute to economic growth but can also cause notable damage to the ecology. In particular, animals, namely, rabbits, goats, cows, buffaloes, pigs, donkeys, horses, and camels, destroy natural habitats through intense or selective grazing. Animals

having strong hoofs compress the soil and prevent vegetation from regenerating, thus helping to erode it. Also, environmental weeds compete with native plants for large invasions of space, nutrients, and sunlight, thereby altering the natural diversity and dynamics of ecological communities and threatening the survival of many plants and animals. Specifically, invasive pests and pathogens can lead to the extinction of many species. Some invasive plant and woody species, such as *Robinia pseudoacacia*, *Ailanthus altissima*, and *Acacia* sp., can also adversely affect native biodiversity, as they may compete with each other or with native species (Young et al. 2017).

24.2.3 Pollution

Atmospheric deposition has severe impacts on biodiversity. While acid emissions have declined in recent decades, the emissions of sulfur dioxide, ammonia emissions, and nitrogen oxide from livestock, industry, or traffic in large parts of the planet are very high. Nitrogen deposition causes the extinction of specialized species in nutrient-poor areas, especially lichens and plants. Other dependent species, such as insects, lose their habitat with the extinction of these species (Young et al. 2017). Soil acidification has affected food networks due to atmospheric inputs. Furthermore, marine habitats and river estuaries are affected by runoff from urban, agricultural, and industrial areas. It can transport herbicides, pesticides, fertilizers, sewage, oil, industrial effluents, and sediments into the water (Young et al. 2017).

24.2.4 Overexploitation

Some plant and fungal species are overexploited, especially if they are slow-growing. For instance, stocks of wild ginseng, which is valued for its health benefits, or cactus pivot due to the hallucination effect and usage in sacred ceremonies, are declining. Also, with the increase in population and individuals' demand for food, the number of livestock is increasing, which causes uncontrolled grazing and destruction of vegetation (Gaujour et al. 2012).

24.2.5 Climate Change Associated with Global Warming

Climate change is one of the serious current and future threats to biodiversity that directly affects species as well as their habitats. Climate change is in favor of some species with specific characteristics, while the abundance of other species may be reduced or even extinct, at least locally. Pests and diseases may also benefit from climate change through increased winter temperature or more extended periods of

vegetation. These effects on the species increase and may lead to change. Climate change is also shifting species that try to stay within the consistent temperature range to higher latitudes and altitudes. In this regard, it should also be noticed that the range of species and habitats cannot be moved freely. Species, for example, found at the heights of low-lying mountains cannot move to higher altitudes as warming progresses. Finally, climate change will also cause changes in the composition and functional characteristics of plants (Pötzelsberger et al. 2021).

24.3 Types of Tissue Culture

There are different types of tissue cultures depending on their applications. Here, some of them are defined briefly.

24.3.1 *Callus Culture*

Plant tissue culture can be traced back to Gottlieb Haberlandt (1854–1945), who established the first callus culture at the beginning of the twentieth century (Bonner 1936). Callus culture can be defined as the production and maintenance of a mass of nonspecialized, organized, and divisible plant cells, tissues, or organs and their growth on artificial nutrient media in glass vials under controlled aseptic conditions. More specifically, calluses are formed when the explants are grown in a suitable medium. Callus culture involves the growth of differentiated and non-differentiated cells, which is then followed by a method that induces organ differentiation. Culture is often performed on a medium, which consists of agar and a mixture of macro- and micronutrients depending on the cell type (Sharma et al. 2015; Baday 2018).

24.3.2 *Organ Culture*

In vitro culture refers to the maintenance of the entire or part of an amputated organ according to its function. Any organs, including branches, roots, leaves, and flowers, can be employed as explants for the cultivation process. Organ culture is used to maintain the structure or function of the plant and allows the organ to retain its features. The growth of distinct structures continues as the organ retains its physiological characteristics (Sharma et al. 2015; Baday 2018).

24.3.3 *Single-Cell Culture*

A single-cell culture is an approach for the growth of isolated cells on a nutrient medium under controlled conditions (aseptically). That is a typical technique for obtaining critical medical substances from plant cells. Specifically, the callus is stimulated from the known part to produce the required materials. Then, the callus is transferred to a liquid nutrient medium that is constantly circulated with a shaker at a certain speed to separate the cells. This method has negative points such as high costs, rapid contamination, and lack of appropriate output compared to the other techniques. Likewise, in suspension culture, a cell or small cell masses proliferate while suspended in a stirred liquid medium. Suspension cultures are used in the induction of embryos and somatic shoots, production of secondary metabolites, mutagenesis and selection of mutants, and genetic transformation studies (Sharma et al. 2015; Baday 2018).

24.3.4 *Embryo Culture*

Embryo culture is the most successful technique of tissue culture. Embryo culture is the aseptic separation of embryos at different stages of development from the mother tissue of the mature seed or capsule and in vitro culture under controlled physical conditions in glass vials containing semisolid or liquid nutrients. The term embryo culture is used to refer to the culture of a sexually produced zygote embryo. Embryo culture may involve the use of mature or immature embryos. Mature embryos for cultivation are obtained mainly from mature seeds. Immature embryos involve the use of immature embryos from immature seeds or hybrids that have failed to germinate. By doing this, the fetus can produce a living plant. The ovule, seed, or fruit from which the fetus emerges becomes infertile. Sucrose and salt are used to supply the fetus with nutrients. Abortion is one of the most common cases that occur sometimes after a cross between species. In cases where the abortion happens in the very early stages after direct fertilization, the fetus cannot be separated due to its small size. The researchers, thus, divide the entire ovule and fertilize it until the fetus grows (Sharma et al. 2015; Baday 2018).

24.3.5 *Anther Culture*

Anther culture is one of the oldest and most widely used techniques for producing single chromosomal plants. This method was first applied in the datura plant. It was utilized for several crops, such as rice, wheat, corn, potatoes, and tobacco. Pollen growth is only achieved with minimal growth of surrounding tissue. The pollens regularly turn into embryos or, like seeds, into irregular masses of calluses. In this

process, the branches containing buds are removed at a suitable sterile surface, and the petals and leaves are separated. The stems grow directly on an appropriate nutrient media. Cultivation of this method is much easier than pollen; however, some precautions can be taken using the anther wall. Thus, the resulting plants are diploid dual chromosomes as well as plants containing half the number of haploid chromosomes that originate accordingly (Sharma et al. 2015; Baday 2018).

24.3.6 Pollen Culture

One in vitro method is pollen culture. In this procedure, pollen grains are separated from intact anthers. Afterward, they are cultured in a nutrient medium in which the microspores are located without producing male gametes (Sharma et al. 2015; Baday 2018).

24.3.7 Somatic Embryogenesis

Somatic embryogenesis is a process in which one or a group of cells that begin the growth pathway contributes to the reproduction of non-zygote embryos. These embryos can germinate to form complete plants. This approach is one of the fastest and most widely used methods. Another advantage is producing a large number of plants. It is possible to create callus tissue from any part of the plant in a food center containing auxin and replant it in a new dioxin-free environment, which induces physical embryogenesis (Sharma et al. 2015; Baday 2018).

24.3.8 Protoplast Culture

Protoplast cultures isolated from wall-less cells surrounded by a plasma membrane are called protoplast cultures. Protoplast culture is an effective strategy that provides multiple single cells and can be utilized for various studies. The protoplast regenerates the cell wall and then undergoes a process of cell division to form a callus. It is then cultured for continuous growth in a suitable environment under aseptic conditions. The technique of isolation, culture, and integration of protoplasts is the solution to the problem of species incompatibility and the growth of embryos that are not found naturally. It also allows the transfer of new genetic material from one cell to another. This technique is done by the cell wall, either chemically or by cell membrane enzymes such as cellulose, which then separates the protoplast in the presence of certain chemicals such as sodium nitrate, calcium ions, or polyethylene glycol (PEG) (Sharma et al. 2015; Baday 2018).

24.3.9 Shoot Tip and Meristem Culture

The tips of shoots containing apical meristems can be grown in vitro to produce shoots from axillary or acquisitive buds. This technique can be employed for clonal proliferation. Although this method is not guaranteed, it is common. The results are accepted in terms of obtaining healthy plants from contamination. The length of the developed summit is usually between 0.1 and 20 mm. A plant, thus, grows relatively slowly, and its success depends on the size of the cultivated area. The larger the size, the greater the chance of success and the less chance of obtaining healthy plants (Sharma et al. 2015; Baday 2018).

24.4 Plant Tissue Culture Media

Plant tissue culture media are divided into three main categories including nutrients, chelating agents, and solidifying agents. The nutrients are separated into organics and inorganics (Fig. 24.2). Tissue culture media provide water, minerals, vitamins, hormones, plant growth regulators (PGR), organic compounds, and other nutrients desirable for the further growth and development of cells or tissues in the undifferentiated callus mass, as well as the optimum pH for the substrate. They also act as a discharge site for secondary metabolites. They provide access to the atmosphere for gas exchange.

Plant tissue culture chemicals must be free of impurities. The preparation of tissue culture medium is a significant step to induce desirable characteristics or to study the growth and development of selected tissue. The preparation of the stock solution is the initial step, followed by the preparation of the medium. Due to the nature of cells or tissues, the standard combination of media should be used for optimal growth and development and can be optimized for better results. Selecting a new or pre-created environment for new species or varieties requires a great deal of optimization to create a cell line in vitro condition. Not all cells play a role in callus formation, and not all plants can efficiently form cell lines. Therefore, it is vital to optimize the environment for plants that are difficult to grow. Since there is no ideal approach that is efficiently useful for unknown explants, a large number of strategies can be employed to assess the nutritional needs of a newly found plant, which can be manipulated later for further research (Bhatia 2015).

24.5 Stress Factors in Plant Tissue Culture

Conditions are controlled in tissue culture. Hence, abiotic and bio-stresses are eliminated as much as possible. Temperature stress, salinity stress, and heavy metal toxicity do not normally occur in tissue culture. Although high temperatures

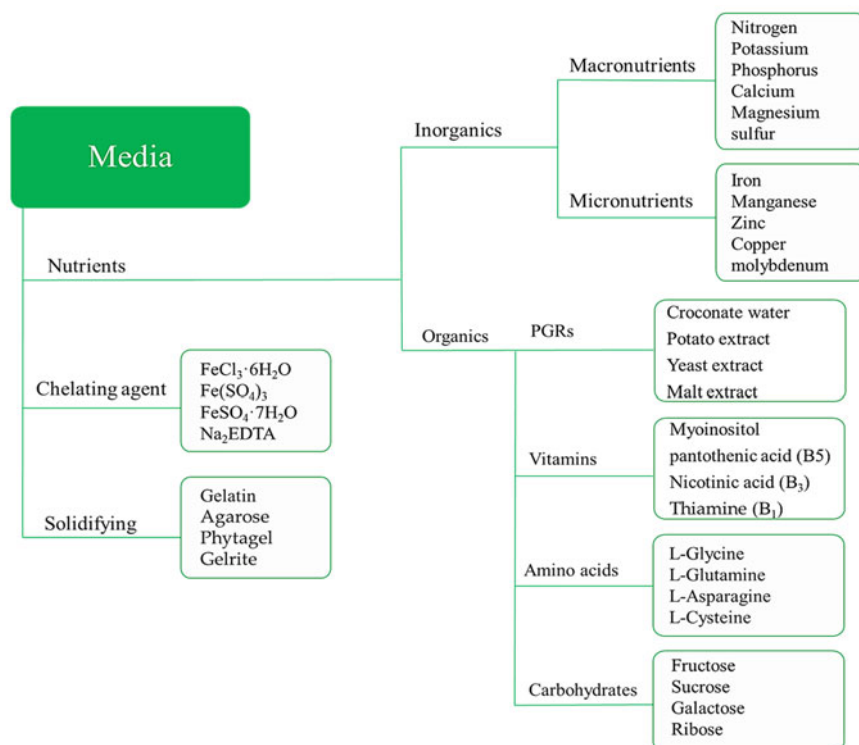


Fig. 24.2 Summary of the main component of tissue culture media

are essential for the *in vitro* thermotherapy, cold stress happens only in certain treatments, such as prolonged maintenance and dormancy disruption. During peeling, tissue culture plants suffer from drought stress due to stomata malfunction. The solidified medium probably has a similar effect on the roots. Stress during adjustment has been fairly examined. In addition to the malfunction of the water storage system, the low rate of photosynthesis is a significant limitation that leads to light inhibition. Both light inhibition and drought stress can produce reactive oxygen species. During adaptation, hydrogen and oxygen levels rise, and tissue culture develops an antioxidant enzyme protection system (De Klerk 2007).

Plants are deformed through *Agrobacterium* attack. Pathogens trigger an oxidative explosion that may damage tissues or cells. The deformity is fixed by a stress pre-treatment known to reduce the harmful effects of significant stress by adding antioxidants. Manipulating operators at the beginning or during culture often means getting injured. This is probably experienced as a herbivorous attack. Cutting a small meristem is believed to coincide with an oxidative explosion that damages the meristem. Isolation of protoplasts can also be considered a wound, and the development of ROS is observed. Hyperhydricity is a common disorder in tissue culture. It is driven by various conditions alone or at high humidity, gelite as the solidifying

agent, and high levels of growth regulator. The leaves of the hyperhydric have high water content and chlorophyll deficiency. They have abnormal anatomical features such as reduced cell layers, irregular stomata, chloroplast degradation, and thin or no cuticle. It has been reported that hyperhydric tissue exhibits the characteristics of abiotic stress, especially hydrogen, oxygen, and increased antioxidant enzymatic activity such as catalase and superoxide dismutase activity. In addition to these stresses, various specific therapies in tissue culture, such as thermotherapy and cryopreservation, are stressful (De Klerk 2007).

24.6 Micropropagation of Medicinal Plants

In the following six endangered medicinal plants, including *Withania somnifera*, *Zhumeria majdae*, *Picrorhiza kurroa*, *Ginkgo biloba*, *Swertia chirata*, and *Gymnema sylvestre*, are studied.

24.6.1 Case Study 1

24.6.1.1 Distribution of *Withania somnifera*

W. somnifera belongs to the Solanaceae or nightshade family. It is commonly known as winter cherry, Ashwagandha, poison gooseberry, and Indian ginseng. This plant is native to South Africa, the Mediterranean region, and India. It is distributed in drier and subtropical regions such as Gujarat, Madhya Pradesh, Punjab, Rajasthan, Uttar Pradesh, and the Himalayas such as Jammu and Kashmir (Haq 2011). It is terrestrial in nature and is distributed in drier and warmer regions of countries such as China, Sri Lanka, Pakistan, Afghanistan, Yemen, Palestine, Spain, Egypt, Congo, East Africa, and Madagascar. *W. somnifera* is cultivated in many arid parts of India. Dried rocky soil with sunlight is preferable for this plant compared to partial shade. It can be grown from seed in early spring or cuttings in late spring (Aslam et al. 2017).

24.6.1.2 Habitat

Ashwagandha grows in marginal barren lands and low-fertility areas. This plant is distributed in arid subtropical regions and at the base of hills in deciduous and *Pinus roxburghii* forests. It is a low shrub with a height of 1.50 m and has tall branches. The plant grows well in sandy, red, black, and loamy soils and has a pH of between 6.5 and 8.0 with adequate water drainage. It can be cultivated up to 1000 m above sea level. Ashwagandha prefers subtropical climates (Haq 2011; Aslam et al. 2017).

24.6.1.3 Morphological Description

This plant is short and has straight shrubs with a height of 35–75 cm. It is grayish and has long tuberous roots. The branches grow radially from a central stem. The leaves are matte green and oval in shape and have petioles with a length of 10–12 cm. The flowers are small, bisexual, green, solitary, and bell-shaped. The calyx is gamosepalous and has five inflated lobes of 3–5 mm with a crescent in the fruit. The corolla consists of five greenish-yellow lobes of 5–8 mm. Also, there are five flags. The ovary is glabrous, spherical, and ovoid, with many ovules as well as a two-lobed stigma. The ripe fruit is spherical and reddish-orange and covered with a large calyx. There are a large number of disc-shaped, reniform, and yellow seeds (Mozaffarian 1998; Aslam et al. 2017).

24.6.1.4 Phytochemicals

Chemical investigation of *W. somnifera* revealed that the main constituents are withaferin A, several sitoindosides, about 40 withanolides (withanolides are structurally similar to ginsenosides of *Panax ginseng* leading to the common name of “Indian ginseng”), and 12 alkaloids. Among all these compounds, withanolides are the main components. Withanolides contain a steroid nucleus, a side chain, and a six-membered lactone ring (Padmawar 2004). Ashwagandha’s remarkable pharmacological activity is characterized by two main withanolides, withaferin A and withanolide D (Fig. 24.3) (Mirjalili et al. 2009). Further chemical analysis of the alkaloids such as anaferrine, anahygrine, beta-sitosterol, chlorogenic acid in leaves, cysteine in fruit, iron, cuscohygrine, pseudotropine, somniferinine, scopoletin, tropanol, somniferiene, withananine, withanine, and withanolides A–Y steroidal lactones have been performed (Rohit et al. 2012; Aslam et al. 2017).

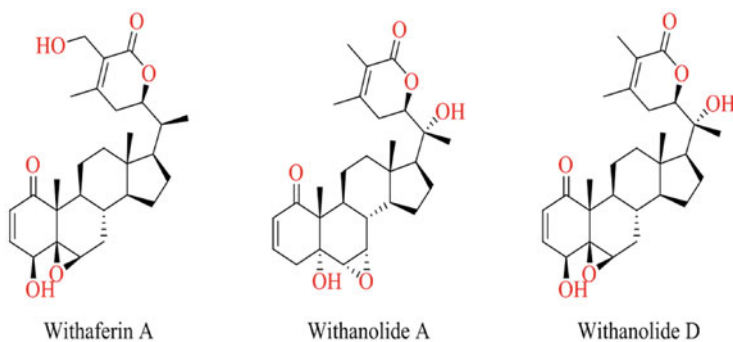


Fig. 24.3 The chemical structure of withaferin A, withanolide A, and withanolide D

24.6.1.5 Pharmacology

Traditionally, it has been used as a medicine against various diseases for thousands of years and is an essential component of more than 200 traditional medicine formulations. *W. somnifera* has versatile medicinal potential. This plant has antimicrobial, anti-inflammatory, anticancer, anti-stress, antianxiety, anti-seizure, immunomodulatory, and cardiovascular activities. The root extract is safe and painless and has blood sugar-lowering, cholesterol-lowering, hypnotic, diuretic, sedative, and disinfectant properties (Shasmita et al. 2018). It is operated on gynecological disorders, bronchitis, arthritis, and drug addiction therapy. The medicinal possessions of *W. somnifera* are predominantly due to various steroid lactones known as withanolides. Withanolide A and withaferin A are key secondary metabolites attributed to the medicinal properties of *W. somnifera*. Apart from these two, there are other significant pharmaceutical compounds such as somniferine, somniferinine, withanine, withananine, withanone, pseudowithanine, tropine, pseudotropine, and withanolide B. *W. somnifera* has different biological properties including a high anti-inflammatory activity. This activity is associated with biologically active steroids. Withaferin A is a major component of these steroids. Its effectiveness is comparable to the dose of hydrocortisone (Aslam et al. 2017). This plant demonstrated the potential to suppress tumor growth. Withanolide E affects only T lymphocytes, while withaferin A potentially affects B and T lymphocytes (Khare 2007). Potential chemical compounds of *W. somnifera* are effective against pathogenic bacteria, fungi, and viruses (Khare 2007). Studies have also shown that Ashwagandha is a potent antimicrobial agent with moderate antifungal and antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Antifungal and antibacterial properties in withanolides isolated from the ethanolic extract of the plant demonstrated that methanolic extract had the maximum inhibitory activity against a range of bacteria. Oral administration of aqueous extract of its fruit successfully eliminated *Salmonella* infection in mice. Methanol, hexane, and diethyl ether extracts from both leaves and roots of *W. somnifera* were also evaluated for antibacterial activity against *Salmonella typhimurium* and *Escherichia coli* (Rohit et al. 2012). *W. somnifera* increases thyroid activity and ultimately affects cellular antioxidant systems. These results characterize Ashwagandha as a valuable plant in the treatment of hypothyroidism (Panda and Kar 1999). The effects of hypocholesterolemia, hypoglycemia, and diuretic of Ashwagandha root in human subjects were investigated. Significant increases in urine volume and urinary sodium and decreased serum cholesterol as well as triglycerides and lipoproteins were observed.

24.6.1.6 Micropropagation

Increased demand for *W. somnifera* has severely damaged its natural reservoirs in the contemporary era. Due to habitat destruction, overexploitation, and illegal collection of this plant, it is on the verge of extinction in various parts of the

world. Unique strategies must be employed to maintain this significant plant because of its medicinal reputation and importance. *Withania somnifera* is on the list of 36 major medicinal plants grown in India as the global demand for raw materials of this species is very high. However, the production of plant materials is partially met. The reason why this is the case is that there are limitations in conventional propagation. This plant is usually propagated only by seeds. Seed survival is limited to 1 year, which makes long-term seed storage ineffective. The presence of inhibitors in the seed and fruit wall is another problem, making seed germination poor. In addition, the disease of young seed and seedling rot also reduce its population. These factors indicate that the conventional propagation method is long and tedious and does not meet commercial needs. Therefore, there is a need for plants to grow in vitro (Aslam et al. 2017).

The micropropagation technique with the auxiliary branching method using the shoot tip as an explant is a quick and effective method. Shoot culture is started on MS medium containing 0.5–2.0 mg/L BA with 0.2–0.5 mg/L NAA (naphthaleneacetic acid) containing 3% commercial sucrose and 0.8% agar. This rapid regeneration protocol can be used for the large-scale production of commercially grown cultivars. This direct regeneration method reducing the genetic flux commonly seen during callus-mediated regeneration helps to produce a large number of selected Ashwagandha varieties that are in high demand in the current pharmaceutical market (Singh et al. 2016).

Callus-mediated somatic embryogenesis was reported for *W. somnifera*. Axenic cotyledonary leaf explants provided embryogenic callus. When the callus was cultured on MS containing 0.3 mg/L Kin, the highest frequency of somatic embryo formation was monitored. In another study, leaf and internodal sections were utilized for somatic embryogenesis in *W. somnifera* by Sharma et al. They discovered that leaf explants were more potent than internodal explants. MS along with 0.5 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) and 1.0 mg/L BAP (6-benzylaminopurine) was used to induce embryogenic callus from leaves. Subculture of embryogenic callus was performed on the same medium and hormonal combination with 10 mg/L casein hydrolysis. It led to embryogenic maturation during different intermediate stages. Notably, there was no single-stage conversion of somatic embryos to seedlings in either report. Shoot emergence was observed when mature embryos were transferred to MS medium which was amplified with 1.0 mg/L BAP and 2.0 mg/L IAA (indoleacetic acid) or 0.5 mg/L BAP. However, by agreement with both groups, there was a need to transfer these branches to MS-based media with 0.1 mg/L IBA (indole-3-butyric acid) for pre-adaptation rooting (Shasmita et al. 2018).

It was found that BAP alone or in combination with Kin or IBA or IAA or NAA gives optimal branch amplification from meristems. TDZ (thidiazuron) was conveyed to be effective in improving the proliferation of axillary shoots in *W. somnifera*, which may be due to its capacity to increase the biosynthesis and accumulation of endogenous adenine cytokinin. The MS and NN (Nitsch and Nitsch) were the most appropriate media for shoot induction and propagation. Sucrose was preferred as the source of carbon for *W. somnifera* meristem culture. Having said that, other carbon sources were not useful. Sucrose at the optimal

concentration (3%) was not as efficient as fructose (2%) for shoot regeneration. Also, glucose was ineffective for shoot initiation and propagation (Shasmita et al. 2018).

A protocol for the production of artificial seeds using sodium alginate and calcium chloride through somatic embryogenesis was developed, which can also be very useful for medicinal plants such as *Withania* (Iqbal et al. 2016).

24.6.2 Case Study 2

24.6.2.1 Distribution of *Zhumeria majdae*

Zhumeria majdae is a relict and small fragrant perennial shrub with gray foliage of the Lamiaceae family. Although the Lamiaceae family is globally distributed, the plant is found only in a limited geographical area in subtropical climates such as Geno, which is a unique mountainous terrain located in southern Iran, northwest of the Persian Gulf port of Bandar Abbas, due to its soil containing sulfur compounds that may affect the germination percentage of seeds (Aghazadeh and Behboodi 2015). Botanically, it was first collected in 1966 and subsequently described as a new genus in 1967 by Rechinger and Per Wendelbo and named in honor of its compiler, *Z. majdae* Rech. f. & Wendelbo (Rechinger and Wendelbo 1967). The plant was known to locals before the botanical description. The natives know this monotype genus by other names such as Mohrekhoosh, Moukhash, and Mourkhoosh. It was wildcrafted severely and was exported to the neighboring nations. It was used as folk medicine by the locals. There are 12 available places for this plant on the slopes of the mountain, where there is a small population. Plant conservation status was assessed based on the IUCN (International Union for Conservation of Nature). The status of *Z. majdae* was defined as “endangered.”

24.6.2.2 Habitat

This plant is distributed from 525 to 1430 meters above sea level in rocky lands and rocks with an extremely steep slope of 85–100% of Geno, Abmah, Fino, Sarchahan, Tangzagh, Keshar, Sarchahan, Zad Mahmoud, Sirmand, and Bastak mountains. The highest altitude is in the Sirmand region at 1430 m, and the lowest is in the Geno region at 525 m from sea level. The plant distribution in all areas is geologically on Miocene limestone and marl lands. This formation has a wide dominance in the northern and western heights of the province. By examining the resource map and the ability of the province's lands, the distribution of the Mohrekhoosh can be seen in the mountainous land type. This unit of land, which consists of relatively calcareous and dolomitic high mountains, is often soilless or with very shallow, pebbled, and nonuniform soil (Soltanipour et al. 2020).

24.6.2.3 Morphological Description

This plant is a 50-cm-tall shrub, very aromatic, with grayish-white skin and branched stems. The entire leaves are almost uniform, ovate, broad, and oval without petioles. The flowers are bluish-purple, and large, with straight petioles and bayonet petals. The gamosepalous calyx has five sepals and is perennial, ovate, and double-edged. Corolla is purple-blue, white, and straight. There are four flags: two fertile and two almost sterile and out of the calyx. Styles are very long, and the stigma has two unequal branches. The seeds are ovate, oval, almost triangular, and pale brown, with a smooth surface (Soltanipour et al. 2020).

24.6.2.4 Phytochemicals

Twenty-nine components (e.g., α -pinene, camphene, limonene, α -terpineol, (E)-caryophyllene) have been identified in *Z. majdae* essential oil by GC-MS analysis. Linalool (39.41%) and camphor (30.94%) were determined as the predominant compounds (Fig. 24.4) (Karami et al. 2021). Figure 24.4 illustrates a diterpene, known as 12,16-dideoxy aegyptinone B isolated from *Z. majdae*. This compound exhibited potent antileishmanial activity with an IC_{50} of 0.75 $\mu\text{g/mL}$ and a selectivity index of 16 compared with renal fibroblasts ($IC_{50} = 12 \mu\text{g/mL}$) (Moein et al. 2008).

24.6.2.5 Pharmacology

Z. majdae is one of the most important and widely used medicinal species in the Hormozgan province, which is very common in traditional medicine. The leaves of the plant have antioxidant properties. Its leaves have been used to treat digestive disorders such as diarrhea, stomachache, flatulence, and indigestion and heal headaches, colds, and wounds. Many studies have conducted various biological activities of *Z. majdae*, including anti-inflammatory, anti-leishmaniasis, antiplasmodial, antinociceptive, antibacterial, and antiviral activity (Fallah et al. 2019; Karami et al. 2021).

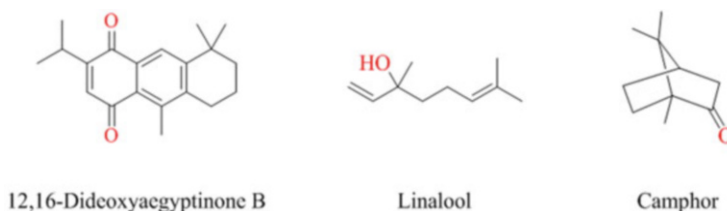


Fig. 24.4 The chemical structure of 12,16-dideoxy aegyptinone B, linalool, camphor

24.6.2.6 Micropropagation

Factors such as limited natural habitats and a low reproductive and irregular harvest have caused this plant to be endangered and destroyed. The importance of the reproduction of this species is more to preserve and revive it in the habitats of southern Iran and its medicinal use.

First, the explants of leaves, stems, and roots are washed with water for 15–30 min to perform this experiment. Next, distilled water and a few drops of dishwashing liquid were used to rinse them for 5–10 min. The explants were sterilized with 80% ethanol solution for 2–4 min and soaked in 10% sodium hypochlorite with Tween 80 (0.05%) for 4–8 min. Afterward, they were washed three times with distilled water and cultured on an MS medium containing different concentrations of 2,4-D (1 mg/L) as a synthetic auxin, auxin IAA (0.1, 0.5 mg/L) as a natural auxin, N-6-benzyladenine (BA) (1, 2, and 3 mg/L) as a synthetic cytokinin, and kinetin (Kin) (0.5, 1, and 3 mg/L) as a natural cytokinin (Aghazadeh and Behboodi 2015).

In a study on this plant, leaf explants were cultured on an MS medium with BA, putrescine, and Kin. Callus formation and organogenesis of leaf, root, and stem parts were obtained by tissue culture on MS, Kin, and IAA medium and organogenesis from leaves on the same medium with 2 mg/L BA. Furthermore, the effects of IBA and NAA auxins were investigated on the rooting of regenerated branches. The most satisfactory results were obtained for the number and length of roots in 1 mg/L NAA and on MS medium with half the concentration of salts. In this regard, it was pointed out that the reduction of mineral salts in the culture medium is effective in facilitating the transfer of tissue culture plants to the soil and their adaptation. Also, in 1987 Chalupa evaluated the effect of auxin family hormones, including IBA and NAA, or a combination of these two hormones on positive rooting (Dalal and Rai 2004).

24.6.3 Case Study 3

24.6.3.1 Distribution of *Picrorhiza kurroa*

Picrorhiza kurroa is an endangered, small, hairy perennial, and evergreen medicinal plant native to India. It is also known as kutki and Indian gentian from the Scrophulariaceae family (Arya et al. 2013). It is found in the states of Himachal Pradesh, Uttarakhand, Ladakh, Jammu and Kashmir, Sikkim, and Arunachal Pradesh at altitudes of approximately 2700–5000 m (Mohi-ud-din et al. 2022). It grows mainly in the humid soil in the western part of Nepal, usually on cliffs and mountains in the fissures of the rocks facing north, during the spring. This medicinal plant is distributed from Kashmir to Kumaon in the Himalayas and Nepal to Garhwal in northern Myanmar, western China, and southeastern Tibet. It is also seen in the Himalayas of China, Pakistan, Bhutan, and Nepal (Bhardwaj et al. 2021; Rawat et al. 2022).

24.6.3.2 Habitat

It grows in the Himalayas from Kashmir to Sikkim at an altitude of 2700–4500 m and in Nepal between 3500 and 4800 m above sea level, away from the communities. It takes hours to reach its growing habitat. This plant grows well in cool and humid climates. Sandy clay is best for its growth. It requires porous soil layers that facilitate the horizontal expansion of the rhizomes and produce aerial sprouts from the nodes. This plant prefers sufficiently moist and shady places. The plant withstands death during heavy and delayed rains.

24.6.3.3 Morphological Description

P. kurroa is a perennial plant consisting of a lifespan of approximately 3 years and needs a whole year to produce seed. *P. kurroa* is a plant with alternate leaves and a base that is smooth, oval, 5–15 cm long, and 0.5–1.2 cm wide. The stem of the plant is small, and hairy, creeping on the ground with straight leaves and flowers. The flowers are small and pale blue or purple with dense terminal clusters. The flowers have five lobes and are about 8 mm with very tall flags. Depending on the height, the flowering period is from June to August. Seeds appear in September and fruits in October. The fruit is spherical, about 13 mm long, oval, and narrow at the top. Rhizomes are woody, brownish-gray, 12–25 cm long, 4–10 mm thick, tubular, and straight. The roots are grayish-brown, straight, and 5–10 cm long. Both roots and rhizomes have a bitter taste but a pleasant odor (Bhardwaj et al. 2021; Mohi-ud-din et al. 2022).

24.6.3.4 Phytochemicals

The phytochemical constituents of *P. kurroa* have been extensively studied. More than 132 secondary metabolites have been isolated from various parts of the plant, including leaves, roots, rhizomes, branches, and seeds. An essential group of bioactive compounds isolated from rhizomes is kutkoside, pikurosides, picroside I–IV (Fig. 24.5), flavonoids, glycosides, and cucurbitacin. The root and rhizome produce the crystalline product (“picroliv” or “kutkin”), which is a mixture of two C9-iridoid glycosides. Herbal compounds such as 4-hydroxy-3-methoxy acetophenone, pikuroside, and veronicoside and various phenolic compounds are also found in various extracts of this plant (Bhardwaj et al. 2021; Mohi-ud-din et al. 2022). Many other active compound derivatives include apocynin and drosine. This endangered plant also contains other significant carbohydrates, such as D-mannitol. Aromatic acids, namely, vanillic acid, cinnamic acid, and ferulic acid, have been elucidated from *P. kurroa*. Aliphatic homocyclic compound (L-shikimic acid), capryl vanillic acid, vanillin- α -D-glucoside, acyl picraldehydes (myristyl

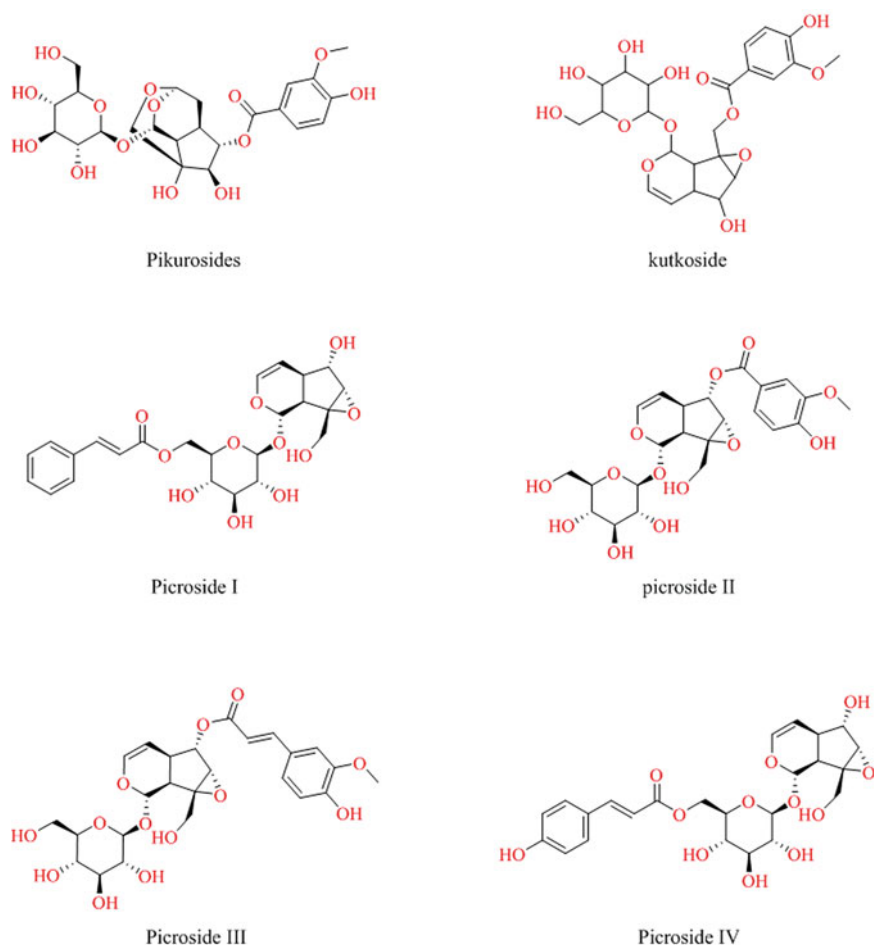


Fig. 24.5 The chemical structure of kutkoside, pikurosides, picrosides I–IV

picroaldehyde), picrortetraglucoside, and picraldehyde α -D-diglucoside are other constituents (Bhardwaj et al. 2021).

24.6.3.5 Pharmacology

P. kurroa is noted for its broad medicinal potential, such as antiviral, anti-inflammatory, antidiabetic, hyperlipidemic, anticancer, cardioprotective, and antioxidant activity. The plant is employed for liver diseases, allergies, and fever. If it is taken orally, it has little effect on the treatment of asthma for up to 12 weeks. A recent study shows that oral doses of *P. kurroa* may reduce the symptoms of anorexia and nausea (Bhardwaj et al. 2021). The antibacterial potency of acetone,

hexane, methanolic, aqueous, and ethanolic extracts of *P. kurroa* rhizome against different gram-negative and gram-positive bacterial strains was evaluated. An ethanolic extract showed a high antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, and *Escherichia coli*. The methanolic extract had a high inhibition of *P. aeruginosa* and *S. aureus*. However, acetone and hexane extracts exhibited a moderate activity against these microorganisms. A report indicated that this plant can be used to treat bacterial and fungal infections based on its antagonistic effects on various pathogenic microbes, including *Bacillus subtilis*, *P. aeruginosa*, *E. coli*, *Micrococcus luteus*, *S. aureus*, *Candida albicans*, and *Aspergillus niger* (Sharma and Kumar 2012; Rathee et al. 2016). *P. kurroa* appeared as a potent antimalarial agent with a significant inhibitory effect on the growth of *Plasmodium falciparum*. Moreover, the methanolic extract inhibited the malaria parasite *P. berghei* (Kr 2012).

24.6.3.6 Micropropagation

The wide range of pharmaceutical activities and the growing interest of people in natural products have led to a high demand for *P. kurroa*. Nearly 300–400 plants are collected to obtain 1 kg of dry roots and rhizomes. The rising demand has put pressure on the natural habitat of *P. kurroa*. Thus, overuse of natural habitat, the lack of awareness of local people, and poor cultivation have led it to the IUCN list of endangered plant species. In vitro breeding techniques are beneficial not only for the rapid and massive reproduction of plant species but also for protecting endangered species.

A study by Lal et al. (1988) is the first report of *P. kurroa* tissue culture using a shoot tip as an explant taken from a natural plant kept under controlled conditions. They discovered that the MS medium with 3.0–5.0 mg/L Kin supported the fast proliferation of multiple shoots of explants. 0.1 mg/L IAA addition to the medium containing Kin showed a significant improvement in the growth of regenerants. Subsequently, Upadhyay et al. (1990) reported in vitro proliferation. Employing BAP at lower concentrations is very useful for inducing multiple shoots. Chandra et al. (2004) also conveyed that those higher concentrations of BAP led to the formation of the hyperhydric shoot during subcultures. Decreasing the concentration of cytokinin in the medium caused the regeneration of regular shoots from the base of vitrified shoots.

Callus culture is a good alternative technique for plant production. Lal and Ahuja (1996) reported the shoot and callus culture for 64 weeks in a hormone-free MS medium at 4–10 °C incubated in the dark. They reported callus induction from leaf explants on MS medium with 0.5–2.0 mg/L 2,4-D. Callus propagation was optimal in the MS medium containing 4.0 mg/L NAA and 1.0 mg/L Kin. Shoot organogenesis was obtained from the callus by its sequential transfer in different environments. Single medium composition for shoot/somatic embryo formation was not found in this species. Regenerated shoots on MS medium containing 0.2 mg/L NAA were successfully established in the soil (Lal and Ahuja 1996).

Jan et al. (2010) stated callus formation in *P. kurroa* in an MS medium containing different concentrations of 2,4-D and direct regeneration with BAP. They found that MS medium at 0.25 mg/L 2,4-D and BAP stimulated callus induction and direct shoot regeneration rate. Indirect and direct production of shoots, propagation, and elongation on MS medium using different concentrations of NAA alone or in combination with IAA and IBA during 10 weeks of culture were reported for the first time.

24.6.4 Case Study 4

24.6.4.1 Distribution of *Ginkgo biloba*

Ginkgo biloba is known as the maidenhair tree from the Ginkgoaceae family. The ginkgo fossil distribution indicates that this genus has been relatively adapted to ecological changes since the Cretaceous. Although *G. biloba* and its other species were once widespread all over creation, its population declined and was limited to a small area in China until two million years ago. It was thought to be extinct in the wild for centuries, but it is now known to grow in at least two small areas in eastern China's Zhejiang Province, in the Tianmushan protected area (Shen et al. 2005). However, there is a high genetic uniformity among ginkgo trees from these areas, which is contrary to the natural origin of these populations. It is suggested that ginkgo trees in these areas may have been planted and preserved by Chinese monks for over a millennium. Cultivated trees of this species are found in various places around the world. However, the occurrence of wild *G. biloba* populations is unknown and has not been established. This study shows greater genetic diversity in southwest China, where several older candidates for wild populations have been reported (Shen et al. 2005; Tang et al. 2012). There is also evidence that the most extensive and oldest *G. biloba* trees may be older than the surrounding human habitats (Shen et al. 2005). Some nations, including China, Germany, and France, have begun efforts to reproduce *G. biloba* trees on a large scale for medicinal purposes. Also, it grows in many parts of the United States and Canada.

24.6.4.2 Habitat

This plant is rarely found in deciduous forests and valleys on acidic fine, sandy, loamy, and even clay soil with good drainage. The soil in which it grows is usually in the pH range of 5.0–5.5 (Patel and Ingalthalli 2013). This plant prefers to grow in warm to temperate climates in areas with humid summers and cold winters. It will not grow in the shade (Lin et al. 2022).

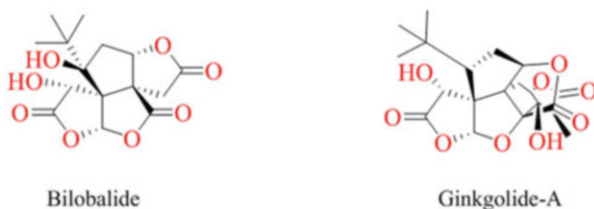
24.6.4.3 Morphological Description

Ginkgo is a large tree usually growing to a height of 20–35 m. Some of them grow up to 50 m in China. This tree has an angled crown and extended, and somewhat irregular, branches (Ansari et al. 2017). The trunk diameter can be about 3–4 m with low branching. Young trees usually have a central trunk in the form of a pyramid, with regular, lateral, ascending, and asymmetrical branches. Its skin is brown and rough. As the tree ages, a wider crown and uneven grooves appear on the trunk. The male tree usually has a narrow columnar shape and is slightly taller. The female tree has a broader crown and a wider body. The live fossil has deep roots and is resistant to wind and snow damage. Young trees are often tall and slender with few branches. The leaves grow slowly, in clusters, and alternately at the ends of the branches in spring (Lin et al. 2022). The leaves are usually 5–10 cm long but sometimes up to 15 cm long. The leaves are unique compared to other trees. They are smooth, leathery, and fan-shaped with holes on both sides, and there are often deep grooves in the middle of the leaf that produce two distinct lobes. The veins are separated from the base of the leaf in pairs and are not connected. They turn green during the summer and turn golden in the fall before being dropped. However, the leaves fall off quickly (Dallimore et al. 1923; Schmid et al. 1993). In ginkgo, short branches allow new leaves to form on old parts of the crown. After a few years, a short branch may turn into a long branch.

24.6.4.4 Phytochemicals

G. biloba leaves have been extensively studied with a wide range of therapeutic activities. The active chemical components in *G. biloba* leaves include 6% terpenoids, 5–24% flavonoid glycosides, 10% organic acids, and other bioactive compounds having a wide range of beneficial health effects. This extract has a variety of medicinal activities, including anti-allergic, antioxidant, anticancer, anti-inflammatory, antibacterial, and cytotoxic activities. Ginkgo leaf extract contains phenolic acids, polyphenols, proanthocyanidins, ginkgo bilirubin, flavanols, alkyl phenols, ginkgolides (A, B, C, J, and M), bilobalide, and flavonoid glycosides such as myricetin, quercetin, kaempferol, isorhamnetin, terpenes, and trilactones (van Beek and Montoro 2009; Noor-E-Tabassum et al. 2022; Fig. 24.6).

Fig. 24.6 The chemical structure of bilobalide and ginkgolide-A



24.6.4.5 Pharmacology

G. biloba possesses various healing properties. It is commonly used to treat asthma and bronchitis. The nuts and leaves have been used as a traditional Chinese medicine for thousands of years. *G. biloba* leaves have traditionally been used to treat skin infections and heart disorders. *G. biloba* has been shown to effectively treat the symptoms associated with cerebrovascular illness, Alzheimer's disease, peripheral vascular disease, epilepsy, and stroke. Evidence suggests that *G. biloba* performs a range of pharmacological activities, such as lowering the risk of platelet antagonization and cardiovascular disease, increasing cerebral blood flow, hepatoprotective functions, and preventing ischemia-induced oxidation (Noor-E-Tabassum et al. 2022).

Antimicrobial activity has been investigated for several components of *G. biloba*, including polysaccharides, proteins, and phenolic acids. Gingolic acids have been experimented with to examine the antimicrobial performance of *G. biloba* seeds. The antimicrobial activity of gingolic acids is supported by the presence of an alkyl side chain. Gingolides have been shown to have antibacterial activity against *S. aureus*, *B. cereus*, and *B. subtilis*. In contrast, gingolic acids displayed potent antibacterial activity against *E. coli* with a minimum inhibitory concentration (MIC) of 7.5×10^{-3} g/mL. Ginkgolides also revealed antimicrobial activity of 2.5×10^{-4} g/mL and 1.5×10^{-4} g/mL against *Penicillium* and *B. subtilis*, respectively. Proteins with broad-spectrum antibacterial activity in *G. biloba* were identified using ammonium sulfate precipitate. *Klebsiella pneumoniae* was inhibited by the protein with MIC 1.0×5^{-10} g/mL and *Aspergillus niger* with MIC 1.0×10^{-10} g/mL. In total, polysaccharides make up 50–80% of the ethanol precipitate of *G. biloba* seeds, and 87.4% of polysaccharides and 1.0% of proteins show antibacterial properties (Afanas'ev 2015).

24.6.4.6 Micropropagation

The IUCN listed this species as an endangered plant due to the rapid decline in its numbers worldwide. A report shows that this plant only exists in cultivated form until it was confirmed that there is a wild population in China. The possible existence of wild *G. biloba* populations has been specifically discussed in China. However, Tang et al. concluded that the population of *G. biloba* in the foothills of the Dalou Mountains shows remnants of the natural range of *G. biloba*, since this species has a very rare and limited wild population and most of the output trees are cultivated.

Camper et al. (1997) cultured the tissues of ginkgo embryo with cotyledon tissue in an MS medium with different concentrations of 2,4-D, NAA, and BA in the light. They found that three embryos were produced, which had the highest callus production. In another study, callus culture and control of callus browning of ginkgo leaf explants were investigated. It was found that MS medium with 1 mg/L BA and

3 mg/L NAA supplementation is the best. Also, phytic acid promotes callus growth and controls browning.

Ghaem and Labafi (2005) researched ginkgo tissue culture showing that the best explants for ginkgo tissue culture are embryo-derived explants, and the best hormonal combination for ginkgo tissue culture is 0.5 μmol of BA and NAA. Another experiment on different hormones (Zeatin; Kin; 2,4-D; BA NAA; IBA; IAA) concluded that the combination of 0.5 IBA and Zeatin is better than the other compounds, and the last experiment deduced that 0.2 and 0.01 μmol of Zeatin and IBA is the best treatment for in vitro culture, respectively.

The response of petiole and embryo microsamples to different hormones was examined by Inoue et al. (1996). They concluded that the addition of 2,4-D or NAA to both explants resulted in the formation of green callus. The addition of IAA or IBA alone increased the germination of immature embryos, and adult embryos germinated in a hormone-free culture medium.

24.6.5 Case Study 5

24.6.5.1 Distribution of *Swertia chirata*

Swertia is a genus of the Gentianaceae family including a large group of annual and perennial plants (approximately 135 species). *Swertia* species contain common compounds in several herbal medicines. In India, 40 species of *Swertia* have been recorded. *S. chirayita* is the most significant one used for its medicinal properties. This plant was first described by Roxburgh as *Gentiana chyrayta* in 1814. Chirayita is also known by a set of names such as Anaryatikta, Bhunimba, Chiratitka, Kairata in Sanskrit, Qasabuzzarrah in Persian, Chiaravata in Urdu, Sekhagi in Burma, and Chiraita in Nepal. *S. chirayita*, commonly known as “Chireta” grows at high altitudes in the temperate Himalayas between 1200 and 2100 m above sea level from Kashmir to Bhutan in the range of moist shady places. Its widespread usage in traditional medicine has led to the overexploitation of natural habitats and is now on the verge of extinction in nature. Some authors have described *S. chirayita* as annual and others as biennial or perennial (Kumar and Van Staden 2016; Jadhav et al. 2022).

24.6.5.2 Habitat

S. chirayita grows in mild and temperate areas of the Himalayas. Fragile soils from loamy to sandy with good drainage are suitable for this plant. The soil should be enriched with manure, and the addition of sand is recommended if the soils are clayey. This medicinal plant can grow in areas with light rainfall of about 100 cm per year and areas with prolonged and cold winters (Jadhav et al. 2022).

24.6.5.3 Morphological Description

S. chirayita is a 1–2-year-old plant that grows to a height of 0.6–1.5 m. It has a vertical stem about 1 m long. The middle part is cylindrical, while the upper part is quadrangular and has a line at each angle. The orange, brown, or purple stem is large and has a continuous yellow pith. The 4 cm leaves are lanceolate and acuminate, have no stalks, are sessile, and have five to seven nerves. The roots are usually 1.2 cm thick and 7–8 cm long, simple, tapering, yellowish, and somewhat oblique. Flowers are tremendous, leafy, small, and yellow with a tinge of purple, green, or white hairs. The calyx has four lobes twisted and stacked on top of each other, united at the base, and each lobe has a pair of nectaries covered with long hairs. It contains four stamens and two stigmas. Capsules are egg-shaped with two valves and a yellowish pericarp. The ovary is unilocular with laminal placentation parietal ovules. The seeds are numerous, small, and dark brown (Kumar and Van Staden 2016; Jadhav et al. 2022).

24.6.5.4 Phytochemicals

The extensive biological activity of *S. chirayita* is due to the presence of a diverse group of bioactive compounds belonging to different classes, including xanthenes and their derivatives (Aamir Quazi et al. 2022), lignans, alkaloids, flavonoids, terpenoids, and iridoids, secoiridoids, and other compounds such as chiratin, ophelicacid, palmitic acid, oleic acid, and stearic acid. Chiratanin was the first isolated dimer xanthenes found in various parts of *S. chirayita*. The drug efficacy of *S. chirayita* has been attributed to the biological activity of major phytochemicals, including sweroside, gentiopicroin, mangiferin, amarogentin, swertiamarin, swerchirin, and amaroswerin (Fig. 24.7) (Kumar and Van Staden 2016).

24.6.5.5 Pharmacology

S. chirayita is employed by locals in various forms for several medicinal purposes. The whole plant is used in traditional medicine, but its root is mentioned as the most active bioactive part. A range of medicinal properties such as treatment of fever, bacterial and viral infections, inflammation, malaria, anemia, asthma, skin diseases, epilepsy, hepatitis, liver disorders, gastritis, constipation, indigestion, ulcers, hypertension, melancholy, and includes certain types of mental disorders, bile secretion, blood purification, and diabetes have been observed by using this plant. Recently, *S. chirayita* extract has shown anti-HBV activity. Herbal formulations such as Ayush-64, Diabecon, Mensturyl syrup, and Melicon V ointment contain various concentrations of *S. chirayita* extract due to its antipyretic, hypoglycemic, antifungal, and antibacterial properties (Kumar and Van Staden 2016; Jadhav et al. 2022). In particular, the compounds mentioned in the previous section have properties that

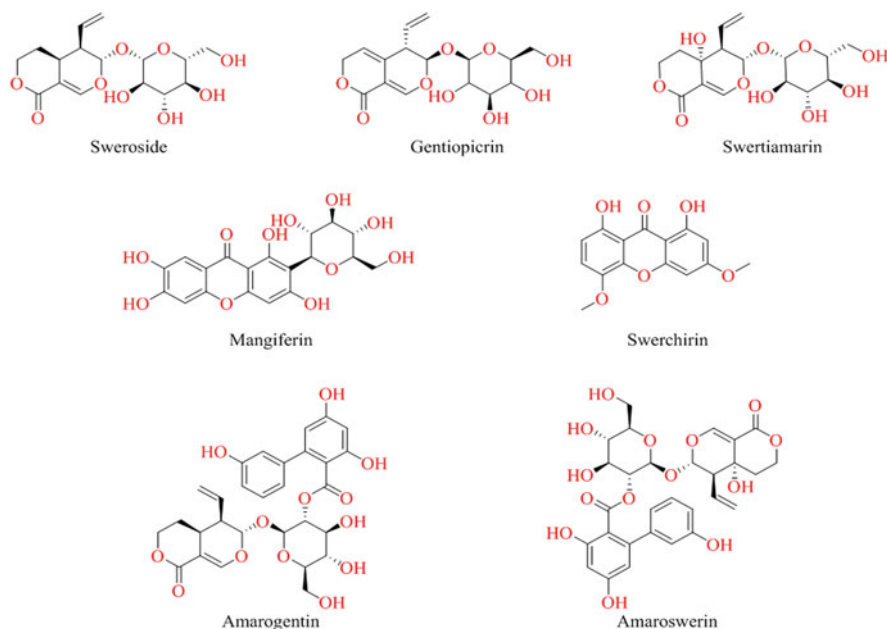


Fig. 24.7 The chemical structure of sweroside, gentiopirin, mangiferin, amarogentin, swertiamarin, swerchirin, and amaroswerin

are discussed below. Antidiabetic, anticancer, and anti-leishmaniasis properties have been reported for amarogentin. Swertiamarin has been tested for anti-hepatitis, anticancer, and anti-arthritis activities. Sweroside has been reported to be an antibacterial, liver protective, preventative in the treatment of hyperpigmentation, and a promising natural product for the treatment of osteoporosis. Mangiferin has also been reported to be antidiabetic, anti-atherosclerotic, anticancer, anti-HIV, and anti-Parkinson's. Swerchirin is known as an antimalarial and hypoglycemic, liver protector, and blood booster, with blood glucose-lowering activity (Kumar and Van Staden 2016).

24.6.5.6 Micropropagation

Micropropagation techniques have played a vital role in creating genetically uniform *Swertia*. The development of micropropagation protocols ensures an adequate supply of *S. chirayita* by reducing the uncontrolled harvesting pressure off wild populations. Several studies on micropropagation methods, physical embryogenesis, and adaptation to the production capacity of many uniform clones have been successfully reported for *S. chirayita* (Kumar and Van Staden 2016).

Many secondary metabolites accumulate in the plant roots, but harvesting these organs is destructive. Therefore, hair root technology has been considered in

medicinal compound production. The high scaling of hair root cultures is essential for biotechnological applications. Attempts have been made to standardize root cultures for the in vitro production of active secondary metabolites in *S. chirayita*. In general, micropropagation performed under a controlled environment will help prevent the current problems of plant biodiversity conservation due to overharvesting practices of wild populations and can profoundly improve the quality of secondary metabolites of *S. chirayita* (Flores et al. 1999; Guillon et al. 2006).

Differential response of isolated leaves was observed when they were exposed to an MS medium with various concentrations of BA, Kin, and NAA. Direct induction of shoot buds occurs solely from the axial surface of the leaf base. The highest number of shoot buds was obtained a month after planting when the leaves were exposed to an MS medium containing 2.22 μ M BA, 11.6 μ M Kin, and 0.5 μ M NAA. Half-strength MS medium with other growth regulators failed to induce direct shoot organogenesis. Lower NAA concentrations between 0.25 and 0.5 μ M played an important role in the direct shoot initiation from *S. chirata* leaves. The combination of cytokinin and auxin caused direct shoot organogenesis from the leaves' basal segment. Detailed observations showed that there was a different expression of the organogenic potential of leaves isolated from different nodes of cultured aseptic shoots on the MS medium. Initial differentiation was observed from a fortnight onward at the basal segment of the leaves adjacent to the apical meristem. These explants were more responsive than the specimens farther away from the meristem. The leaves of the first and second nodes showed a higher morphogenic potential of 70% and 63.33%, respectively (Chaudhuri et al. 2008).

24.6.6 Case Study 6

24.6.6.1 Distribution of *Gymnema sylvestre*

G. sylvestre is a valuable plant belonging to the family Asclepiadaceae. The genus *Gymnema* includes 40 species distributed from West Africa to Australia. *G. acuminatum* (Roxb.) Wall, *G. aurantiacum*, *G. balsamicum*, *G. elegans* W&A, *G. hirsutum* W&A, *G. lactiferum*, *G. latifolium*, *G. montanum* Hook.f., *G. sylvestre* R.Br., *G. tingens* W&A, *G. indorum*, *G. yunnanense*, and *G. spartum* are some of the most important species of *Gymnema* (Vaidya 2011). It is native to the tropical forests of central and southern India and grows in forests at altitudes of 300–700 m. It is widely distributed in India, Malaysia, Sri Lanka, Australia, Indonesia, Japan, Vietnam, tropical Africa, and southwestern China. This plant is well known as Periploca of the woods in English, Gurmar in Hindi, kalikardori in Marathi, Mardashingi in Gujrathi, Meshashringi in Sanskrit, and Sannagerasehambu in Kannada (Saneja and Sharma 2010). The word “*Gymnema*” is derived from the Hindu word “Gurmar,” which means to eliminate sugar, and it is believed that it may neutralize excess sugar in the diabetic body (Keshavamoorthy and Yoganarasimhan 1989).

24.6.6.2 Habitat

G. sylvestre is a wood-climbing shrub native to the tropical forests of Africa, India, and Australia. Its habitat is sub-forests, river forests, secondary forests, and dry shrub savannahs. Sandy and clayey soils are the best soils for this plant. It is scattered and is common locally.

24.6.6.3 Morphological Description

G. sylvestre is a vulnerable and slow-growing species. Propagation by seed is challenging due to the short seed lifetime. Planting root cuttings or planting end cuttings, thus, is an alternative approach in June and July or in February and March, respectively. This plant is very branched and woody and can grow up to the top of trees in the central and southern Indian forests. This medicinal plant is a pubescent-type shrub with young stems and branches. Stems are 0.7–17.2 cm long and 2–10 mm in diameter, internodes terete, rigid, cylindrical, branched, and twin. The leaves have a phyllotactic opposite arrangement and are 2.5–6 cm long, usually oval, simple, and slightly sharp. The plant contains 1–2-cm-long petioles with a round base that are smooth from the top, dense velvet below, and ciliate along the margins and the nerves. Venation is transverse and reticulates with the marginal vein (Kanetkar et al. 2007). Seeds are 1.3 cm long, flat with thin marginal wings, and ovoid-rectangular in shape. The flowers are small and yellow. The follicles are lanceolate and up to 7.5 cm long. The five-lobed calyx is ovate, obtuse, and ciliated in which the corolla is yellow and five-lobed. The flowering of this plant occurs from August to March (Saneja and Sharma 2010; Khan et al. 2019).

24.6.6.4 Phytochemicals

Gymnemic acids and gymnemasaponins are the main chemical compounds of this plant and are classified as oleanane saponins found in the leaves of *G. sylvestre* (Foster 1999). In addition, the plant was found to contain tannins, quinones, flavonoids, and phenols. Other plant compounds include anthraquinones, flavones, hentriacontane, pentatriacontane, phytin, tartaric acid, formic acid, butyric acid, lupeol, β -amyrin-related glycosides, stigmasterol, triterpenoid saponins, and calcium oxalate (Sinsheimer et al. 1970). The major secondary metabolites in *Gymnema* include a group of nine closely related acidic glycosides (Dateo and Long 1973). The main ones are gymnemic acids A–D and are found in all parts of the plant. The maximum content of gymnemic acid is found in the tip of the stem (54.29 mg/g of DW) and the lowest in the seeds (1.31 mg/g of DW). The ester group in the gymnemic acid genin section gives antisweetness effects to triterpene saponins and gymnemic acids. Gymnemic acids A₂ and A₃ have both glucuronic acid and galactose in their molecular structure, while glucuronic acid is the only component in

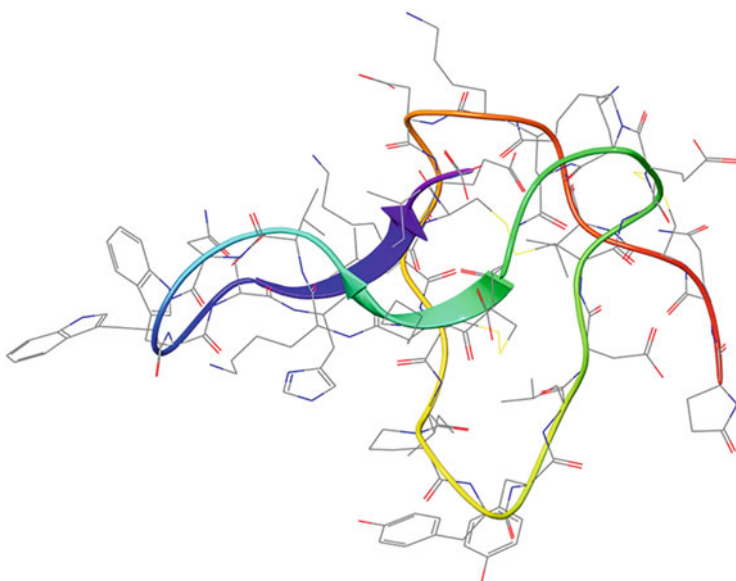


Fig. 24.8 The chemical structure of gurmarin peptide

gymnemic acid A₁ (Chakravarthi 1981). In addition, a series of gymnemic acids (I, II, III, IV, V, VI, and VII) were isolated from the water extract of *G. sylvestre* leaves. Gymnemic acid A is made up of gymnemic acids A₁, A₂, A₃, and A₄ called gymnemagenin. It is a D-glucuronide of hexahydroxy-triterpene that is esterified with acids. The other five gymnastic acids (VIII, IX, X, XI, and XII) were later isolated (Yoshikawa et al. 1989). Gymnemasaponin III is another antisweet compound isolated. A significant 35-amino-acid peptide having an MW of 4209 g/mol called gurmarin (Fig. 24.8) was separated from *G. sylvestre* (Imoto et al. 1991).

The glucose suppression activity of this compound was determined by the electrophysiological method. Other important phytoconstituents isolated from leaves are gymnemasins A, B, C, and D and alkaloids. Several saponins, namely, gymnemagenin, 23-hydroxylgispinogenin, gymnemic acid, deacyl gymnemic acid, and gymnestrogenin, have been purified from this medicinal plant (Tiwari et al. 2014).

24.6.6.5 Pharmacology

In vitro studies have shown several medicinal potentials for this plant, including anticancer, anti-inflammatory, and immunosuppressive, gastrointestinal, hypoglycemic, antimicrobial, and antidiabetic activities. Stigmasterol compound showed therapeutic activity such as antidiabetic, hypoglycemic, antioxidant, and anticancer. Triterpenoid saponins have also displayed their anti-tumor, antifungal, hepatoprotective, and antidiabetic potential in several studies (Khan et al. 2019).

Various extracts and isolated bioactive compounds of *G. sylvestre* have been reported to have antimicrobial potential against several microorganisms. The methanolic extract of the leaves of this plant showed antimicrobial activity against *C. kefir*, *E. coli*, *C. albicans*, and *B. cereus*. Moderate antimicrobial activity against *Staphylococcus aureus*, *C. krusei*, *C. perfringens* type A, and *C. kefir* was revealed by the aqueous extract. The hexane extract inhibited *S. aureus*, *B. cereus*, *S. enterica*, *H. paragallinarum*, and *C. perfringens* type A (Tahir et al. 2017). Moreover, the ethanolic extract is active against the pathogenic species of *S. typhi*, *S. typhimurium*, and *S. paratyphi*. *P. vulgaris*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* were inactivated by chloroform and ethyl acetate extracts (Pasha et al. 2009). In a study in which antimicrobial activity was measured utilizing the disk diffusion technique, the gymnemic acid also showed antimicrobial activity against *E. coli*, *V. cholera*, *S. mutans*, *S. aureus*, *A. niger*, and *C. albicans* with an inhibition area of 8.65 mm, 6.00 mm, 7.12 mm, 9.25 mm, 6.43 mm, and 8.60 mm, respectively (Gupta and Singh 2014).

24.6.6.6 Micropropagation

Various in vitro propagation approaches are employed to propagate this medicinal plant through shoot regeneration from mature *G. sylvestre* explants and large-scale production of gymnemic acids. Somatic embryogenesis was optimized, and whole plant regeneration was obtained in callus cultures derived from cotyledon, hypocotyl, and leaf explants cut from *G. sylvestre* seedlings. Heart-shaped embryos formed torpedo and cotyledon embryos after culture on a medium containing MS salt, B5 vitamins, 0.5 μ M BA, and 2% sucrose (EM8) (Ashok Kumar et al. 2002).

For in vitro reconstruction of adult nodular explants of *G. sylvestre*, MS medium was used to inoculate single-nodal explants with different compounds of BAP or kinetin with NAA and auxins such as IAA alone. MS medium containing 5 mg/L BAP and 0.2 mg/L NAA showed the highest number of shoots. In addition, regenerated shoots were uprooted in the absence of growth regulators (IAA, IBA, and NAA) in a half-strength MS medium. In cultures inoculated with seedling explants on auxin-free half-strength MS medium, root primordia emerged from the shoot base 15–20 days after implanting and subsequently developed into roots without basal callus compared to MS medium supplemented with different concentrations of auxin (Reddy et al. 1998).

Plant cell suspension cultures were produced for the large-scale production of gymnemic acids and antisweetening compounds. The method used led to the development of a new cell culture system for in vitro growth of this species. Gymnemic acids did not accumulate in the callus but were released into the environment. In another study, gymnemic acid extraction was reported through *G. sylvestre* callus culture. Gymnemagenin was extracted, identified, and measured in various cultures of *G. sylvestre* callus (Kanetkar et al. 2006).

Recently, the research attempted to produce gymnemic acids on a large scale under in vitro conditions mediated by fungal elicitors. The use of bioelicitors such as

Aspergillus niger cell extract increased the production of secondary metabolites from the suspension culture of *G. sylvestre*. It is interesting to note that *A. niger* extraction of *Gymnema* suspension culture significantly increased gymnemic acid production compared to non-extracted cultures. This technique is a potential tool for the large-scale production of gymnemic acids through the use of bioreactors (Nagella et al. 2011).

24.7 Conclusion and Prospects

There are many medicinal plant species in the world. In general, it can be concluded that habitat loss (deforestation), invasive species, pollution, overexploitation (overgrazing, population pressure), and climate change (low rainfall, natural and accidental fires, and soil drought) are the main threats around the world. Also, the demand for herbal products has grown dramatically in recent decades due to the lower side effects of herbal medicines. Medicinal plants are the resources that most people in the world prefer for a healthy life and are also used as preventive and therapeutic agents. Despite the success of antibiotics, infectious diseases have always been the second leading cause of death worldwide. In addition, new and emerging diseases caused by viruses, including the COVID-19 virus, threaten human existence. The collection and export of these plant species, namely, *Withania somnifera*, *Zhumeria majdae*, *Picrorhiza kurroa*, *Ginkgo biloba*, *Swertia chirata*, and *Gymnema sylvestre*, for medicinal purposes destroy flora and threaten some plant species. If new methods such as tissue culture are not replaced, the affluent parts of medicinal plants will disappear quickly. Plant tissue culture methods play an essential role in producing secondary metabolites and meeting the growing needs of the pharmaceutical industry in an environmentally friendly manner. Swift, mass-produced, high-quality, disease-free, and uniform production is possible only through micropropagation. Plant production can be done throughout the year regardless of the season and climate. However, micropropagation technology is expensive compared to conventional methods such as seed, cutting, and grafting propagation. It is, therefore, necessary to take measures to reduce production costs. As a result, in addition to using these techniques, raising public awareness is very consequential for locals in caring for plant species.

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Chapter 25

Insights into the In Vitro Approaches for the Production of Secondary Metabolites Towards The Conservation of Medicinal Orchids



Nutan Singh and Suman Kumaria

Abstract Orchids are unique plants that flourish in epiphytic, terrestrial, and saprophytic conditions. They are not only well known for their ornamental worth but are also being tremendously used in various ailments worldwide. Orchids form the potential source for the discovery of plant-based drugs which are widely used in pharmaceutical companies. The phytochemicals/secondary metabolites produced from orchids have remarkable values in various domains with diverse applications. Numerous phytoconstituents have been isolated from orchids and studied revealing their diverse structures and functions. Looking into the importance of orchids for the presence of various important secondary metabolites and to meet the demand of the pharmaceutical industry, it is extremely important to multiply the plants on a large scale so as to reduce the pressures on their natural populations. Plant tissue culture techniques have emerged as a powerful tool for propagating orchids on mass scale and have multidisciplinary benefits including the development of elite cultivars, varietal improvement, and the endless enrichment of the secondary metabolites in demand. Mass scale-up of orchids and the production of value-added natural products through tissue culture reduce the pressures exerted on their natural populations. Various aspects have been taken into account using in vitro systems for the improvement of plant phytoconstituents. This chapter focuses on the different approaches taken for the production of secondary metabolites in orchids under in vitro conditions and the genes involved in their biosynthesis.

Keywords Orchids · Tissue culture · Elicitors · Secondary metabolites

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25.1 Introduction

Orchids form an important part of vegetation across the world, and globally approx. 30,000–35,000 species in 750 genera have been reported (Dressler 1993; Hossain 2011). They are widely distributed ranging from the tropics to high alpine and occur in different types of habitats (epiphytic, terrestrial, and saprophytic) (White and Sharma 2000). About 70% of the world's orchids are epiphytic and/or lithophytic, 25% are terrestrial, and 5% are found growing in mixed habitats (lithophytic, epiphytic, and terrestrial) (Arditti and Ernst 1993). According to the reports, the main genera of orchids reported are *Angraecum* (200 spp.), *Bulbophyllum* (1000 spp.), *Calanthe* (150 spp.), *Catasetum* (100 spp.), *Dendrobium* (900 spp.), *Encyclia* (235 spp.), *Eulophia* (200 spp.), *Epidendrum* (800 spp.), *Habenaria* (600 spp.), *Eria* (500 spp.), *Lepanthes* (460 spp.), *Liparis* (350 spp.), *Maxillaria* (420 spp.), *Masdevallia* (380 spp.), *Malaxis* (300 spp.), *Oberonia* (300 spp.), *Oncidium* (420 spp.), *Pleurothallis* (1120 spp.), *Phreatia* (160 spp.), *Polystachya* (150 spp.), *Stelis* (370 spp.), *Taeniophyllum* (170 spp.), and *Vanilla* (100 spp.). Asia forms the dominating reservoir of orchids in the world, housing mostly *Cymbidium*, *Dendrobium*, *Oncidium*, and *Phalaenopsis* species (Hew et al. 1994; Laws 1995). However, in India, approximately 158 genera with 1331 orchid species have been reported (Kong et al. 2003; Hossain 2011). The reported native orchid genera in India are *Arachnis*, *Cymbidium*, *Dendrobium*, *Paphiopedilum*, and *Vanda* (Sarmah et al. 2017). The Northeastern region of India is known to house epiphytic orchids, growing mostly in Arunachal Pradesh (558 spp.) followed by Sikkim (557 spp.) and Meghalaya which consists of about 439 species (Luckson 2007; Rao 2010; Rao and Singh 2015). Other than Northeastern India, orchids are also distributed in the natural habitats of Northwestern India and Western Ghats (Chowdhery 2009; Sarmah et al. 2017).

25.2 Orchids: The Magnificent Plants in Plant Kingdom

Orchids have diverse flowers which are in high demand due to their large size, long lasting multicolored appearances, and fragrance that makes them occupy the top position under the flowering plants in the global market (De et al. 2014). They are largely grown for their ornamental worth with high demands for the cut flowers and potted plants leading to their fabulous fabrication in the recent years (Tokuhara and Mii 2003). In terms of export, Taiwan, Brazil, Italy, Thailand, Japan, New Zealand, and the UK are the largest exporter of orchids.

Other than their ornamental and horticultural importance, orchids are also known for their pharmacological attributes. The important genera of orchids which contribute to medicinal attributes are *Anoectochilus*, *Calanthe*, *Coelogyne*, *Cymbidium*, *Cypripedium*, *Dendrobium*, *Ephemerantha*, *Eria*, *Galeola*, *Gastrodia*, *Gymnadenia*, *Habenaria*, *Ludisia*, *Luisia*, *Nervilia*, and *Thunia* (Szlachetko 2001). Orchids have

been used since the ancient times in herbal medicine system for the treatment of various ailments. The use of orchids, namely, *Bletilla striata*, *Gastrodia elata*, and *Dendrobium nobile*, in Traditional Chinese Medicine (TCM) system is well documented. According to the report of Kong et al. (2003), approx. 365 plants including orchids reported under TCM from different divisions of the plant kingdom have been used in Chinese “Materia Medica.”

In India, orchids have been used since the Vedic time in Ayurvedic medicine system. They are even used as a source of food and also form raw materials for industries. Almost all parts of orchids are used in traditional and herbal medicine systems in India and different parts of the world. The best example of Ayurvedic use is the tonic “*Chavyanprasa*” which contains four important orchids, viz., *Habenaria edgeworthii*, *H. intermedia*, *Malaxis muscifera*, and *M. acuminata* (Singh and Duggal 2009). Orchids such as *Dendrobium nobile*, *D. fimbriatum*, *D. moschatum*, *Eulophia campestris*, *Eria musicucola*, *Cymbidium giganteum*, *C. willomsoni*, *C. aloifolium*, *Coelogyne cristata*, *C. ovalis*, *C. fimbriata*, *Liparis odorata*, *Orchis latifolia*, *Phaius tancarvilleae*, *Papilionanthe teres*, *Satyrium nepalense*, *Vanda cristata*, *V. tessellata*, *V. spathulata*, and *V. coerulea* have been reported to be used in traditional medicine system for the treatment of various human diseases and disorders (Kong et al. 2003; Hossain et al. 2009; Medhi and Chakraborti 2009). Various species of the genus *Dendrobium* are well known for their medicinal importance across the globe. Approximately 40 *Dendrobium* species are reported to be used in TCM (Tsering et al. 2017). “*Jivanti*,” an Ayurvedic formulation, is reported to have *D. macraei* as one of the constituent herbs (Kasera and Shukla 2001).

In Trans-Himalayan region, the commonly growing medicinal orchids reported are *Brachycorythis obcordata*, *C. cristata*, *Dactylorhiza hatagirea*, *Otochilus porrectus*, *Rhynchostylis retusa*, *S. nepalense*, and *V. cristata*. Previous reports had revealed the medicinal importance of orchids in the treatment of ailments such as stomach worms, joint pains, eye infection, headache, fatigue, and fever (Lewis and Elvin-Lewis 1977; Bulpitt 2005). Different studies have also shown the biological properties of orchids such as anti-inflammatory, anticancer, antioxidant, and antimicrobial (Ho and Chen 2003; Kumar et al. 2005; Behera 2008; Hoang et al. 2017; Assédé et al. 2017). The medicinal properties of orchids could be due to the presence of different secondary metabolites such as phenolics, flavonoids, alkaloids, anthocyanins, ayapin, β -sitosterol, coelonin, crepidatin, confusarin, dendrobine, fimbriatone, gigantol, hircinol, moscatalin, tannins, etc. These metabolites have helped in improving the status of the pharmaceutical industries (Majumder and Laha 1981; Sachdev and Kulshreshtha 1986; Kong et al. 2003; Bhattacharyya et al. 2016a; Nag and Kumaria 2018). Due to their overexploitation for various reasons, most orchids are listed under the Appendix II of CITES under the rare and endangered categories (Anonymous 2017). Moreover, few orchids such as *Aerangis ellisii*, *D. cruentum*, *Laelia jongheana*, *Laelia lobata*, *Paphiopedilum* spp., *Peristeria elata*, *Phragmipedium* spp., and *Renanthera imschootiana* are listed under the Appendix I of CITES to control illegal collections.

25.3 Medicinal Orchids: The Source of Secondary Metabolites

Plants can produce numerous chemical compounds naturally and also under stress conditions. The chemical compounds are classified under two broad categories, i.e., primary and secondary metabolites. Primary metabolites include those organic compounds which are necessary for the normal growth and development of the plants, whereas secondary metabolites are the products of the primary metabolites. These metabolites are not directly involved in the growth and developmental processes of the plants (Fig. 25.1). Approximately 250,000 plant species have been known in the plant kingdom, which serve as the repository of low molecular weight chemical compounds known as secondary metabolites.

The plant metabolites are multifunctional in nature which are basically synthesized via single or in combination of multiple pathways such as phenylpropanoid, mevalonate, amino acid, glucose, acetate-malonate, etc. (Narayani and Srivastava 2017). These metabolic pathways help in the production of diverse sets of secondary metabolites which help the plants in defense and stress responses in their habitats. The secondary metabolites exert different ranges of effects on plants and other living organisms and are also reported to have various important roles in flower induction, fruit set, and abscission. They are also used as antimicrobials, attractants, and repellents and are also known for their diverse functional roles in signaling and catalytic activities.

Medicinal herbs and many modern medicines rely on plant secondary metabolites for their remedial actions. The secondary metabolites have diverse commercial applications in the fabrication of value-added active compounds that form the important constituents of many pharmaceutical drugs. Several factors such as environmental condition, physiology of plants, type of genotypes, and pathogen attack are responsible for the production of metabolites in nature. Specialized metabolites are produced only during specific developmental stages in plants (Shitan 2016; Isah 2019). The several types of secondary metabolites produced in plants are alkaloids,

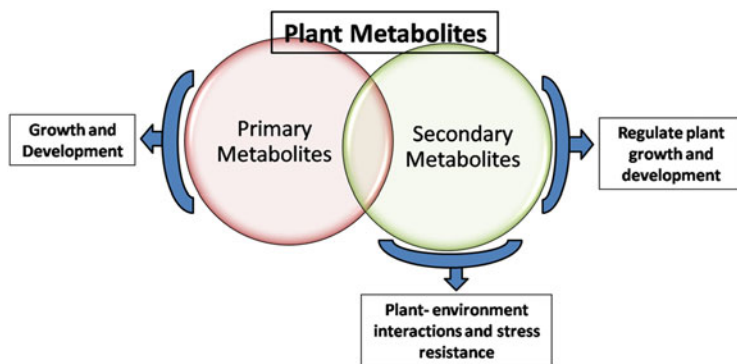


Fig. 25.1 Plant metabolites, primary and secondary, with their respective roles

anthocyanins, bibenzyl derivatives, flavonoids, flavonols, flavidin, phenanthrenes, stilbenes, etc.

Orchids like other plants also contain important phytoconstituents of commercial needs (Sut et al. 2017). The most common secondary metabolites in orchids are stilbenes, alkaloids, anthocyanins, bibenzyl derivatives, and phenanthrenes (Ye et al. 2002). Stilbenes, 1,2-diphenylethylene with hydroxyl groups on the aromatic ring, are basically synthesized through the phenylpropanoid pathway using CoA ester as precursor (Pecyna et al. 2020). Moreover, dihydrostilbenoid compounds mimic the properties of resveratrol which are used for the treatment of neurological ailments (Majumder et al. 1998a; Rueda et al. 2014). Also, a number of different types of alkaloids such as imidazole, indolizidines, phthalides, pyrrolidines, and sesquiterpenoid derivatives have been reported from orchids (Yang et al. 2014). The secondary metabolites such as dendrobine (*D. candidum*, *D. nobile*), dendrochrysanene (*D. chrysanthum*), fimbriatone (*D. fimbriatum*), moscatilin (*D. moschatum*), sesquiterpenoids and indolizidine (*D. nobile*, *D. crepidatum*, and *D. polyanthum*), coeloginanthridin, coeloginanthrin (*C. cristata*), rutinoid (*Anoectochilus roxburghii*), cymbidine A (*C. goeringii*), blestrianol A (*B. striata*), heptacosane, octacosanol (*V. tessellate*), and phenanthrenes (*Bulbophyllum*, *Cymbidium*, *Coelogyne*, *Dendrobium*, *Epidendrum*, *Maxillaria*) have been reported to occur in orchids (Majumder et al. 1998b; Shiao et al. 2005; Kovács et al. 2008; Naing et al. 2010; Mohanty et al. 2012, 2013; Xu et al. 2013; Rueda et al. 2014; Wang et al. 2016; Paul et al. 2017; Jin et al. 2017; Park et al. 2018; Zhang et al. 2018a; Manokari et al. 2021). These medicinal orchids have been proved to have anti-tumor, antipyretic, antiplatelet aggregation, anti-oxidative, hypotensive, diuretic, anti-inflammatory, antimicrobial, and cardiovascular properties (Shiao et al. 2005; Moyo et al. 2010; Naing et al. 2010; Hossain 2011; Mohanty et al. 2012, 2013; Paul et al. 2017; Jin et al. 2017; Park et al. 2018; Zhang et al. 2018b; Manokari et al. 2021).

Furthermore, the production of secondary metabolites is under the control of different genes involved in the biosynthesis of secondary metabolites (Nag and Kumaria 2018; Singh and Kumaria 2020). The secondary metabolite enzymes, namely, phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), are the key regulatory enzymes in the phenylpropanoid and flavonoid pathway. All the downstream secondary metabolite products are dependent on these two key enzymes' regulation. PAL is the first key enzyme in the phenylpropanoid pathway which catalyzes the conversion of primary metabolites to secondary metabolites, i.e., L-phenylalanine to ammonia and trans-cinnamic acid (Camm and Towers 1973). The two important substrates, malonyl-CoA and 4-coumaroyl-CoA, in the presence of CHS enzyme lead to the production of naringenin chalcone. CHS is the first key enzyme in the flavonoid biosynthesis. The pathways lead to the production of different types of secondary metabolites of medicinal importance, namely, flavonoids, phenylpropanoids, and lignins. Various enzymes of this pathway have been studied in different plants including orchids such as *Apostasia shenzhenica*, *Coelogyne ovalis*, *Dendrobium catenatum*, *Phalaenopsis equestris*, and *Vanda coerulea* (Nag and Kumaria 2018; Singh and Kumaria 2020; Vishwakarma et al.

2021). Studies have shown that the expression of transcripts of *PAL* and *CHS* genes is enhanced with an increase in the contents of secondary metabolites in the different tissues of *C. ovalis* and *V. coerulea* (Nag and Kumaria 2018; Singh and Kumaria 2020).

25.4 Plant Tissue Culture: A Continuous Source of Secondary Metabolites' Accumulation

Plant tissue culture serves as an important platform for sustainable production and utilization of plant-based chemical resources. These techniques also help in the conservation and protection of the rare, endangered, and threatened wild plants by increasing the production of plantlets which not only are disease-free but also multiply fast as compared to naturally growing plants (Tandon and Kumaria 2005). The continuous demand of plants in various sectors has resulted in the overexploitation and disappearance of plants from their natural habitats. The diminution of plant populations can affect the essential productivity of plants especially the total production of secondary metabolites of interest. Plant tissue culture has emerged as one of the novel researches for the discovery of new bioactive compounds of interest and is also significant for driving research towards commercialization with the planned production of defined chemical products. The continuous demand of secondary metabolites and the capability of tissue culture techniques for the production of desired compounds have refocused the attention on the in vitro raised plantlets for the growth of pharmaceuticals and cosmetic industries. Different strategies have been used in order to uplift the productions of specialized metabolites in the tissue-cultured plantlets (Rao and Ravishankar 2002; Nag and Kumaria 2018; Singh and Kumaria 2020; Paul and Kumaria 2020).

Orchids can be propagated using in vitro approach through asymbiotic seed germination and explants such as internodes, nodes, pseudobulbs, thin cell layers, roots, rhizomes, shoot meristems, leaves, and protocorms (Arditti and Ernst 1993; Kumaria and Tandon 1994; Nayak et al. 1997; Chen and Chang 2000; Vij et al. 2000; Malabadi et al. 2005; Sheelavanthmath et al. 2005; Dohling et al. 2007; Nag and Kumaria 2018; Singh and Kumaria 2020; Oliya et al. 2021; Tikendra et al. 2021). The regeneration of orchids through asymbiotic seed germination ensures heterogeneity in the propagules and is the most common propagation method wherein different media have been used (Arditti and Ernst 1993; Stewart and Kane 2006; Manners et al. 2011; Pavasupree et al. 2021; Kumar et al. 2022) (Fig. 25.2).

The secondary metabolites are the utmost important constituents in the biochemical profiles of the medicinal orchids, because of their medicinal or therapeutic uses. The continuously increasing demand for specialized metabolites has created a serious problem of extinction of orchid species from their natural habitats due to their extensive exploitation for medicinal and commercial purposes. The balance between the naturally growing orchids and the demand for their secondary



Fig. 25.2 In vitro propagation of *Coelogyne ovalis* through asymbiotic seed germination (Singh and Kumaria 2020). (a) Mature plant in greenhouse, (b) 8-month-old capsule, (c) seed germination in B₅ medium (60 days; bar = 0.1 cm), (d) seed germination in Mitra medium (after 60 days; bar = 0.1 cm), (e) seed germination in KC medium (after 60 days; bar = 0.1 cm), (f) development of protocorm in KC medium (bar = 0.5 cm), (g) PLBs with leaves (bar = 1 cm), (h) well-developed plantlets at 15 µM NAA, (i) well-developed plantlets with root at 15 µM of NAA, (j) well-developed plantlets in KC medium supplemented with 10 µM NAA and 20 µM SA, (k) rooting in KC medium with 15 µM NAA and 30 µM PG, (l) hardened plantlets in net house

metabolites can be maintained by large-scale propagation of medicinal orchids using in vitro approaches. The enhancement of secondary metabolites in in vitro cultures has been reported from many plants where differentiated and undifferentiated tissues have been used as the source for the alteration of secondary metabolite production (Murthy et al. 2014; Isah et al. 2018; Paul and Kumaria 2020). Moreover, the production of metabolites in the in vitro propagated plantlets is considered as a secure method for the constant production to maintain the yield and uniform quality. The production of the metabolites in culture is dependent on both internal and external factors, viz., age of the explants, culture conditions, quality and intensity of light, effect of plant growth regulators, and so on. Orchids are among one of the most important reservoirs of secondary metabolites in the plant kingdom.

Also, secondary metabolite production in tissue culture-raised plantlets can be enhanced by the overexpression of genes coding for regulatory enzymes which are involved in their biosynthesis. However, overexpression of particular genes may not always enhance the production of secondary metabolites. Therefore, understanding

the specific pathways is of utmost importance (Verpoorte et al. 2002). Tremendous research and development efforts have been made towards bringing the production of secondary products to semicommercial level, e.g., vanillin and taxol production in cell cultures. In a large number of cases, the secondary metabolite productions have fallen far short of expectations and have never reached the commercial status. In recent years, various techniques have been used for enhancing the yield of secondary metabolites in plant cell cultures including screening and selection of high cell lines, improving culture strategies, media optimization, elicitation, culture of different cells (organ cultures), and immobilization (Singh and Kumaria 2020; Paul and Kumaria 2020).

The production of secondary metabolites in in vitro cultures has been studied in many plants which can be regulated by the exogenous application of various elicitors such as methyl jasmonate, salicylic acid, heavy metals, and chitosan (Rao and Ravishankar 2002; Poulev et al. 2003; Espinosa-Leal et al. 2018; Singh and Kumaria 2021). Elicitors, additives, and precursors play essential roles in the accumulation of specialized metabolites through defense mechanisms against different stimuli/stresses, resulting in the activation of different organic metabolites in plant cells (Table 25.1). The concentration of metabolites is found to be higher in in vitro grown treated plantlets than the plants growing in nature. Therefore, the requirement of abundant quantities of metabolites can be fulfilled by the elicitation process in the cultured plantlets grown in vitro (Marchev et al. 2020; Salmerón-Manzano et al. 2020). The elicitor is the specific foreign molecules which has the capability to stimulate the desired compounds in the plants' system and also arouse stress-induced or defense responses in the plants. Elicitation is one of the common methods used for the enhancement and accumulation of secondary metabolites in many plants as well as in orchids (Zhao et al. 2005; Nag and Kumaria 2018; Singh and Kumaria 2020). The elicitation of in vitro raised plantlets has been reported to be beneficial to orchids such as *V. coerulea*, *C. ovalis*, and *D. fimbriatum* (Nag and Kumaria 2018; Singh and Kumaria 2021; Paul and Kumaria 2020). Among the different techniques used for plant tissue culture, the cell suspension culture has been reported to be one of the simplest and cost-effective methods for the production of secondary metabolites on a large-scale. Paul and Kumaria (2020) induced the higher accumulation of the secondary metabolites by the precursor treatment in the suspension cultures of protocorm-like bodies (PLBs) of *D. fimbriatum*. The cultures of *D. fimbriatum* were treated with different precursors, namely, caffeic acid, ferulic acid, and *p*-coumaric acid. The results obtained by them showed that the PLBs treated with caffeic acid in the medium showed the highest accumulation of phenolic and flavonoid contents. Also, the highest antioxidant activity (DPPH and FRAP assay) was recorded in the cultures treated with caffeic acid. On the other hand, the cultures treated with *p*-coumaric acid reported the highest tannin contents, and the cultures treated with ferulic acid showed the highest alkaloid contents (Fig. 25.3).

Elicitors act as signaling molecules which are recognized by specific elicitor receptors present on the cell membrane of plants that subsequently help in the transcriptional activation of genes leading to the biosynthesis of secondary metabolites (Torkamani et al. 2014; Halder et al. 2019). The production of metabolites in

Table 25.1 Effect of different elicitors/precursors/additives on the production of secondary metabolites in orchids

Elicitors or precursors/additives	Orchid species	Secondary metabolites	References
L-Phenylalanine	Callus-derived plantlets of <i>D. ovatum</i>	Moscatilin	Singh and Kumaria (2021)
Chitosan, salicylic acid, and methyl jasmonate	<i>D. ovatum</i>	Moscatilin	
Salicylic acid	<i>C. ovalis</i>	TPC, TFC, TAC	
Light-emitting diodes	<i>Dendrobium</i> Enopi × <i>Dendrobium</i> Pink Lady PLBs	Accumulation of high phenolics	Yeow et al. (2020)
Caffeic acid, <i>p</i> -coumaric acid	<i>D. fimbriatum</i>	TPC, TFC, TTC	Paul and Kumaria (2020)
PGRs	PLB cultures of <i>A. africana</i>	Phenolics	Bhattacharyya et al. (2019)
Chitosan	<i>V. coerulea</i>	Increased TFC, TPC, TTC, and TAC after chitosan treatment	Nag and Kumaria (2018)
10 mM AgNO ₃	<i>D. aphyllum</i>	Increase in TPC, TFC, and TAC	Bhattacharyya et al. (2018)
2% Casein + 2% AC	<i>H. lanceum</i>	Higher amount of GA and SA in leaf extract	Singh and Babbar (2016)
Putrescine	<i>D. nobile</i>	Increased TPC and TTC in methanolic stem extracts and TFC and TAC in methanolic leaf extracts	Bhattacharyya et al. (2016a)
100 myo-inositol + 0.8 mM putrescine	<i>D. crepidatum</i>	Increased TFC and TAC in methanolic leaf extract and TPC in methanolic stem extract	Bhattacharyya et al. (2016b)

TPC total phenolic content, TFC total flavonoid content, TAC total alkaloid content, TTC total tannin content, SA salicylic acid, GA gibberellic acid, PGR plant growth regulators, AC activated charcoal

the tissue culture raised plantlets depends not only on the elicitors but on many other factors, viz., plant growth regulators, nature of elicitors, and time of exposure to elicitors on which the productions of desired metabolites can be dependent (Zhao et al. 2005; Baenas et al. 2014; Singh and Kumaria 2021). Elicitors from non-biological origin are called abiotic elicitors which include metals, light, osmotic stress, drought, salinity, thermal stress, and hormones, whereas biotic elicitors are the substances of biological origin including polysaccharides (e.g. chitin, pectin, and cellulose) and microorganisms (Fig. 25.4).

The effects of elicitors, precursors, and additives on the production of various secondary metabolites have been reported in the tissue culture-raised plantlets of

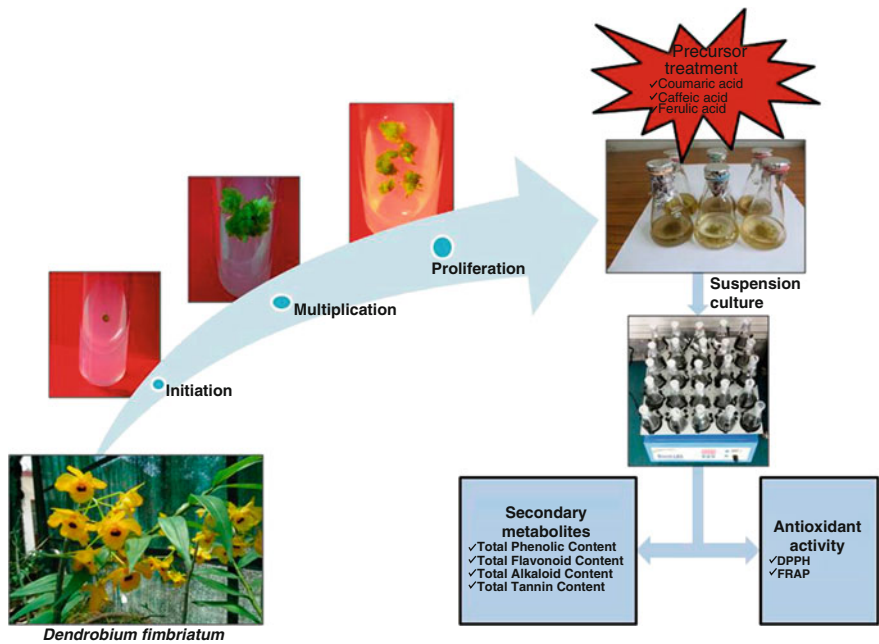


Fig. 25.3 Production of secondary metabolites in *Dendrobium fimbriatum* (Paul and Kumaria 2020)

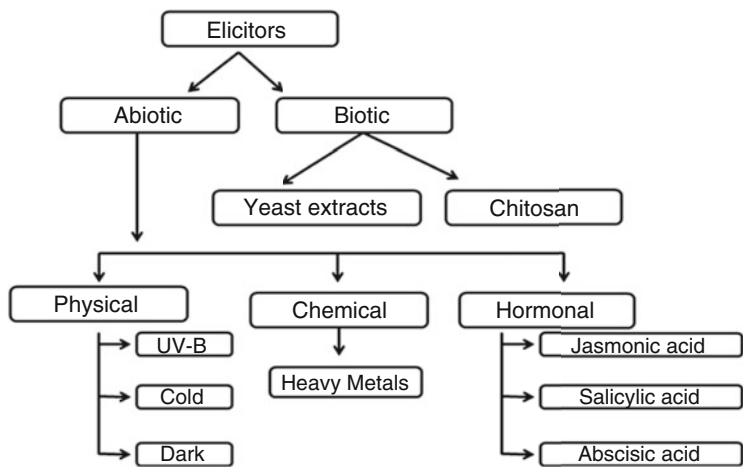


Fig. 25.4 Different types of elicitors

orchids, namely, *Ansellia africana*, *C. ovalis*, *D. ovatum*, *Dendrobium* Enopi $\times$ *Dendrobium* Pink Lady, *D. fimbriatum*, *D. aphyllum*, *D. nobile*, *Herminium lanceum*, and *D. crepidatum* (Table 25.1) (Singh and Babbar 2016; Bhattacharyya

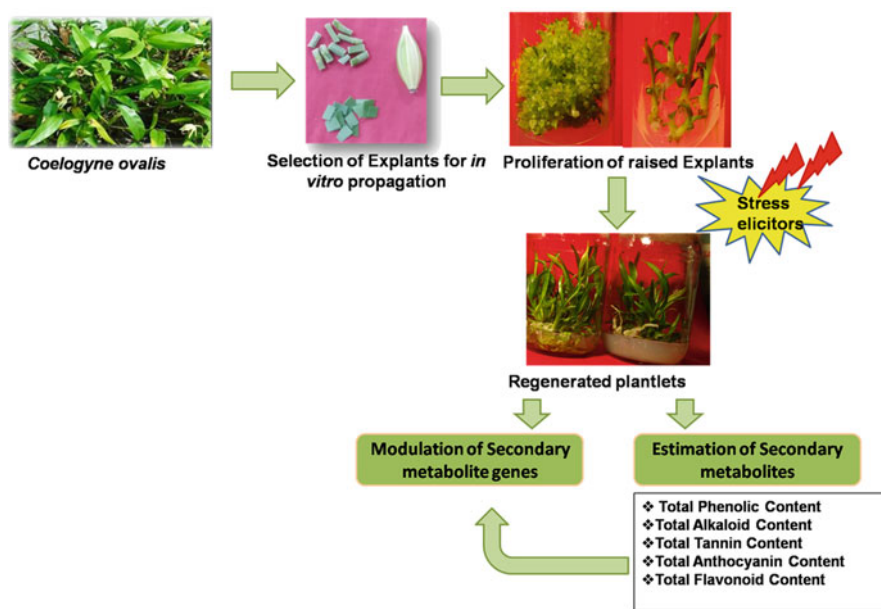


Fig. 25.5 Differential modulation of *CHS* gene and subsequent production of secondary metabolites in micropropagated plantlets of *C. ovalis* Lindl (Singh and Kumaria 2020)

et al. 2016a, b, 2018, 2019; Nag and Kumaria 2018; Paul and Kumaria 2020; Yeow et al. 2020; Singh and Kumaria 2021; Pujari et al. 2021). Elicitation is proven to be a faster and effective method for positive alteration of specialized metabolites and plays a vital role in transcripts' expression of the different genes involved in secondary metabolite production. Furthermore, the expressions of transcripts are also dependent on both the types of elicitors used and the time of exposure. Elicitors play a significant role in the upregulation of different genes by controlling the range of cellular activities both at molecular and biochemical levels (Yue et al. 2016). Various reports have confirmed the use of wounding treatment, low temperature, salinity, fungal infection, PGRs, chitosan, UV-B light, and saline conditions to enhance the expressions of the genes involved in the biosynthesis of secondary metabolites in plant species (Leyva et al. 1995; Richard et al. 2000; Pang et al. 2005; Zhou et al. 2011; Dehghan et al. 2014; Lijuan et al. 2015; Obianom et al. 2019; Singh and Kumaria 2020; Inyai et al. 2021). The expression of secondary metabolite genes in many plant species subjected to various stress conditions with the enhanced transcript expression has been studied (Ito et al. 1997; Richard et al. 2000; Pang et al. 2005; Nag and Kumaria 2018). The enhancement of *CHS* expression and the specialized secondary metabolites (phenolics, flavonoids, and anthocyanins) in the in vitro raised plantlets of the orchid *C. ovalis* subjected to elicitors' treatment (chitosan, yeast extracts, salicylic acid, methyl jasmonate, and abscisic acid) has been reported (Singh and Kumaria 2020) (Fig. 25.5).

25.5 Conclusion

Orchids form the huge reservoir of secondary metabolites which can serve as a good opportunity for the discovery of new classes of bioactive compounds. In the natural conditions, the growth of orchids is very slow, and it takes many years for them to become fully developed which becomes a limiting factor in their propagation. The alternative mode for large-scale propagation of rare or exotic orchids is through plant tissue culture. This approach is important for not only mass scale-up of orchids but also for obtaining desirable bioactive compounds. Various strategies have been used in order to increase the content of desirable metabolites. Also, plant tissue culture improves secondary metabolites' enrichment in the in vitro raised plantlets. Exploring the biosynthetic pathway of specialized metabolites can give a better understanding for the upregulation of the secondary metabolites in the in vitro cultured plantlets. Knowledge on the biosynthesis pathway of targeted compounds is still rudimentary in orchids, and better strategies have to be developed at cellular and molecular level.

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Chapter 26

Biotechnological Approaches for Ex Situ Conservation of Medicinal Plants



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Abstract Medicinal plants are natural store houses of a wide range of pharmacologically important plant secondary metabolites with diverse therapeutic properties. With the rise in global population, the demand for these metabolites from their natural plant sources is ever increasing. As a result, indiscriminate collection of these plants from the wild for the extraction of metabolites has threatened the very existence of a number of these plants; some are even on the verge of extinction. So, various conservation measures have been adopted to ensure the survival of these species. Plants preserved ex situ in field gene banks are subject to various diseases, pest infestation, natural calamities, and also anthropogenic error, leading to the ultimate extinction of a huge amount of germplasm with elite characters. On the other hand, biotechnological approaches appear to be attractive alternatives for the ex situ conservation of medicinally important plant species. Various techniques like regeneration of plants through in vitro somatic embryogenesis (i.e., development of embryo-like structures from somatic cells of the plant body), encapsulation of these somatic embryos by various agents to produce artificial seeds, and subsequent storage at ultralow temperatures have emerged as suitable alternatives for ex situ conservation of plant germplasm. Apart from a very few examples of plant species where somatic embryogenesis occurs naturally, the process, which is the ultimate exhibition of plant totipotency, is observed in vitro, either directly from an explant or indirectly via callus formation. A number of factors control the process, of which the effect of phytohormones, amino acids, light, and type of explant on somatic embryogenesis of some plants of pharmaceutical interest are discussed in this review. By definition, synthetic seeds are artificially encapsulated somatic embryos, or any other somatic tissue, which are stored and sown as seeds and

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upon germination either under *in vitro* or *in vivo* condition develop into complete plant. Here, we focus on factors that affect the quality of the artificially produced seeds and its germination efficiency, with special emphasis on the instances of medicinal plant synseed production and its role in conservation. In the last section of the review, we discuss the role of different cryopreservation techniques and factors controlling a successful cryopreservation program and enlist a few of the medicinal plants where this method has been successfully applied for germplasm conservation.

Keywords Medicinal plants · Conservation · Somatic embryo · Artificial seed · Cryopreservation

26.1 Introduction

Human beings relied on plants since the dawn of civilization as a source of food, fodder, medicine, and many more. According to the World Health Organization (WHO), 80% of the world population still relies on herbal medicine for primary treatments in their daily lives. With the increasing demand for medicinally important plants, uncontrolled exploitation is leading many plant species on the verge of being critically endangered (Sharma et al. 2010). Thus, conservation and management of medicinally important plants via different methods, including various biotechnological tools, has gained immense importance. Importance of different tissue culture methods in conservation of medicinally important plants has been discussed in details repeatedly (Cruz-Cruz et al. 2013; Rather et al. 2022). Conservation of plant genetic resources based on *in vitro* methodologies targets short-, mid-, and long-term storage as mentioned by Chauhan et al. (2019). Kulak et al. (2022) recently reviewed how *in vitro* technologies can serve as a powerful tool in the total bio-cultural conservation if employed methodically. Plant regeneration via somatic embryogenesis, artificial seed generation, and subsequent cryopreservation has emerged as a suitable method of biotechnology-mediated conservation (Micheli and Standardi 2016a, b; Ozudogru and Lambardi 2016). Generation of genetic clones, preservation of elite traits, low cost of production, surpassing steps of cutting and rhizogenesis, and ability of long-term preservation without repeated sub-culturing are some of the benefits associated with somatic embryo explants (Micheli and Standardi 2016a, b; Khajuria et al. 2021). Analysis of factors regulating somatic embryo development including role of participatory genes provides the basic necessity to exploit this technology. The role of explant type, hormonal, amino acid, and other biochemical additives as well as role of light has been discussed in this chapter while highlighting the major breakthroughs achieved with respect to somatic embryo development in medicinal plants. Scientists like Ali and Ray (2022) and Sharma et al. (2019) have dealt with the role of synthetic seeds in conservation of rare, threatened, and endangered (RET) medicinal species. Once developed, somatic embryos are subjected to regeneration or encapsulation by synseed technology. Overcoming recalcitrance, desiccation sensitivity, and

pathogen infectivity are the advantages of artificial seeds over conventional seeds. These factors along with ease in handling and identical metabolite profile of plants regenerated from artificial seed have led to its successful exploitation in case of almost all categories of economically important plants (Rai et al. 2009). Here, we discuss the role of encapsulating agents and laboratory parameters that play a role in synseed production and subsequent germination efficiency and viability after short-term or long-term storage. The ability to preserve live cells over an extended period was achieved by the emergence of cryopreservation mechanisms involving encapsulation-vitrification and encapsulation-dehydration protocols (Jang et al. 2017; Débora de Oliveira Prudente et al. 2019). Choice of explants, selection of protectants, protocol, and physical parameters associated with storage as applied to medicinal plants are elaborated here. Making the tissue cold tolerant, degree of tissue organization, and nature of cell lines must be carefully manipulated if successful regeneration is to be achieved.

Biodiversity is under the control of several endogenous and environmental factors (Roque-Borda et al. 2021), which has an inadvertent effect on ecological balance, evolution, and adaptation of all species. Anthropogenic factors causing uncontrolled exploitation particularly of medicinal plants are a serious threat to biodiversity. Thus, the necessity of biotechnology-mediated conservation of the same is the need of the hour (Gantait et al. 2015a). In this chapter, an attempt has been made to discuss the pros and cons of three such tools, namely, somatic embryos, artificial seeds, and cryopreservation, thereby pointing out the parameters to be kept in mind for future research related to medicinal plant conservation through biotechnology.

26.2 Somatic Embryogenesis

In angiosperms, reproduction mainly takes place by sexual reproduction with the fusion of gametes, followed by formation of zygotic embryo, ultimately leading to formation of whole plant. In addition to sexual reproduction, plants can also propagate via vegetative and asexual means. Development of embryo-like structure from cells other than gametes or its fertilized product (i.e., from somatic cells) is known as somatic embryogenesis. The concept of somatic embryo formation as a mean of apomixis was first described by Strasburger in 1878. The detailed description of the process of somatic embryogenesis was first described in callus culture of *Daucus carota* by Steward et al. (1958). Since early years, somatic embryogenesis has been reported in a large number of plant species.

Somatic embryogenesis is naturally observed in some plants like in *Kalanchoe* leaf margins, while in most others, this adventitious embryo formation is noted in vitro (Garcês et al. 2014; Salaün et al. 2021). The process of somatic embryogenesis can be described as the ultimate exhibition of plant totipotency. While in some plant species direct somatic embryogenesis is observed (Balaraju et al. 2011; Cantelmo et al. 2013; Khilwani et al. 2016; Sedaghati et al. 2019; Patel et al. 2021), in others indirect somatic embryo formation takes place from the explants via

intervening callus tissue formation (Rukh et al. 2019; Khajuria et al. 2021; Verma et al. 2022). Reprogramming of expressing genes is needed during induction of somatic cells to embryogenic cells. Some cells are predetermined to enter into the embryogenic pathway and only need appropriate conditions to do so; such cells are known as PEDC or pre-embryogenic determined cells. In this case, providing appropriate stimulus is sufficient for the formation of somatic embryos from the explants and thus known as direct somatic embryogenesis. In contrast, IEDC or induced embryogenic determined cells need to undergo multiple cycles of mitotic division in the presence of phytohormones resulting in callus formation before inducing somatic embryogenesis. This process is additionally described as indirect somatic embryogenesis (Wann 1988). Following the process of induction, development of somatic embryos takes place, which is very similar to stages observed in zygotic embryo development. In dicotyledonous plants and coniferous trees, the histological development takes place through globular, heart, torpedo, and cotyledonary stages, whereas in monocotyledons, the somatic embryo develop through globular, coleoptiles, and scutellar stages (Zimmerman 1993; Thorpe and Stasolla 2001). Becwar et al. (1989) divided regeneration of plants from somatic embryo into three stages: maturation (immature somatic embryo to cotyledonary stage), germination (growth of radical), and conversion (growth and survival of in vitro derived plantlet in soil).

26.3 Somatic Embryogenesis in Conservation of Medicinal Plants

Somatic embryogenesis is one of the most important biotechnological tools with various applications including ones in conservation strategy (Cheruvathur et al. 2013a; Khilwani et al. 2016; Salma et al. 2019). The advantages of somatic embryogenesis over conventional micro-propagation methods include: (a) regeneration of large number of somatic embryos (nearly one million) per liter of culture medium is possible with proper standardization of protocol; (b) somatic embryo being bipolar structure eliminates the need of root induction step, which is common for conventional micropropagation methods; (c) the process allows easy scale-up with minimum labor; (d) synchronization of somatic embryo formation and germination process can be achieved with minimum manipulation of culture conditions, hence increasing plant output; and (e) long-term storage of somatic embryos is possible through induction of dormancy period. Hence, the process can be successfully implemented in large-scale multiplication of plants within small time directly in vitro or via production of synthetic seeds by encapsulation of somatic embryos (discussed in details in the next section) for propagation in more stable way (Deo et al. 2010). Direct regeneration protocol is advantageous for conservation of any endangered species as the intervening callus phase in indirect methods brings high chances of introducing somaclonal variations. Regeneration of plants via both direct

and indirect somatic embryogenesis is reported for a large number of medicinal plants in the last decades (Table 26.1). The process of somatic embryogenesis is achieved by manipulation of nutrients, phytohormones in medium, and growth conditions in different plant species with variety of explants as discussed below.

26.3.1 Explants in Somatic Embryogenesis

Direct somatic embryogenesis is reported for a number of medicinal plants from different explants like leaf (Thomas and Jacob 2004; Balaraju et al. 2011; Cantelmo et al. 2013; Khilwani et al. 2016; Sedaghati et al. 2019; Patel et al. 2021; Khan et al. 2022), leaf base (Lincy et al. 2009), root (Chaudhuri et al. 2004; Filipović et al. 2015), zygotic embryo (Raomai et al. 2014), hypocotyle segments (Verma et al. 2012), stem segments (Lincy et al. 2009), petiole segments (Choffe et al. 2000), etc. Leaf explants seem to be the most responsive tissue for direct somatic embryogenesis. In *Rheum spiciforme* (Khan et al. 2022), *Portulaca oleracea* (Sedaghati et al. 2019), and *Swertia chirata* (Balaraju et al. 2011), intact axillary leaves from in vitro germinated seedling showed direct somatic embryo formation on hormone supplemented medium. In *S. chirata*, leaf explants scored better than root explants from the germinated seedlings for direct somatic embryogenesis (Balaraju et al. 2011). In *Leptadenia reticulata*, leaf explants from field-grown plant showed higher direct somatic embryo formation compared to nodal explants from the same (Patel et al. 2021). Leaves harvested from in vitro propagated micro-shoots were used successfully as explants for direct somatic embryogenesis in *Bacopa monnieri* (Khilwani et al. 2016), *Curculigo orchioides* (Thomas and Jacob 2004), and *Petiveria alliacea* (Cantelmo et al. 2013). Khilwani et al. (2016) investigated response of leaves excised from third to eight nodes from top of 6-week-old micro-shoots and concluded fourth leaf from the top to be the most responsive explants for direct somatic embryo formation in *B. monnieri*. Similar study with response of leaf explants in relation to its age (explants age: 10–40 days) for direct somatic embryogenesis was determined in the endangered medicinal plant *C. orchioides*, and the study showed highest frequency (89%) and number of somatic embryos formed per explants (~16) in 20-day-old leaf explants (Thomas and Jacob 2004). Intrinsic physiological conditions play important role in induction of somatic embryogenesis as portrayed by low somatic embryo induction and frequency in immature explants in plants like *C. orchioides* and *B. monnieri* (Thomas and Jacob 2004; Khilwani et al. 2016).

Indirect somatic embryogenesis is also established from different explants, namely, leaf (Kumar and Chandra 2014; Sharmin et al. 2014; Filipović et al. 2015; Keshvari et al. 2018; Rukh et al. 2019; Khajuria et al. 2021; Ali et al. 2021; Bogdanović et al. 2021), hypocotyle segments (Dhir et al. 2014; Verma et al. 2022), nodal explants (Salma et al. 2019), intermodal segment (Sharmin et al. 2014), corm slices (Kumar et al. 2017), and scale tissue (Zhang et al. 2016). Similar to direct somatic embryogenesis, indirect process was also initiated in most of the

Table 26.1 Somatic embryogenesis in some medicinal plants reported in the past decade

Name of plant	Family	Explants	Culture media	Culture condition	Direct/indirect SE	SE to plantlet conversion	Reference
<i>Lycium barbarum</i> L.	Solanaceae	Hypocotyle, leaf and root explants from in vitro germinated seedlings	MS + 2,4-D (0.25 mg/L) + TDZ (0.5, 1, 3 mg/L); CS: sucrose (3%); SA: agar (0.8%); pH: 5.8	Temperature: 25 ± 2 °C; photoperiod: light: dark = 16 h:8 h	Spontaneous ISEG observed with hypocotyle explants (6SE/callus) on MS + TDZ (1 mg/L) + 2,4-D (0.25 mg/L) followed by leaf explant (2.5 SE/explant)	SEs developed into plantlets on hormone-free MS with 100% survival rate after acclimatization	Verma et al. (2022)
<i>Rheum spiciforme</i> Royle	Polygonaceae	Intact axillary leaves from in vitro germinated seedlings (6 week-old)	MS + BAP (5–30 µM) + NAA (0–1 µM) + GA ₃ (2–8 µM) + 2,4-D (2–8 µM) + glutamine (25–100 µM) + AdSO ₄ (25–100 µM); CS: sucrose (3%); SA: agar (0.8%); pH: 5.8 ± 0.2	Temperature: 25 ± 2 °C; humidity: 55% photoperiod—dark for 1 week followed by light: dark = 12 h:12 h	DSEG with maximum germination (~91%) observed on leaf explants cultured on MS + BAP (25 µM) + NAA (2 µM) + GA ₃ (1 µM) + glutamine (2 µM) + AdSO ₄ (50 µM) (50 µM)	Maximum number of plantlets/explant 3.9 ± 1 obtained on the same medium	Khan et al. (2022)
<i>Viola canescens</i> Wall. ex. Roxb.	Violaceae	Sterilized leaf explants from field grown plants	CIM: MS + 2,4-D (1.5 mg/L) + KN (1.5 mg/L); SEIM: MS + 2,4-D/IBA (0.1–0.25 mg/L)/KN (0.1–0.5 mg/L); MS + 2,4-D (0.1–0.25 mg/L) + KN (0.5 mg/L) + /glutamine (200–400 mg/	Temperature: 25 ± 2 °C; photoperiod: light: dark = 16 h:8 h	High number of SE (19.15 ± 2.66) induced from embryogenic calli on MS + 2,4-D (0.15 mg/L) + KN (0.05 mg/L). Number of SE increased to 27.66 ± 2.67 on MS + 2,4-D (0.15 mg/L) + KN	Mature SE developed on MS + AgNO ₃ /ABA converted into plantlet (86.67% and 83.33%, respectively)	Khajuria et al. (2021)

<i>Leptadenia reticulata</i> (Retz.) Wight and Arn	Apocynaceae	Leaf and nodal explants harvested from 1 year old field grown plant	L) + CH (1–4%); SEMM: MS + ABA (0.5–0.2 mg/L)/AgNO ₃ (1–3 mg/L); CS: sucrose (3% w/v); SA: agar (0.8% w/v); pH: 5.8 ± 0.2	Temperature: 26 ± 2 °C; photoperiod: light: dark = 16 h:8 h	(0.05 mg/L) + glutamine (2%) and CH (50 mg/L). Maximum number of mature SE (35.96 ± 3.68) observed on MS + ABA (1.5 mg/L)	on MS + BAP (0.2 mg/L)	Patel et al. (2021)
<i>Bacopa monnieri</i> (Linn.) Wettist	Scrophulariaceae	Leaf, node, and inter-nodal explants (0.5–1 cm) from field-grown healthy plants	MS + BAP, KN, AdSO ₄ (5–20 µM) + IAA, NAA, 2,4-D (0.1–1.0 µM); SEMM: static and liquid MS + BA (15 µM) + AdSO ₄ (15 µM)/GA ₃ (0.5–2.0 µM)/CW (4%); CS: sucrose (3% w/v); SA: agar (0.8% w/v); pH: 5.8	Temperature: 25 ± 2 °C; For CI dark for 5 days then transferred to photoperiod—light: dark = 16 h:8 h; humidity: 70–80%	DSEG observed on leaf explants with MS + BAP (15 µM) + KN (5 µM). 100% leaf explants showed ISEG (21.70 ± 2.06 SE) on MS + BA (15 µM) + AdSO ₄ (15 µM)	SE developed into healthy plantlets on static MS + GA ₃ (1.0 µM)	Ali et al. (2021)

(continued)

Table 26.1 (continued)

Name of plant	Family	Explants	Culture media	Culture condition	Direct/indirect SE	SE to plantlet conversion	Reference
<i>Centaurium erythraea</i> Rafn.	Gentianaceae	Root and leaf explants (5–7 mm segments)	MS + 2,4-D (0.5–2 mg/L) + CPPU (0.5–3 mg/L)	Temperature: 25 ± 2 °C; photoperiod—light: dark = 16 h:8 h and dark	DSEG reported in root explants. Leaf explants showed ISEG on MS + 2,4-D (0.2 mg/L) + CPPU (≥0.1 mg/L) both under light and in dark conditions. Cyclic SEG (both direct and indirect) observed from primary SE developed from leaf explants cultured on MS + 2,4-D (0.1 mg/L) + CPPU (0.25 mg/L) for three cycles	SE developed into healthy plantlets on hormone-free MS medium	Filipović et al. (2015); Bogdanović et al. (2021)
<i>Ajuga bracteosa</i> Wall. ex Benth.	Lamiaceae	Leaf explants (3–4 mm ²) from in vitro grown plantlets (40 days old)	MS + 2,4-D (0.5–2 mg/L) + BAP (0.5–2 mg/L); CS: sucrose (3%); SA: agar (0.7–0.8%)	Temperature: 25 ± 2 °C; photoperiod: light: dark = 16 h:8 h. To study effect of light: green, blue, red, yellow and	MS + 2,4-D (1 mg/L) + BAP (2 mg/L) was most effective in CI and SEG. Highest SEG (92.75%) was observed on the same medium with red light	NA	Rukh et al. (2019)

<i>Portulaca oleracea</i> L.	Portulacaceae	Leaf and stem segments from in vitro germinated seeds	For DSEG: MS + BAP (1.5 mg/L); CIM: MS + BAP (0.5–2 mg/L) + 2,4-D (0.5–2 mg/L); SEIM: MS + KN/BAP (0.5–6 mg/L) alone or with IAA/NAA (0.1–0.4 mg/L); CS: sucrose (3% w/v); SA: agar (0.7% w/v); pH: 5.8 ± 0.02	white light (control) Temperature: 25 ± 2 °C; humidity: 55% Photoperiod—light: dark = 16 h:8 h or dark	followed by green light (90.31%) DSEG observed on leaf explants cultured on MS + BAP (1.5 mg/L) under both dark and light conditions. Under continuous dark (3 weeks) more SE produced (~133 SE/explant) compared to explants cultured under light: dark condition (~39 SE/explant). ISEG also reported from callus, highest rate of SE recorded on MS + BAP (1.5 mg/L) + NAA (0.2 mg/L)	SE developed into healthy plantlets on hormone-free MS medium	Sedaghati et al. (2019)
<i>Eclipta alba</i> (L.) Hassk.	Asteraceae	Nodal segment, shoot tip and leaf from in vitro plants	CIM: MS + PIC/2,4-D/NAA (0.1–3 mg/L); SEIM: MS + PIC (2 mg/L) + KN/BAP/Zeatin/TDZ (0.1–1 mg/L) + ABA (0.1–1 mg/L); CS: sucrose (3% w/v); SA: agar (0.8% w/v); pH: 5.7 ± 0.2	Temperature: 25 ± 2 °C; for CI: complete dark; for regeneration Photoperiod—light: dark = 16 h:8 h	Nodal segments showed highest CI on MS + PIC (2 mg/L). Maximum frequency (88.4%) and number of SE (34.83/callus) were recorded in MS + PIC (2 mg/L) + TDZ (0.5 mg/L) + ABA (0.25 mg/L)	SE attached with mother tissue showed highest (~86%) conversion into plantlets on MS + BA (0.75 mg/L)	Salma et al. (2019)

(continued)

Table 26.1 (continued)

Name of plant	Family	Explants	Culture media	Culture condition	Direct/indirect SE	SE to plantlet conversion	Reference
<i>Stevia rebaudiana</i> (Bert.)	Asteraceae	Leaf and internodal explants from field-grown plants	MS/B5 + 2,4-D + BAP + NAA (0.1–4 mg/L); SA: agar	Temperature: 25 ± 2 °C; photoperiod: light: dark = 16 h:8 h	Highest CI (100%) was observed on both MS and B5 + BAP (1 mg/L) + NAA (1 mg/L) for both leaf and internodal explants. Highest frequency (63%) of SEG was noted in MS + NAA (0.5 mg/L) + BAP (0.5 mg/L) + 2,4-D (2 mg/L)	NA	Keshvari et al. (2018)
<i>Hypoxis hemerocallidea</i> Fisch. & C.A. Mey.	Hypoxidaceae	Corm slices (1 cm ³) from field grown plants sterilized and used as explants	CIM: MS + 2,4-D (5, 10, 15, 20, 25 µM); SEIM: MS + 2,4-D + BAP (varied conc.); CS: sucrose (3%); SA: agar (0.8%); pH: 5.8	Temperature: 25 ± 2 °C; photoperiod: light: dark = 16 h:8 h	Embryogenic calli obtained on MS + 2,4-D (15 µM) + BAP (2.5 µM). Different stages of SE developed and matured on the same medium on subsequent subculture	Mature SE developed into plantlets on ½ MS on isolated culture. Germination frequency increased significantly on ½ MS + GA ₃ (1.44 µM)	Kumar et al. (2017)
<i>Carum copiticum</i> L.	Apiaceae	Hypocotyle explants excised from 5, 10, 15 days old in vitro	CIM: MS + 2,4-D (1, 1.5, 2 mg/L) + KN (0.25, 0.5, 0.75 mg/L); SEIM: calli transferred to basal MS medium; CS: sucrose	Temperature: 21 °C; photoperiod: light: dark = 16 h:8 h	Age of explant and ecotype played important factor in the indirect SEG in addition to PGRs. Highest frequency of SE	NA	Niazian et al. (2017)

<i>Bacopa monnieri</i> (L.) Wettst.	Scrophulariaceae	germinated seedlings from three ecotypes (Ghoom, Shahedye-Yazd and Shiraz)	(3% w/v); SA: phytigel (0.38% w/v); pH: 5.8 ± 0.2	Temperature: 25 ± 1 °C; photoperiod: light: dark = 16 h:8 h	(28.33) observed with explants excised from 15 days old seedling (Ghoom ecotype) on transfer of calli initiated on MS + 2,4-D (1.5 mg/L) + KN (0.5 mg/L) to MS basal medium	Isolated SE cultured on ½ MS basal showed 100% conversion of SE to plantlets	Khilwani et al. (2016)
		Leaf explants (third to eighth from top) excised from microshoots (3 weeks old)	MS + BA (0, 1, 5, 12.5 µM) + 2,4-D (0, 1, 5, 12.5 µM); CS: sucrose (58 mM); SA: agar 0.7% (w/v); To study effect of sucrose concentration: MS + 2,4-D (1 µM) + BA (12.5 µM) + Sucrose (10–250 mM)	Maximum frequency (47.1%) was noted in MS + 2,4-D (1 µM) + BA (12.5 µM). SE induction frequency was inversely related to 2,4-D concentration. Sucrose had positive effect on SE induction, highest frequency of leaf explants inducing SE (77%) observed in MS + 2,4-D (1 µM) + BA (12.5 µM) + sucrose (250 mM) in fourth leaf from top.			

(continued)

Table 26.1 (continued)

Name of plant	Family	Explants	Culture media	Culture condition	Direct/indirect SE	SE to plantlet conversion	Reference
<i>Lilium pumilum</i> Delile	Liliaceae	Transversely cut scale tissue (1 cm ²) from in vitro grown bulb (15 weeks old) used as explants	MS + PIC (0.5–2 mg/L) + INAA (0.2–0.5 mg/L) CS: sucrose (3% w/v); SA: agar (0.5% w/v); pH: 5.8	Temperature: 25 ± 1 °C; photoperiod: continuous darkness	Light yellowish, granular, friable embryogenic calli observed on concave side of explant cultured on MS + PIC + NAA. Highest SE (90.7% frequency; 11 SE/explant) on MS + PIC (1.0 mg/L) + NAA (0.2 mg/L)	Mature SE transferred to basal MS successfully transformed into plantlets with root and shoots within 4 weeks.	Zhang et al. (2016)
<i>Paris polyphylla</i> Sm.	Melanthiaceae	Excised immature zygotic embryo from sterilized seeds	B5, MS, N6, and SH media (full strength and half strength), 1/2 MS + NAA/2,4-D (0.1, 0.2, 0.5, 1.0 mg/L); CS: sucrose (3% w/v) SA: gelrite (0.3% w/v)/agar (0.8% w/v); pH: 5.8	Temperature: 25 ± 2 °C; Photoperiod: dark (for SE induction)/light: dark = 14 h:10 h (for plantlet conversion)	DSEG observed. Maximum frequency (32.6%) and number of SE/explant (28.7) was noted in 1/2 MS medium. Both parameters increased (40.7% SE induction and 32.6 SEs/explant) with gelrite as SA compared to agar (1.4 times)	Late cotyledonary SE cultured vertically in 1/2 MS ± GA ₃ converted into small plantlets. Highest germination was found in 1/2 MS + GA ₃ (0.5 mg/L) after 2 months	Raomai et al. (2014)
<i>Anethum graveolens</i> L.	Apiaceae	Hypocotyle explants (1 cm) excised from	CIM: MS + 2,4-D (0.23–2.26 µM)/NAA (0.26–2.68 µM); SEIM:	Temperature: 25 ± 2 °C; photoperiod: light: dark = 16 h:8 h	Highest EC (87%) was induced on MS + 2,4-D (1.13 µM) with the	Maturation of SE and subsequent development into	Dhir et al. (2014)

			germinated seedlings	MS + 2,4-D (0.23–0.34 µM); CS: sucrose (3%); SA: agar (0.8%); pH: 5.8		hypocotyle explants. Maximum number of SE (40–45 globular stage and 16–20 heart stage per 10 mg EC) observed on MS + 2,4-D (0.34 µM). Further development noted only on basal MS medium with maximum 128.3/flask	plantlets observed on MS basal medium		
<i>Wedelia calendulacea</i> (L.) Less	Compositae	Leaf disc (2r = 3–5 mm) and internode (2–3 mm) explants from in vitro grown shoots		MS + 2,4-D, NAA, BAP (alone or in combinations); CS: sucrose (3%); SA: agar (0.8%); pH: 5.7	Temperature: 25 °C; Photoperiod: light: dark = 16 h:8 h	CI noted from Leaf and internodes on MS + 2,4-D (2 mg/L). Reduced 2,4-D facilitated SEG. SE developed on 76% of cultures on MS + 2,4-D (0.5 mg/L).	Cotyledonary SE germinated into plantlets on MS + BAP (1 mg/L) + GA ₃ (0.5 mg/L)	Sharmin et al. (2014)	
<i>Swertia chirata</i> Buch-Ham. ex Wall.	Gentianaceae	In vivo leaf explants harvested from 6- to 8-month-old plants		MS + 2,4-D (0.5–3 mg/L) + /KN (0.5 mg/L) + BAP (1 mg/L); for improvement of embryogenic calli regular subculture (2–3 weeks) on MS + 2,4-D (0.5 mg/L) + KN (0.5 mg/L); CS: sucrose (3%)	Temperature: 25 ± 2 °C; photoperiod: light: dark = 16 h:8 h	Highest frequency (76%) EC observed on MS + 2,4-D (0.5 mg/L) + KN (0.5 mg/L). Globular SE matured to subsequent stages on the same medium. Highest number of SE (48.83 ± 4.6) noted	Via synthetic seed	Kumar and Chandra (2014)	(continued)

Table 26.1 (continued)

Name of plant	Family	Explants	Culture media	Culture condition	Direct/indirect SE	SE to plantlet conversion	Reference
<i>Petiveria alliacea</i> L.	Phytolaccaceae	Leaf segments (1 cm ²) from in vitro grown shoots	w/v); SA: agar (0.7% w/v); pH: 5.8 Primary SEIM: MS + zeatin, TDZ, 2,4-D (2.2–22.8 µM), PIC (2–20 µM); SA: agar (0.7% w/v); secondary SEIM: liquid MS ± PIC (20 µM)/ 2,4-D (22.6 µM); CS: sucrose (3% w/v); pH: 5.8	Temperature: 30 ± 2 °C; photoperiod — light: dark = 16 h:8 h; liquid cultures kept in rotary shaker (100 RPM) under same photoperiod	after 6 weeks on same medium DSEG observed on leaf explants cultured on MS + PIC (20 µM). These primary SE showed cyclic SEG on similar inductive medium or even basal liquid MS and ½ MS	The SE developed into plantlets (100%) on solid basal MS.	Cantelmo et al. (2013)
<i>Hemidesmus indicus</i> (L.) R. Br. ex Schult.	Apocynaceae	Sterilized nodal explants from field-grown plants	CIM: MS + IBA/2,4-D (1–4 µM); SEIM: MS + IBA (2 µM); ½ MS + IBA/NAA (1–4 µM); SEGIM: MS + BAP (1–5 µM) ± GA ₃ (0.5–2 µM); CS: sucrose (3% w/v); SA: agar (0.8%); pH: 5.8	Temperature: 25 ± 2 °C; photoperiod: light: dark = 16 h:8 h	Maximum ISEG (92%) observed from nodal explants on ½ MS + IBA (2 µM)	Development further torpedo stage and germination of SE took place on MS + BAP (4 µM) + GA ₃ (1.5 µM)	Cheruvathur et al. (2013a)
<i>Digitalis trojana</i> Ivanina	Plantaginaceae	0.5–0.8 cm segments of hypocotyle excised from in vitro germinated	MS + BAP/KN/TDZ/zeatin (0.1, 0.5, 1.0 mg/L), MS + TDZ (0.5, 1.0, 2.0 mg/L)/BAP (1.0 mg/L) + IAA/NA/IBA	Temperature: 23 ± 1 °C; photoperiod: light: dark = 16 h:8 h	DSEG observed. After 5 weeks, highest number of SEs (13.8 SE/explant) noted in MS + TDZ (1.0 mg/L) + IAA (0.5 mg/L)	SEs cultured on ½ MS for 4 weeks developed plantlets	Verma et al. (2012)

<i>Swertia chirata</i> Buch-Ham. ex Wall.	Gentianaceae	Leaf and root explants from in vitro grown seedlings	(0.1, 0.25, 0.5 mg/ L); CS: sucrose (3% w/v); SA: agar (0.8%); pH: 5.8	Temperature: 22 ± 2 °C; photope- riod: light; dark = 16 h:8 h	DSEG observed. Number of SE noted in leaf explants was higher than in root explants. Highest SE (>60 per explant) noted in leaf explants cultured on MS + 2,4- D (1.0 mg/L)	SEs cultured on MS + 2,4-D (1.0 mg/ L) + BAP (0.1 mg/L) showed maxi- mum germina- tion and conversion	Balaraju et al. (2011)
<i>Pulsatilla koreana</i> Nakai ex T. Mori	Ranunculaceae	Leaf explants (0.5 cm ²) excised from surface steril- ized leaves of field-grown plants	SEIM: MS + zeatin (0, 0.25, 0.5, 0.75, 1.0 mg/L) + IAA (0.05 mg/L); SEMM: MS + zeatin (1 mg/ L) + IAA (0.05 mg/ L); CS: sucrose (3% w/v); SA: agar (0.8%); pH: 5.8	Temperature: 24 ± 1 °C; photope- riod: light; dark = 16 h:8 h	Best EC was noted on MS + zeatin (0.5 mg/ L) + IAA (0.05 mg/ L). Different stage of SE developed on MS + zeatin (1 mg/ L) + IAA (0.05 mg/L)	Mature SEs converted to plantlets on basal ½ MS medium	Lin et al. (2011)

2,4-D 2,4-diphenoxyacetic acid, 2IP 2 isopentenyl adenine, AdSO₄ adenine sulfate, B5 Gamborg et al. (1968) medium, BAP 6-benzylamino purine, CH casein hydrolysate, CI callus induction, CIM callus induction medium, CPPU N-(2-chloro-4-pyridyl)-N'-phenylurea, CS carbon source, CW coconut water, DSEG direct somatic embryogenesis, EG embryogenic calli, IAA indole-3-acetic acid, IBA indole-3-butyric acid, ISEG indirect somatic embryogenesis, KN kinetin, MS Murashige and Skoog (1962) basal medium, N6 Chu et al. (1978) basal medium, NAA α naphthalene acetic acid, OM Owen and Miller (1992) basal medium, PIC picloram, SA solidifying agent, SE somatic embryo, SEG somatic embryogenesis, SEGM somatic embryo germination medium, SEIM somatic embryo induction medium, SEMM somatic embryo maturation medium, SH Schenk and Hildebrandt (1972) basal medium, TDZ thidiazuron

medicinal plants from leaf explants. Sterilized leaf explants from field-grown plant showed embryogenic callus production in *Viola canescens* (Khajuria et al. 2021), *S. chirata* (Kumar and Chandra 2014), and *Pulsatilla koreana* (Lin et al. 2011). Among leaf, node, and internodal explants harvested from field-grown plants of *B. monnieri*, leaf explants showed highest embryogenic callus induction (Ali et al. 2021). In *Centaurium erythraea*, while root explants showed direct somatic embryo formation, leaf explants developed indirect embryogenic callus formation (Filipović et al. 2015; Bogdanović et al. 2021). Leaf explants from in vitro grown plants also proved to be very responsive to induce indirect somatic embryogenesis in *Ajuga bracteosa* (Rukh et al. 2019). In *Eclipta alba*, nodal segments showed better induction of indirect somatic embryos compared to leaf and shoot tip explants (Salma et al. 2019). Hypocotyle segments scored better for indirect somatic embryo formation compared to leaf explants followed by root explants in *Lycium barbarum* (Verma et al. 2022). Niazian et al. (2017) reported earlier that age of germinated seedling from which hypocotyle is being excised to be used as explants plays an important role in addition to ecotype in *Carum copticum*. The study showed hypocotyle from 15-day-old seedling to be most responsive in Ghoom ecotype. Dhir et al. (2014) also used hypocotyle segment from germinated seedlings of *Anethum graveolens* successfully for generation of embryogenic calli. In some plants, for example, *Hypoxis hemerocallidea*, special structures like sterilized corm slices were successfully used as explants for embryogenic callus induction (Kumar et al. 2017). Similarly, in *Lilium pumilum*, transversely cut scale tissue (1 cm²) from in vitro grown 15-week-old bulb is used as explants (Zhang et al. 2016).

In addition to different types of explants mentioned above, primarily formed somatic embryos often work as explants for subsequent cultures. When primary somatic embryo is used as an explant to induce somatic embryogenesis, it is known as secondary somatic embryogenesis or cyclic embryogenesis or recurring embryogenesis (Cantelmo et al. 2013; Raomai et al. 2014; Bogdanović et al. 2021). Secondary embryogenesis can occur both directly and indirectly from the primary somatic embryos as shown histologically in *C. erythraea* (Bogdanović et al. 2021). Both types of embryos showed impressive germination and conversion rate leading to the development of complete plantlets, which opens a new avenue for conservation of endangered medicinal plants via in vitro micropropagation (Bogdanović et al. 2021). Direct somatic embryogenesis observed on leaf explants of *Petiveria alliacea* showed cyclic somatic embryogenesis on similar inductive medium or even basal liquid MS and one-half MS medium (Cantelmo et al. 2013).

Selection of suitable explants is one of the major important factors for callus induction and subsequent somatic embryogenesis as pointed out by Lema-Rumińska and Kulus (2014). Different explants are found to have varied response and regenerative potential under similar conditions for each plant species. The variation in response by different explants of same species can be due to specific physiological and biochemical makeup of the tissue in addition to interaction between explants and exogenously supplied phytohormones in the medium regulating the success of somatic embryogenesis (Moon et al. 2013).

26.3.2 *Phytohormones in Somatic Embryogenesis*

Somatic embryos can develop directly or indirectly from different explants, and exogenous hormones play an important role in reprogramming the cells of the explants, specifically auxins and cytokinins. The type and concentrations of growth regulators effective in induction and maturation of somatic embryos vary with plant species. In majority of the plant species including medicinal ones, auxins, commonly 2,4-dichlorophenoxyacetic acid (2,4-D), plays an important role in the induction process of somatic embryogenesis from plethora of explants. However, it is commonly observed that for further development of the embryos, removal of auxins from the media or sometimes reduction of its concentration is important. In some exceptional cases, the induction and development of the somatic embryo is observed to take place on the same media composition.

While in some plants like *Paris polyphylla*, half strength Murashige and Skoog's (1962) basal medium (MS) was sufficient for induction of somatic embryos from zygotic embryo explants and addition of commonly used phytohormones showed negative effect (Raomai et al. 2014), in other plants, cytokinin alone is sufficient to induce direct somatic embryogenesis, for example, in *Portulaca oleracea*, where MS medium supplemented with 1.5 mg/L 6-benzylaminopurine (BAP) showed ~133 somatic embryo/explants (Sedaghati et al. 2019). For direct somatic embryogenesis induction, combination of auxin and cytokinin supplementation showed good result for different explants in different medicinal plants. Higher concentration of cytokinin (25 μ M BAP) compared to auxin (1 μ M 1-naphthaleneacetic acid or NAA) induced direct somatic embryos in *Rheum spiciforme* (Khan et al. 2022). Similar results were seen for *B. monnieri*, where maximum frequency of direct somatic embryogenesis (47.1%) was noted in MS + 2,4-D (1 μ M) + BAP (12.5 μ M) by Khilwani et al. (2016). In this study, induction of somatic embryo was inversely related to the auxin concentration. Hypocotyle explants of *Digitalis trojana* showed highest direct somatic embryo formation on MS medium supplemented with 1.0 mg/L thidiazuron (TDZ) and 0.5 mg/L indole-3-acetic acid (IAA) followed by MS + TDZ (1.0 mg/L); thus, cytokinin alone is capable of somatic embryo formation in this species, but the number of somatic embryos is significantly augmented by addition of low concentration of auxin (Verma et al. 2012). Contrastingly, auxin alone showed highest frequency and number of direct SE in *S. chirata* with 1 mg/L 2,4-D (Balaraju et al. 2011). Patel et al. (2021) reported direct embryogenesis from leaf of *Leptadenia reticulata* stimulated by a combination of two cytokinins, that is, MS + BAP (15 μ M) + kinetin (5 μ M). BAP in combination with kinetin is rarely reported to induce somatic embryogenesis.

Dedifferentiation and redifferentiation of a plant cell are well known to be regulated by levels of different auxins and cytokinins in the system. Auxin-induced callus culture often showed onset of somatic embryogenesis on addition of cytokinins in the presence of the auxin. A combination of auxin and cytokinin is found to increase somatic embryogenesis in many plants (Dam et al. 2010). Among the cytokinins, TDZ is found to be more inductive than other cytokinins at an optimum

level of 0.5 mg/L, as reported for indirect somatic embryogenesis in *Murraya koenigii* (Paul et al. 2011), *Pelargonium sidoides* (Kumar et al. 2015), *E. alba* (Salma et al. 2019), etc. High concentration of cytokinin in combination with low concentration of auxin increases somatic embryo development in many plants like *P. oleracea* L. (Sedaghati et al. 2019), *L. barbarum* (Verma et al. 2022), and *A. bracteosa* (Rukh et al. 2019). In *L. barbarum*, high cytokinin with low level of auxin, namely, MS + TDZ (1 mg/L) + 2,4-D (0.25 mg/L) demonstrated high frequency of somatic embryo formation from hypocotyle explants followed by leaf explants (Verma et al. 2022). In *A. bracteosa*, the most effective combination of 2,4-D and BAP to induce indirect somatic embryogenesis was found to be with MS + 2,4-D (1 mg/L) + BAP (2 mg/L) by Rukh et al. (2019). Augmentation of somatic embryo with auxins can be due to the role of these phytohormones on *wus* gene expression, which is proved to be involved in meristem formation during somatic embryogenesis process. In some plants along with direct somatic embryogenesis, indirect embryo formation is also noted on a different phytohormone supplementation. For example, in *Leptadenia reticulata*, cent percent leaf explants showed indirect somatic embryogenesis on MS medium supplemented with a combination of BAP and another cytokinin-type synthetic plant growth regulator adenine sulfate or AdSO₄ in equal concentrations (15 µM). Proliferating embryogenic calli from the leaf explants was also observed in this plant, on supplementation with a combination of low concentration of auxin and higher concentration of cytokinin, namely, MS + BAP (10 µM) + IAA (0.5 µM), but the number of somatic embryo formation was significantly lower (Patel et al. 2021). Auxins alone showed very weak response to induced callus from leaf explants of *L. reticulata*. Cytokinins are known to play an important role in stimulation of pro-embryogenic cells (Feher et al. 2003). Among the different cytokinins, BAP plays a very important role in both induction and development of somatic embryos (Salma et al. 2019; Patel et al. 2021). AdSO₄ in combination with cytokinins has been reported to induce somatic embryogenesis in other plants as well, by checking degradation of cytokinin in medium (Van Staden et al. 2008). In *P. oleracea*, indirect somatic embryogenesis was noted on MS + BAP (1.5 mg/L) + NAA (0.2 mg/L). Optimal concentration of BAP is important for somatic embryo induction, and while lower concentration (<1.5 mg/L) failed to induce somatic embryo, higher concentration led to the development of abnormal somatic embryo in *P. oleracea* L. (Sedaghati et al. 2019). Imbalance in phytohormones has been pointed out to be one of the main key factors responsible for abnormal somatic embryo development in various other species (Ramage and Williams 2004).

A combination of auxin and cytokinin with higher concentration of auxin compared to cytokinin showed induction of embryogenic calli in some medicinal plants like *B. monnieri*, *Viola canescens*, *Stevia rebaudiana*, etc. In *B. monnieri*, MS + 2,4-D (2.0 mg/L) + kinetin (1.5 mg/L) resulted in highest (84%) embryogenic calli formation (Ali et al. 2021). In *V. canescens*, callus cultured on MS + 2,4-D (0.15 mg/L) + kinetin (0.05 mg/L) showed highest frequency of somatic embryogenesis (88%) compared to auxin or cytokinin used alone. When callus was cultured on MS supplemented with auxins or cytokinins alone, maximum frequency for somatic

embryogenesis was noted in 2,4-D (0.15 mg/L) supplemented medium with 77% frequency and in kinetin (0.2–0.25 mg/L) supplemented medium with 55% frequency (Khajuria et al. 2021). Higher concentration of cytokinin (>0.25 mg/L) induced shoot organogenesis from the calli in *V. canescens*. Higher concentration of auxin/cytokinin [MS + 2,4-D (2 mg/L) + NAA (0.5 mg/L) + BAP (0.5 mg/L)] favoring somatic embryogenesis is also noted in *S. rebaudiana* (Keshvari et al. 2018). The disagreement between auxin favoring or inhibiting somatic embryogenesis might be dependent of plant species and type of auxins used.

In *C. erythraea*, leaf explants cultured on MS medium supplemented with auxin and synthetic cytokinin—*N*-(2-chloro-4-pyridyl)-*N'*-phenylurea—CPPU, that is, MS + 2,4-D (0.2 mg/L) + CPPU (≥ 0.1 mg/L), formed indirect somatic embryogenesis. Such primarily formed somatic embryos showed capability to produce cyclic embryogenesis on reduced 2,4-D and increased CPPU concentration, that is, MS + 2,4-D (0.1 mg/L) + CPPU (0.25 mg/L) for three cycles (Filipović et al. 2015; Bogdanović et al. 2021). The number of cycles for successful embryo formation generally depends on media and phytohormone supplementation. Increasing 2,4-D concentration (0.1–0.2 mg/L) and CPPU (0.25–0.5 mg/L) reduced secondary somatic embryogenesis from three to two cycles in *C. erythraea* (Bogdanović et al. 2021).

For somatic embryogenesis induction, 2,4-D is observed to be superior to other auxins, specifically over indole-3-butyric acid (IBA) as reported in large number of plants including medicinally important ones (Balaraju et al. 2011; Khajuria et al. 2021; Bogdanović et al. 2021). 2,4-D can be the most common choice of auxin for induction of calli for indirect somatic embryogenesis (Ali et al. 2021); however, it is not true for all species. In *E. alba*, different explants like nodal segments, leaf and shoot tips showed reduced calli induction with less biomass compared to Picloram (PIC) (Salma et al. 2019). Picloram (PIC) is systemic herbicide acting as auxin in plant tissue culture that promotes loosening of cell walls, cell growth, stem curvature, and root initiation (Bekheet et al. 2014). Concentration of PIC played important role in callus biomass generation and subsequent embryogenic potential. In *E. alba*, PIC (≤ 2 mg/L) promoted friable light-colored calli with embryogenic potential, whereas a higher concentration of PIC produced compact dark brown callus with reduced biomass (Salma et al. 2019). Similar effect of PIC compared to 2,4-D for induction of callus was reported in plants like *Elaeis guineensis* (Scherwinski-Pereira et al. 2010). PIC also induced direct somatic embryos in *Petiveria alliacea* alone with MS medium at a concentration of 20 μ M (Cantelmo et al. 2013).

Many investigations supported the role of abscisic acid (ABA) in maturation and germination of somatic embryos in different species, including medicinal plants, crop plants, and ornamental ones. ABA is found to have an enhancing effect on somatic embryo induction as well when supplemented in combination with cytokinins in certain plants. At a lower concentration, ABA is reported to enhance somatic embryogenesis and has a negative effect at higher levels in plants like *Picea abies*, *Picea omorika*, *E. alba*, etc. (Hazubska-Przybył et al. 2016; Salma et al. 2019). In *E. alba*, callus cultured on MS + PIC (2 mg/L) + TDZ (0.5 mg/L) + ABA (0.25 mg/L) showed highest somatic embryogenesis rate with approximately 35 somatic

embryos per callus having synchronous development (Salma et al. 2019). However, supplementation of higher concentration of ABA (>0.5 mg/L) in the same medium showed negative effect with death of somatic embryos. Similar observation is reported for *Picea* species, where ABA at a concentration of $10\text{ }\mu\text{M}$ showed enhanced rate of somatic embryo maturation and germination (Hazubska-Przybył et al. 2016). In *V. canescens*, Khajuria et al. (2021) reported significantly enhanced somatic embryo maturation rate (average 36 somatic embryos) in ABA (1.5 mg/L) supplemented MS medium. ABA and osmotic stress are major factors in determining proper maturation and germination of somatic embryos by playing an important role in accumulation of reserve materials in developing embryos and synchronization during maturation (Hazubska-Przybył et al. 2016). Méndez-Hernández et al. (2019) described the role of ABA in somatic embryo maturation, which takes place through transcriptional reprogramming involving regulators like ABA-insensitive 3.

In some cases, the maturation and germination took place on the same inductive medium as in *R. spiciforme* (Khan et al. 2022). Most investigations show that the removal of phytohormones from the medium facilitates maturation and germination of the somatic embryos (Dhir et al. 2014; Zhang et al. 2016; Khilwani et al. 2016; Sedaghati et al. 2019; Hazubska-Przybył et al. 2016; Bogdanović et al. 2021; Verma et al. 2022). There were also cases where supplementation of medium with hormones or related synthetic substances favored the process. Gibberellic acid or GA_3 -supplemented solid basal MS medium supported somatic embryo germination in some plants (Raomai et al. 2014; Kumar et al. 2017; Patel et al. 2021). In *L. reticulata*, somatic embryos developed into healthy plantlets on static MS media supplemented with $1.0\text{ }\mu\text{M}$ GA_3 (Patel et al. 2021). In *Wedelia calendulacea*, Sharmin et al. (2014) reported cotyledonary SE germinated into plantlets on MS + BAP (1 mg/L) + GA_3 (0.5 mg/L). In some plants, hormone-supplemented medium facilitated conversion of somatic embryo into plantlets like in *V. canescens*, maturation of SE took place on MS supplemented with ABA/ AgNO_3 , and germination was favored by BAP-supplemented medium (Khajuria et al. 2021). Similar high-frequency germination of SE was noted in *E. alba* in medium supplemented with low concentration of BAP (Salma et al. 2019). Ali et al. (2021) observed germination of SE on MS supplemented with combination of auxin and cytokinin in *B. monnieri*. Hence, just like induction of somatic embryos, maturation and germination conditions also vary with plant species, although removal of hormone seems to be favorable in most cases.

26.3.3 Light and Somatic Embryogenesis

Light conditions significantly affect the somatic embryogenesis process. While in some plants direct somatic embryo formation takes place under 16 h:8 h light/dark condition, for example, *P. oleracea* (Sedaghati et al. 2019), in certain plants like *R. spiciforme*, pre-conditioning in dark condition for a week followed by culture in 16 h:8 h light/dark photoperiod facilitated direct somatic embryogenesis (Khan et al.

2022). In *P. oleracea*, direct somatic embryogenesis was noted both under dark and light/dark conditions; however, continuous darkness (3 weeks prior to transfer in light) produced more somatic embryos (~133 somatic embryo/explant) compared to explants cultured under light/dark condition (~39 somatic embryo/explant) by Sedaghati et al. (2019). Similarly, in *Paris polyphylla*, direct somatic embryo induction took place in dark condition, and for conversion, 14 h:10 h photoperiod was used (Raomai et al. 2014).

For indirect somatic embryogenesis, some plants showed good results under light, while others performed better with pre-culture under dark condition prior to transfer in light. In *V. canescens* (Khajuria et al. 2021), *Hypoxis hemerocallidea* (Kumar et al. 2017), *Carum copticum* (Niazian et al. 2017), *Anethum graveolens* (Dhir et al. 2014), and *W. calendulacea* (Sharmin et al. 2014), embryogenic calli was induced, and somatic embryo formation took place under 16:8 light/dark photoperiod. In *C. erythraea*, indirect somatic embryogenesis was observed both under light and in dark conditions (Bogdanović et al. 2021). Ali et al. (2021) reported pre-culture of explants in dark condition for 5 days before transferring to 16 h:8 h light/dark photoperiod for induction of embryogenic calli in *B. monnieri* plants. In *E. alba*, callus induction is initiated under dark condition, and subsequent embryogenesis is observed under light/dark photoperiod (Salma et al. 2019). Some plants even favored continuous darkness for induction of indirect somatic embryogenesis as in *Lilium pumilum* (Zhang et al. 2016).

Light and phytohormones play important role in the differentiation and developmental process either independently or dependent on one another. Different investigations showed modulation of auxin synthesis pathway and gene regulation via auxins to be regulated by light; however, the molecular mechanism is yet not clear. Zelená (2000) reported that light affects auxin synthesis, namely, IAA, and hence, it can be possible that light positively influences auxin synthesis and high level of auxin inhibits or reduces somatic embryogenesis as reported in the above examples. Thus, pre-conditioning in dark condition improves cytokinin/auxin ratio and thus facilitates somatic embryogenesis. Very limited literature is present investigating the effect of light quality on somatic embryogenesis. Rukh et al. (2019) studied the effect of different wavelengths of light (green, blue, red, and yellow along with white light as control) on induction of somatic embryogenesis from callus of *A. bracteosa*. The study revealed highest somatic embryogenesis (92.75%) under red light followed by 90.31% under green light in this plant.

26.3.4 Amino Acids and Other Biochemical Factors in Somatic Embryogenesis

Biochemical factors like amino acids are reported to enhance somatic embryogenesis in certain medicinal plants as well. In *R. spiciforme*, direct somatic embryo with maximum germination (~91%) was observed on leaf explants cultured on MS + BAP

(25 μM) + NAA (1 μM) + GA_3 (2 μM) + glutamine (50 μM) + adenine sulfate (50 μM) by Khan et al. (2022). Similarly, Khajuria et al. (2021) reported significant enhancement in number of indirect somatic embryo with supplementation of glutamine (2%) and casein hydrolysate (50 mg/L) in MS medium containing 2,4-D and kinetin in *V. canescens*. The number of somatic embryos increased from ~19 on MS + 2,4-D (0.15 mg/L) + kinetin (0.05 mg/L) to ~27 on MS + 2,4-D (0.15 mg/L) + kinetin (0.05 mg/L) + glutamine (2%) + casein hydrolysate (50 mg/L). The enhancing effect due to addition of amino acids can be due to the improvement in cell signaling pathways, acting as precursor to phytohormone or by regulating DNA synthesis (Deo et al. 2010).

In addition to different phytohormones, additives like coconut water (4%) are reported to promote maturation and germination of somatic embryos (Patel et al. 2021). Similar enhancement by other complex organic extracts, like corn, papaya, potato, and taro extracts, is reported in some plants (Deo et al. 2010). Inclusion of these organic compounds might be advantageous being less mutagenic and hence minimizing chances of somaclonal variation compared to conventional phytohormones; however, maintaining consistency of concentration in culture and getting reproducible results might be difficult.

Another important factor influencing somatic embryogenesis is known to be sucrose concentration. Sucrose concentration in the medium is reported to have a positive influence on somatic embryogenesis in many plants including medicinal ones. Khilwani et al. (2016) studied the effect of 10–250 mM sucrose on indirect somatic embryo formation and reported highest frequency of somatic embryo in MS + 2,4-D (1 μM) + BAP (12.5 μM) + sucrose (250 mM). At lower concentration, sucrose is found to induce organogenesis, while at higher concentration, it induces somatic embryogenesis as observed in *B. monnieri* (Khilwani et al. 2016), *Chlorophytum borivilianum* (Kumar et al. 2010), etc. Contrastingly, the number of somatic embryos increased with reduction of sucrose concentration in *C. orchoides* with decrease in embryogenic frequency (Thomas and Jacob 2004). Osmoticum has been reported to have important role in the production and maturation of somatic embryos in certain plants. Hazubska-Przybył et al. (2016) reported increasing sucrose concentration to 68 g/L in maturation medium significantly increased germination of SE in *Picea* species. Sucrose is the most common osmotic agent used for maturation of somatic embryos. It not only works as carbon source but also participates in gene regulation required for somatic embryo maturation. Moreover, sucrose also helps in increasing the water stress in the culture medium and hence promotes maturation of the embryos.

Hence, it is evident that successful protocol for somatic embryogenesis can be achieved by standardization of various factors mentioned above, as already established for a wide variety of plant species including medicinal ones. Although with great potential, somatic embryogenesis has some drawbacks like (a) intervening callus in the indirect process can involve somaclonal variations and (b) somatic embryos develop asynchronously. Methods have been established to overcome the asynchrony, for example, by grouping cell aggregates of similar size and density, with reduced yield; however, with the kind of large number of somatic embryos

(>1 million/L) possible, sub-grouping cells still make a huge number of regenerants compared to other micropropagation methods. To overcome the problem of somaclonal variation, frequent initiation of new cultures and limiting subculture to maximum 1 year are also recommended. Thus, somatic embryos can be successfully utilized for the conservation of endangered medicinal plants either directly as a micropropagation method or via production of artificial seeds, as discussed in the next section.

26.4 Artificial Seed Production

Natural seeds produced by sexual reproduction are genetically different from the individual parent as a result of crossing over. In contrast, synthetic seed-germinated plantlets are clones of their parents. Micropropagation, conservation, preservation of biodiversity, and exchange of plants have been successful by the development of these artificially encapsulated seeds. The technique was conceptualized by Murashige in 1977 and primarily reported by Kitto and Janick in 1982 wherein water-soluble resin coat and polyoxyethylene glycol were used to coat somatic embryos of *Daucus carota*. The most commonly method practiced today involves the use of somatic embryo encapsulated with alginate coating, which was reported first by Redenbaugh et al. (1984) in alfalfa. Since then, promising reports were obtained for a wide variety of plants ranging from ornamentals and crop plants to pharmaceutical ones (Rai et al. 2009; Maqsood et al. 2020; Mangena 2021). This technology is particularly beneficial for medicinal plants, which have become endemic or endangered due to low seed set and poor germination caused by environmental modifications or edaphic factors (Reddy et al. 2012).

According to the definition of Capuano et al. (1998), synthetic seeds are artificially encapsulated somatic embryos, or other somatic tissues such as shoot tips, axillary buds, or meristematic tissue, which are stored and sown as seeds and upon germination either under in vitro or in vivo condition, develop into whole plant. Detailed description of artificial seed technology including its mechanism and application was done by several researchers including Rihan et al. (2017), Pond and Cameron (2017), and Sharma et al. (2013). Here, we focus on the factors that affect the quality of the artificially produced seeds and its germination efficiency. Special emphasis is given on the instances of medicinal plant synseed production and its role in conservation.

Interestingly, synthetic seed technology has been extended to pteridophytes as well. Bracken fern *Pteridium aquilinum* var. *latiusculum* spores were encapsulated in alginate and upon germination formed gametophyte and sporophytes. This report by Jang et al. (2020) paves way for easy transportation and commercialization of the fern spores in spite of their small size. Successful encapsulation of *Splachnum ampullaceum* moss bud (Mallón et al. 2007) and *Osmunda regalis* gametophyte (Makowski et al. 2016) have also been targeted for their conservation. In the last few decades, success has been achieved in artificial seed production of medicinal plants (Table 26.2).

Table 26.2 Some reports on optimal conditions of artificial seed production and germination in the past decade

Plant	Explant	Optimum encapsulating agent (supplementary additives and Sodium alginate w/v + CaCl ₂ · 2H ₂ O or CaCl ₂)	Synseed germination media	Maximum regeneration percentage in vitro	Hardening mixture	Reference
<i>Nyctanthes arbor-tristis</i> L.	NS	MS + 3% SA 100 mM + CaCl ₂ · 2H ₂ O	MS + 5 mg/L BAP	65	Garden soil, soil-rite, sand (1:1:1)	Mishra et al. (2022)
<i>Dendrobium crumenatum</i> Sw.	PLB	MS + 3% SA + 100 mM CaCl ₂	MS + 0.2% activated charcoal	100	Coconut husks	Klaosheed et al. (2021)
<i>Asparagus officinalis</i> L.	AB, SE	MS + 2% SA + 100 mM CaCl ₂ · 2H ₂ O	MS ^{1/2} MS + BA or NAA	85.05	Peat, sand (1:1)	Abbasi et al. (2020)
<i>Crinum malabaricum</i> Lekhak & Yadav	Longitudinally dissected bulb	2.0% SA + 100 mM CaCl ₂	MS + 2.0 mg/L BAP + 0.5 mg/L + additives	92.5	Soil-rite followed by red-soil, vermicompost (1:1)	Priyadharshini et al. (2020)
<i>Decalepis salicifolia</i> (Bedd. ex Hook.f.) Bruyns	ST	MS + 3% SA + 100 mM CaCl ₂	MS + 2.2 µM BAP + 5.7 µM IAA	53.33	Soil, vermicompost (1:1/2)	Rodrigues et al. (2020)
<i>Hedycheium coronarium</i> J.Koenig	ST	MS + 3.0 mg/L mT + 3% SA + 100 mM CaCl ₂	MS	90	Soil, sand (1:1)	Behera et al. (2020)
<i>Simmondsia chinensis</i> C.K.Schneid.	AB	MS + 4% sucrose + 3% SA + 100 mM CaCl ₂	MS	80	Sand, soil, FYM (1:2:1)	Bala et al. (2020)
<i>Celastrus paniculatus</i> Willd.	NS	1/2 MS + 4% SA + 100 mmol/L CaCl ₂	MS + 2 mg/L BA + 0.2 mg/L NAA	80	–	Fonseka et al. (2019)
<i>Eclipta alba</i> L. ex B.D. Jacks.	SE	1/2 MS + 2.5% SA + 75 mM CaCl ₂ · 2H ₂ O	1/2 MS	40–52	Soil, sand, vermicompost (1:1:1)	Salma et al. (2019)
<i>Athaea officinalis</i> L.	NS	1/2 MS + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	MS + 7.5 µM BA + 0.5 µM NAA	66	Sandy soil, garden soil (1:1)	Naz et al. (2018)

<i>Ansellia africana</i> Lindl.	PLBs	MS + 3% SA + 100 mM CaCl ₂	Supplemented with 7.5 µM memTTHP	88.21	Vermiculite, sand, decaying litter (1:1:1)	Bhattacharyya et al. (2018)
<i>Coriandrum sativum</i> L. varieties (Rajendra Swathi 'RS' and Co-1)	SE	MS + 3% SA + 100 mM CaCl ₂	MS + 1 mg/l BA + 0.5 mg/l GA ₃	RS—74.0 Co-1—70.6	Soil	Ali et al. (2018)
<i>Gynemna sylvestre</i> (Retz.) R.Br. ex Sm.	NS	MS + 0.56 mg/L BA + 0.86 mg/L GA ₃ + 18.41 mg/L ADS + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	MS + 5.0 µM BA	88.2 ± 0.48	Soil-rite, vermicompost (3:1)	Saeed et al. (2018)
<i>Plumbago zeylanica</i> L.	callus	MS + 6% SA + 50 mM CaCl ₂	MS + 0.25 mg/L NAA + 1 mg/L BAP	76.6	—	Jain et al. (2018)
<i>Urginea altissima</i> (L.f.) Baker	ST	MS + 2.42 mg/L metatopolin + 0.37 mg/L NAA + 3% SA + 100 mM CaCl ₂	MS + 10 µM metatopolin + 2 µM NAA	91	Vermiculite: soil (1:1)	Baskaran et al. (2018)
<i>Acorus calamus</i> L.	Microrhizome	½ MS + 3% SA + 3% CaCl ₂	MS + 100 mg/L citric acid + 1 g/L PVP-40	100	Coco peat	Quraishi et al. (2017)
<i>Rauvolfia serpentina</i> (L.) Benth. Ex Kurz	ST	½ MS + 3% SA + 75 mM CaCl	½ MS	88	—	Gantait et al. (2017b)
<i>Rauvolfia serpentina</i> (L.) Benth. Ex Kurz	ST	½ MS + 3% SA + 75 mM CaCl	½ MS	52	—	Gantait and Kundu (2017)
<i>Tylophora indica</i> (Burm.f.) Merr.	NS	½ MS + 3% SA + 75 mM CaCl ₂ · 2H ₂ O	½ MS Liquid	70	—	Gantait et al. (2017a)
<i>Vitex trifolia</i> L.	NS	MS + 3% SA + 100 mM CaCl	MS + 2.5 µM kin + 1 µM NAA	89.6	Garden soil	Alatar et al. (2017)
<i>Bacopa monnieri</i> (L.) Wettst.	SE	MS + 3% SA + 125 mM CaCl ₂ · 2H ₂ O	MS + 12.5 µM BA + 1 µM 2,4-D	41.66	—	Khiwani et al. (2016)

(continued)

Table 26.2 (continued)

Plant	Explant	Optimum encapsulating agent (supplementary additives and Sodium alginate w/v + CaCl ₂ · 2H ₂ O or CaCl ₂)	Synseed germination media	Maximum regeneration percentage in vitro	Hardening mixture	Reference
<i>Citrus</i> spp.	SE	½ MS + 0.25 g/L malt extract + 0.25 g/L ascorbic acid + 1 mg/L GA ₃ + 0.02 mg/L NAA + 68 g/L sucrose + 2.5% SA + 1.1% CaCl ₂	MS + 0.5 g/L malt extract + 0.5 g/L ascorbic acid + 68 g/L sucrose	–	–	Micheli and Standardi (2016a, b)
<i>Curcuma amada</i> Roxb.	SE	½ MS + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	1/2 MS solid medium supplemented with 3% (w/v) sucrose and 0.25 mg/L GA ₃	88.10 (sucrose-dehydrated seed)	Sand, soil, vermiculate (1:2:1)	Raju et al. (2016)
<i>Digitalis davisiana</i> Heywood	ST	MS + 0.25 mg/L NNA + 1.0 mg/L BAP + 4% SA 100 mM CaCl ₂	MS + 0.25 mg/L NAA + 1 mg/L BAP	>25	Soil, manure, sand (1:1:1)	Verma et al. (2016)
<i>Bacopa chamaedryoides</i> (Kunth) Wettst.	MSt	MS devoid of Ca ²⁺ 3% SE 3% (calcium nitrate)	MS + 3 mg/L TDZ + 15 mg/L AvG	>60	Soil, organic manure (2:1)	Haque et al. (2015)
<i>Bacopa monnieri</i> (L.) Wettst.	NS	MS + 2.5% SA + 75 mM CaCl ₂ · 2H ₂ O	½ MS	–	–	Gantait et al. (2015b)
<i>Ocimum kilimandscharicum</i> Gürke	ST	MS + 3% SA + 75 mM CaCl ₂ · 2H ₂ O	MS + 1.5 mg/L IBA.	83.33	Soil, vermiculite (1:1)	Saha et al. (2015)
<i>Tecomella bulweri</i> F. Muell.	NS	MS + 3% SA + 100 mM CaCl ₂	MS + 10.0 M BA	58.5	Garden soil	Shaheen and Shahzad (2015)

<i>Vitex trifolia</i> L.	NS	MS + 3% SA 100 mM CaCl ₂ · 2H ₂ O	MS + 5.0 µM BA + 0.5 µM NAA	84.9	Soil	Ahmed et al. (2015)
<i>Anethum graveolens</i> L.	SE	MS + 3% SA + 100 mM CaCl ₂	MS + 0.2 mg/L TDZ	95	Soil, sand, vermiculite (1:1:1)	Dhir et al. (2014)
<i>Balanites aegyptiaca</i> (L.) Delile	NS	MS + 3% SA + 100 mM CaCl ₂	MS + 12.5 µM BA + 1 µM NAA	80	Garden soil	Varshney and Anis (2014)
<i>Cassia angustifolia</i> Vahl	NS	MS + 3% SA + 100 mM CaCl ₂	MS + 2.5 µM BA + 0.4 µM NAA	94	Garden soil, garden manure (1:1)	Parveen and Shahzad (2014)
<i>Cassia angustifolia</i> Vahl	NS	MS + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	½ MS + 5 µM TDZ + 1 µM IAA	93	Soil-rite	Bukhar et al. (2014)
<i>Centella asiatica</i> (L.) Urb.	AB, NS	MS + 0.01% meso-ino- sitol + 3% sucrose + 11.61 M kinetin + 4% SA + 75 mM CaCl ₂ · 2H ₂ O	MS	87	Soil, vermiculite, FYM (2:1:1)	Prasad et al. (2014)
<i>Mimosa pudica</i> L.	ST, NS	MS + 2.0 mg/L BAP + 0.2 mg/L NAA 4% SA + CaCl ₂	MS + 2.0 mg/L BAP + 0.2 mg/L NAA	100	–	Banu et al. (2014)
<i>Ochradenus baccatus</i> Delile	NS, ST	MS + 3% SA + 100 mM CaCl ₂	MS + 1 µM BA (shooting), MS + 100 µM IBA (rooting)	86	FYM, garden soil	Al-Qurainy et al. (2014)
<i>Ocimum gratissimum</i> Forssk.	MSt	MS + 3% SA + 75 mM CaCl ₂ · 2H ₂ O	MS + 1 mg/L BA	98.6	Soil, vermiculite (1:1)	Saha et al. (2014)
<i>Phyllanthus fraternus</i> G.L. Webster	NS	MS + 3% SA + 75 mM CaCl	½ MS + 0.5 mg/L BAP	92.5	Soil, sand (1:1)	Upadhyay et al. (2014)
<i>Salvia splendens</i> Sellow ex Nees	NS	MS + 5.0 µM BA and 2.5 µM IAA + 4% SA 100 mM CaCl ₂ · 2H ₂ O	MS	63.3	Soil, farmyard soil, sand (2:1:1)	Sharma et al. (2014)

(continued)

Table 26.2 (continued)

Plant	Explant	Optimum encapsulating agent (supplementary additives and Sodium alginate w/v + CaCl ₂ · 2H ₂ O or CaCl ₂)	Synseed germination media	Maximum regeneration percentage in vitro	Hardening mixture	Reference
<i>Sterculia urens</i> Roxb.	NS	MS + 4% SA + 100 mM CaCl ₂ · 2H ₂ O	MS + 0.2 mg/L TDZ	95	–	Devi et al. (2014)
<i>Stevia rebaudiana</i> Bertoni	ST	MS + 4% SA + 100 mM of Ca (NO ₃) ₂ · 4H ₂ O	½ MS	69	Not mentioned	Nower (2014)
<i>Swertia chirayita</i> (Roxb.) Buch.-Ham. ex C.B. Clarke	SE	MS + SA 4% + 100 mM CaCl ₂ · 2H ₂ O	MS + 1.0 mg/L BA and 0.5 mg/L NAA	84	Soil-rite, sand (1:2)	Kumar and Chandra (2014)
<i>Terminalia arjuna</i> (Roxb. ex DC.) Wight & Arn.	ST	Modified MS + BAP (0.5 mg/L) + NAA (0.1 mg/L) + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	MS + 0.5 mg/L BAP + 0.1 mg/L NAA	91.6	Soil-rite	Gupta (2014)
<i>Tuberaria major</i> (Willk.) P. Silva & Rozeira	ST	MS + 3% SA + 75 mM CaCl ₂	MS	44	–	Coelho (2014)
<i>Aristolochia tagala</i> Cham.	MSt	MS + 3% SA + 1% CaCl ₂	MS + 3 µM BAP + 0.5 µM KIN	–	Coir pith, vermiculite, soil (1:1:1)	Remya et al. (2013)
<i>Artemisia vulgaris</i> L.	SE	MS + 2–4% SA + 50–80 mM CaCl ₂	MS + GA ₃ (1.5 mg/L) + IAA (0.5 mg/L) + Ascorbic acid (40 mg/L)	90	Soil	Sudarshana et al. (2013)
<i>Centaurium erythraea</i> Rafn.	HR	MS + 3% SA + 50 mM CaCl ₂	WPM + 3% sucrose + 0.5 mg/L BAP	100	Soil, sand, peat (4:3:3)	Piąteczak and Wysockńska (2013)

<i>Ceropegia bulbosa</i> Roxb.	NS	MS + 3% SA + 100 mM CaCl ₂	MS + 8.88 µM BA	100	Sterile soil, sand, vermiculite (1:1:1)	Dhir and Shekhawat (2013)
<i>Ceropegia spiralis</i> Hook.f. & Thomson, <i>C. pusilla</i> Hook.f. & Thomson	ST, NS	MS + 3 mg/L BAP + 3% SA + 50 mM CaCl ₂	MS medium supplemented with 3.0 mg/L BAP	<i>C. spiralis</i> -90, <i>C. pusilla</i> -86.6	–	Murthy et al. (2013)
<i>Dendrobium nobile</i> Lindl.	PLB	MS + sucrose and mannitol (0.0–15.0%) + 3% SA + 100 mM CaCl ₂	½ MS + 1 mg/L BAP + 0.1 mg/L NAA	78.2	–	Mohanty et al. (2013)
<i>Hemidesmus indicus</i> (L.) R.Br. ex Schult.	SE	MS + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	MS + Kn (2 µM) and IBA (0.5 µM).	88	Garden soil, sand (1:1)	Chervuvathur et al. (2013a)
<i>Rauwolfia tetraphylla</i> L.	NS	½ WPM + 3% sodium alginate + 100 mM CaCl ₂	WPM + 7.5 µM BA and 2.5 µM NAA	72	Garden soil	Faisal et al. (2013)
<i>Rhinacanthus nasutus</i> (L.) Kurz	SE	MS + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	MS + 0.2 mg/L GA ₃	92	Garden soil, sand (1:1)	Chervuvathur et al. (2013b)
<i>Withania somnifera</i> (L.) Dunal	NS	MS + 3% SA + 100 mM CaCl ₂	MS + 2.5 µM BA + 0.5 µM NAA	86.2	Soil	Fatima et al. (2013)
<i>Aranda Wan Chark Kuan</i> 'Blue' × <i>Vanda coerulea</i> Griff. Ex. Lindl.	PLB	½ MS + 3% SA + 75 mM CaCl ₂ · 2H ₂ O	MS	96.2	Garden soil	Gantait et al. (2012)
<i>Catharanthus roseus</i> (L.) G.Don	SE	MS + 2.5% SA + 100 mM CaCl ₂ · 2H ₂ O	MS + 1.34 µM NAA + 1.1 µM BA	84.33	–	Maqsood et al. (2012)
<i>Clitoria ternatea</i> L.	SE	MS medium + 3% sucrose + 1 mg/L BA + 0.2 mg/L NAA + 4% SA + 100 mM CaCl ₂	MS + 2 mg/L BA + 0.5 mg/L NAA	92	Soil, sand, dry cow-dung (1:1:1)	Kumar and Thomas (2012)

(continued)

Table 26.2 (continued)

Plant	Explant	Optimum encapsulating agent (supplementary additives and Sodium alginate w/v + CaCl ₂ · 2H ₂ O or CaCl ₂)	Synseed germination media	Maximum regeneration percentage in vitro	Hardening mixture	Reference
<i>Corymbia torelliana</i> (F. Muell.) K.D.Hill & L. A.S.Johnson × <i>Corymbia citriodora</i> (Hook.) K.D. Hill & L.A.S.Johnson	ST, NS	MS + 2.2 µM BA + 3% SA + MS + 100 mM CaCl ₂	MS + 3% sucrose + 2.2 µM BA	62–100	Organic compost (vegetables and saw dust)	Hung and Trueman (2012a, b)
<i>Curcuma amada</i> Roxb.	MS with rhizome	½ MS + 1.5% sucrose + 1.0 mg/L Rosebengal/bavistin + 3% SA + 3% CaCl ₂	MS	50–75	–	Banerjee et al. (2012)
<i>Decalepis hamiltonii</i> Wight & Arn.	NS	MS + 4% SA + 100 mM CaCl ₂ · 2H ₂ O	MS + 5 µM BA + 0.5 µM IAA + 30 µM ADS	77	Garden soil, green manure (2:1)	Sharma and Shahzad (2012)
<i>Mentha arvensis</i> L.	ST, NS	½ MS + 2.0 mg/L BAP + 0.2 mg/L NAA + 4% SA + CaCl ₂	MS + 2.0 mg/L BAP + 0.2 mg/L NAA	80	–	Islam and Bari (2012)
<i>Rauvolfia serpentina</i> (L.) Benth. ex Kurz	NS	WPM + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	WPM + 5.0 µM BA + 1.0 µM NAA (for sprouting) and ½ liquid MS + 0.5 µM IAA	80	Garden soil, soil-rite (75% Irish peat moss and 25% horticulture grade expanded perlite) or vermiculite	Faisal et al. (2012)
<i>Rauvolfia tetraphylla</i> L.	NS	WPM + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	WPM + 7.5 µM BA + 2.5 µM NAA	90.3	Soil	Alatar and Faisal (2012)

<i>Rauvolfia vomitoria</i> Wennberg	AMS	MS + 4% SA + 100 mM CaCl ₂	MS + 1.0 mg/L BAP and 0.1 mg/L NAA	69.6	Sand, soil, FYM (1:2:1)	Mehrotra et al. (2012)
<i>Ruta graveolens</i> L.	NS	MS + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	MS + 10 µM BA and 2.5 µM NAA	80 approx	Garden soil	Ahmad et al. (2012)
<i>Stevia rebaudiana</i> Bertonii	ST, NS	MS + 3% SA + 100 mM CaCl ₂	MS + 1.0 mg/L BA	100	—	Ali et al. (2012)
<i>Cannabis sativa</i> L.	NS	MS + 0.5 µM TDZ + 2.5 µM IBA + 0.3–0.55 PPM + 5% SA + 50 mM CaCl ₂	MS + 0.5 µM TDZ	60	—	Lata et al. (2011)
<i>Citrus sinensis</i> (L.) <i>Osbeck</i> × <i>Poncirus</i> <i>trifoliata</i> (L.) Raf.	NS	½ MS + 50 g/L sucrose + 2.5% SA + 1.1 % CaCl	MS	100 (sprouting)	—	Germanà et al. (2011)
<i>Flickingeria nodosa</i> (Dalzell) Seidentf.	PLBs	Burgeff's N3F + 2% SA + 100 mM CaCl ₂ · 2H ₂ O	1 × Burgeff's N3F medium, 2% sucrose fortified with 2 mg/L adenine sulfate and IAA 1 mg/L	95	Soil	Nagananda et al. (2011)
<i>Khaya senegalensis</i> A. Juss.	ST, NS	½ MS medium + 2% sucrose + 3% SA + 100 mM CaCl ₂ · 2H ₂ O	MS + 245 µM IBA	52–98	Organic compost (veg- etables and saw dust)	Hung and Trueman (2011)
<i>Picrorhiza kurroa</i> Royle	ST, NS	½ MS + 1.5% sucrose + 3% SA + 3% CaCl ₂	MS	21.4	Not mentioned	Mishra et al. (2011)
<i>Pogostemon cablin</i> Benth.	NS	MS + 4% SA	MS + 0.5 mg/L BA	43.3	Soil	Patil et al. (2011)
<i>Stevia rebaudiana</i> Bertonii	ST	MS + 3% SA + 2.5% CaCl ₂ · 2H ₂ O	MS + 2 mg/L BAP + 1 mg/L IAA	70	—	Andlib et al. (2011)

µM micro molar; AB, apical bud; ADS, adenine sulfate; AMS, axillary microshoot; BAP, 6 benzene aminopurine; CaCl₂ · 2H₂O or CaCl₂; FYM, Farm Yard manure; GA₃, gibberellic acid; HR, hairy root; IAA, indole acetic acid; memTTHP, meta-methoxy topolin 9-tetrahydropyran-2-yl; mM, mili molar; MS, Murashige and Skoog basal media (1962); mT, meta-Topolin; NAA, naphthoxy acetic acid; NS, nodal segment; PLB, protochorm like bodies; PPM, plant preservative mixture; PVP-40, polyvinyl pyrrolidone-40; SA, sodium alginate; SE, somatic embryo; ST, shoot tip; WPM, Woody Plant Media

26.4.1 *Explant Selection*

Plant parts such as unipolar or bipolar vegetative propagules (including somatic embryos), rhizomes, protocorms, nodal regions, shoot tips, micro-cuttings, and differentiating tissue aggregates are commonly used as the initial living material for medicinal plant synthetic seed production (Rihan et al. 2011; Sharma and Shahzad 2012; Gantait et al. 2015a). Embryos of *Arnebia euchroma* in early cotyledonary stage were used by Manjkhola et al. (2005). The artificial seeds thus developed showed a germination rate of 60.6% and high field survival (72%). Approximately, 3–5 cm long explants from both embryogenic and somatic in vitro plantlets were converted to synthetic seeds (Gantait et al. 2012; Gantait and Sinniah 2013; Sharma et al. 2013). Genetically transformed roots of *Scutellaria baicalensis* and *Ruta graveolens* also served as explants (Vdovitchenko and Kuzovkina 2011a, b).

26.4.2 *Encapsulation Agents and Synthesis Conditions*

The artificial seed matrix is similar to the endosperm of natural seeds and remains in direct contact with the explant. Survival and nutrition of the encapsulated tissue depends upon the nature of the encapsulating agent (Antonietta et al. 1999). In comparison to naked explants, synthetic seeds are either desiccated or hydrated (Bhojwani and Razdan 2006). Hydrated artificial seed production involves the use of hydrogel as the capsule. Benelli (2016) observed better propagation of alginate-coated explant due to the nutritive and protective nature of the coat. Polymers having favorable gelling properties, cost-effective, and nontoxicity are used. Other requirements include imparting protection during storage and handling, effectively bearing nutrients and growth regulators, non-interfering with the biochemical processes of the explant, and being favorable for germination and biodegradability (Khor and Loh 2005; Nongdam 2016). While preparing the artificial seeds, the explants are mixed with a polymeric solution, dispensed dropwise in a liquid having divalent metal ions, which results in a cross-linking effect, giving rise to the hydrogel. The resulting bead shields the explant without putting pressure on it (Gantait et al. 2015b). The rigidity of the calcium alginate hydrogel depends on the number of Na^+ (from sodium alginate) exchanged with Ca^{2+} (from $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ solution) (Daud et al. 2008). Hydrated synthetic seeds may either be single-layered, double-layered (prepared by coating a single-layered bead with a similar concentration of matrix), or hollow bead-type (prepared by complete protection of somatic embryos in a sterile core, thereby allowing its continued development) (Sharma et al. 2013).

Sodium alginate has emerged as the substance of choice with concentrations varying from 1.5% to 6.0%. The concentration of the encapsulating agent as well as the duration for which the explant is exposed to it determined the quality of the seed and its germination frequency (Singh and Chand 2010). The concentration has been

standardized in a variety of plant species including *Cacicus carota* (Latif et al. 2007), *Spartina alterniflora* (Utomo et al. 2008), *Catharanthus roseus* (Maqsood et al. 2012), *Rhinacanthus nasutus* (Meena et al. 2013), and (Ali et al. 2018). From experimentations carried out in *Coelogyne breviscapa* (Mohanraj et al. 2009), *Stevia rebaudiana* (Ali et al. 2012), *C. sativum* (Ali et al. 2018), and *Vitex trifolia* (Ahmed et al. 2015; Alatar et al. 2017), sodium alginate at a concentration of 3% and along with 100 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ seems to be mostly suitable. The resulting beads are compact, transparent, and isodiametric. The combination of 3% sodium alginate and 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was also optimal for the encapsulation of the medicinal plant *Mondia whitei* providing 95.7% survival and germination 73% germination frequency (Baskaran et al. 2018). From various reports, it can thus be stated that higher frequency of germination of synthetic seed bears correlation with lower concentration and exposure time to $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Cheruvathur et al. 2013a, b). Longer incubation time to allow the ion exchange process is normally preferred in case of lower concentration of calcium chloride. Scientists have used exposure time ranging from 5 min (Nagesh et al. 2009) to 50 min (Nirala et al. 2010) for 100 mM concentration of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Prolonged exposure to $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and its absorption by the enclosed tissue impairs germination due to excessive absorption (Malabadi and Van Staden 2005).

Other gelling agents include agar, carrageenan, alginate, carboxy methyl cellulose, gelrite, sodium pectate, etc. Supplementing encapsulating agent with plant growth regulators contributes to increased viability and germination. Adjuvants incorporated into the gelling agents help the explant to tide over desiccation, microbial infection, and mechanical injury (Bhatia et al. 2015). In preparation of the desiccated type, somatic embryos are treated with ABA or stress to induce desiccation tolerance (Helal 2011) followed by removal of moisture either progressively or rapidly (Reddy et al. 2012).

26.4.3 Factors Affecting Storage and Germination

Seed viability and germination potential depended on environmental conditions and duration of storage. Temperature had an effect on synthetic seed conservation and regeneration efficiency. Storage at 4 °C was optimal than 20 and 25 °C temperature conditions in case of *Coriandrum sativum* (Ali et al. 2018). Exposure to low temperature is detrimental probably due to inhibition in nutrient absorption and formation of ice crystals leading to frost-induced stress and injury (Mittler 2006). Temperature optimization however seemed to be species-dependent as a higher temperature (25 °C) and medium temperature (15 °C) were a better choice (Sharma et al. 2013; Haque and Ghosh 2014, 2016) than low temperature (4 °C). In case of *Tylophora indica* 15 ± 1 °C was appropriate for storage and germination of synthetic seeds (Gantait et al. 2017a). Higher temperature storing was reported to be beneficial for *Zingiber officinale* (Sundararaj et al. 2010), *Khaya senegalensis* (Hung and Trueman 2011), *Centella asiatica* (Prasad et al. 2014), and *Bacopa monnieri*

(Khilwani et al. 2016) probably due to susceptibility of these plants to cold conditions. As reported by Andlib et al. (2011), *Stevia rebaudiana* artificial seed storage was optimum at 48 °C. Increased ABA/gibberellic acid (GA) ratio under light condition caused physiological dormancy under light condition. Lower light was thus beneficial for explant survival and growth (Kamińska et al. 2018). The optimal abiotic condition for in vitro artificial seed germination in majority of explants was deduced to be 23–27 °C temperature, 50–70% relative humidity, and 16 h photoperiod at 30–60 $\mu\text{mol}/\text{m}^2/\text{s}$ light intensity. These factors are essential for maintenance of enzyme function and physiological processes (Gantait and Kundu 2017; Das et al. 2021).

Long-term storage had inverse effect on germination potential possibly due to depletion in carbohydrate levels (Ding et al. 1998; Parveen and Shahzad 2014). While 100% germination was reported up to 7 days storage, a sharp decrease to 66% happened when storage period was extended to 28 days in *Cymbidium aloifolium* (Pradhan et al. 2014). Baskaran et al. (2018) reported a drop in germination frequency of synthetic seeds of *Mondia whitei* from 95.7% to 51.6% after storage for 50 days at 4 °C. Lower germination upon storage than non-stored seeds was attributed to drying and oxygen deficiency. While short-term storage is ideal under favorable storage conditions, long-term storage is best achieved by dehydration and cryopreservation (Das et al. 2021). Synthetic seeds were stored at room temperature (25 ± 2 °C) for up to 6 months using sponge derived from dried and processed fruits of *Luffa aegyptica* treated with fungi rose bengal/bavistin. This was a cost-efficient way of storing the encapsulated explants of *Curcuma amada* (Banerjee et al. 2012).

26.4.4 Germination and Acclimatization

Percentage of artificial seeds undergoing increase in size along with emergence of shoot or root is designated as regrowth rate, while the percentage of seeds undergoing both shooting and rooting to form entire plantlets is termed as regeneration or conversion. Pre- and post-storage conditions in terms of basal media, duration of storage, abiotic conditions, and presence of PGRs normally determine germination of synseeds (Das et al. 2021).

Optimal regeneration system is needed for high germination frequency at a fast rate. Combinatorial role of cytokinin and auxin has been reported to play a role in shoot and root development. Germination response was triggered by supplementation with BA and GA₃, while rooting of plantlets was induced by IBA (Godishala et al. 2011; Swarna and Ravindhran 2013). Of the different combinations of PGRs, the highest fresh weight and seed penetrance percentage was on media with 0.035 GA₃, while the highest percentage of germination was on 0.035 mg/L GA₃ and 1.7 mg/L IBA (Muslihatin et al. 2018). Artificial seeds of medicinal orchid *Cymbidium aloifolium* were subjected to different experimental conditions to study viability and germination rate. Nutrient absorption in artificial seed takes place from the basal media in the same way as a natural seed derives its nourishment

from the soil. Unsupplemented liquid MS used at quarter, half, and full strength supported 100% germination in contrast to 83.33% germination in PGR supplemented MS and 2–5% germination of natural seeds. Fungicide treatment reduces chances of contamination during long-term storage; however, a higher concentration or combination of different fungicides seems to have an adverse effect by early rupture of bead (Pradhan et al. 2014). Woody plant medium (WPM) was selected as the media of choice for *Rauvolfia tetraphylla* (Alatar and Faisal 2012) and olive medium modified (OMM) for *Olea europaea* (Micheli et al. 2019). Supplementation of 40–50 mg/L adenine sulfate was suitable for *Gymnema sylvestre* by reducing shoot tip necrosis and leaf abscission of the regenerated plantlets (Saeed et al. 2018; Naaz et al. 2014). Plant preservative mixtures such as *Aloe vera* gel and activated charcoal support germination by providing nourishment and removal of toxic substances, respectively (Haque et al. 2015).

26.4.5 Post-storage Viability and Stability of Regenerants

Successful ex vitro acclimatization up to flowering stage has been reported in *Aloe*, *Bacopa*, *Ledebouria*, and *Drimiopsis* (Haque and Ghosh 2013a, b, 2016, 2017). The genetic stability of plantlets obtained by germination of synseeds (stored for short term at 4 °C) of *R. serpentina* and its subsequent hardening was confirmed by random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) analysis (Faisal et al. 2012). Similar case has been reported in *Ananas comosus* (Gangopadhyay et al. 2005), *Cineraria maritima* (Srivastava et al. 2009), and *Picrorhiza kurroa* (Mishra et al. 2011). ISSR marker study further confirmed genetic stability in ex vitro germinated plantlets from synseeds of *G. sylvestre* (Saeed et al. 2018). Reports on genetic stability of artificial seed-derived plantlets were furnished by Saha et al. (2015) in *Ocimum kilimandscharicum* and by Khilwani et al. (2016) in *B. monnieri*. Genetic stability of artificial seed-derived plantlets was also reported in *Cineraria*, *Drimiopsis*, *Glycyrrhiza glabra*, and *Picrorhiza* (Srivastava et al. 2009; Mishra et al. 2011; Mehrotra et al. 2012; Haque and Ghosh 2014). Shilpha et al. (2021) confirmed genetic stability among *Solanum trilobatum* synseed regenerants and in vitro parent plant by start codon-targeted polymorphic (SCoT) markers even after 12 week storage. Acclimatized plantlets developed from encapsulated nodal explants of *Withania somnifera* revealed monomorphic RAPD and ISSR bands when compared with in vitro grown mother plant. Hardened plants showed enhanced pigment profile (chlorophyll, carotenoids, and net photosynthetic rates), antioxidant enzymes (superoxide dismutase, catalase, and peroxidase), and lipid peroxidation suggest that the plants undertake these mechanisms to combat stress (Fatima et al. 2013).

Establishment of cytological stability by mitotic and meiotic chromosomal analysis along with molecular stability by RAPD fingerprinting analysis was carried out in *Ledebouria revoluta* by Haque and Ghosh (2016). However, genetic variability was detected in hardened plants derived from synseeds of *B. monnieri* and *Beta*

vulgaris probably due to passing through stress conditions during formation of artificial seeds and its storage (Ramesh et al. 2011; Rizkalla et al. 2012).

Chemical stability in terms of wound healing activity and centelloside profile (asiaticoside, madecassoside, asiatic acid, and madecassic acid) of artificial seed-regenerated plantlets of *Centella asiatica* was reported by Prasad et al. (2014). Similarity in metabolite pattern was also reported in *Cannabis sativa* (Lata et al. 2011) and *B. monnieri* (Muthiah et al. 2013). Genetic stability at the level of phenotype and cytology was proved due to the absence of polymorphic bands using start codon-targeted polymorphism (SCoT) analysis (Mishra et al. 2022).

26.4.6 Importance/Limitations and Applications Related to Conservation of Medicinal Plants

Conventional storage methods involving natural seeds are often challenging in case of medicinal plants due to recalcitrant or desiccation-sensitive seeds and pathogen infection. Synthetic seeds developed from in vitro or in vivo explants are suitable for preservation and exchange of seedless medicinal plants as well as plants having sterile genotypes, mycorrhiza-dependent germination, reduced endosperm, and rare genotypes (Saiprasad 2001; Gantait et al. 2015a, b). Successful commercialization and transport across international borders depend on standardization of storage conditions. Furthermore, low temperature storage eliminates the need of subculturing in fresh media (Sharma et al. 2013; Haque and Ghosh 2017). Nyende et al. (2003) attributed the success of synthetic seeds in terms of biotechnology-mediated conservation to genetic stability of regenerants, being cost-effective and relative simplicity in preparation and storage.

Retention of high level of secondary metabolite in artificial seeds is particularly suitable for medicinal plants (Lata et al. 2009). Clonal propagation and short-term storage are effectively dealt with by using artificial seeds (Kikowska and Thiem 2011). This complementary technology is widely used for in vitro plant propagation, germplasm exchange, and seed banks (Palanyandy et al. 2015; Rai et al. 2009) and in ex vitro conservation via cryopreservation (González-Benito et al. 2016).

In spite of several advantages, the artificial seed technology has certain limitations, which necessitates further fine-tuning of the methods for enhanced practical application (Fig. 26.1). Artificial seeds involving non-embryogenic tissues encountered difficulties in the rooting stage (Hung and Trueman 2012a, b). Germination is often unpredictable due to variability of embryo maturation time. Dormancy and stress tolerance are often compromised. Further, the cost encountered during production may be high (Bekele 2021; Magray et al. 2017; Ara et al. 2000).

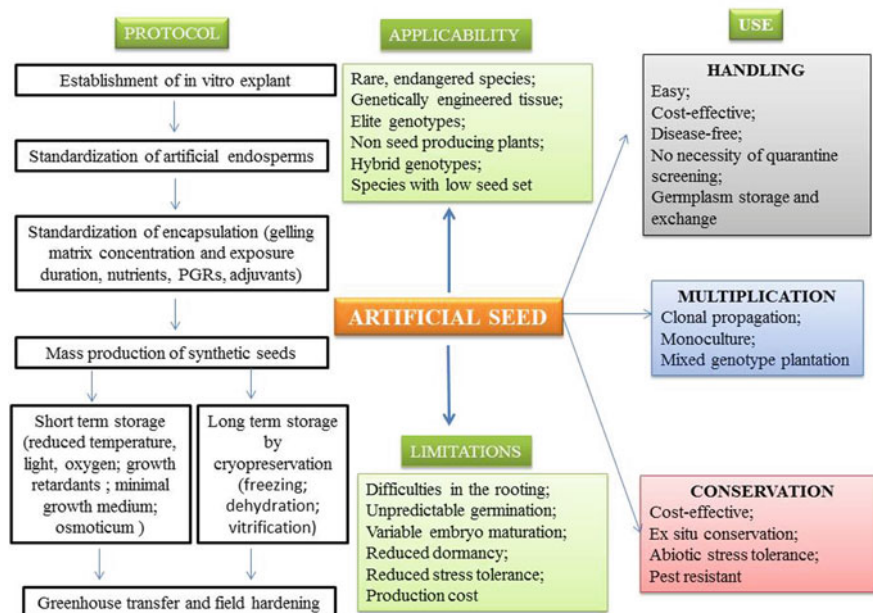


Fig. 26.1 Artificial seed as conservation strategy

26.5 Cryopreservation

Cryopreservation or freeze preservation refers to the technique of preserving structurally intact, living cells, tissues, or other biological constructs at ultralow, cryogenic temperatures [below -130°C , usually at -196°C in liquid nitrogen (LN)] for long periods of time (Panis and Lambardi 2005; Popova et al. 2015; Jang et al. 2017). The method is a suitable substitute to field and in vitro germplasm collection for the conservation of plant genetic resources. Collection and preservation of germplasm are important for the maintenance of biodiversity and prevention of genetic erosion. Traditionally, genetic resources have been conserved in field gene banks or botanical gardens (Hong and Yin 2012). Although the most convenient method to conserve germplasm of plants is by storing desiccated seeds at low temperature, the method is not suitable for seedless plants or many tropical plants with recalcitrant seeds (otherwise known as non-orthodox seeds, which cannot be dried enough for storage) as well as for those species that are vegetatively propagated to preserve their genetic makeup (Panis and Lambardi 2005). Genetic resources of endangered plant materials preserved ex situ in field gene banks are subject to various diseases; pest infestation; other natural calamities such as floods, drought, and frosts; and anthropogenic error, leading to genetic erosion (Engelmann and Engels 2002), ultimately causing the disappearance of huge amount of germplasm with elite characters. In addition, maintenance of clonal orchards conserving

woody plants requires intensive labor and large land areas, and running costs are huge (for pruning, pest and weed control, irrigation etc.). Similarly, maintenance of germplasms *in vitro* has its own inherent limitations—apart from being similarly labor intensive, it is also time-consuming, expensive, and subject to human error. Subculturing for prolonged duration can again result in contamination and somaclonal variation (Panis and Lambardi 2005; Yin and Hong 2010). Cell characteristics may be lost due to epigenetic instability (*viz.*, structural changes in chromosome morphology, ploidy, cell morphology, and the biosynthetic capacity of a cell line) (Deus-Neumann and Zenk 1984). Cryopreservation is a sound alternative for long-term conservation of diverse germplasm for prolonged duration (Vasanth and Vivier 2011; Maria et al. 2011), preserving the genetic stability of the conserved tissues. It is safe and cost-effective, and space saving; does not require regular maintenance; and reduces the risks of contamination and somaclonal variation or genetic alteration (Engelmann 2004; Pence 2011; Volk et al. 2014; Tolga and Yeşim 2017; Matsumoto 2017). It has become an important backup tool for plant tissue culture for the conservation of economically and pharmaceutically important, endemic, rare, and/or threatened species (Reed 2008; González-Benito and Martín 2011; Heywood 2014), as well for the conservation of cultured cells and embryos with unique attributes. The loss of embryogenic potential of cells or culture productivity, frequently observed when cultures are maintained for extended periods under standard growth conditions, can also be avoided by cryopreservation (Reinhold et al. 2000; Park et al. 2011). Cryogenic cooling is also considered an attractive alternative for long-term storage of undifferentiated plant cells, hairy root cultures, and genetically transformed cultures as sources of important phytochemicals and for other research purposes (Bekheet et al. 2020).

The essence of cryopreservation is the storage of cells and tissues at ultralow temperature (-196°C) (Wen and Wang 2010). Long-term storage and preservation of cells and tissues by cryopreservation rely on the fact that ultralow temperatures can put the biological, enzymatic, and chemical reactions occurring inside living cells to a halt (Jang et al. 2017), leading to temporary arrest in cellular divisions and metabolic processes (Bekheet et al. 2020). Thus, tissues can be kept suspended in time, unaltered, as long as these temperatures are maintained.

The technique of cryopreservation uses physical or osmotic dehydration, followed by ultra-rapid freezing to eliminate all freezable water from tissues (Reed et al. 2004). Most of the living organisms cannot tolerate the frozen condition due to the formation of ice crystals both intra- and extracellularly, which is normally lethal. The conversion of the water molecules, constituting about 80% of the mass of the tissue, to the crystalline ice phase is critical for living cells (Pegg 2007). Many of the structures constituting the living organisms as well as their metabolism/function are dependent on temperature. Plant cells are big in size, containing a large amount of water in the central vacuole, occupying more than 90% of the cell, making cryopreservation difficult (Popov 1993; Bachiri et al. 2000). The formation of ice crystals leads to mechanical constraints in terms of volume expansion and damage of the organelles and membranes, causing loss of cellular compartmentation, structural integrity, and cytolysis; ice crystals can also pierce and tease apart the cells (Pegg

2007; Engelmann 2011; Santos and Salomão 2017). The semipermeable nature and fluidity of cell membranes are damaged, and accumulation of ice crystals may also lead to concentration and precipitation of dissolved solutes (viz., sodium, potassium, chlorides) disrupting signaling processes, enzyme activity, and other macromolecular interactions (Panis and Lambardi 2005; Pegg 2007; Benson 2008; Reed 2011). Membranes of thylakoids, mitochondria, and tonoplast are also affected (Uemura and Steponkus 1999). To some extent, plant cells can endure the formation of extracellular ice crystals but not the intracellular crystals, which can damage the cells both during freezing and thawing. High water content will lead to formation of ice crystals, affecting the survival of cells; crystal formation and freezing injury can thus be logically avoided by reducing the cellular water content. Cryopreservation protocols are designed to minimize the damages imparted to cells by low temperature freezing by controlling dehydration and by preventing the formation of ice crystals (Pegg 2007). Unprotected freezing is usually lethal for cells and tissues (Pegg 2007). The freezing behavior of cells and tissues can be altered by adding a chemical additive, a cryoprotectant (or a cryoprotective agent/CPA), affecting water transport rates and reducing freezing temperatures of solutes, nucleation, and growth of ice crystals and their shape (Fuller 2004; Jang et al. 2017). The choice or selection of a suitable CPA is an important factor controlling the viability of the cryopreserved sample. The technique of cryopreservation of plant parts was introduced in the late 1950s and early 1960s (Sakai 1960), and research for decades has enabled the long-term storage of plant parts in LN without intracellular ice formation (Uchendu et al. 2019).

Theoretically, different types of plant materials (viz., apical or axillary shoot tips, meristems, scions, seeds, spores, gametophytes, rhizomes, zygotic and somatic embryos, pollens, embryogenic callus lines, cell suspensions, etc.) can be cryopreserved unmodified for unlimited span of time (Benson 2008; Lisheng and von Patrick 2011; Wang et al. 2014; Tolga and Yeşim 2017; Matsumoto 2017). It has been proposed as one of the best possible methods to maintain genetic stability and preserve cell lines, meristems, and transgenic plant materials for long term (Withers 1995). Seeds of many species are most efficiently cryopreserved because of simple application and the huge variety conserved (Engelmann 2004; Pritchard and Nadarajan 2008). The method is often regarded as the most suitable alternative for conservation of seedless species, non-orthodox seeds, and vegetatively propagated cultivars (Engelmann 2014; Reed 2001). Fruit trees, ornamental plants, forestry, and plantation crops as well as plants of pharmaceutical interest from tropical and temperate regions have been successfully conserved by cryopreservation (Engelmann 2004).

26.5.1 Factors Affecting Cryopreservation

Several factors are crucial for a cryopreservation protocol to become successful. Plant type, cell and tissue type, age and size of the explants, genotype, cell density,

selection of correct growth phase, method of cryopreservation selected, etc. are all important factors for a fruitful cryopreservation program. Parameters need to be optimized for different plant species, types of cells, tissues and organs, and even for each single cell line (Mustafa et al. 2011).

26.5.1.1 Type of Plant

Techniques of cryopreservation have been used successfully to preserve germplasms from all types of plants ranging from herbs and shrubs to trees, growing across the world in tropical, subtropical, temperate, and arctic regions with varying responses (Noor et al. 2019). With the exception of certain recalcitrant species, temperate plants are the most responsive to cryopreservation treatments because of their natural cold hardiness, acclimatization, and dormancy responses, which are sometimes used to cryopreserve them (Benson et al. 2007; Reed 2008). Shoot tips of such plants can tolerate cold temperatures more, and hence, these can be cold hardened and precultured at a low temperature (Reed 2001). In the tropical regions, plants are not adapted to go through extreme cold conditions and are thus sensitive to freezing/chilling conditions and desiccation (Noor et al. 2019). Recalcitrant tropical and temperate trees are mostly propagated by seeds, which are cold and/or desiccation sensitive. Thus, for these plants, mostly the embryos and embryonic axes are cryopreserved with exceptions (Noor et al. 2019).

26.5.1.2 Age and Size of the Explants

The age of the explant or the correct developmental stage is another decisive factor for the success of a cryopreservation protocol. For some species, young explants of a few weeks are preferable (Al-Ababneh et al. 2002), whereas in other cases, long-term cultures are more amenable to cryopreservation (Liu et al. 2004; Hong and Yin 2009). To be cryopreserved successfully, embryos should be in their right developmental stage. Selection of the proper size of the explant often plays a significant role in post cryopreservation recovery/regrowth rates; shoot tips should be large enough so that they can easily regrow after cryopreservation (Engelmann 2012). The size however varies with the plant material and the technique of preservation used. Commonly, 1 mm shoot tips are used, although shoot tips in the range of 0.5–3 mm have been used for cryopreservation of different plant species (Noor et al. 2019).

26.5.1.3 Genotype

The genotype of the explant can affect the outcome of the cryopreservation procedure. Reed and his coworkers (Reed et al. 2003; Reed 2008) suggested that different

genotypes of a single genus can have variable recovery rates; different genotypes can respond differently.

26.5.1.4 Type of cells, Tissues, and Organs

Optimal cryopreservation protocols are dependent on the type of plant cells and tissues selected. The response of the cells/tissues and their survival during the freezing and thawing cycles depend strongly upon the type of cell used (Jang et al. 2017). Cells and tissues respond differently to different cryopreservation protocols in terms of toleration to desiccation. Meristematic tissues, displaying the features of homogeneous cell populations composed of small, actively dividing cells with few vacuoles and high nucleo-cytoplasmic ratio, make the cells of this region more tolerant to desiccation than highly vacuolated differentiated cells (Engelmann 2004). These tissues (e.g., shoot tips, obtained from apical or axillary buds) are the most frequently used explants for cryopreservation of species, which propagate vegetatively (Panis and Lambardi 2005). Embryonic tissues similarly are composed of small sized, relatively homogeneous cells with less vacuoles and therefore less intracellular water, making these tissues withstand ultralow temperatures (Gonzalez-Arnao et al. 2014), after proper cryogenic protective treatments (Engelmann 2000). Seeds or embryos isolated from seeds are mainly used for the preservation of plants reproducing by non-orthodox seeds, which are not amenable to storage in seeds banks (Pence 1995). In some cases, however, the root pole of the embryo behaves differently from the shoot pole, displaying differential sensitivity to freezing and desiccation (Engelmann 2004). Sometimes the sensitivity of embryos to desiccation is extreme, leading to structural damages with slight decrease in moisture content (Chandel et al. 1995).

Cells and tissues cultured *in vitro* are somewhat synchronous because of culture conditions; thus, chances of positive responses to cryopreservation are high as tissues used for freezing are more or less homogeneous in terms of cell size, composition, physiological state, and growth responses (Engelmann 2004). A wide variety of plant tissues and organs have been tested for cryopreservation because of the advances made in the field of plant in tissue culture technology. Callus cultures can be derived from various plant parts like roots, stems, petioles, leaves, flowers, young seedlings, etc. when these are exposed to a combination of auxins and cytokinins *in vitro*. Cell suspensions are derived from callus cultures. The heterogeneous origin of cell lines from suspension cultures can affect various parameters, namely, cell wall characteristics, vacuolarization, rate of proliferation, metabolism, and formation of secondary products in the cell lines, which in turn dictate whether the cultures will be responsive to the cryopreservation protocol or not (Reed 2011). Differences in heat and mass transfer effects occur during cryopreservation of bulk tissues in single cell suspensions, making rapid cooling and warming and equal distribution of CPAs difficult (Jang et al. 2017). Cryopreservation of embryogenic callus, an important *in vitro* morphogenetic system, can be

looked upon as an attractive alternative to the long-term conservation of woody plants and also for the conservation of non-orthodox seed-producing plants.

However, in case of organized tissues, chances of somaclonal variation are less. Many plant species have large-sized seeds that are difficult to be frozen as such, and hence for such species, embryos and embryonic axes are cryopreserved. Responses from recalcitrant seeds are variable, frequently resulting in callusing or incomplete plantlet development. Methods of cryopreservation are limited for species for recalcitrant seeds as majority of the species are wild, and consequently, knowledge about their seed biology and seed storage behavior is little. Still in cases where information is available, tissue culture protocols required for regrowth of embryos after cryopreservation are unsuccessful (Engelmann 1999, 2000). Also, large differences are observed in moisture content and stages of maturity among seeds of different lots and harvests, thus making application of cryopreservation protocols difficult (Engelmann 2004). Cryopreservation of shoot apices, adventitious buds, or somatic embryos obtained from embryogenic cells has been suggested as an alternative where freezing of whole embryos or embryonic axes has not been successful (Engelmann 2004).

Most of the plant cells or organs do not possess inherent capacity to endure freezing and/or desiccation conditions (Popova et al. 2011). Such tolerance can be induced artificially by cold-hardening of mother plants (Paul et al. 2000; Tanaka et al. 2004). Explants can also be preconditioned by treatment with high concentrations of sugars, sugar alcohols, amino acids, and ABA for improving cryopreservation tolerance (Popova et al. 2011). Apart from changes in gene expression, such treatments result in increase in content of fatty acids, soluble sugars, and changes in protein composition (Bachiri et al. 2000; Wang et al. 2004; Zhu et al. 2006; Carpentier et al. 2007; Popova et al. 2009).

26.5.1.5 Cell Density

Cell density is another important parameter that can affect cryopreservation efficiency. *PCVs* (ratio between the volume of cryopreservation medium and the volume of cells) ranging from 20% to 30% were found to be suitable to allow 80% regrowth in slow freezing or encapsulation-/dehydration-based protocols, while 40–50% *PCVs* are required for vitrification to be successful (Huang et al. 1995; Chen and Wang 2003). *PCVs* have been standardized to achieve higher regrowth rates in various plant species like *Picea abies* and *Vitis vinifera* (Dussert et al. 1991; Find et al. 1998).

26.5.1.6 Growth Phases

The stage of development or the growth phase of the explant is a key deciding factor for the survival of recovered cryopreserved cells (Mustafa et al. 2011). Plant cells grown in batches pass through a sequence of *growth phases*—lag phase,

acceleration phase, exponential phase during which cells proliferate at the highest rate, deceleration phase, and a stationary phase. In the exponential phase, plant cells have least volume and less water and normally contain smaller vacuoles, resulting in a higher cytosol-to-vacuole volume ratio, which in turn makes the solute concentrations high (Mustafa et al. 2011). These solutes may function as CPAs, resisting freezing stress compared to cells in the other growth phases. Thus, cells in the *exponential phases* of growth are preferred for cryopreservation as they are better adapted to freezing compared to cells in the lag or stationary phases (Yoshida et al. 1993; Jain et al. 1996; Reinhoud et al. 2000; Winkelmann et al. 2004; Mustafa et al. 2011). In *Beta vulgaris*, better viability after cryopreservation was noted when explants were taken from young, freshly subcultured roots (Benson and Hamill 1991).

26.5.1.7 Choice of CPA

Cryoprotectants are compounds that can increase the total concentration of all solutes in the system, and thus, the quantity of ice formed at any given temperature is minimized (Pegg 2007). At the same time, these compounds should be nontoxic to the cells. So, the addition of CPAs during cryopreservation can circumvent the damage caused by freezing and can thus protect the cells. Broadly, CPAs can be divided into two types—pCPAs or those that can penetrate membranes (viz., glycerol, propylene or ethylene glycol, and dimethyl sulfoxide) and npCPAs, that is, those that do not possess this property, namely, sugars and sugar alcohols (sucrose, mannitol, and sorbitol) (Benson 2008; Heine-Dobbernack et al. 2008; Mustafa et al. 2011; Elliott et al. 2017). CPAs reduce the water availability by forming hydrogen bonds with water or interact via dipole forces (Benson 2008). npCPAs induce dehydration at the cellular level by efflux and create a hypertonic extracellular medium, whereas pCPAs do not allow the formation of ice crystals intracellularly during freezing (Benson 2008; Elliott et al. 2017). A total of 90–100% cell viability, with a growth rate comparable to untreated control cultures, can be achieved by using CPAs in cryopreservation protocols (Menges and Murray 2004; Mikula 2006; Yamazaki et al. 2009; Ogawa et al. 2012; Häkkinen et al. 2018). Thus, CPAs are widely used in almost all cryopreservation protocols to improve post cryopreservation growth of plant tissues. Preconditioning with npCPAs can prepare the cells to withstand stress induced by cryopreservation and is usually done by adding sugars and sugar alcohols (viz., sorbitol, mannitol). In many cases, it was seen that cell viability reduced by 90–100% when the preconditioning step was omitted (Huang et al. 1995; Tsukazaki et al. 2000; Chen and Wang 2003). However, many CPAs used in the concentrations required for cryopreservation protocols (1.0–8.0 M) may become toxic for plant cells (Best 2015); exposing cells to a high concentration of cryoprotectant can cause osmotic dehydration (Pegg 2007). Hence, a meticulous balance of CPAs is essential for the survival of plant cells. Also, because plant cells in general are sensitive to CPAs, incubation time of samples with CPAs are minimized, and the CPA should be withdrawn rapidly after thawing, by transferring

the cells repeatedly to CPA-free fresh solid or liquid medium (Dussert et al. 1991). Although solid media can be used for removing CPAs from cells cryopreserved by encapsulation-dehydration or slow freezing, vitrified cells have been mostly washed in liquid medium (Nausch and Buyel 2021). Concentrations of cryoprotectants used as well as duration and temperature of cryoprotection treatment need to be optimized for separate species for dehydration of samples, avoiding cytotoxicity (Sakai et al. 2008; Kim et al. 2009). Wide use of mixed cryoprotectants, neutralizing the cytotoxicity of a single cryoprotectant by lowering the total cryoprotectant concentration, is reported (Withers 1995; Sakai et al. 2008; Benson 2008). The temperature of the CPA treatment can also be lowered to avoid toxicity (Popova et al. 2011). However, for optimal regrowth of individual cell lines/tissues, specific CPA combinations must be determined (Nausch and Buyel 2021).

A crucial factor causing cell injury in cryopreserved materials is oxidative stress (Benson and Bremner 2004). Thus, antioxidants have been added to pretreatment or cryoprotective solutions to promote regrowth of cells and tissues post cryopreservation. Examples include addition of glycine betaine, ascorbic acid, glutathione, ABA, vitamin E, ascorbic acid, lipoic acid, glutathione and glycine betaine, and melatonin as antioxidants, improving regrowth in cryopreserved plant samples (Uchendu et al. 2010, 2013; Zhao et al. 2011; Ren et al. 2014).

26.5.1.8 The Technique of Cryopreservation Selected

Protocols selected for cryopreservation of plants materials are often dependent on the plant species and are specific for cell lines (Nausch and Buyel 2021). Better regrowth rate was achieved in Madagascar periwinkle after slow freezing, compared to encapsulation-dehydration (Chen et al. 1984). In case of *Gentiana tibetica*, cells did not regrow after slow freezing but was successfully achieved using vitrification and encapsulation-dehydration (Mikula 2006). When different methods like controlled-rate cooling, PVS2 vitrification, and encapsulation dehydration were compared for diverse species of mint, it was observed that regrowth was significantly better using controlled-rate cooling (Uchendu and Reed 2008). A two-step freezing was effective for the cryopreservation of important medicinal plant *Catharanthus roseus* with a survival rate of 69.5% (Samar et al. 2009), whereas for *Sapindus mukorossi*, known for its antimicrobial, antidermatophytic, and antihelminthic properties, somatic embryos were cryopreserved by vitrification, achieving 80% survival (Kim et al. 2006b). For cryopreservation of root cultures, vitrification- and encapsulation-based methods appeared to be suitable than ultrarapid freezing or desiccation (Popova et al. 2011). Root tips are very delicate, tiny, with highly cytoplasmic cells, making them sensitive to desiccation. Thus, encapsulation-based methods are advantageous as the explants are coated, reducing the risk of mechanical damage (Popova et al. 2011). In contrast, undifferentiated cell cultures of medicinal plants have been mostly cryopreserved by slow and two-step freezing processes (Popova et al. 2011). Reports indicate that different types of cultures of the same species can be cryopreserved using different methods, and interestingly, various

methods can be applied for the same type of explant from the same plant species with comparable regrowth rates; the choice of method usually depends on the physiological state of the explant as well as many times on the laboratory facilities available, equipment, expertise, etc. (Mustafa et al. 2011). Complex organs containing different types of cells such as shoot tips or somatic embryos can be preserved by both slow-freezing and vitrification-based cryopreservation techniques (Popova et al. 2011). Normally, regeneration is fast and direct, and a callus phase is only observed in such cases where the protocol is not optimized (Engelmann 2004). However, for freeze preserving embryos and embryonic axes, the desiccation technique is mostly used (Engelmann 2004).

The *cooling* and the *warming rates* (Pegg 2007) are important. Mazur in 1963 discovered that the transport of water molecules across the cell membrane was determined by the rate of change of temperature, which indirectly controlled the probability of the formation of intracellular ice crystals and thus was an important determinant in the survival of cells following cryopreservation (Mazur 1963). The rate at which water is transformed into ice is controlled by the rate of cooling, which in turn controls the rate of change of solution concentration surrounding the cells (Pegg 2007), affecting the survival of cells. When vials of CPA-treated cells are plunged into liquid nitrogen, cooling rates of ~ 200 °C/min are achieved, which reduce ice crystal formation and thus minimize cell damage (Reed 2008). Just like rapid freezing, rapid warming rates are important to avoid cellular damage. This is due the formation of transient ice crystals during the reversal of glass-like amorphous cytosol to the liquid phase (Nausch and Buyel 2021). In most cases, cells have been thawed in water baths at 35–45 °C with a heating rate of ~ 250 °C/min (Nausch and Buyel 2021).

Other factors important for the success of cryopreservation protocols are hardening of stock cultures, composition of preculture, regrowth medium, exogenous plant growth regulators added to the medium before and after cryopreservation, kind of container used for storage, etc. (Ozudogru et al. 2010; Popova et al. 2011).

26.5.2 Techniques of Cryopreservation

The process of cryopreservation is complicated, comprising of a number of steps including selection of explant and excision, preculture or preconditioning, osmoprotection and cryoprotection, freezing, storage, thawing, and recovery (Benson 2008; Popova et al. 2015). Successful regrowth of plant tissues after cryopreservation depends on meticulous optimization of each step as well as the physiological condition of the explants. From the initial experiments in the 1970s, methods of cryopreservation have become more refined, making it possible to cryopreserve a wide range of plants from herbs to trees from the tropical, subtropical, and temperate regions (Reed 2008; Normah et al. 2016).

As mentioned, a successful cryopreservation technique aims at inhibiting the formation of intracellular ice crystals. In nature, some plant species synthesize

sugars, proline, or specific proteins at subzero temperature, which lower the freezing point of the cell and avoid ice crystal formation (Panis and Lambardi 2005). But to keep the cells viable, minimum hydration is required; thus, it is not possible to avoid crystal formation at ultralow temperatures of -196°C required for cryopreservation. The only way to avoid ice crystal formation without reducing the cellular water content to an extreme is through “vitrification”—the transition of water directly from the liquid phase into an amorphous phase or glass, avoiding the formation of crystalline ice (Fahy et al. 1984). Some orthodox seeds or dormant buds dehydrate naturally and can be cryopreserved as such. However, calli, cell suspensions, shoot tips, and embryos contain high proportions of cellular water and are sensitive to injury caused by freezing. Thus, the cryopreservation protocols employ artificial dehydration to protect the cells from injury caused by intracellular ice crystal formation (Engelmann 2004). The cryopreservation protocols are different and have been variously divided into the older classical protocols (involving freeze-induced dehydration of cells) and the new methods (vitrification-based), depending upon the techniques employed and the physical mechanisms upon which they are based (Withers and Engelmann 1998). In the classical cryopreservation techniques, cells and tissues are cooled down slowly to a defined prefreezing temperature, followed by rapid immersion in liquid nitrogen (Engelmann 2004), whereas in vitrification-based protocols, cells are dehydrated before freezing by exposing samples to cryoprotectants and/or desiccation, followed by fast cooling (Engelmann 2004). In addition to preserving calli and cell suspensions, these protocols are suitable for complex plant organs with different types of cells, each with its unique requirement to tolerate the conditions of freeze-induced dehydration (viz., shoot tips, embryos), while on the contrary, the classical techniques have been applied for cryopreserving undifferentiated cultures like calli and cell suspension cultures (Kartha and Engelmann 1994; Withers and Engelmann 1998; Engelmann 2004). Also, vitrification-based methods are less complex as compared to the classical ones and can be applied to a broader range of plant materials (Engelmann 2004). In the classical techniques, cells and the external medium initially supercool, followed by formation of ice in the medium during slow cooling. With further decrease in temperature, more amount of extracellular solution turns into ice, concentrating the intracellular solutes. Equilibrium is reached by the loss of intracellular water to external ice, thus reducing the formation of intracellular ice when the sample is immersed in LN (Engelmann 2004). Pre-growth of explants, cryoprotection, slow cooling (around -40°C), immersion of samples in LN, storage, thawing, and recovery are the steps normally included in classical freezing programs (Engelmann 2004). Dehydration is a critical factor in the newer techniques, with extensive dehydration protein conformation may be changed leading to their denaturation (Samygin 1994). Other effects are membrane and macromolecule destabilization, rise in cytoplasmic viscosity, and increase in the concentration of potentially hazardous intracellular solutes (Mazur 2004).

Combining the classical and new techniques, widely used cryopreservation protocols for plant cells include programmed (or slow) freezing, vitrification, dehydration, encapsulation-dehydration, encapsulation-vitrification, droplet-vitrification,

pre-growth (Sakai et al. 2008; Reed 2008). These methods are governed by explant dehydration, a crucial step in cryopreserving hydrated biological samples (Mazur 1984). The techniques share some common basic approaches, namely, pre-conditioning of the explant, pretreatment with a suitable CPA, freezing, and a recovery treatment after thawing (Benson 2008). Preconditioning or preculture of samples in the presence of a cryoprotective substance is important for the plant tissue to tolerate desiccation (William and Pushpangan 1999) and is normally applied before slow freezing or vitrification (Nausch and Buyel 2021). Each step of the cryopreservation protocol should be carefully optimized for successful regrowth.

26.5.2.1 Programmed Freezing

Also referred to as the *slow freezing* or *controlled rate cooling*, it is a two-step freezing process, the first cryopreservation technique to be applied successfully to plant cells (Withers and King 1980) and is based on dehydration induced by freezing in the presence of extracellular ice (Reed and Uchendu 2008). Cryoprotected plant samples are dehydrated while being frozen slowly (0.3–1 °C/min) to –40 to –70 °C in an automated programmed freezer and then plunged quickly into liquid nitrogen (LN) to deep freeze the cells. Only the cell contents vitrify to an amorphous glassy state, while ice crystal formation takes place extracellularly, reducing ice crystal formation (Mustafa et al. 2011; Popova et al. 2011). A mixture of npCPAs and pCPAs is used before cooling the cells at a controlled rate to an intermediate low temperature (Mustafa et al. 2011). npCPAs cause the water to flow out, whereas intracellular ice crystal formation is suppressed by pCPAs (Reed 2008). Cells are recovered by thawing and preferably inoculated onto solid/semisolid medium. Risk of contamination is low in slow freezing, and a high manipulation skill is not required (Jang et al. 2017). But an expensive program freezer is required, and exposure of samples to subzero temperatures for a relatively long duration can have deleterious effects on cold-sensitive species, which are the major disadvantages of this method (Popova et al. 2015, 2011). Also, formation of extracellular ice can induce freeze injury (Jang et al. 2017).

26.5.2.2 Vitrification

This technique, now being successfully applied to more than 200 plant species from the tropical as well as temperate regions, was originally developed by Sakai in the late 1980s (Sakai et al. 2008; Matsumoto and Niino 2014). Vitrification or the transition/solidification of water present in the cells or tissues to a noncrystalline, metastable, amorphous, or glassy phase can be attained by dehydration; thereby, the formation of lethal and intracellular ice crystals can be avoided allowing regrowth of frozen samples (Fahy et al. 1984; Sakai 2000; Tolga and Yeşim 2017; Nausch and Buyel 2021). In this process, plant samples are treated with cryoprotective substances, dehydrated osmotically with highly concentrated solutions, cooled, and

thawed rapidly, and the cryoprotectants are removed for recovering the samples. Samples are sequentially dehydrated in loading solution (LS) and vitrification solutions (VS) on ice or at ambient temperatures. Loading, also known as osmoprotection, involves exposure of samples to cryoprotectants at a moderate concentration of 35–45%, a step that prepares the samples to withstand extensive dehydration with vitrification solutions, which are highly concentrated. Explants have also been exposed stepwise to 20% and 65% vitrification solutions to initially avoid exposure of samples to toxic concentrations (Fahy et al. 1984; Yamada et al. 1993; Chen and Wang 2003; Mikula 2006). PVS2 or plant vitrification solution 2, comprising of w/v, 30% glycerol, 15% ethylene glycol, 15% DMSO, and 0.4 M sucrose (Sakai et al. 1990), and PVS3 comprising, w/v, 50% glycerol, and 50% sucrose (Nishizawa et al. 1993) are the two most widely used vitrification solutions. Dehydrated samples are then rapidly immersed in LN (Popova et al. 2015). Cells are then recovered by warming quickly, preventing devitrification during thawing (Mustafa et al. 2011). Although the use of PVS prevents the formation of ice crystals in the cytoplasm, its toxicity is higher than that of the CPA mix used for slow freezing protocol (Benson 2008; Reed 2008; Best 2015). Thus, the time of exposure to PVS is limited to 5–10 min at 24–26 °C (Fahy et al. 1984; Yamada et al. 1993; Reinhoud et al. 1995; Chen and Wang 2003; van Eck and Keen 2009).

Vitrification-based protocols are suitable for large-sized plant samples, namely, shoot tips, 0.5–3 mm, which have the capability to regrow directly with ease (Engelmann 2004). In order to vitrify, a cell must possess rapid freezing rates and a concentrated cellular solution. Rapid freezing rates (6 °C/s) are usually achieved by plunging cryovial enclosed explants into LN. Air-drying, freeze dehydration, application of penetrating (e.g., DMSO, glycerol, etc.) or non-penetrating cryoprotectants (sugars, sugar alcohols, PEG, etc.), and adaptive metabolism (hardening) are some of the techniques used for concentrating the cytosol (Panis and Lambardi 2005). Attributes such as simplicity, reproducibility, applicability to a wide range of tissues and plants, and high survival rate post cryopreservation have made vitrification a better option than the conventional slow cooling and the most widely used cryopreservation protocol in the present times. As mentioned, apart from large-sized plant samples, it is the method of choice for cryopreserving embryogenic callus lines, cell suspensions, meristems, somatic and zygotic embryos, etc. and has been used for cryopreserving plant samples from the tropics and subtropical regions, which naturally cannot endure ultralow temperatures (Engelmann 2011; Suhartono et al. 2005; Santos and Salomão 2017; Tolga and Yeşim 2017). However, high cytotoxicity of CPAs used make regular application of this process difficult (Shin et al. 2012). For a vitrification protocol to be successful, application of the cryoprotectant should be carefully controlled so as to dehydrate the cells and simultaneously prevent toxicity and sudden osmotic stress, injuring the cells (Panis et al. 2001).

26.5.2.3 Dehydration

The main hurdle faced by cryobiologists while preserving plant cells is the formation of ice crystals due to the large amount of water present in plant cells, damaging the cellular structure and preventing the recovery of tissue (Engelmann 2004; Reed 2008; Kaczmarczyk et al. 2012). In the simplest of all techniques, that is, dehydration, samples are dehydrated and frozen quickly by plunging them in LN. This technique of desiccation, either over silica gel or in a laminar airflow cabinet, is used for cryopreserving synthetic seeds, embryonic axes, or zygotic/somatic embryos (Engelmann 2004; Tolga and Yeşim 2017). Samples are desiccated for 1–2 h to retain 8–20% moisture depending on the plant species and the type of explant (Tolga and Yeşim 2017). Dehydration beyond a certain extent can trigger stress responses, namely, membrane changes and lipid peroxidation, affecting cell function and recovery of plant materials; thus, protective dehydration is important (Fang et al. 2009).

26.5.2.4 Cryopreservation by Encapsulation

Based on the technology of artificial seed production, in this protocol, Ca-alginate beads (~4 mm diameter) (Bachiri et al. 1995; Yin et al. 2012) are used to encapsulate plant materials, which are then desiccated by airflow or silica gel (encapsulation desiccation/dehydration) to a water content of 20% (fresh weight) or dehydrated and vitrified (encapsulation vitrification), coupled with rapid immersion in LN (Popova et al. 2015). It has been suggested that the diffusion of CPAs and changes in osmotic pressure can be limited by alginate matrix, thus minimizing physical and chemical stresses (Draget et al. 1988). The residual water content (rwc) can be reduced to <20% by air-drying the beads before freezing (Bachiri et al. 1995). Cells can be recovered on solid or liquid medium after thawing or slowly warming the beads (Nausch and Buyel 2021; Mustafa et al. 2011). Cryopreservation by encapsulation is particularly useful while preserving small, fragile plant materials or samples that are sensitive to desiccation (Escobar-Pérez 2005; Mikula 2006; Popova et al. 2011; Gupta 2014). Encapsulation protects dehydrated tissues from mechanical and oxidative damages as well as osmotic pressure changes during cryostorage and also makes the handling of plant parts easy during cryopreservation procedures (Niino and Sakai 1992). Survival rate is high, and nearly 80% regrowth after thawing is achieved when plant materials are encapsulated in alginate (Bachiri et al. 1995; Blakesley et al. 1995; Wang et al. 2002; Ogawa et al. 2008); growth of recovered samples is fast and direct without an intervening callus phase (Engelmann 2004). The method is also applied to plant cell suspension cultures, although it was first developed for shoot tips (Dereuddre et al. 1990; Bachiri et al. 1995).

26.5.2.5 Droplet Vitrification

Here, osmoprotected samples are cryopreserved on aluminum plates or foil stripes in microliter drops of vitrification solutions and cooled rapidly in LN. A direct contact of the samples with LN is ensured, facilitating cooling and rewarming of the plant material (Towill and Bonnart 2003), which results in improved regrowth and high recovery percentage for a number of species (Halmagyi et al. 2004; Kim et al. 2006a; Kaczmarczyk et al. 2011).

In yet another technique, *pregrowth*, samples are dehydrated under the laminar air flow cabinet or with silica gel after being cultured in presence of a cryoprotectant and then frozen fast (Engelmann 2004).

Novel cryopreservation methods named *V-cryoplate* and *D-cryo-plate techniques* have been developed based on osmotic dehydration inducing vitrification and evaporative dehydration, respectively, of explants placed on aluminum cryo-plates (Tolga and Yeşim 2017). Explants attached to aluminum plates are put through the steps of dehydration, cryoprotection, and rewarming after withdrawing liquid nitrogen. A number of explants can be manipulated at the same time through this technique, which also lessens the disparities occurring during individual treatment of (Engelmann 2014). Factors such as thermal conductivity of aluminum, promoting ultra-fast cooling and heating rates, thus minimizing tissue damage, ease of handling of explants and absence of toxic cryoprotectants have made the cryoplate technique successful (Volk and Caspersen 2007; Matsumoto 2017). Table 26.3 enlists some of the medicinal plants, which have been successfully cryopreserved in the past decade.

26.5.3 Determination of Survival and Assessment of Genetic Stability Post Cryopreservation

Although considered as an attractive alternative for plant germplasm conservation, cryopreservation techniques may result in cryoinjury of plant cells and tissues because of exposure to physical, chemical, and physiological stresses. Also, the use of cryoprotectants and some reactions (viz., free radical formation, molecular damage imparted due to ionizing radiation), which still occur at ultralow temperatures, results in genetic instability in treated explants (Panis and Lambardi 2005). Regrowth, retention of biosynthetic capacity, and “true-to-type” characteristic of the preserved samples are of primary importance in cryopreservation of medicinal plants. The “true-to-type” nature of samples post cryopreservation could be ascertained by morphological, cytological, biochemical, and molecular parameters (Harding 2004). Growth characteristics, retention of T-DNA fragments, and accumulation of secondary metabolites of pharmaceutical interest have been often evaluated in recovered root cultures of medicinally important plants post cryopreservation (Touno et al. 2006; Xue et al. 2008; Oh et al. 2009). Similarly, in case of embryogenic cultures, “retention of the embryogenic potential and regeneration of

Table 26.3 Cryopreservation of medicinal plants in the past decade

Plant species	Tissue/plant part cryopreserved	Method of cryopreservation	Post CP survival rate/regrowth/regeneration	References
<i>Petiveria alliacea</i> L.	Somatic embryos	D-cryoplate	8.6–32.2 secondary embryos/SE treated	Gonçalves et al. (2021)
<i>Sideritis leucantha</i> Cav.	Shoot tips from in vitro cultured plantlets	Vitrification	5–70%	Juan-Vicedo et al. (2021)
<i>Stevia rebaudiana</i> Bertoni	Apical and axillary shoot tips	Droplet-vitrification	43–93%	Benelli et al. (2021)
		D-cryoplate	46–93%	
<i>Valeriana jatamansi</i> Jones	Shoot tips from in vitro grown shoot cultures	Vitrification	73–91%	Sharma et al. (2021)
<i>Bunium persicum</i> B. Fedtsch.	Shoot buds and somatic embryos from in vitro cultures	Encapsulation-dehydration	45.8%	Sharma et al. (2020)
<i>Cynara scolymus</i> L.	Shoot apices from in vitro grown cultures	Vitrification	56%	Bekheet et al. (2020)
<i>Rauwolfia serpentina</i> (L.) Benth. ex Kurz	Shoot tips	Encapsulation-dehydration	26.66%	Kisku et al. (2020)
<i>Cannabis sativa</i> L.	Shoot tips from in vitro grown shoot cultures	Droplet vitrification	5–63%	Uchendu et al. (2019)
<i>Cleome spinosa</i> Jacq.	In vitro grown shoot tips	Vitrification	83.3%	Vilardo et al. (2019)
		V-cryoplates		
<i>Clitoria ternatea</i> L.	Axillary buds from in vitro multiplied shoots	Encapsulation-dehydration	50–65%	Nair et al. (2019)
<i>Hypericum perforatum</i> L.	Roots	Vitrification	45–87%	Yang et al. (2019)
<i>Panax ginseng</i> C.A.Mey.	Adventitious root cultures	Droplet vitrification	15%	Le et al. (2019)
<i>Pfaffia glomerata</i> (Spreng.) Pedersen	Shoot tips from in vitro grown plants	Vitrification	19–63%	Oliveira et al. (2019)
<i>Petiveria alliacea</i> L.	Somatic embryos	Vitrification	85%	Pettinelli et al. (2017)
		Encapsulation-dehydration	70%	
<i>Satureja spicigera</i> Boiss.	Callus	Desiccation, vitrification, DMSO freezing	66–98.7% regrowth	Ghaffarzadeh-Namazi et al. (2016)

(continued)

Table 26.3 (continued)

Plant species	Tissue/plant part cryopreserved	Method of cryopreservation	Post CP survival rate/regrowth/regeneration	References
<i>Schisandra chinensis</i> (Turcz.) Baill.	Callus	Slow freezing Step-wise freezing Fast freezing	10–70%	Sun et al. (2016)
<i>Byrsonima intermedia</i> A. Juss.	Shoot tips from in vitro grown cultures	Droplet vitrification	67–90%	Silva et al. (2013)
<i>Kaempferia galanga</i> L.	Shoot tips from in vitro grown shoot cultures	Vitrification	50–60%	Preetha (2013)
<i>Anemarrhena asphodeloides</i> Bunge	Embryogenic calli	Vitrification	63.8%	Hong and Yin (2012)
<i>Kalopanax septemlobus</i> Koidz.	Embryogenic callus	Vitrification	15–99%	Shin et al. (2012)
<i>Bacopa monnieri</i> (L.) Wettst.	Shoot tips from in vitro grown shoot cultures	Vitrification	20%	Sharma et al. (2011)
<i>Catharanthus roseus</i> (L.) G. Don	Embryogenic cell suspension	Vitrification	16–69%	Fatima et al. (2009)
<i>Dioscorea bulbifera</i> L.	Embryogenic callus	Vitrification	60–70%	Hong et al. (2009)
<i>Ginkgo biloba</i> L.	Callus	Simple desiccation	54%	Popova et al. (2009)
<i>Maesa lanceolata</i> Forssk.	Hairy roots	Encapsulation-dehydration	90%	Lambert et al. (2009)
<i>Trichilia emetica</i> Vahl	Shoot tips from in vitro grown plants	Vitrification	38–71%	Varghese et al. (2009)
<i>Astragalus membranaceus</i> Bunge	Hairy roots	Encapsulation-vitrification	6%	Xue et al. (2008)
<i>Eruca sativa</i> (L.) Cav.	Hairy roots	Encapsulation-vitrification	73%	Xue et al. (2008)

healthy plants” are the chief aims of cryopreservation protocols. Regrowth of cells is the key indicator of a successful cryopreservation protocol (Nausch and Buyel 2021). The survival of cells post cryopreservation can be determined by certain methods like viability test using FDA or 2,3,5-triphenyltetrazolium chloride staining and measuring regrowth (Verleysen et al. 2004; Škrlep et al. 2008). Apart from preservation of germplasm, another goal of cryopreservation is to maintain the genetic stability of plant materials. Different techniques like the flow cytometry have been used to analyze the nuclear DNA content, and NMR-based metabolic

profiling has been carried out to determine the metabolomic stability after cryopreservation (Suhartono et al. 2005). Molecular markers like randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), randomly amplified DNA fingerprinting (RAF), amplified DNA methylation polymorphism (AMP), and methyl-sensitive amplified polymorphism (MSAP) have been used to assess genetic stability (Kaity et al. 2008, 2009; Fernandes et al. 2008; Mikula et al. 2011).

26.6 Conclusion

Somatic embryogenesis is the process of development of embryo-like structures from cells other than gametes and its fertilized product. Similar to zygotic embryo, a mature somatic embryo is a bipolar structure, which can be successfully converted into whole plant. The process is a well-known biotechnological tool for micropropagation as well as for conservation of different plant species. In this chapter, we observed how the regeneration procedure is established for large number of medicinal plants as well as recent reports suggesting the continuous process of protocol standardization and improvement for more medicinal plants including woody trees. Leaf explants from in vitro as well as field-grown medicinal plants seemed to be most responsive for both direct and indirect somatic embryogenesis, followed by hypocotyle segments from germinated seedlings. Phytohormones are known to play an important role in dedifferentiation and redifferentiation of tissue, and hence, it also has significant influence on induction, development, maturation, and germination of somatic embryos. The type and concentration of phytohormones optimum for establishment and maturation of the embryos are very much plant specific. In direct somatic embryogenesis from explants, a combination of cytokinin (higher concentration) and auxin (lower concentration) or cytokinin alone seems to be most successful. For indirect somatic embryogenesis, auxins imply to play the most important role, although with some exceptions. For further development and maturation, the common protocol involved reduction or complete removal of auxin and sometimes supplementation with cytokinins. A combination of auxin and cytokinin is also observed to be successfully employed for a number of medicinal plants for the purpose. Pre-incubation in dark condition before culturing under light condition improved somatic embryo frequency in a number of medicinal species. For maturation of the embryos, increase in osmoticum and ABA supplementation helped in some cases. Germination of mature embryos in most of the medicinal plant species was observed on basal medium or on supplementation with GA₃. Hence, by manipulation of different factors, like type of explants, media composition, phytohormone supplementation, culture conditions, and other biochemical factors, somatic embryos can be successfully established and used for conservation purpose either directly or via encapsulation and artificial seed production.

Artificial seed or synthetic seeds can be successfully used for those medicinal plants as well, where low seed set and poor germination are the main causes for

making the species endangered/threatened. Modern definition of artificial seed does not limit it to encapsulated somatic embryo alone, and synthetic seeds are defined as artificially encapsulated somatic embryos or any other somatic tissues (shoot tip, axillary bud, meristematic tissues, etc.), which can be stored and sown as seed and can be germinated to develop into whole plant. This chapter provides an up-to-date summary of protocols devised for successful establishment of artificial seeds and its germination protocol for medicinal plants in the last decade. Bipolar as well as unipolar vegetative propagules of medicinal plants are encapsulated preferably with 1.5–6% sodium alginate and calcium alginate being the second-best choice. Different storage protocols are suggested for different medicinal plants including storage at room temperature ($25 \pm 2^\circ\text{C}$) for up to 6 months using fungicide treated *Luffa* in *Curcuma amada*. Optimum germination rate in these plants was achieved by supplementation with GA_3 or BAP or even on half strength basal medium. Additionally, fungicide treatment, plant preservative mixtures like *Aloe vera* gel and activated charcoal, or biochemical agents like adenine sulfate improved germination rates in many plants. Successful field transfer and subsequent flowering are observed in different plant species. Genetic, cytological, and biochemical stability among the plants regenerated via somatic embryogenesis and artificial seed production is reported for many medicinal plants like *Rauvolfia serpentina*, *Cineraria*, *Drimiopsis*, *Glycyrrhiza glabra*, *Picrorhiza*, *Ledebouria revoluta*, etc. However, genetic variability is also known in some plants like in *Bacopa monnieri*. Moreover, retention of high level of secondary metabolite in artificial seeds makes it suitable for medicinal plant conservation and propagation.

Another biotechnological tool successfully employed for conservation of medicinal plants is cryopreservation, which is a safe and cost-effective substitute to field and in vitro germplasm collection. A suitable technique for long-term storage of various plant parts, undifferentiated plant cells, hairy root cultures, genetically transformed cultures, etc. uses ultralow, cryogenic temperatures for preserving structurally intact living cells and tissues. Cells and tissues conserved through this method remain genetically stable, and embryogenic potentiality and productivity of cultures are also retained. Various protocols of the technique, namely, vitrification, slow freezing, and V- or D-cryoplate techniques, are designed to minimize the damages imparted to cells by low temperature freezing by controlling dehydration and by preventing the formation of intracellular ice crystals. Several factors, namely, type of plant, organs, tissues and cells, age and size of explants, growth phase, genotype, protocol selected, choice of chemicals added, etc., are crucial for any kind of plant material to be cryopreserved successfully. A part of this review describes the different protocols and factors responsible for effective cryopreservation of plant species and lists some of the medicinal plants, which have been successfully cryopreserved through various techniques in the past decade.

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Chapter 27

Conservation of Medicinal Plants by Tissue Culture Techniques



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Abstract Currently, the tendency to produce and consume medicinal plants has increased a lot. Medicinal plants have been used in traditional medicine practices in many nations. The high requisition for medicinal plants and enhancing anthropogenic activities have accelerated the extinction of species and the destruction of habitats. Therefore, there is an instant need for the conservation of endangered medicinal plants. Different approaches extended to the conservation of medicinal plants. Biotechnology is one of the methods that have emerged as a potent method for this goal without affecting the major traits of plants. In biotechnology, there are multiple techniques for the preservation of these plants. One of the most important techniques is tissue culture. Since in plants, unlike animals, there is the ability to proliferate and differentiate cells and produce a complete plant in all organs, as a result, a whole plant can be produced with a piece of leaf, bud, stem, etc. through tissue culture. Tissue culture has provided a tool for the rapid reproduction of a large number of uniform plants while maintaining their genotype. Plant tissue culture can be a good way to conserve native or endangered species and preserve valuable germplasm resources. This chapter focuses on reviewing efforts on tissue culture for the preservation of medicinal plants.

Keywords Biotechnology · Maintaining genotype · Maintaining species

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27.1 Introduction

Medicinal plants (MPs), also called medicinal herbs, can be defined as any plant that is used for therapeutic purposes, contains secondary metabolites, and can also be applied as a precursor for the production of new medicines (Castillo-Pérez et al. 2021). These species have a pivotal and significant role as sources of valuable chemicals and drug-lead compounds in the health of both plants and humans (Singh 2018; Gusain et al. 2021; Ur Rehman et al. 2021). Various parts of herbs and plants are used for curative as well as therapeutic aids, such as fruits, leaves, barks, seeds, flowers, resins, rhizomes, roots, and stems (Ur Rehman et al. 2021; Alqethami et al. 2017), which have a special and distinct physiological impact on the human body (Kumar and Satapathy 2011; Borokini and Omotayo 2012). In addition to being used as medicine to treat human and plant diseases, MPs are also an important source of substances that are useful for maintaining the health and condition of the body. About two-thirds of the world's people use MPs for safety maintenance (Ur Rehman et al. 2021).

Currently, population growth has increased the demand for herbal drugs and natural medicines. The application of MPs is developing quickly over the universe, which has resulted in the loss of MP species 100–1000 times the natural overthrow rate. Although plants have been in danger of extinction for decades, species extinction and habitat destruction are now on the rise around the world due to the high demand (Gusain et al. 2021).

According to a report by the International Union for Conservation of Nature and the World Wildlife Fund, there are about 50,000–80,000 floral species used in therapeutic applications worldwide. It is estimated that nearly 15,000 of these species are endangered due to overharvesting and habitat destruction (Barboza et al. 2009). At present, almost 20% of the wild resources of MPs are depleted by enhancing population and plant utilization. Therefore, the cultivation and propagation of different species of MPs are the most efficient way to bridge the gap between supply and demand (Wang et al. 2020).

Due to the importance of MPs in human health and the growing need for these plants, the preservation and sustainable utilization of these plants is an important issue now that there are different methods for this work. The use of biotechnological methods such as tissue culture is a suitable and efficient way to reproduce and preserve important medical and economic plants, synthesize bioactive compounds for use in pharmaceuticals, and produce important secondary metabolites for use in medical activities. This chapter highlights the application of tissue culture for in vitro regeneration of rare endangered or threatened medicinal plants.

27.2 Importance of Medicinal Plants and Their Conservation

Evaluation of the multiple publications on MPs shows an increasing desire for research in this field. Research on medicinal plants has been on the rise since 1960, and in general, from 1960 to 2019, more than 110,000 studies on medicinal plants have been published. Three periods have been identified:

- (a) From 1960 to 2001, more than 1300 studies were published, and there was a steady increase.
- (b) It grows faster from 2002 to 2011 when it reaches a maximum of more than 6200 releases.
- (c) From 2011 to 2019, publications stabilized at around 5000 per year (Salmerón-Manzano et al. 2020).

The importance of using MPs to maintain human health is well known. Currently, it is known that two-thirds of the world's plant species have medicinal value (Krishnaiah et al. 2011). The greater availability, cost-effectiveness, and nontoxic nature of medicinal plants have led to the utilization of these drugs as a good source of therapeutic agents (Agbor and Ngogang 2005). According to the WHO,¹ traditional medicine meets the basic healthcare requirements of 80% of the world's population, and the use of plant extracts and their active ingredients for treatment is more prevalent (World Health Organization 1993).

Medicinal plants are considered an essential resource in the treatment/inhibition of different types of sickness (Rakotoarivelo et al. 2015). Because medicinal plants have several components with therapeutic value, therefore, these plants can be used to treat various human diseases in the form of drugs or formulations (Joshi et al. 2011). In the past, medicinal plants were applied by our ancient ancestors to preserve and flavor food, relieve pain, treat headaches, and even prevent a variety of diseases. Knowledge on the healing properties of different plants has been passed down from generation to generation over the centuries. The biological attributes of plant species are due to the active compounds synthesized in secondary metabolism, which are used all over the world for several aims, such as the treatment of infected sickness (Mohammed 2019).

Different parts of MPs are applied, including various kinds of seeds, roots, leaves, fruits, bark, flowers, or even the whole plant. In most ingredients of MPs, there are active compounds that are directly or indirectly involved in the treatment of diseases and are used as herbal medicines in medicine. Active compounds (substances) are synthesized and stored in different parts of these plants, which can have different uses by having different physiological effects on living organisms (Jamshidi-Kia et al. 2018).

¹World Health Organization.

It has been found that one of the causes of some diseases such as cancer, dementia, arthritis, asthma, mongolism, and Parkinson's disease is the presence of free radicals and ROS including hydrogen peroxide, superoxide radicals, and hydroxyl radicals. These compounds are produced through aerobic respiration or from exogenous sources in the human body (Halliwell and Gutteridge 1990). Free radicals are harmful in higher concentrations. The reaction of ROS with several biological molecules, including lipids, proteins, and deoxyribonucleic acids (DNA), causes an imbalance between oxidants and antioxidants. Herbs have a strong antioxidant activity due to their flavonoids, terpenoids, alkaloids, diterpenes, tannins, glycosides, and other phenolic compounds; they can scavenge these free radicals and, by guarding cells against oxidative damage, can protect human health against several diseases (Singh et al. 2016).

Medicinal plants are not uniformly distributed throughout the world, and the number of species of these plants varies from country to country or region to region. China, India, Colombia, South Africa, the USA, Paraguay, Mexico, Thailand, Vietnam, Pakistan, Malaysia, Korea, France, Nepal, the Philippines, Bulgaria, Sri Lanka, and Chile have the highest number of plant species, respectively (Chen et al. 2016). Improper use of natural resources for medicinal plants causes the extinction of these species, which is one of the challenges facing these important plants. About 15,000 species of 50,000–80,000 flowering plant species around the world are endangered due to overharvesting and habitat destruction (Barboza et al. 2009), and due to the growth of the human population and excessive consumption of plants, 20% of their wildlife resources are declining.

Generally, with population growth, increasing demand, and inadequate supply of plant medicines, the use of plant materials as a source of medicine is developing quickly all over the world (Cole et al. 2007). Considering that the abundance of medicinal plants in nature is low, and also that these plants grow slowly, overharvesting these plants from nature causes the destruction and loss of their species and leads to the depletion of resources and even extinction of species (Baker et al. 2007).

Different parts of the plant have different sensitivities to harvest; for example, root and whole plant harvesting is more destructive to medicinal plants (such as plants, shrubs, and trees) than the harvesting of leaves and flowers or their buds. Several biological characteristics are associated with the risk of extinction, such as habitat characteristics, distribution range, population size, species diversity, growth rate, and reproductive system (Chen et al. 2016). Therefore, various strategies must be adopted to preserve and promote agricultural biodiversity.

27.3 Conservation Strategies

The emergence of new challenges to human health has increased the need for medicinal plants. Studying plants and fungi to find medicinal properties or other uses is a useful way to determine the importance and value of these plants and makes

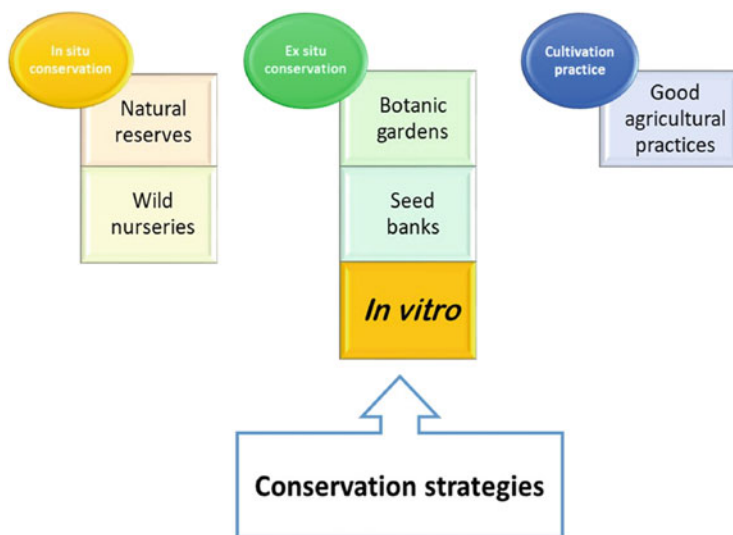


Fig. 27.1 Several conservation strategies for medicinal plants (Kadam and Pawar 2020)

more efforts to preserve valuable species of these plants around the world. Recently, in 2019, 1955, and 1886, new plant and fungal species were reported, respectively (Cheek et al. 2020 #31). Some may produce beneficial compounds for humanity (Cheek et al. 2018 #32).

In recent decades, the increasing demand for medicinal plant resources in Europe, North America, and Asia has led to overexploitation, mainly from wild populations (Ross 2005). Due to this overharvesting, plant medicinal resources are gradually reduced. There are several conservation strategies for medicinal plants, such as providing both in situ and ex situ conservation (Fig. 27.1).

Preservation of endangered medicinal plants and fungi in endemic regions is more significant than focusing on globally endangered species. Therefore, considering plant habitats and conserving them are better ways than preserving individual species.

27.3.1 *In Situ Conservation*

This type of conservation includes areas such as natural reserves and national parks (collectively referred to as “protected areas”) as well as the preservation of wild plants in managed habitats such as farms and plantation forests (World Health Organization 1993). On the other hand, preservation of ecosystems and natural habitats is defined as in situ conservation. In this method, the genetic resources present in their natural environment, as well as the conservation of domesticated or

cultivated forms, in an environment where they have developed distinctive characteristics.

In most regions, medicinal plants are native species of the region, and the secondary metabolites present in their different parts, which have medicinal properties, may not be expressed under cultural conditions and respond only to cultivation in natural environments (Coley 1983).

27.3.2 *Ex Situ Conservation*

When different species are protected in areas outside their natural habitat and the genetic material is transferred to an area far from the place where it is found, this conservation method is called ex situ conservation. This method facilitates access to germplasm for evaluation and use (Salgotra and Chauhan 2023).

One of the simplest methods to maintain more plant germplasm is seed storage. In this method, seeds are reserved as active (medium-term) or base (long-term) collections. In an active collection, the seeds remain above 65% for 10–20 years, while in a base collection, the seeds are stored at -20°C and can survive for a long time (more than 50 years) (Salgotra and Chauhan 2023).

Another method of ex situ conservation is botanical gardens. Botanical gardens contain live plants that grow in greenhouses and gardens. They are used for growing and showing plants generally for scientific and educational aims. Currently, there are more than 2000 botanical gardens that keep 80,000 plant species in their living collections (Brütting et al. 2013). In botanical gardens, plants can grow in relatively improved environmental media (intensive cultivation, respectively high fertility, and high levels of disturbance) (Brütting et al. 2013).

Effective conservation requires the use of different strategies; also, the appropriate conservation method varies from species to species. Therefore, in addition to the method of seed bank and botanical garden, the use of new methods such as in vitro culture is also necessary for conservation. It is an effective method for the ex situ preservation of species of medicinal and economically important plants (Fay 1994).

Tissues such as meristems, axillary buds, and zygote embryos are used for tissue culture. Adventitious buds and somatic embryos derived from leaves, stems, roots, or calluses are also used, but these undifferentiated tissues should be conserved with caution because of their potential for somaclonal diversity (Engels et al. 2006). In vitro methods for the conservation and use of the plant germplasm can have various advantages and disadvantages (Fig. 27.2).

27.3.3 *Cultivation Practice*

Cultivation practices relieve harvesting pressure on scarce and endangered plants, and they can help to standardize or improve genotypes (Chen et al. 2016). The use of

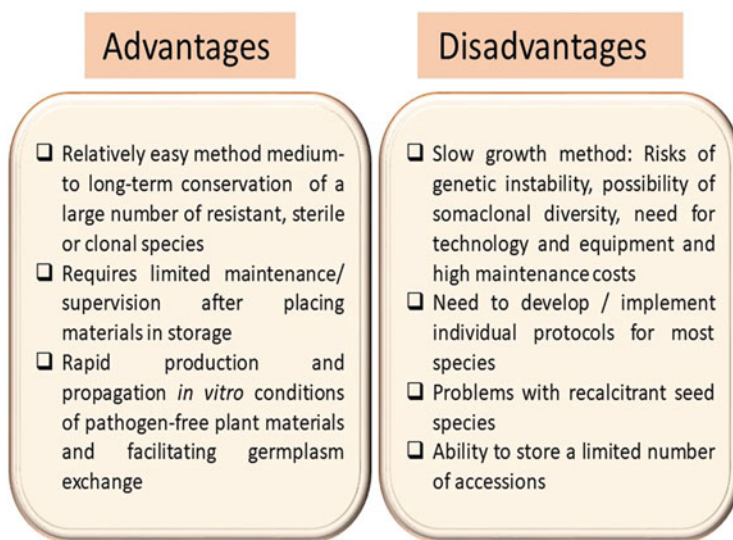


Fig. 27.2 Different advantages and disadvantages of *in vitro* methods for medicinal plant conservation (Thormann et al. 2006)

herbicides and pesticides may be dangerous for the environment, for those who grow plants, and for those who are treated with these drugs. Therefore, the use of chemicals should be reduced to an absolute minimum, and there should be necessary regulations strictly observed for the use of chemicals (World Health Organization 1993).

27.4 Plant Tissue Culture

One of the newest and most efficient methods for the reproduction and preservation of important plant species is the use of plant tissue culture. In addition to being used as research tools, these methods have been used industrially in recent years for plant propagation, production of disease-free plants, plant breeding, and synthesis of secondary metabolite. A major advantage of plant tissue culture is that hundreds and many plants are produced from one plant in a continuous process using small pieces of tissue (called explants). Also, a single explant can be propagated out of season and regardless of the weather throughout the year in a short period and placed under controlled conditions (Idowu et al. 2009).

In vitro culture is a very effective instrumentation for long-term germplasm preservation in controlled and contaminated conditions (Hussain et al. 2012). The tissue culture method is used successfully to conserve endangered species, especially for plants that are not easily propagated by conventional propagation methods. Using plant tissue culture methods, genotypes with high reproduction rates can be

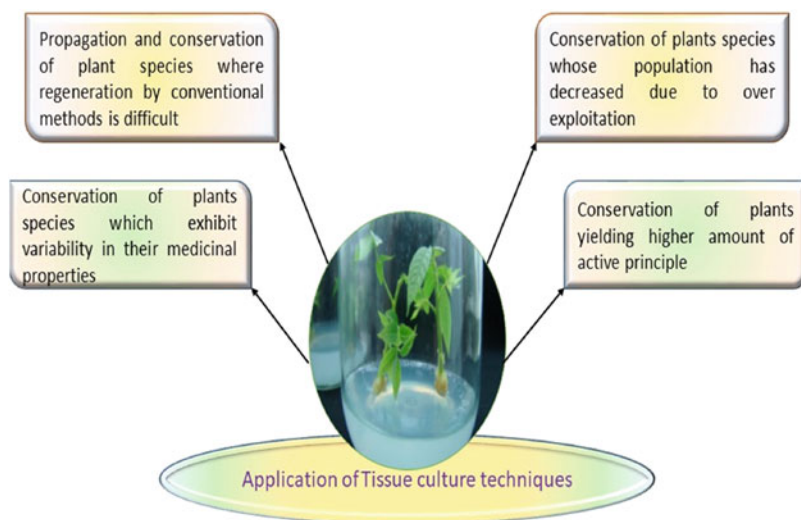


Fig. 27.3 Different utilizations of tissue culture in medicinal plant conservation (Kadam and Pawar 2020)

multiplied by creating a controlled growth environment. Because in this system the explants are small, it is possible to reduce the space requirement and thus reduce labor costs to maintain germplasm. Plant materials can be conserved in the *in vitro* conditions using slow-growing media at normal room temperature for a longer duration or by cryopreservation methods (Sudharsan et al. 2003). Due to the high propagation coefficient and low need for the number of primary plants and space, so far, many species of endangered plants have been easily grown and conserved via micropropagation (Oseni et al. 2018). Tissue culture techniques are used for various plants (Fig. 27.3).

Various factors affect the success of tissue culture in plants (Fig. 27.4). One of the effective factors in tissue culture is the culture medium. The presence of all nutrients required for the natural growth and development of plants, including macronutrients, micronutrients, vitamins, other organic compounds, carbon sources, plant growth regulators, and some gelling agents (in the solid media), is required in the plant culture medium. The most widely used medium for vegetative micropropagation of most plant species is Murashige and Skoog (MS). Another important factor is the pH of the medium, which affects plant growth. Also, the activity of plant growth regulators is usually adjusted from 5.4 to 5.8. There are two types of culture medium: solid culture medium and liquid culture medium. The composition of the medium, especially plant growth regulators and the nitrogen source, has deep impacts on the initial explant reaction (Oseni et al. 2018).

Plant growth regulators or phytohormones of culture medium are one of the main agents in regulating the growth of cells and plant tissues. The most common growth regulators include auxins, cytokinins, and gibberellins. Usually, depending on the

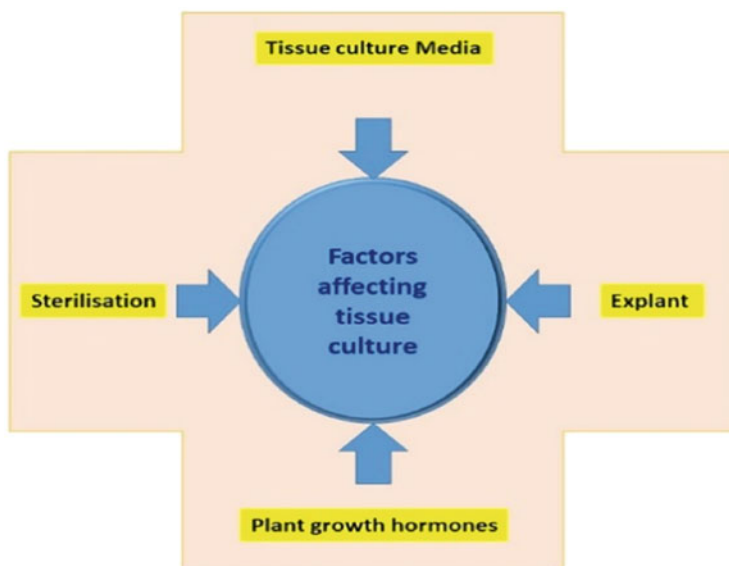


Fig. 27.4 Various factors affecting plant tissue culture

type of plant, tissue or organ cultivated, and the purpose of the experiment, different types and concentrations of hormones are used in the culture medium (Oseni et al. 2018). Figure 27.5 represents the various types of plant growth regulators and their functions in plants.

27.4.1 Medicinal Plant Conservation by Tissue Culture

27.4.1.1 Medium-Term Conservation Through Slow Growth Method

The slow-growing storage method is used commercially in laboratories to preserve storage cultures at low temperatures (Moriguchi and Yamaki 1988). Slow-growing storage (also known as medium-term protection) can be a storage method by reducing metabolic activity. In this method, the growth rate of laboratory cultures is reduced by keeping them in a modified growth medium, and they can be stored in this condition for a long time, from a few months to 1 year or more, depending on the species (Lambardi and Ozudogru 2011).

Generally, there is an observed decrease in the growth of in vitro cultures when the culture medium and/or culture conditions were changed (Engelmann 1998, 2004). Lowering the temperature is one of the most widely used methods of changing the culture medium, which can usually be accompanied by a reduction in light intensity (Engelmann 2011).

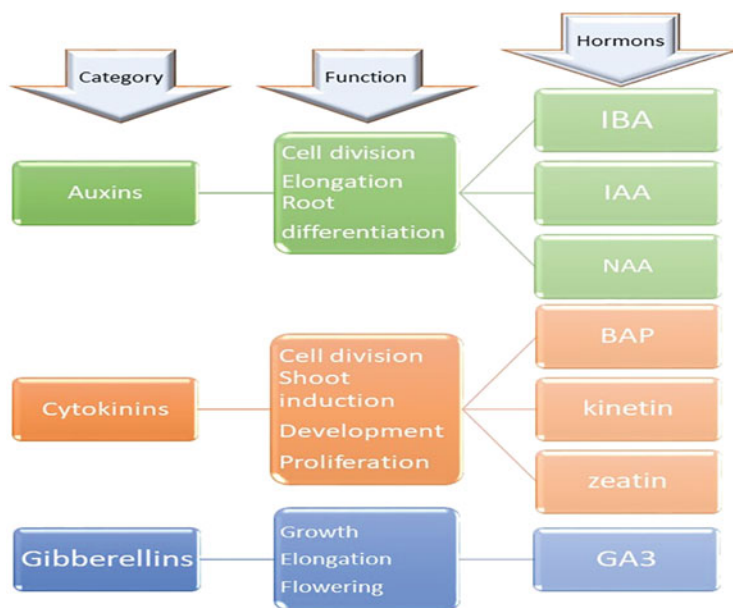


Fig. 27.5 Types of important plant growth regulators and their functions. Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), benzyl amino purine (BAP)

Also, reducing the growth of in vitro cultures through the use of minimal media and growth retardant, resulting in longer distances between subcultures, can lead to medium-term plant protection. A variety of plant species, such as endangered plants, of tropical as well as temperate origin can be protected through medium-term conservation strategies (Chauhan et al. 2019). The use of osmotic regulators, such as sucrose and mannitol, by causing osmotic stress to the substances under protection, reduces the water potential, limits the access of water to the explants, and acts as an inhibitory factor for plant growth. In addition, growth regulators are also commonly applied to preserve laboratory germplasm, of which ABA is one of the most widely utilized (Oseni et al. 2018).

Currently, slow-growing cultivation is used for a variety of commercial purposes including crop production, storing plant material for future needs, and when there is a shortage of labor for transplanting and limited space (Kozai et al. 1997). This method is also used for conservation projects, where the storage of seed-derived plants ensures the preservation of the genetic variability of the species (Pence 2010). For the protection of endangered species, this method is more economical and valuable due to the reduction of the number of crops and their long-term storage (de Carvalho et al. 2014).

The results showed that *Bacopa monnieri* (Thakur et al.) Pennell stem tips, nodal sections, and multiple-shoot clumps could be grown on MS media with 20 g l^{-1} sucrose and free of growth regulator, in culture tubes with polypropylene caps, or in culture bottles conserved for more than 20 months. After storage, the plants were

propagated well in MS media containing 0.5 mg l^{-1} N6-benzyladenine. Using MS medium without a growth regulator, stem growth and rooting were achieved, and rooted plants with 100% establishment were successfully transferred to the soil. A comparison of chemical profiles of mother plants and protected plants in vitro (after field establishment) using HPLC showed the similarities of chemical profiles (George et al. 2007). In another research, the conservation of *B. monnieri* under tissue culture conditions was performed using a single-node explant on MS media with 0.2 mg l^{-1} BA. Regenerated shoots were maintained for 12 months with up to a 100% survival rate. These plants did not show any morphological changes compared to the native plants after transfer to soil (Sharma et al. 2007).

The lack of carbon source in the culture medium reduced the cell and shoot proliferation of *Capparis spinosa* which was able to survive 17 weeks in the control treatment. After 17 weeks, the survival of *C. spinosa* seedlings with 3% sorbitol was 100%, if stored in light conditions (Al-Mahmood et al. 2012). An effective protocol for maintaining the shoot culture of *B. monnieri* under slow-growth conditions was presented. The results showed that multiple-shoot clumps survived up to 20 months in half-strength MS medium with 20 g l^{-1} sucrose in polypropylene cap culture bottles with 90% viability. Also, these plants were successfully planted in the soil (George et al. 2007).

Recently, in research on *B. monnieri*, explants of shoot tips and nodal segments, in combination with a mineral oil coating, were used for in vitro regeneration and effective medium-term preservation. For medium-term protection, a standard propagation medium MS containing 0.2 mg l^{-1} benzyladenine (BA) was used. The results showed that mineral oil coating led to a very significant reduction in growth and increased survival of cultures. After 12 months of protection in vitro, nodal segment explants encapsulated in combination with mineral oil coating showed the highest survival rate ($91.6 \pm 5.7\%$) and the number of branches (21.4 ± 1.6) (Sharma et al. 2020).

In vitro plantlets and in vitro plantlets without tips of *C. spinosa* were successfully stored using an MS culture medium with an appropriate concentration of osmoticum (sucrose, sorbitol, mannitol, or glucose) at different concentrations (0, 3, 6, 9, or 12%) at $24 \pm 2^\circ\text{C}$, for up to 17 weeks. In addition, conservation under light was found to be more beneficial than in dark (Hana et al. 2012). In another research, the basic medium dilution method was used to protect *Hypericum perforatum* L. In this experiment, sterile shoots were cultivated in MS media with reduced macronutrients. Investigating the growth rate of the plant after 3 months indicated that the use of dilution of the base media affects the growth of *H. perforatum*. The application of $\frac{1}{4}$ MS + 0.1 mg l^{-1} BA combination can suppress the growth of the culture without showing symptoms of necrosis up to 3 months after storage (Syahid 2021).

Slow-growth conditions conserved the nodal parts of *Nyctanthes arbor-tristis* L. for up to 180 days in one-eighth strength MS medium with 0.5% sucrose with a satisfactory survival percentage ($40.28 \pm 2.04\%$). It was also found that subsequent adding of 0.5 mg l^{-1} of ABA had a positive effect on maintenance and increased the viability of nodal parts by $40.36 \pm 1.01\%$ (Mishra et al. 2022). The explant of *Ruta*

graveolens was conserved without serious damage on MS media by various levels of sucrose, glucose, fructose, or sorbitol at a temperature of 24 ± 2 °C for up to 16 weeks (Al Shhab et al. 2021). *Stevia rebaudiana* microshoots were successfully stored for 16 weeks on MS medium containing sucrose, sorbitol, or mannitol, at a temperature of 24 ± 2 °C, with a survival rate of 93.6% after 32 weeks, and 89.3% were able to grow again (Shatnawi et al. 2011).

Stem tips and nodal segments obtained from in vitro propagated shoots of *Silene schimperiana* were encapsulated in calcium alginate beads for medium-term preservation. The encapsulated plants were kept for 5 months at 4 °C with 100% survival. The most efficient gel complex to form uniform beads was 3% Na-alginate and 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. About 90% of regenerated seedlings were successfully adapted to greenhouse conditions (Ghareb 2021). Utilization of mannitol or sorbitol as osmoticum and reducing sucrose to 1.5% (w/v) in 1/2 MS media for tissue cultures of *Asparagus racemosus* shoots conserved these plants for 6 months at 25 ± 2 °C without subculture. Surviving shoots from slow-growing cultures were regenerated with 100% efficiency (Thakur et al. 2015). 1/2 MS culture medium with 3% sorbitol and 2% sucrose was selected as the best medium for storage with slow growth of *Arnica montana* cultures, which effectively delayed the growth of seedlings, thus conserved 6 months without subculture (Petrova et al. 2021).

Using nodule explants cultured on MS media with mannitol, sorbitol, and ABA and on 1.2 MS stored at 15 ± 2 and 26 ± 2 °, *Spilanthes acmella* grows slowly under in vitro conditions. The maximum percentage of survival was obtained in MS + 2% mannitol at a temperature of 15 ± 2 °C at the end of 8 months. Mannitol 2% and sorbitol 2% were effective in slowing the growth of the cultures in terms of the number and length of the stem (Joshi and Jadhav 2013). Other researchers showed that 20 g l^{-1} of mannitol was suitable for maintaining the slow growth of *Glycyrrhiza glabra* with only one subculture per year. Shoots showed 100% survival and full recovery after protection. Modified 1/2 MS media contain 0.25 mg l^{-1} BA, 1.0 mg l^{-1} IAA, and 10 mg l^{-1} B-9 along with 10 mg l^{-1} AdS for shoot growth and leaf growth as well as root growth (Srivastava et al. 2013).

27.4.1.2 Long-Term Conservation Through Cryopreservation

For long-term preservation of plant species, cryopreservation (storing in liquid nitrogen at -196 °C) is usually used. It is a conservation method for the long-term preservation of plant genetic resources safely and cost-effectively. It was previously thought that tropical plant tissues could not be cryopreserved, but using these cryopreservation methods, these tissues were also successfully conserved in liquid nitrogen (LN) (Oseni et al. 2018).

This method enables the indefinite storage of endangered plants and ensures the survival of specimens after thawing. In the cryopreservation method, all freezable water is removed from tissues using physical or osmotic dehydration followed by ultrafast freezing. The metabolic and biochemical reactions of the cell are stopped

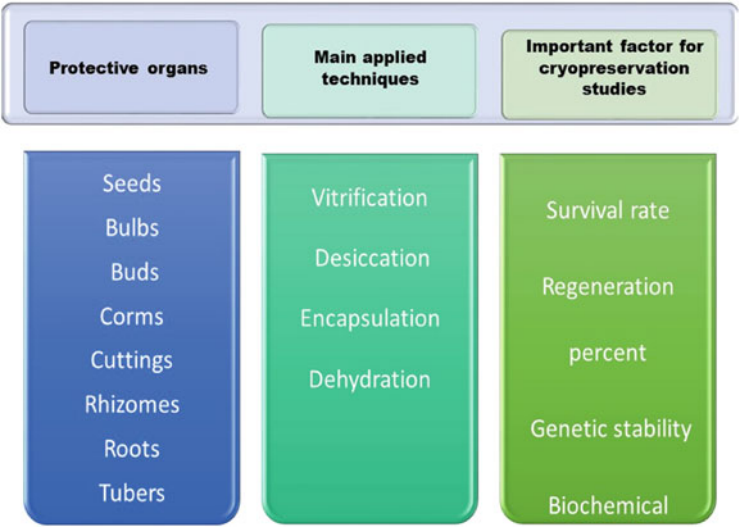


Fig. 27.6 Cryopreservation protective organ, techniques, and evaluation factors

due to a large decrease in temperature, thus preventing the formation of intracellular ice crystals that can cause cell death and destruction of cell organelles during the freezing process. Currently, one of the most innovative and cost-effective biotechnological approaches is the use of cryopreservation, which allows long-term safe protection of plant biodiversity without the risk of genetic changes (Benelli 2021).

Since the 1970s, research on cryopreservation techniques using various plant organs, tissues, and cells has begun, and so far, various cryopreservation methods have been introduced and developed (e.g., slow pre-freeze method, vitrification method, dehydration method) (Oseni et al. 2018). The following figure briefly shows the plant organs and the methods used and the attributes investigated in preservation by the freezing cryopreservation (Fig. 27.6).

In research, the tips of *Picrorhiza kurroa* were cryopreserved. Shoot tips from 4-week-old propagated cultures were pre-cultured on MS medium before dehydrating using PVS2 solution at 0 °C. Dehydrated shoot tips were immediately swallowed in liquid nitrogen (LN). The average survival in terms of normal stem formation after 4 weeks of plating was about 20% without callus formation (Sharma and Sharma 2003).

In vitro, cryopreservation of shoot tips of *Dioscorea deltoidea* was done successfully. Organ regeneration after being placed in liquid nitrogen (LN) up to 83%, and by encapsulation-dehydration, the most regeneration frequency recorded was 76% (Mandal and Dixit-Sharma 2007). In another study, *Hypericum perforatum* L. middle and basal root sections successfully cryopreserved and froze at 10 days after isolation indicate the highest plant regrowth after freezing (Yang et al. 2019).

A cryopreservation protocol was developed for *Valeriana jatamansi* Jones, a medicinal plant of high commercial value in the Himalayan region. Cryopreservation of shoot tips with PVS2 at 0 °C for 110 min showed the highest shoot recovery (91.6%) after transfer to LN (Sharma et al. 2021). The shoot tips of *Dioscorea deltoidea* were frozen using the vitrification and encapsulation-dehydration method. The results of HPLC analysis showed that the diosgenin content of regenerated plants from frozen shoot tips was similar to control plants (Dixit-Sharma et al. 2005). Overnight pre-culturing of isolated *Kaempferia galanga* L. shoot tips in MS media with 0.4 M sucrose, osmotic protection for 20 min, and dehydration with PVS2 for 20 min at 0 °C was optimal. Cryoprotected shoots gave 50–60% survival and 30–40% regeneration rates after flash freezing in LN followed by rapid thawing. The incorporation of GA3 (gibberellic acid) into the culture medium after thawing was necessary to induce shoot greening in the early phase (Preetha et al. 2013).

After cryopreservation of *Atractylodes macrocephala*, survival and growth rates of 76% and 62% were reported (Zhang et al. 2015). Seeds of *Satureja bachtiarica* were treated at –196 °C and transferred to LN and stored for 90 days. According to the results, growth indices (germination percentage, germination rate) were not affected by liquid nitrogen (Ghaffarzadeh-Namazi et al. 2015). Also, cryopreservation with PVS3 is recommended as the most appropriate protocol for *Satureja spicigera* callus freezing (Ghaffarzadeh-Namazi et al. 2017). Embryogenic calli from in vitro grown tillers of *Anemarrhena asphodeloides* Bunge were successfully cryopreserved by the cryo-encapsulation method. Survival of frozen embryogenic calli, including after storage, reached 80%. The plants obtained from control and frozen embryogenic callus did not show any significant difference in morphological growth (Sen-Rong and Ming-Hua 2012).

Encapsulation-vitrification is a combination of two methods of encapsulation-dehydration and vitrification. Explants are encapsulated in alginate beads and then incubated in LS and/or PVS and exposed to liquid nitrogen (Gantait et al. 2017). This system has gained popularity because it is a rapid method that is physically and chemically harmless. This method is more effective than conventional cryopreservation systems and has a larger utilization for germplasm preservation because it is effective for smaller shoot tips and protocorm-like bodies of medicinal orchids (small embryo-like tissues that can potentially develop into whole plants) (Kundu et al. 2018).

As a result, the utilization of tissue culture techniques in the protection of rare and threatened medicinal plant species can lead to the preservation of natural germplasms and protection against natural calamities that cause the loss of wild species and thus reduce biodiversity and affect the ecosystem (Oseni et al. 2018).

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Chapter 28

Current Status of Metabolic Engineering of Medicinal Plants for Production of Plant-Derived Secondary Metabolites



Mihir Halder  and Shreyasi Roy

Abstract Availability of both medicinal plants and their diverse groups of secondary metabolites is often significantly low in the natural habitats due to low in planta yield, structural complexity, lack of geographic access, cultivation obstacles, tissue, organ or developmental stage-specific production, complicated extraction operations, destruction of natural habitats, risk of overexploitation, and seasonal variation. It results in high production costs and recurring supply issues in the traditional, herbal, and modern medicine-based application of primary healthcare of 70–80% of the global human population. Currently, most of the 50,000–70,000 medicinal plant species are used globally, suffering great biodiversity loss as a consequence of climate changes and anthropogenic interference. The combined effects of these challenges force humanity to explore and exploit alternative sources and approaches, including chemical synthesis, searching for alternative sources, in vivo and in vitro production using tissue culture techniques, and transgenic approaches. In addition, metabolic pathway manipulation approaches are applied to find innovative ways to create superior production systems to secure the global need for the desired phytochemicals. Medicinal plant metabolic engineering is a powerful and promising gateway for improved production of secondary metabolites by intentional manipulation of pre-existing intrinsic metabolic pathways in native species or the introduction of heterologous reconstruction of the novel cellular metabolic pathways into heterologous host systems followed by optimization for de novo production. Several strategies, such as redirecting flux by overexpression of single or multiple key enzyme coding genes, over or downregulation of transcription factors, gene silencing of the competitive pathway, overexpression membrane transporter proteins, modification in cis-regulatory elements, metabolic channeling of the substrate through the formation of enzyme complexes or multifunctional enzymes by the construction of scaffolds or fusion of genes, co-localization of related enzymes to

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specific cell compartments by tagging with signal sequence, and fine-tuning of cofactor, are commonly applied in genetically modified microbial, whole plant, cell suspension culture, callus, and hairy root culture systems in metabolic engineering. This review highlights the recent major achievements in metabolic engineering-mediated homologous and/or heterologous production of phytochemicals in higher plant systems, particularly medicinal plants, along with basic knowledge about currently available host systems, general steps, techniques, and strategies.

Keywords Metabolic engineering · Medicinal plant · Secondary metabolite · Overexpression · Transcription factors

28.1 Introduction

Plants represent an essential component of the ecosystem that provides a valuable reserve of atmospheric oxygen, foods, nutrients, biofuels, drugs, and secondary metabolites (SMs) to its ecosystem since their evolution. Besides agricultural crops, medicinal plants are the primary source of a seemingly endless chemically and functionally diverse group of high-value natural phytochemicals with a myriad of fascinating pharmaceutical properties. It includes antimalaria artemisinin from *Artemisia annua*, anticancerous taxol from *Taxus brevifolia*, anticancerous vinblastine and vincristine from *Catharanthus roseus*, gout and anti-inflammatory drug colchicines from *Colchicum autumnale*, anticancerous camptothecin from *Camptotheca acuminata*, acetylcholinesterase inhibitor galanthamine from *Narcissus pseudonarcissus*, analgesic morphine and codeine from *Papaver somniferum*, cardioprotective resveratrol from *Vitis vinifera*, tanshinones from *Salvia miltiorrhiza*, anticholinergic atropine and scopolamine from *Datura*, and so on (Guerriero et al. 2018; Ramawat et al. 2020). These therapeutic molecules are synthesized in medicinal plants through plant-specific complex secondary metabolic pathways and enable plants to survive against different biotic and abiotic factors, acting as attractants and/or stimulants during pollination, seed dispersal, and symbiotic association with nitrogen-fixing bacteria and mycorrhiza (Halder et al. 2021). An estimated 50,000–70,000 plant species are used in traditional, herbal, and modern medicine throughout the world to meet the livelihood and primary healthcare needs of 70–80% of the global human population. With few exceptions, wild medicinal plants grown in the field are the major commercial source for the extraction of these plant-derived secondary metabolites.

In addition to natural crisis and pandemics, which can dramatically impact the yield of plant-derived SMs from natural resources, low bioavailability due to low in planta productivity, structural complexity, lack of geographic access, cultivation obstacles, seasonal/tissue/organ or developmental stage-specific production, complicated extraction operations, destruction of natural habitats, and risk of overexploitation of wild plant species are the major challenges in the way of their sustainable commercial production and supply. For example, artemisinin biosynthesis and accumulation occur exclusively in glandular secretory trichomes of *A. annua*

leaves (Zhou et al. 2020), vinblastine and vincristine are produced in the aerial parts of *C. roseus* (Barrales-Cureño et al. 2019), ginsenosides accumulate primarily in the roots of *S. miltiorrhiza* (Kim et al. 2015b), and production of reserpine predominantly occurs in roots of *Rauwolfia serpentina* (Tiwari et al. 2021).

Despite the fact that medicinal plants are renewable resources, they are currently suffering great biodiversity loss as a consequence of climate changes and anthropogenic interference, resulting in high production cost and recurring supply issues. As a result, researchers are continuously and extensively exploiting different alternative sources and approaches with the goal of finding innovative ways to create superior production systems to secure the global need for the desired phytochemicals. These alternative approaches include chemical synthesis and semisynthesis, searching for alternative natural plant genera or fungus or microorganisms, in vivo and in vitro production using tissue culture techniques, transgenic approaches and metabolic pathway manipulation in native plants, and introduction of a biosynthesis pathway into heterologous systems followed by optimization for de novo production. However, most of the time, the chemical structure and stereo-chemistry of these phytochemicals are far too complex that their industrial chemical synthesis and semisynthesis is either not possible or possible, but economically not viable due to the higher cost and synthesis of toxic byproducts during multistep production. Different modern techniques, such as tissue culture (Halder et al. 2019; Chandran et al. 2020), plant breeding (Faehrich et al. 2021), polyploidization (Iannicelli et al. 2020; Kumar 2021), and mutational breeding (Lal et al. 2020; Shah et al. 2020), have been evaluated for this purpose. Although highly productive superior lines, cultivars, or varieties have been established in a few cases by such efforts, they face instability in yield over generations or the long term.

In this context, metabolic engineering is a powerful and promising tool that involves intentional manipulation of pre-existing intrinsic metabolic pathways in native species or heterologous reconstruction of the novel cellular metabolic pathways in heterologous host. In classical metabolic engineering, the expression of pre-existing metabolic pathway enzyme(s) or regulatory protein(s) is altered in plants or reconstruction of novel metabolic pathways in the heterologous hosts using knowledge of modern recombinant DNA technology, transcriptomics, proteomics, metabolomics, bioinformatics, etc. It offers significant improvement or optimized de novo production of commercially important specific value-added phytochemicals, including biofuels, pharmaceuticals, natural products, recombinant proteins, polymers, etc., in a more efficient and cost-effective manner (Barone et al. 2020; Courdavault et al. 2021; Fig. 28.1). Besides these, metabolic engineered systems have been effectively developed utilizing metabolic machinery of different crop plants, medicinal plants, and microbes that serve as a biofactory for the production of desirable agronomic traits, improvement of resistance against biotic and abiotic stress, improvement in nutritional quality as well as reduction of unwanted compounds in food and fodder and degrading xenobiotics (Fig. 28.1).

Previously, metabolic engineering was predominantly restricted to a few microbial species like yeast and *E. coli*, and the entire process was difficult, laborious, time-consuming, and very expensive. There is a huge gap between the potentiality

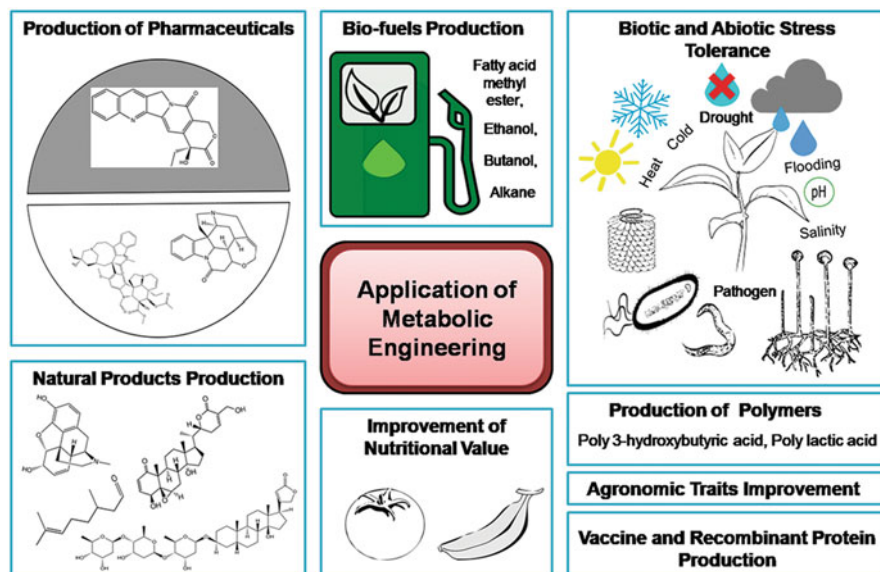


Fig. 28.1 Major application of metabolic engineering

and achievement of metabolic engineering due to several challenges associated with it (Li et al. 2020; Lynch et al. 2021). Plant metabolic engineering is a dynamic and complicated process that demands an in-depth understanding and full elucidation of endogenous plant biosynthetic pathways for ensuring the knowledge of key metabolic enzymes coding genes, their sophisticated regulation mechanism, and the point of intervention, metabolic bottlenecks, transcription factors, and the complex cross-talk for metabolic flux among other biosynthetic pathways. Metabolic engineering has grown rapidly in the previous two decades as a result of recent development and progress in omics technologies (genomics, transcriptomics, interactomics, proteomics, metabolomics, fluxomics), computational system biology, genetic engineering, and synthetic biology that extended knowledge about the genes, enzymes involved in the complex multistep biosynthetic pathway of plants, their regulation, reconstruction, analysis, and annotation. The investigation of biochemical processes allows the discovery of many novel plant metabolites, whereas potential bioinformatics tools accelerated the discovery of the genes for manipulations of the selected biochemical pathways.

More efficient gene editing toolkits are currently available to remove bottleneck (s) of existing biosynthesis pathway(s) or offer new insights into the genetic reconstruction of the novel metabolic pathway(s) by genetic manipulation (Bahadur et al. 2015; Adli 2018; Grohmann et al. 2019; Mao et al. 2019). Nowadays, a combination of numerous engineering strategies, techniques, and processes, such as rational metabolic engineering, evolutionary engineering, synthetic biology, system biology, inverse metabolic engineering, engineering of secretory pathways, and process optimization along with classical metabolic engineering, is implemented to achieve

several goals (Erb et al. 2017; Choi et al. 2019; Ko et al. 2020). Apart from microbial metabolic engineering, several types of research have been conducted in medicinal plant-based systems, either through the alteration in native pathway gene expression for the generation of the more effective and efficient platform of desired SM production or through the introduction of the heterologous gene(s) for generation of de novo production platform.

There are numerous recent reviews on harnessing microbial systems for metabolic engineering-mediated heterologous production of phytochemicals (Chen et al. 2020; Rahmat and Kang 2020; Yang et al. 2020; Iacovelli et al. 2022; McElwain et al. 2022; Romero-Suarez et al. 2022). However, the review that highlights the recent status of metabolic engineering-mediated homologous and/or heterologous production of phytochemicals in higher plant systems, particularly medicinal plants, is either very limited or absent (Vásquez et al. 2022). Thus, the primary objective of this review is to highlight major achievements in metabolic engineering in the field of plant secondary metabolite production in medicinal plant systems along with basic knowledge about currently available host systems, general steps, techniques, and strategies applied in metabolic engineering for the production of potential therapeutic secondary metabolites.

28.2 Systems Used for Metabolic Engineering

Metabolic engineering commonly employs three different systems, namely, micro-organisms, regenerated transgenic whole plants, and in vitro grown plant cells, callus, or organs, as chemical biofactories for generating desired phytochemicals (Fig. 28.2).

28.2.1 *Prokaryotic Microbial System for Metabolic Engineering*

Prokaryotic microbes, such as *Escherichia coli*, *Corynebacterium glutamicum*, and *Synechocystis* sp. PCC 6803 (cyanobacteria), have been selected as a system in several metabolic engineering research during the last two decades for heterologous production of plant-derived phytochemicals (Pontrelli et al. 2018; Yang et al. 2020; Dietsch et al. 2021; Wolf et al. 2021). These microbes attract scientists owing to their simple genetic and metabolic structure, higher growth rate with a short generation time, reduced space and resource requirement, and high adaptability. In general, the introduction, expression, and optimization of reconstructed plant biosynthetic pathways in suitable microbial hosts are very promising and effective in the de novo synthesis of therapeutically important SM to meet high supply demands. In contrast to transgenic plant-mediated heterologous SM production, microbial SM

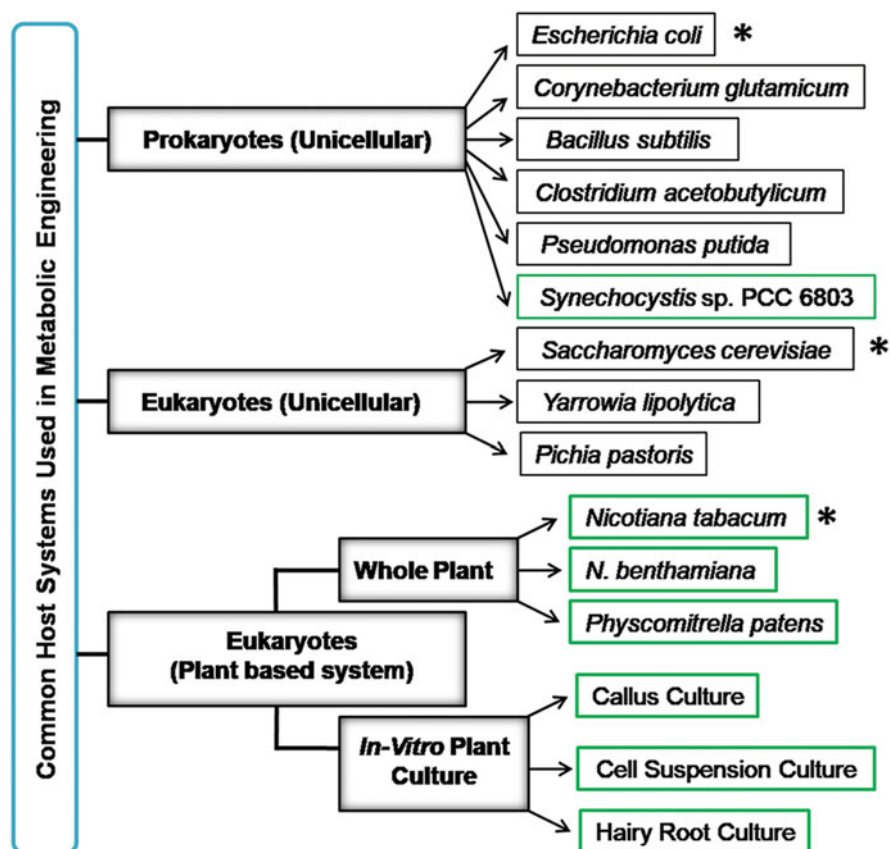


Fig. 28.2 Common host systems available for metabolic engineering for the production of secondary metabolites. “*” marked organisms are being considered as model system for metabolic engineering

accumulation does not involve a time-consuming, plant species-specific regeneration process. Numerous approaches, such as heterologous expression of multiple gene clusters, engineering regulatory networks, redirecting metabolic pathways, stimulation by precursors, and quorum sensing have been used for secondary metabolite production in microbes (Kumar and Prasad 2011; Lyu et al. 2019; McElwain et al. 2022; Romero-Suarez et al. 2022).

E. coli is the foremost commonly exploited prokaryote in metabolic engineering for specific heterologous production of phytochemicals due to several advantages, such as high growth rate, easy to genetic modifications, reduced space, and resource requirement, shorter generation time, simple genetic and metabolic structure (Sangwan et al. 2018; Ma et al. 2021). Metabolic-engineered *E. coli* strain able to produce alkaloids (Zhao et al. 2021), artemisinin (Carqueijeiro et al. 2020), cinnamaldehyde (Pereira et al. 2021), flavonoids (Kim et al. 2015a, b), hyperoside

(Li et al. 2022), pinosylvin (van Summeren-Wesenhagen and Marienhagen 2015), resveratrol (Wu et al. 2017), terpenoid (Wang et al. 2018), valerena-1, 10-diene (Nybo et al. 2017), etc. In spite of the tremendous potential of *E. coli* as a prokaryotic platform in metabolic engineering for specific SM production, it has been greatly challenged by the absence of eukaryotic cellular organization and protein posttranslational modification processes in contrast to native plants. The expression of some fungi- and plant-origin genes like prenyl transferase and P450 suffers protein misfolding, inactivation, and degradation-related issues in this foremost and commonly exploited system (Li et al. 2020). This may cause lower productivity via interruption in the metabolic flow of the reconstructed route. The deletion and substitution of N-terminal sequences that encode hydrophobic transmembrane domain may be applied to solve these problems (Ma et al. 2021).

Metabolic-engineered strains of *C. glutamicum* also have been developed, and they showed the capacity for the ectopic synthesis of natural phytochemicals (Kallscheuer et al. 2017; Zha et al. 2018; Wolf et al. 2021). Advantageous features like rapid spreading ability, ability to grow on low-the cost substrate, such as lignocellulosic biomass at a high growth rate with a short generation time, exhibiting no autolysis, and being safer for human utilization as they do not secrete any endotoxin make this host system a good choice for a variety of industrial compound production.

The unique autotrophic or mixotrophic lifestyle, simplicity in structural organization, and flexible metabolic capacity with rapid-growth of *Synechocystis* sp. PCC 6803 have been increasing their utility in metabolic engineering as alternative economic heterologous chassis that allows light-driven production of diverse valuable phytochemicals from simple inorganic carbon dioxide (Lin and Pakrasi 2019; Dietsch et al. 2021; Lin et al. 2021; Rodrigues and Lindberg 2021; Wolf et al. 2021).

28.2.2 *Eukaryotic Microbial System for Metabolic Engineering*

In the majority of the cases, the potentiality of prokaryotic microbe-based heterologous SM biosynthesis platforms, especially in *S. cerevisiae* and *E. coli*, suffers very low productivity (in microgram per liter range) due to the absence of native biosynthetic pathways in microorganisms and the lack of cellular compartmentalization (Buyel 2019). Multigene insertion, extensive genetic background optimization, and codon optimization are necessary for expression and functional protein production, which are often expensive and time-consuming and need extensive knowledge to achieve the goal. Additionally, the production of complex, less soluble transmembrane proteins often faces problems in prokaryotic hosts. As a result, researchers are emphasizing the need for continued exploration of alternative host systems.

The eukaryotic biosynthesis platform includes microbial systems, such as *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Pichia pastoris*, as well as plant cell systems (Birchfield and McIntosh 2020). They are more appealing to overcome the limitations of prokaryotic microbial systems by providing cellular compartmentalization and cofactors required for the proper expression of genes derived from higher fungi and plants.

The presence of a native MVA pathway in *S. cerevisiae* (yeast) makes it an outstanding and extensively exploited eukaryotic chassis for the heterologous synthesis of specific SMs, such as ginsenoside compound K (Dai et al. 2013), linalool (Amiri et al. 2016) citronellol (Jiang et al. 2021), geraniol (Jiang et al. 2017), astaxanthin (Kildegaard et al. 2017), limonene (Cheng et al. 2019), lycopene (Ma et al. 2019), hyperoside (Li et al. 2022), strictosidine (Brown et al. 2015), and cannabinoids (Luo et al. 2019), and other in this organism by reconstitution of these pathways.

Some scientists consider a nonconventional, oleaginous yeast *Y. lipolytica* as a promising valuable cell factory for producing a wide range of lipid, lipid-derived compounds (Abdel-Mawgoud et al. 2018; Chattopadhyay et al. 2021), recombinant proteins (Vandermies and Fickers 2019), and different SMs (Liu et al. 2019a; Lv et al. 2019; Arnesen et al. 2020; Tang et al. 2021; Zhang et al. 2022) utilizing a diverse range of low-cost substrates via simple pathway modulation and culturing strategies. However, some challenges, such as poor basic knowledge of its metabolism and physiology and a lower titer yield in *Y. lipolytica* than *S. cerevisiae*, limits harnessing their full production potential during heterologous terpenoid production (Worland et al. 2020).

28.2.3 Eukaryotic Plant Cell-Based System for Metabolic Engineering

Introduction of a large number of heterologous enzymes from the plant biosynthesis pathway in metabolic-engineered microorganisms often causes a metabolic burdening-mediated limited productivity via an imbalance in flux, accumulation of harmful intermediates, and genetic instability. Thus, extensive background information, genetic tools, engineering methodologies, and efficient enzymes are required to fully explore their capability as a system for plant SM production (Zhang et al. 2022). In contrast to microbe-based or animal cell-based heterologous production platforms, plant-based heterologous SM biosynthesis platforms are superior in terms of simplicity, scalability, and/or the speed of transient expression, potentially providing gram quantities of product, rapid growth and high biomass, total cost-effectiveness, and safety. Plant cells already have evolutionary evolved a substantial amount of endogenous native cellular, genetic, and biochemical framework similar to native-producing plants. Thus, minimal number of gene insertion and optimization are required for the de novo production of SMs in these systems. Moreover,

genetic manipulation of existing pathways (overexpression of bottleneck rate-limiting enzymes, inhibition of competitive pathways, etc.) may show drastic enhancement in desired SMs.

Nowadays, plant tissue culture-based *in vitro* cultures, such as cell suspension cultures (CSCs), callus cultures, and hairy root cultures (HRCs) of various medicinal plant species, are very widely used as a promising alternative platform for the accumulation of plant-derived phytochemicals in a more defined cultivable condition. HRCs are preferred due to genetic and biochemical stability, rapid growth, and accumulation of similar or higher amounts of phytochemicals in the phytohormone-free medium. In metabolic engineering, they are quite commonly explored as excellent model systems for genetic manipulation of existing metabolic pathways or introduction and ectopic expression of the gene(s) or regulatory factor involved in the targeted biosynthesis pathway for obtaining the enhanced amount of phytochemicals in the least time and space. These native or heterologous *in vitro* cultures can also be used in the discovery of new genes and TFs, characterization of gene function, and introduction of new steps and/or pathways to produce *de novo* molecules. In comparison to prokaryotic microorganisms, they offer distinct platforms that are more close to the natural host plant. Additionally, plant-derived heterologous gene-encoded proteins showed proper folding in HRC than in microbes.

Genetically engineered whole plant systems are also used in metabolic engineering to improve native *in planta* metabolite synthesis or *de novo in planta* SM production. The presence of naturally evolved native genetic and biochemical background, tissue and cellular differentiation, compartmentalization, the requirement of manipulation of only desirable key steps, and fewer optimization steps make the transgenic whole plant system advantageous (Sangwan et al. 2018). In contrast, the need for a huge cultivation area, numerous trials and approval, complicated metabolic cross-talk, and difficulties in the purification of desired phytochemicals are major problems associated with this system. When complete knowledge about the genes and regulation of biosynthetic pathways is unavailable, the utility of this system is proffered to meet the huge clinical demand for natural SMs in comparison to genetically engineered microbial systems. However, the current progress and advances in genetic and metabolic engineering make possible *de novo* productions of different SMs in various transgenic plant species.

Similar to microbe-based biopharming, the selection of the most suitable host organism referred to as “chassis” is a key component. In recent times, *Nicotiana tabacum*, *N. benthamiana*, and *Physcomitrella patens* (moss) have emerged as a new attractive and promising photosynthetic chassis for stable heterologous production of commercial products by heterologous expression of reconstructed plant natural product pathways (Wang et al. 2016; Khairul Ikram et al. 2017; Molina-Hidalgo et al. 2021). *Nicotiana* sp. is at the forefront of synthetic biology and metabolic engineering because of several advantages, including nonfood status, abundant seed availability, genetically and biochemically well-known, availability of a wide array of omics information, well-established methods of nuclear and chloroplast genetic modification (agroinfiltration, virus-mediated overexpression/homology-dependent gene silencing, and gene editing) for alter endogenous metabolite levels or to express

non-native metabolites. The potential of *N. benthamiana* for both stable and transient expression of heterologous genes of structurally diverse plant metabolic pathways like alkaloids (Dudley et al. 2022), terpenoid (Reed and Osbourn 2018), C-glucosylated octaketide anthraquinones (Andersen-Ranberg et al. 2017), glucosinolates (Crocoll et al. 2016), and lignans (Lau and Sattely 2015) has been evaluated by heterologous reconstruction.

Over the past 6 years, significant progress in metabolic engineering and optimization of the artemisinin pathway has led to the successfully produced artemisinin in *Nicotiana* spp. and *P. patens* (Han et al. 2016; Wang et al. 2016; Khairul Ikram et al. 2017) via the introduction of genes involved in artemisinin production pathway. Despite multiple attempts have been made to explore *Nicotiana* for the enhancement of artemisinin production, the engineering of the gene expression and regulation in the artemisinin biosynthesis pathway and complicated glycosylation response has made it difficult to do so and suffering relatively low artemisinin yield. Malhotra et al. (2016) reported the production of ~ 0.8 mg g⁻¹ DW artemisinin in metabolically engineered *N. tabacum* by engineering two mega-metabolic pathways separately into two different cellular compartments. The first elevated the IPP pools by introducing six genes from the MVA pathway into *N. tabacum* chloroplast followed by the artemisinin pathway genes into the nuclear genome with subcellular targeting of *DBR2*, *CPR*, and *CYP71AV1* via chloroplast transit peptide (Malhotra et al. 2016).

On the other hand, a fully sequenced 511 Mb genome, access to huge omics data, their highly efficient and accurate genome engineering, relatively limited chemical and metabolic complexity compared to higher plants, scalability, excellent batch-to-batch homogeneity and stability of products by predominant haploid growth phase, certified good manufacturing in bioreactors, along with the presence of subcellular compartmentalization in *P. patens* allow demarcated biosynthesis steps, and specific storage in organelles, which may compensate the advantages of a short doubling time and available advanced genetic tools in *E. coli* or *S. cerevisiae* (Reski et al. 2018; Banerjee et al. 2019). Furthermore, their highly efficient and precise homologous recombination-based multigene integration or knockout toolkits, development of new high-throughput transformation techniques, and rescue of episomally replicating DNA similar to *E. coli* are amenable to metabolic engineering and have accelerated their use as a biotechnological chassis for the improvement of end metabolite biosynthesis in complex biosynthesis pathway. Continuous considerable progress in culture media optimization, protoplast culture, regeneration, genetic transformation, and development of new auxotrophic moss mutants (Ulfstedt et al. 2017) strongly support moss as a metabolic cell factory (King et al. 2016). The capability of *P. patens* as a photosynthesis-driven green factory for heterologous SM production via metabolic engineering has been tested in the last decades, and a few compounds, such as artemisinin (Khairul Ikram et al. 2017; Ikram et al. 2019), patchoulol and α/β -santalene (Zhan et al. 2014), and sclareol (Pan et al. 2015). In vivo assembly of multiple genes of the artemisinin biosynthesis pathway followed by efficient transformation, their ectopic expression, and optimization of precursor in *P. patens* showed significantly improved production of amorphadiene and artemisinin (Khairul Ikram et al. 2017; Ikram et al. 2019). Banerjee et al. (2019) mimicked the

modular nature of diterpene biosynthetic pathways found in modern land plants by developing a flexible pipeline to install three combinations of class II and class I diterpene synthases in *P. patens* and able to produce diterpenes matching the reported activities in the angiosperm sources.

28.3 General Steps and Techniques Used in Metabolic Engineering

Metabolic engineering, similar to other engineering fields, follows the fundamental steps of designing, building, testing, and learning to manipulate endogenous biochemical pathways (Whitford et al. 2021; Fig. 28.3). The design step involves identifying the problem and selecting desired metabolic pathway and appropriate host; the building step entails developing and assembling of in silico reconstructed pathway as well their incorporation into the host; test step validates the efficacy of constructed host for desired metabolites or product production, and in the learning step, some conclusions are drawn based on the available results about the constructed design, and the learning knowledge is implemented to improve the existing system in more effective ways in subsequent cycle (Fig. 28.3).



Fig. 28.3 The fundamental steps (design–build–test–learn cycle) of metabolic engineering with major events of each step (red, blue, yellow, and purple color box for design, build, test, and learning step events, respectively)

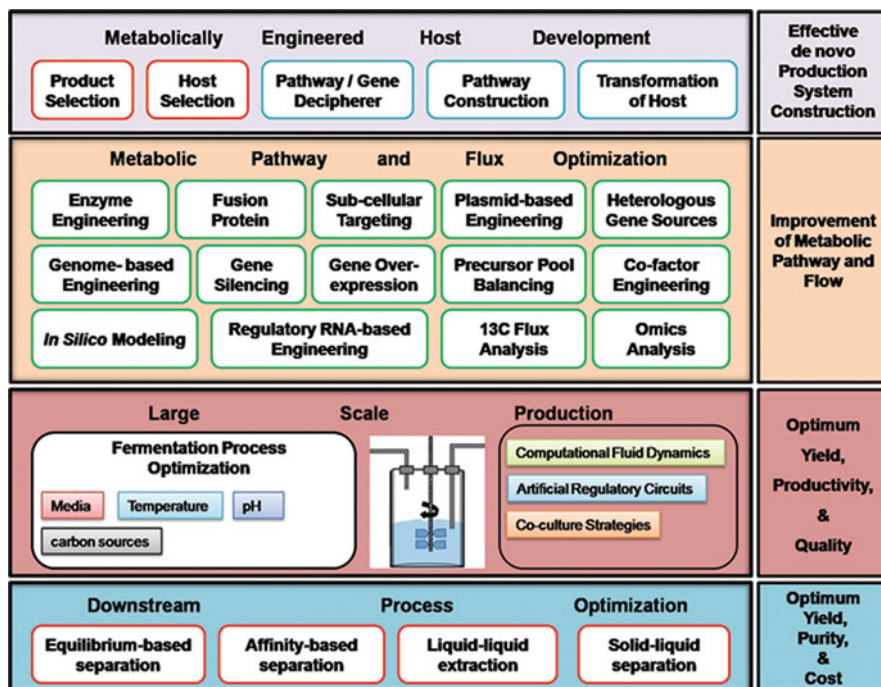


Fig. 28.4 Different steps of de novo bio-pharming in engineered heterologous hosts include the knowledge of enzymes, their regulation, and encoding gene of the particular secondary metabolic pathway; the selection of a suitable heterologous host organism relying on the host's characteristics; isolation, cloning, and reconstruction of heterologous biosynthetic pathways; metabolic flux and process optimization; scale-up of the system and optimization of downstream process

In general, de novo bio-pharming in engineered heterologous hosts necessitated the discovery of unknown enzymes of the particular secondary metabolic pathway along with knowledge of their regulation and encoding gene; the selection of a suitable heterologous host organism relying on the host's characteristics; isolation, cloning, and reconstruction of heterologous biosynthetic pathways; metabolic flux and process optimization; scale-up of the system; and optimization of downstream process to increase yield and reduce cost of the product (Fig. 28.4).

28.3.1 *Discovery of Unknown Enzymes of SM Production Pathway*

The comprehensive knowledge of the diversity and uniqueness of the biosynthetic pathway of the desired product in the native plant is very much a prerequisite for metabolic engineering during the designing of the metabolic pathway, which

includes knowledge of each enzyme, their encoding gene, the mechanism of the enzymatic reaction, and the regulation of the pathway. Numerous metabolic pathways have been discovered and described, and their metabolic information is stored in various public repositories, such as BioCyc, KEGG, MetaCyc, PlantCyc, and Plant Reactome (Baghalian et al. 2014; Caspi et al. 2018; Kanehisa et al. 2019; Karp et al. 2019). However, numerous metabolic pathways are still not well characterized, and several gaps exist as certain components have not been recognized. Researchers use precursor feeding, over- or down-expression of the gene, mutant selection, or differential gene expression to discover and characterize genes involved within an unelucidated pathway. Nowadays, several next-generation genome sequencing platforms (Roche 454/GS FLX, Illumina Hiseq, Applied Biosystems (ABI) SOLiD sequencing), quantitative trait loci (QTL) analysis, genome-wide association studies (GWAS), microarray, transcriptome-based analysis (RNA-seq), expressed sequence tags (ESTs), and suppressive subtractive hybridization (SSH) are used to identify biosynthesis pathway genes and their metabolites (Scossa et al. 2018; Arya et al. 2020). Further, bioinformatics tools may be used to annotate and identify the complete metabolic pathway following that the transcriptome or genome sequencing data has been assembled. Several proteome-based analyses (2D electrophoresis, shotgun proteomics, MALDI-TOF, LC-MS) and metabolome-based analyses (GC-MS, LC-MS, and NMR) further strengthen the knowledge acquired from genomics and transcriptomics studies (Lian et al. 2018; Presnell and Alper 2019).

Although lots of progress have been taken in recent times, still the lack of total information related to plant metabolic pathway is the key barrier in pathway reconstruction in heterologous hosts. Contemporary technical advances, such as single-cell metabolomics, homology-based cloning, and plant iSMASH have speeded up their enzyme discovery (Pandita et al. 2021). Following the selection of potential enzymes, the enzymatic activities of selected enzymes are assessed utilizing functional analysis in combination with metabolomics methods to identify desired product production.

28.3.2 Selection of Suitable Host

The selection of effective and reliable heterologous hosts is critical for maintaining the optimum biosynthesis of target phytochemicals. The genetic and metabolic makeup, codon bias, and availability of the host organisms should be taken into account during reconstructing the new pathway(s) and enhancing the pathway flows (Lanza et al. 2014; Ko et al. 2020). The use of a native high-yielding host is the best effective strategy for the production of desired phytochemicals by metabolic engineering, but their restricted number, availability, and public acceptance are the main barrier to this system. *E. coli* and *S. cerevisiae* have been preferentially used as heterologous hosts for yielding phytochemicals owing to their advantageous characteristics. Interestingly, the number of heterologous hosts has expanded

unprecedentedly in the last few years due to several breakthroughs in metabolic engineering techniques, including *E. coli*, *C. glutamicum*, *B. subtilis*, *P. putida*, *S. cerevisiae*, *Nicotiana tabacum*, *N. benthamiana*, and *Physcomitrella patens* (Calero and Nikel 2019; Carqueijeiro et al. 2020; Ko et al. 2020; Fig. 28.2). A suitable heterologous host may be chosen based on the host's characteristics. Recently, minimal synthetic genome organisms having only functionally defined genetic components have been established in microbial organisms that considerably reduce the likelihood of metabolic flux shifting (Calero and Nikel 2019).

28.3.3 *The Heterologous Reconstitution of Plant Biosynthetic Pathways*

After the selection of desired product, metabolic pathway, and appropriate host, the reconstruction of a heterologous plant biosynthesis pathway in the non-native host is a challenging task that involves designing, building, and incorporating the heterologous pathway in the host system. Computer models of different parts of the pathway were constructed using existing knowledge, and then the entire reconstruction of the heterologous metabolic pathway was assembled. Gene cassettes are constructed with the help of computational programs and synthetic biology, and in silico expression study was conducted conditionally, which is very important for the effective production of desired molecules inside the cell.

Targeted genes are isolated from various plants, and gene cassettes are constructed with the help of recombinant DNA technology and cell-free expression systems. Depending on the strategies, the desired foreign genes are cloned into an appropriate expression vector under different types of regulatory elements (constitutive or inducible or strong promoters) and integrated into the selected host organism genome via different reliable and stable gene transfer methods. Electroporation, conjugation, and protoplast transformation are the most frequently used methods along with other methods like phage-enabled transfer, sonoporation, nanofiber piercing, and liposome-mediated fusion for gene transfer in microbes. In contrast, *Agrobacterium*-mediated transformation and biolistic approaches are often utilized for the integration of heterologous genes or gene cassettes in plant host. The natural ability of gram-positive soil bacterium, *A. tumefaciens*, and *A. rhizogenes*, is utilized to introduce heterologous DNA into plant cells in *Agrobacterium*-mediated transformation. Most commonly applied *A. tumefaciens*- and *A. rhizogenes*-mediated genetic transformation results in the development of crown gall and hairy roots (HR), respectively (Halder and Jha 2021). Transgenic plant development may be possible from the Ti-induced crown galls (undifferentiated calli) or Ri-induced hairy roots via spontaneous or induced regeneration. The co-transformation method allows the simultaneous insertion of multiple genes in a stable manner. On the contrary, DNA- (i.e., gene/vector) coated high-speeded tungsten or gold molecules are used for penetrating and delivering the DNA inside the nuclear or cellular

compartments under vacuum conditions to transform plant tissues or cells in a species-independent manner in the biolistics method. Application of *Agrobacterium*-mediated transformation and particle bombardment techniques are often associated with problems like nonspecific gene integration, high copy number, the threat of unintended transgene escape, marker-free transgenic system production, and regulatory and biosafety measurements.

Different marker gene-or reporter gene-assistance selection methods are applied to select transformed cultures and PCR and quantitative real-time PCR; blotting techniques may also be conduct to verify the transgenic insertion as well as their expression at transcriptional and posttranscriptional levels. After the successful introduction of the heterologous pathway in the host organism, the effectiveness of metabolic engineering is assessed in terms of the expression of heterologous genes with a high-throughput screening or by monitoring the metabolic output through spectroscopic analysis (HPLC, GC-MS, or LC-MS), expression data, RNA seq data, or gene expression data. Manipulation of genes can be performed via site-directed mutagenesis, gene shuffling, or recombinant DNA technologies to optimize the pathway, if necessary. Combinations of techniques are usually more effective as they provide better resolution and applicability.

28.3.4 Metabolic Pathway and Flux Optimization

The optimization of metabolic flow in the direction of the desired phytochemical is a very essential step to maximize the bio-production after the reconstruction of metabolic pathways in host organisms. Plasmid-based expression systems are commonly used to express target genes in metabolic engineering due to their inherent advantages, such as being easy to manipulate and capable of transforming a host strain. Metabolic pathway optimization can also be achieved by the proper selection of a specific copy number plasmid and the effective designing of the promoter, terminator, and ribosome binding site of the gene expression cassette in the plasmid (Ko et al. 2020).

The correlation between high-copy number plasmids and enhancement in the metabolic fluxes is not always true, as sometimes they lead to impaired cell growth, formation of inclusion bodies, and toxic intermediate accumulation. This may lead to a reduction in yield. To ensure accurate and stable gene expression, a variety of synthetic promoters with a wide range of strengths has been developed by using error-prone PCR, site-directed mutagenesis, hybrid promoters, randomization of nonconserved regions, design of synthetic transcription factor attaching regions, incoherent feed-forward loop (iFFL) using a transcription activator-like effector (TALE) and other approaches (Ko et al. 2020). Furthermore, selecting the correct terminator is essential to ensuring gene transcription stability. UTR designer, ribosome binding site designer, ribosome binding site calculator, and ribosome binding site library calculator are some computational modeling tools used to predict the

translation initiation rate (TIR) of RBSs that can be used as an effective strategy to optimize the translation process by engineering RBS sequences (Ko et al. 2020).

In metabolic engineering, pathway optimization can also be possible by several available genome or gene editing tools like hairpin, virus-induced gene silencing vectors, meganuclease, RNAi, ZFN-Zinc finger nucleases, and TALEN-transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR) along with the CRISPR-associated protein (Cas) system (CRISPR/Cas). In contrast to classical homology-based entire gene families, silencing strategies, TALENs, ZFNs, and CRISPR/Cas9 gene editing systems are more efficient genome editing and targeted mutagenesis tools in plant metabolic engineering. CRISPR-Cas-mediated genome editing toolkits have been used for direct genomic modifications without using any plasmids that include site-specific gene mutagenesis, replacement, deletion, or insertion of specific DNA sequence through non-homologous end joining or homologous recombination (Zhao et al. 2020). Furthermore, the development of numerous variants of the CRISPR/Cas9 systems, such as CRISPR interference (CRISPRi), CRISPRa, CRISPR-AID system, and CRISPR-Base Editing SysTem (CRISPR-BEST), allows expression modification of specific gene (inactivation, downregulation, or upregulation) of certain SM biosynthesis pathways (Ko et al. 2020; Zhao et al. 2020). Although these gene-editing methods are still in their infancy, new progress in genome editing techniques provides an amazing potential for bioproduction of pharmaceuticals, nutraceuticals, and other secondary metabolites via genome engineering.

Recent advances in molecular techniques and novel genome editing technologies, such as the Easy-Clone system, Golden Gate cloning, Gibson assembly, CRISPR-Cas, etc., have greatly aided in the complex process of integration of longer heterologous reconstruction of plant biosynthetic pathways into the genome of a heterologous host. The common problems associated with the use of plasmids are the metabolic burden, plasmid instability, and use of high value of selection markers, which can be overcome through these approaches (Ko et al. 2020). RecABCD system-based homologous recombination, 1 Red recombination method, Cre-lox, and Flippase-Flippase Recombinase Target (Flp-FRT) are also used in metabolic engineering (Ko et al. 2020). Several trans-acting regulatory RNA-mediated tools, such as synthetic small regulatory RNA, RNA interference, antisense RNA, and small transcription-activating RNAs, are extensively utilized for genome-wide engineering and fine-tuning of gene expression due to their modular, tunable, and designable characteristics (Ko et al. 2020).

A variety of sophisticated algorithms (MOMA, minimization of metabolic adjustment; MILP, mixed-integer linear programming; RobustKnock; BiMOMA; OptReg; OptStrain; OptGene; FSEOF, Flux scanning based on enforced objective flux; FVSEOF, flux variability-based scanning of enforced objective flux) and simulation methods (flux response analysis, flux-sum analysis, and elementary flux mode analysis) can be utilized for a comprehensive understanding of the complicated metabolic network, identifying further engineering targets and pathway optimization by *in silico* genome-scale metabolic stimulation (Badri et al. 2017; Ko et al.

2020). All the necessary steps should be considered to ensure maximum expression of transgenes to increase the flux in host organisms.

28.3.5 Scale-Up Production

Scale-up production generally involves a subsequent increase in the culture volume from laboratory-scale fermentor/bioreactors (0.5–30 L) to full-scale production fermentors (20,000–2,000,000 L) through pilot-scale fermentors (30–3000 L) and sometimes demo plant-scale fermentors (3000–20,000 L). In every step of the scale-up process, validation of the stability and efficacy of the engineered strain and determination of optimal fermentation conditions are critical as the lab-scale bioreactors have different physical properties compared to other large-scale bioreactors to ensure the optimum yield, productivity, and quality of desired molecules. Under certain circumstances, high-performing strains are unable to handle increased metabolic burden or product toxicity, and sub-performing or non-producing populations can emerge. To combat genetic heterogeneity problems, artificial regulatory circuits that control cellular metabolism in relation to extracellular and intracellular perturbations could be implemented (Ko et al. 2020). Adaptive laboratory evolution could be used to enhance tolerance capacity.

Inputs from the computational fluid dynamics can be used to calculate the effects of mixing mode or shear stress on cell morphology in a particular fermentation condition, which enables the efficiently homogeneous mixing of oxygen and feed substrate without disrupting cells, decreasing cell growth and enhancing byproduct synthesis. Considering the high expenses of large-scale fermentation, the next steps are only taken after achieving satisfactory product quality. Several strategies, such as stable maintenance of plasmids without selection pressure, integration of all the biosynthetic pathways under constitutive promoters of desired strength in the genome, or plasmid-based expression systems, are adapted to reduce capital expenditure without compromising yield in scale-up fermentation.

Further metabolic engineering may be required to resolve any yield-related issues encountered during the scale-up process. Impurities in low-cost crude carbon sources or commercial-grade medium ingredients may cause massive accumulation of inhibitors and non-fermentable substances in the fermentor that may inhibit the growth of the microbes or result in growth of undesirable organisms in the fermentor.

28.3.6 Downstream Process

The downstream process is the last but very vital step of commercial production of any compound as it ensures the ultimate yield, purity, and cost of a target chemical. This step is expected to account for 20–50% of the entire production cost. Thus, any

commercial bioprocessing greatly relies on the development of a downstream process that can recover the high yield and purity of a target chemical from a liquid medium or produce organisms in an economically friendly and energy-efficient manner.

In the beginning, centrifugation or filtration is used for the purification of both intracellular and extracellular phytochemicals. Centrifugation or filtration is followed by the disruption of cells by a bead mill or French press. Various recovery and purification techniques, like equilibrium-based separation (e.g., absorption, distillation, and liquid-liquid extraction), affinity-based separation (e.g., adsorption, ion exchange, and chromatography), membrane separation (e.g., reverse osmosis, microfiltration, ultrafiltration, and electrodialysis), solid-liquid separation (e.g., conventional filtration, direct crystallization, and precipitation), and liquid-liquid extraction (e.g., solvent extraction and two-phase aqueous extraction), could be applied for effective recovery of desired products based on the product-containing solution's properties (Ko et al. 2020). Minimized byproduct production in metabolically engineered microbes has the potential to cause a significant reduction in downstream processing costs as well as improve the yield of the desired product.

28.4 Common Strategies Applied in Metabolic Engineering

28.4.1 Redirecting Flux by Overexpression of Single Gene

Precursor and/or substrate availability impacts the production of target compounds. The overexpression of a specific enzyme coding gene of a particular metabolic pathway is the simplest and most common strategy of metabolic engineering to redirect the precursor/substrate availability by metabolic flux toward a specific SM (Fig. 28.5). This strategy can be used in both homologous and heterologous systems for the hyper-production of key enzymes (Fig. 28.5(1)). Identification of target gene(s) and step(s) in the large plant biosynthetic pathway is often challenging due to limited information about the pathway and genes.

28.4.2 Shift in Metabolic Flux by Overexpression of Multiple Genes

The complexity of plant biosynthetic pathways with multiple rate-limiting steps, branching points, and limited knowledge decrease the effectiveness of the overexpression of a single gene strategy. Several studies have revealed that a multigene engineering approach to increase SM production is more effective due to the push-pull effect created by a synergistic effect of simultaneous expression of several genes (Fig. 28.5(2)). The techniques of multiple gene transfer and their

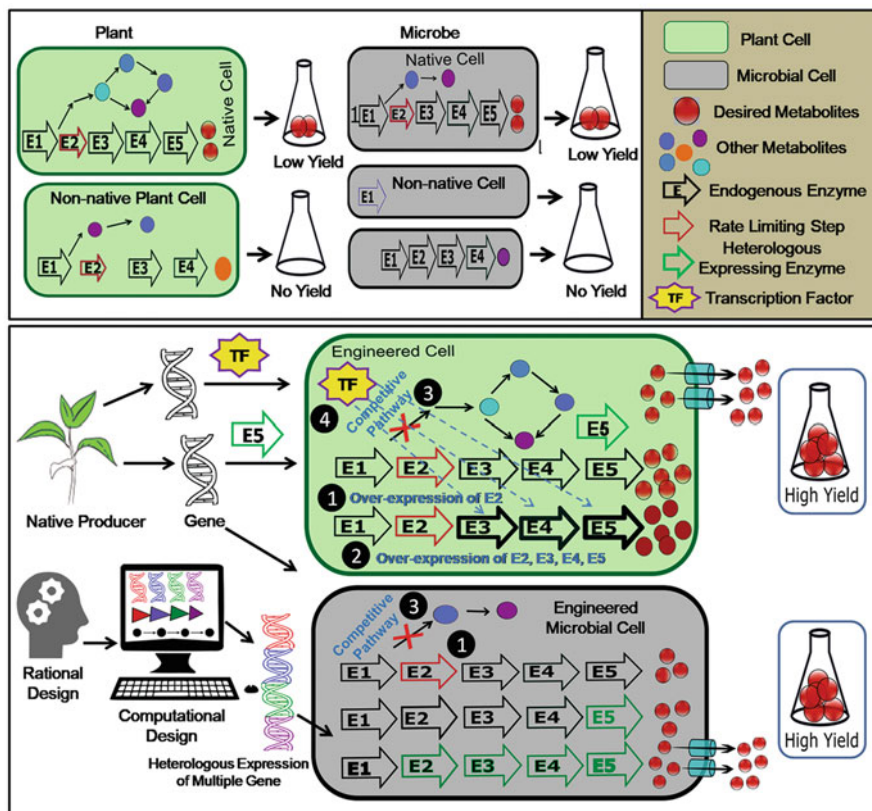


Fig. 28.5 General strategies of metabolic engineering for the production of desired plant metabolite in homologous and heterologous plant and microbial cells. (1) Overexpression of single rate-limiting enzyme (brown color), (2) overexpression of multiple enzymes (brown color and dark black color), (3) inhibition of competitive pathway by gene silencing of branch point enzyme (red color cross), (4) expression modulation of transcription factor improve the expression of multiple enzymes that improve desired molecule production

regulation are often difficult and time-consuming but very effective to achieve definite goals in metabolic engineering. Multiple genes may be overexpressed to improve metabolic flux toward desired metabolites.

28.4.3 Shift in Metabolic Flux by Gene Silencing

Plant metabolic pathways are mostly complicated and diversified or branched at one or more positions. The main pathway is often limited in its productivity as enzymes compete for the common substrate at each branch point. This parallel competitive pathway is responsible for the diversion of the common precursor pool resulting in

decrease metabolite production. Silencing or knockout of the expression of branch point enzyme coding gene of the competitive pathway has also been used as an alternative or with a combination of overexpression of selected genes of the precursor pathway for improving targeted metabolite synthesis by redirecting (inhibiting or decreasing) the precursor pool/metabolic flux toward it (Fig. 28.5(3)).

Moreover, the negative effects of cellular production of undesirable toxic metabolites may be prevented effectively via metabolic engineering-mediated minimization of the precursor flux toward the specific step in the metabolic pathway. It can be achieved through the downregulation of the upstream enzyme of the pathway and suppression of gene expression of the particular enzyme, enhancing their catabolism and increasing flux into competitor pathways by antisense, co-suppression, and RNA interference (RNAi) methods. RNA interference (RNAi) and antibody overexpression are more effective approaches producing better outcomes than classical approaches. Nowadays, more stable knockdown ensures the elimination or reduction of undesirable compounds by the knockdown of a specific gene in specific plant tissue and organs.

28.4.4 Over- or Downregulation of Transcription Factors

Transcription regulation proteins or transcription factors (TFs) are regulatory DNA binding proteins with an activator domain, DNA binding domain, and trans-activation domain that recognize and bind with a specific cis-regulatory element (promoter or enhancer) located at the upstream region of a gene and control decoding of genomic information via regulation of transcription rates of the particular gene. Alteration in expression (over- or downregulation) of endogenous or foreign transcription factor coding gene is a novel emerging powerful approach in metabolic engineering and synthetic biology to deal with the issue of multigene regulation (Zhao et al. 2015; Ma et al. 2017b; Alfieri et al. 2018; Cao et al. 2018; Deng et al. 2019; Wang et al. 2019; Hao et al. 2020; Luo et al. 2020; Wang et al. 2021). This approach manipulates multiple steps of metabolic pathways or modulates the entire complex metabolic pathway in the homologous or heterologous system due to their gene expression regulatory ability in an integrated and coordinated fashion via their attachment to thousands of nucleotide upstream from the transcription initiation point of the gene by sequence-specific DNA binding and protein-protein interactions (Sun et al. 2019; Shi et al. 2020b).

Manipulation of metabolite flux can be possible via regulation or modulation of transcription factors that either activate or repress the transcription of multiple enzymes involved in specific SM biosynthesis (Fig. 28.5 (4)). Thus, unlike structural genes, over- or downregulation of a single transcription factor is termed a global approach of gene regulation as it commonly governs synchronized expression of vital genes of multistep metabolic pathways at a time to manage the flow of metabolites (Fig. 28.5(4)). The use of transcription factors necessitates the integration of data from genomics, transcriptomics, proteomics, and metabolomics (Kayser

and Warzecha 2012). Identification and characterization of several transcription factors expand the knowledge of the transcription factors that open up a new horizon of ME. Several transcription factors, such as *MYB1*, *MYB12*, *MYC2*, *TCP4*, *WRKY1*, *WRKY2*, *WRKY3*, *WRKY37*, *WRKY40*, *MYB98*, *ERF1L1*, *ERF115*, *ORCA3*, *PAP26*, etc., were already identified and tested in several medicinal plants, such as *C. roseus*, *Isatis indigotica*, *Lycium ruthenicum*, *L. erythrorhizon*, *S. miltiorrhiza*, etc., to alter the metabolic flow in several biosynthetic routes and significant alterations in secondary metabolite production. Artificial transcription factors are now used in addition to natural transcription factors. The utilization of transcription factors in conjunction with expression change in the enzyme-encoding gene may be a more promising strategy in metabolic engineering (Sangwan et al. 2018).

28.4.5 *Transporter Engineering-Mediated Metabolic Channeling to Secondary Sites*

Secondary metabolites synthesized in plants are transported into different subcellular compartments or sink organs via sophisticated intra- or intercellular transport mechanisms to regulate the metabolite efflux, which helps them mitigate cytotoxic effects caused by overaccumulation. In spite of the initiation of extensive research on membrane transporters of economically important therapeutic SMs and their genes in plants and microbes, little information currently available on it. Systematic, targeted metabolic flow via transporter engineering required a comprehensive understanding of the route of specific secondary metabolite trafficking and their molecular interaction with membrane transporters. Numerous secondary metabolite transporters have been discovered in plants, including PDR1, ABCG1.1, NUP1, MATE-1, 2, JAT1, 2, MATE-2, 3, 5,7,8,9, AcrB, MacAB, MsbA, MfsA, ABC2, 3, ADP1, etc., and genes of these transporters have been used in heterologous microbial and plant hosts to study their effects (Kell et al. 2015; Lv et al. 2016). According to Wang et al. (2013), sesquiterpene amorphadiene and diterpene kaurene were heterologously produced in *E. coli* by successful overexpression, assembly, and precise tuning of several PDR pumps. Overexpression of *AcrB* and *TolC* transporter genes enhanced 31% and 37% amorphadiene and kaurene production, respectively, whereas expression of *TolC-TolCAcrB* enhanced 118% amorphadiene yield (Wang et al. 2013). The overexpression of the *TPT2* gene of *C. roseus* that encodes an ATP-binding cassette transporter for catharanthine in HRC of *C. roseus* caused fivefold higher catharanthine production compared to the control (Wang et al. 2019). Transporters are also used in the secretion of metabolites from microbial and plant cells into culture medium.

28.4.6 *Modification in Cis-Regulatory Elements*

In metabolic engineering, changing key cis-regulatory elements, like promoters, can create fine-tuned metabolic flow and synchronization among different pathways by altering its gene expression. Natural or modified promoters with suitable strength and regulatory features are used in metabolic engineering to alter the gene expression at the quality and quantity level (Hubmann et al. 2014). The nature and strength of the promoters, whether constitutive or tissue-specific, weak or strong, used during the tailoring of metabolic pathways have a great impact on transgene expression and desired phytochemical production (Bilas et al. 2016). Several constitutive (*35S*, *actin*, *ubiquitin 1*, *PD1-PD9*), inducible (*hsr203J*, *adh*), and tissue-specific promoters (*CYP71AV1*, *2A11*, *TobRB7*, β -*conglycinin*) are commonly used in metabolic engineering.

28.4.7 *Substrate Channeling*

Substrate channelling is an enzyme engineering-mediated metabolic flow improvement strategy where the product of one enzyme directly transfers to subsequent enzymes of the same metabolic pathway through the formation of enzyme complexes or multifunctional enzymes by the construction of scaffolds or fusion of genes (Han et al. 2016). This strategy has great potential to increase reaction rates in metabolic engineering. For example, Han et al. (2016) overexpressed fused genes of amorpha-4,11-diene synthase (ADS) and farnesyl diphosphate synthase (FDS) under the constitutive *CaMV35S* promoter or the recombinant trichome-specific promoter of amorphadiene 12-hydroxylase (*CYP71AV1*) in two relatively high artemisinin-producing varieties of *A. annua* to investigate the impact of substrate channelling in artemisinin biosynthesis pathway. ADS is responsible for converting farnesyl diphosphate (FDP) into the aliphatic sesquiterpene amorpha-4,11-diene at a very low turnover rate in the first rate-limiting step of the artemisinin biosynthesis pathway. An elevated amount of amorpha-4,11-diene production was possible by the fusion of two enzymes that resulting in higher local FDP concentration and proximity of two enzymes than the native ADS present in the cytosol (Han et al. 2016).

28.4.8 *Signal Sequence Tagging Improves Localization of Enzyme*

Expression of the transgene tagged with organelle targeting or retention signals facilitates the transport or retention of enzymes to a particular cellular compartment or organelle having the precursors, which may boost the concentration of the desired

chemicals. Interestingly, a fivefold improvement in yield of the target compound achieved by the indirect effect of silencing of SNARE proteins in combination with transient expression of linalool and caryophyllene synthases in *N. benthamiana* compared with non-silenced control might be due to reducing proteasome function that enhances the stability of the ectopically expressed terpene synthase proteins (Ting et al. 2015).

28.4.9 Cofactor Manipulation

The combined inputs of all enzymes, cofactors, and metabolites involved in a particular metabolic pathway act as a system to regulate the metabolic flow in biochemical processes. Each component of the metabolic pathway impacts metabolic flow, although their effects are not equal. Changes to any component frequently have an impact on others. Several studies showed that modulation in the production and availability of cofactors also could improve metabolic flux redistribution in metabolic engineering (Cui et al. 2014; Hao et al. 2016; Liu et al. 2021).

28.5 Secondary Metabolite Production in Metabolic Engineered HRC of Medicinal Plants

28.5.1 Homologous Overexpression of Single Gene of SM Biosynthetic Pathway

The overexpression of a single key gene of the target biosynthesis pathways is a common genetic manipulation strategy used in metabolic engineering to improve the production of desired secondary metabolites in HRs. To evaluate the impact on internal JA pool via upregulation of allene oxide cyclase (*AOC*) gene on secondary metabolite production, *AOC* gene was isolated from *Salvia miltiorrhiza*, cloned, characterized, and incorporated into HRs of *S. miltiorrhiza* by Gu et al. (2012). *AOC*-upregulated HRC of *S. miltiorrhiza* significantly enhanced tanshinone IIA, RA, and LAB production along with alteration in the transcriptional expression of diterpene and phenolic acid biosynthesis pathway-related genes (Gu et al. 2012).

Genetically modified HRCs of *Valeriana officinalis* showed increased valerenic acid (two to fourfold), sesquiterpene hydrocarbon (1.5- to fourfold), and oxygenated sesquiterpenoids (five- to ninefold) accumulation by overexpressing farnesyl pyrophosphate synthase, valerendiene synthase, or germacrene C synthase genes of *V. officinalis* in comparison to the non-transgenic root lines (Ricigliano et al. 2016).

Overexpression of one of the *W. somnifera* sterol glycosyltransferase-4 (*Wsgt14*) gene enhanced the production of total withanolide yield and withanolides-A in the HRC of *W. somnifera* in response to the elicitors (Pandey et al. 2016).

Targeted metabolic engineering of glycyrrhizin pathway in a well-known medicinal herb *Glycyrrhiza glabra* by integration of constructed plasmid (pBI121^{GUS-9}; *CYP88D6*) using *A. rhizogene* ATCC 15834 leads to overexpression of β -amyrin 11-oxidase (*CYP88D6*) gene, which catalyzes two sequential oxidation steps of intermediate β -amyrin molecule, enhance or similar glycyrrhizin production in transgenic HRCs compared with control HRs (Shirazi et al. 2018).

Rosmarinic acid synthase (RAS) and *CYP98A14* (a cytochrome P450-dependent monooxygenase) genes derived from *S. miltiorrhiza* have been effectively transferred to the HRC of *S. miltiorrhiza*. HR lines overexpressing RAS or *CYP98A14* gene have increased phenolic acid accumulation (up to threefold) and antioxidant activity (Fu et al. 2020).

Significant boosting of tanshinone yield through overexpression of the *S. miltiorrhiza* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*MDS*) gene in HRC of *S. miltiorrhiza* compared to the control and total tanshinone content in *SmMDS*-overexpressing lines increased after elicitor treatment (Wei et al. 2019).

Ophiorrhiza pumila is a well-known natural source of DNA topoisomerase I inhibited anticancer drug camptothecin (CPT). The HR lines of *O. pumila* overexpressing geraniol 10-hydroxylase (*G10H*) gene (designated as G line) or secologanin synthetase (*SLS*) gene (designated as S line) increased CPT biosynthesis up to 2.40 mg g⁻¹ and 3.28 mg g⁻¹ in G line and S line, respectively (Shi et al. 2020a). Moreover, CRISPR-Cas9-mediated knockout of *G10H* gene or *SLS* in HR lines showed significantly reduced camptothecin content suggesting their important role in CPT biosynthetic pathway (Shi et al. 2020a).

The HR line of *W. somnifera* (Ws9), which overexpresses the *Squalene synthase* gene, exhibits enhanced withanolide A (2.82 mg g⁻¹ DW) synthesis. Interestingly, these HRCs also showed improved production of withanolide B (1.34 mg g⁻¹ DW), withaferin A (1.83 mg g⁻¹ DW), and withanone (1.97 mg g⁻¹ DW) (Sivanandhan et al. 2020).

Similarly, the overexpression of chalcone synthase (*CHS*) gene from *Glycyrrhiza uralensis*, which encodes the first rate-limiting enzyme of flavonoid biosynthetic pathway, in HR lines of Chinese herbal medicinal plant *G. uralensis* by transfer of a constructed expression vector pCA-*CHS* using *A. rhizogenes* strain ACCC10060, showed improved content of total flavonoid as well as liquiritigenin, isoliquiritigenin, and isoliquiritin (Yin et al. 2020). Interestingly, the maximum flavonoid concentration was found in the S20 transgenic line, harbouring nine copies of CHS (Yin et al. 2020).

Similarly, HRCs of *S. miltiorrhiza* that overexpress their kaurene synthase-like (*KSL*) gene showed maximum 2.7-fold increased total tanshinone production compared to wild-type control lines (Wei et al. 2020). Application of elicitation strategy by application of yeast extract treatment further increases total tanshinone production (4.2 mg g⁻¹ DW) in the *KSL*-overexpressing HR line, which represents a 3.5-fold improvement over control (Wei et al. 2020).

28.5.2 *Heterologous Expression of Single Gene of SM Biosynthetic Pathway*

The introduction of heterologous genes and/or pathways and their ectopic expression in HRCs have been implemented to alter the native metabolic flow for the production of benzylisoquinoline alkaloid, ginsenoside, reserpine, ajmalicine, abietane diterpenoid, and aethiopinone via metabolic engineering (Mehrotra et al. 2013; Sharafi et al. 2013a, b; Cui et al. 2015; Zhang et al. 2015; Vaccaro et al. 2019).

Six transgenic HRCs of *R. serpentina* were established that overexpress tryptophan decarboxylase gene isolated from *C. roseus* utilizing *A. rhizogenes*-mediated transformation (Mehrotra et al. 2013). This work indicates that overexpression *tryptophan decarboxylase* gene is an excellent approach for increasing the reserpine and ajmalicine biosynthetic capacity of HRC up to 0.1202% DW and 0.0064% DW, respectively, after ten weeks of culture (Mehrotra et al. 2013).

Enhanced flavonolignan biosynthesis is also reported in the HRCs of *Silybum marianum* through a metabolic engineering approach of upregulation of the *chalcone synthase* (*chsA*) gene derived from *Petunia* (Rahnama et al. 2013). These HRCs showed sevenfold and tenfold higher silymarin and silybin content in comparison to non-transgenic hairy roots (Rahnama et al. 2013).

Codeinone reductase (CodR) and salutaridinol 7-*o*-acetyltransferase (SalAT) are important enzymes of the benzylisoquinoline alkaloid (BIAs) biosynthesis pathway. CodR enzyme catalyzes the reversible NADPH-dependent penultimate reduction step of morphine biosynthesis in *Papaver somniferum* (Carr et al. 2021), whereas SalAT is a highly substrate-specific enzyme that involves in the stereo-selective transfer of the acetyl group from acetyl coenzyme A to the 7-OH group of salutaridinol yielding salutaridinol-7-*O*-acetate. The overexpression of the *CodR* gene isolated from *P. somniferum* considerably increased codeine (~11-fold) and morphine in HRCs of *Papaver bracteatum* in comparison with HRCs without *CodR* overexpression and wild-type roots (Sharafi et al. 2013a). A novel peak of 0.28% DW morphine was detected in transgenic HRCs overexpressing the *CodR* gene. Transgenic HRC overexpressing *SalAT* transgene of *P. somniferum* showed comparatively ameliorated quantities of thebaine, codeine, and morphine relative to control (Sharafi et al. 2013b).

Roots of *Salvia sclarea* are the source of bioactive abietanic diterpenes, such as aethiopinone, 1-oxoaethiopinone, salvipisone, and ferruginol, but at a very low amount (about 1% of root DW). The heterologous expression *DXS* (D-xylulose 5-phosphate synthase) or *DXR* (1-deoxy-D-xylulose 5-phosphate reductoisomerase) gene from *A. thaliana*, encoding the upstream enzymes of the plastidial 2-*C*-methyl-D-erythritol 4-phosphate (MEP)-dependent terpenoid pathway, in HRC of *S. sclarea*, revealed a significantly higher content of abietanic diterpenes compared to the control HR line transformed with the empty vector (Vaccaro et al. 2014).

Anticancerous monoterpenoid indole alkaloid camptothecin production significantly increased by heterologous overexpression of individual *G10H* gene (G lines) and *STR* gene (S lines) of *C. roseus* in comparison to NT HRC (Cui et al. 2015).

Interestingly, the enhancement in camptothecin production was much higher in G HR lines than in S lines suggesting that *G10H* plays a more important role in stimulating CPT accumulation than *STR* in *O. pumila* (Cui et al. 2015).

The overexpression of α -L-rhamnosidase gene of *Bifidobacterium breve* in the HRC of *P. ginseng* using *A. rhizogenes* strain A4 showed 2.2-fold increased ginsenoside Rg1 production ($3.6 \text{ mg g}^{-1} \text{ DW}$) in HRC than the control (Zhang et al. 2015).

Metabolic manipulation of tropane alkaloid metabolic pathway in HRCs of the native Tibetan medicinal plant *Scopolia lurida* using heterologous expression of hyoscyamine 6-hydroxylase gene derived from *Hyoscyamus niger* (*HnH6H*) and homologous expression of *H6H* gene derived from *S. lurida* (*SlH6H*) resulted in a significant increase in scopolamine and anisodamine accumulation and decrease in hyoscyamine content (Lan et al. 2018). *HnH6H*-overexpressing HRCs contained a higher amount of scopolamine than *SlH6H*-overexpressing root cultures, indicating that *HnH6H* expression was superior to *SlH6H* expression in terms of engineering scopolamine synthesis.

Metabolically engineered plastidial 2-C-methyl-D-erythritol 4-phosphate-derived isoprenoid pathway through ectopic expression and plastid targeting of cyanobacterial genes encoding 1-deoxy-D-xylulose 5-phosphate synthase or 1-deoxy-D-xylulose-5-phosphate reductoisomerase significantly enhance the synthesis of abietane diterpenoid (threefold) and aethiopinone (sixfold) without any negative effect on biomass accumulation in HRCs of *Salvia sclarea* (Vaccaro et al. 2019).

28.5.3 Homologous Expression of Single Transcription Factor

Zhao et al. (2015) effectively established HRCs of *L. erythrorhizon* that overexpress *MYB1* transcription factor via transformation of seedling node explants with *A. rhizogenes* strain ATCC15834 carrying the *pBI121-LeMYB1* plasmid. A significant increase in the total amount of shikonin and its derivatives was noted in *MYB1* overexpressing transgenic HRC of *L. erythrorhizon*, which was positively correlated with the modulation in expression of shikonin biosynthesis pathway-related enzyme-coding genes such as *PAL*, *HMGR*, and *PGT* and regulators like *DI-2* and *PS-2* (Zhao et al. 2015).

The endogenous *ORCA* gene cluster and *MYC2* gene act downstream of a MAP kinase cascade, including MAP kinase, *MAPKK1*. Considerable enhancement of TIA production in HRC of *C. roseus* is reported by overexpression of *ORCA4* gene or *MAPKK1* gene of *C. roseus* via upregulation of TIA pathway genes (Paul et al. 2017).

Transgenic hairy roots of *Isatis indigotica* overexpressing one novel AP2/ERF response factor gene, named *Ii049*, showed high expression levels of lignan/lignin

biosynthetic genes and 8.3-fold higher lariciresinol accumulation ($425.60 \mu\text{g g}^{-1}$) than the wild type (Ma et al. 2017b).

Overexpression of *WRKY1*, a transcription factor derived from *S. miltiorrhiza*, significantly enhanced transcription of MEP pathway-associated enzyme-encoding genes, especially *DXS* and *DXR*, resulted in a fivefold increase in tanshinone synthesis in transgenic HRCs (up to $13.7 \text{ mg g}^{-1} \text{ DW}$) than the non-transformed lines (Cao et al. 2018).

The overexpression of the plant-specific transcription factor *TCP4* gene isolated from *Lycium ruthenicum* in HRC of *L. ruthenicum* resulted in higher production of kukoamine A along with 17 other SMs in transgenic HRs than in the control (Chahel et al. 2019).

Tanshinone accumulation was enhanced significantly in HRCs of *S. miltiorrhiza* through overexpression of the *WRKY2* transcription factor of *S. miltiorrhiza* belonging to the WRKY family's subgroup I (Deng et al. 2019). This enhancement in tanshinone production is also correlated with subsequent overexpression of *DXS2* and *CPS* genes. The highest total tanshinone content was detected in *WRKY2-1* overexpressing line, which was 1.83-fold in comparison with the control HRC (Deng et al. 2019). *WRKY2* transcription factor acts as a positive regulator of tanshinone biosynthesis via interaction with the *CPS* gene (Deng et al. 2019).

Huang et al. (2019) successfully isolated a new JA-responsive gene that encodes AP2/ERF transcription factor, *ERF1L1*, from *Salvia miltiorrhiza*, and performed its functional characterization. They found a significant increase in tanshinone accumulation in transgenic HRC of *S. miltiorrhiza*, overexpressing *ERF1L1* gene by positive transcriptional activation of the *DXR* gene of tanshinone biosynthetic pathway (Huang et al. 2019).

Sun et al. (2019) isolated and identified a new MeJA-responsive AP2/ERF transcription factor, that is, *ERF115* in *S. miltiorrhiza* and used to study its potential impact in metabolic engineering of phenolic acid biosynthesis in *S. miltiorrhiza*. Overexpression of transcription factor *ERF115* in HRCs of *S. miltiorrhiza* increased phenolic acid and salvianolic acid B production by upregulation of *PAL3*, *4CL5*, *TAT3*, and *RAS1* genes but decreased tanshinone content (Sun et al. 2019). In contrast, HR line *SmERF115*-RNAi showed reduced phenolic acid and enhanced tanshinone accumulation (Sun et al. 2019).

The total camptothecin production and the concentration of camptothecin precursors, including loganin and secologanin, were distinctly altered in transformed HR lines of *O. pumila* that overexpressed a novel tryptophan-arginine-lysine-tyrosine (*WRKY3*) transcription factor (Wang et al. 2019).

Hao et al. (2020) found a R2R3-MYB subgroup 20 TF, *SmMYB98*, mainly expressed in the lateral roots of *S. miltiorrhiza*. The HR lines that overexpress *SmMYB98* showed higher quantities of primary liposoluble bioactive tanshinone and water-soluble salvianolic acid via significant transcriptional activation of *GGPPS1*, *PAL1*, and *RAS1* genes of *S. miltiorrhiza* (Hao et al. 2020). In contrast, *SmMYB98* knockout HR lines showed reduced accumulation of tanshinone and salvianolic acid production.

Hao et al. (2021) decipher a positive association between the expression patterns of transcriptional regulator WRKY2 with camptothecin production in HRC of *O. pumila* in a time-course expression evaluation. Upregulated expression of the WRKY2 transcription factor in HRC of *O. pumila* causes threefold boosting overaccumulation in camptothecin production, suggesting its role as a direct positive regulator in camptothecin biosynthesis. In contrast, gene silencing resulted in reduced camptothecin levels in the transgenic *O. pumila* plant (Hao et al. 2021). In contrast, Xu et al. (2020) reported that transcription factor WRKY1 isolated from *O. pumila* acts as a negative regulator of camptothecin biosynthesis as its overexpression significantly repressed CPT accumulation in *OpWRKY1*-overexpressing HRC of *O. pumila* by inhibiting the expression of several CPT biosynthesis genes, especially *CPR*.

The overexpression of transcription factor WRKY37 isolated from *Rehmannia glutinosa* increased acteoside and total phenylethanoid glycosides in HRCs of *R. glutinosa* by upregulating the transcription of seven enzyme genes involved in the acteoside biosynthesis pathway (Wang et al. 2021).

28.5.4 Heterologous Expression of Single Transcription Factor

Heterologous expression of different transcription factors in HRC of the targeted host showed improved SM production, mostly via overexpression of several key genes of particular SM biosynthesis pathway. Some of the reports published in the last ten years are given below.

Park et al. (2012) reported enhanced rutin production in HRCs of *Fagopyrum esculentum* that overexpress flavonol-specific transcription factor, MYB12, of *A. thaliana*. Moreover, a positive correlation between the enhanced rutin accumulation and upregulation of several genes involved in the flavonoid biosynthetic pathway was observed in *AtMYB12* overexpressing HRCs of *F. esculentum* (Park et al. 2012).

ORCA3 is a jasmonate-responsive APETALA2-domain transcript factor with a high potential to cause overexpression of various critical genes participating in the TIA biosynthesis pathway (Ni et al. 2011). In HRCs of *C. acuminata*, a significant (1.5 times) increase in camptothecin synthesis has been reported in comparison to control via the establishment of transgenic HR lines, which upregulate heterologous transcription factor ORCA3 isolated from *C. roseus* (Ni et al. 2011).

Plant abietane diterpenoids, such as aethiopinone, 1-oxoaethiopinone, salvipisone, and ferruginol, are well known for their antibacterial, antifungal, sedative, and antiproliferative properties. Heterologous overexpression of WRKY40 transcription factor of *A. thaliana* in HR lines of *S. sclarea* showed a maximum fourfold increase in production of aethiopinone, an abietane diterpene derived from the plastidial MEP-derived terpenoid pathway (Alfieri et al. 2018).

The heterologous expression of *MYB4* transcription factor of *Trifolium repens* in HRCs of a traditional medicinal plant *Fagopyrum cymosum* significantly upregulates the amount of overall flavonoids (twofold) and rutin (fourfold) compared to non-transgenic HRs (Luo et al. 2020).

The *PAP26* gene isolated from *A. thaliana* was cloned under CaMV-35S promoter, and *PAP26*-overexpressing HRC of *Echinacea purpurea* was established via transformation with *A. rhizogenes* strain R15834. These transgenic root cultures showed a 16-fold increase in chicoric acid yield under Pi starvation condition (Salmanzadeh et al. 2020).

Heterologous expression of basic helix-loop-helix transcription factor *MYC2* isolated from *A. thaliana* within HRC of *S. miltiorrhiza* showed a positive impact in both tanshinones and phenolic acid biosynthesis (Shi et al. 2020b). A maximum of $\sim 14 \text{ mg g}^{-1}$ DW tanshinone production is noted in the *AtMYC2-3* root line that represent ~ 5.5 -fold higher than the control (Shi et al. 2020b). Enhanced production of these bioactive SMs was correlated with the upregulation of some (*DXS2* and *TAT*) genes of *S. miltiorrhiza* associated with tanshinones and phenolic acid biogenesis.

28.5.5 Expression of Multiple Genes of SM Biosynthetic Pathway

Simultaneous overexpression of two key enzyme genes, that is, *HMGR* (3-hydroxy-3-methylglutaryl CoA reductase) and *DXR* isolated from *S. miltiorrhiza* in HR lines of *S. miltiorrhiza*, significantly enhances the total tanshinone (3.25 mg g^{-1} DW) yield than the non-transformed and single gene transformed lines (Shi et al. 2014).

A similar metabolic engineering strategy was successfully implemented to improve camptothecin accumulation in HRC of *O. pumila* by combined overexpression of strictosidine synthase (*STR*) and *G10H* genes isolated from *C. roseus* due to their synergistic effect on CPT biosynthesis (Cui et al. 2015). SG hairy root lines that co-overexpressed both *CrG10H* and *CrSTR* genes resulted in a 56% increase in CPT yield compared to the non-transformed and single gene transgenic lines.

In order to improve the accumulation of the health-promoting diterpenoid tanshinones, when geranylgeranyl diphosphate synthase (*GGPPS*) and *DXSII* genes of *S. miltiorrhiza* are co-expressed in HRCs of *S. miltiorrhiza*, they showed a higher level of tanshinone (12.93 mg g^{-1} DW) production than control (0.61 mg g^{-1} DW) (Shi et al. 2016a). Interestingly, the co-expression of both *DXS2* and *GGPPS* genes of *S. miltiorrhiza* showed a better effect on tanshinone biosynthesis than the co-expression of *HMGR* and *GGPPS* genes of *S. miltiorrhiza*.

Sun and Peebles (2016) established HRCs of *C. roseus* that overexpress transcriptional regulator *ORCA3* (octadecanoid-responsive *Catharanthus* AP2-domain protein) gene and a key pathway gene, that is, strictosidine glucosidase gene (*SGD*),

under the control of a glucocorticoid-inducible promoter, which resulted in 47% increase in root-specific TIAs along with an increase in serpentine (44%), ajmalicine (32%), catharanthine (38%), tabersonine (40%), lochnericine (60%), and hörhammericine (56%) production.

Shi et al. (2020a) adopted a similar approach to improve camptothecin production (3.5 mg g^{-1} in SG-17 line) in HRC of *O. pumila* by subsequent co-introduction of *G10H* and *SLS* (secologanin synthase) (SG line) genes of *O. pumila* via the cooperative effect of a single gene.

28.5.6 Gene Silencing

HR lines of *S. miltiorrhiza* (*anti-SmJAZ3-3* line and in *anti-SmJAZ9-23* line) that downregulate two new JASMONATE ZIM-DOMAIN (JAZ) proteins, namely, *JAZ3* and *JAZ9*, derived from *S. miltiorrhiza* showed 2.48- and 1.35-fold increase in tanshione production, respectively (Shi et al. 2016b). It suggests that JAZ proteins act as repressive transcriptional regulators in the JA signaling pathway.

The enhanced production of an anesthetic and antispasmodic drug, scopolamine (8.84 mg g^{-1}), in the HRCs of *Duboisia leichhardtii* achieved via RNAi-mediated silencing of *quinolinate phosphoribosyl transferase* (*QPT*) gene of the nicotine biosynthetic pathway using the *QPT*-RNAi construct under the control of CaMV 35S promoter that resulted in efficient inhibition of nicotine biosynthesis (Singh et al. 2018). Additionally, the application of MJ as an elicitor increased the yield of scopolamine 2.2-fold (19.344 mg g^{-1}) than the non-elicited culture (Singh et al. 2018).

The overexpression of miR160a in HRCs of *S. miltiorrhiza* promotes IAA production and growth (1.90-fold in HR line 4) by targeting *ARF10*, *ARF16*, and *ARF17*, whereas it inhibits the total (in HR line 4) or partial (in HR line 1) tanshinone production. The major four tanshinone production also decreased to 9.06% (dihydrotanshinone I), 12.56% (cryptotanshinone), 12.02% (tanshinone I), and 8.52% (tanshinone IIA) in miR160a-overexpressing HR line 1, although biomass was increased 1.72-fold compared to the wild type (Zhang et al. 2020).

Although the medicinal plant *Symphytum officinale* has anti-inflammatory, analgesic, and proliferative properties, presence of pyrrolizidine alkaloids (PAs) in its tissues makes medicinal applications difficult. CRISPR/Cas9-based silencing of homospermidine synthase (*hss*) gene in HRCs of *S. officinale* encodes the first pathway-specific enzyme of toxic pyrrolizidine alkaloid biosynthesis (Zakaria et al. 2021).

The biosynthesis of hyoscyamine, anisodamine, and scopolamine was achieved by decreasing in putrescine and *N*-methylputrescine accumulation through limiting in transcriptional expression of *AbODC* transgene in transgenic *AbODC*-RNAi HRCs of *A. belladonna* through RNAi (Zhao et al. 2022).

28.6 Secondary Metabolite Production in Metabolic Engineered Callus and Cell Suspension Culture of Medicinal Plants

By metabolic engineering, significant improvement in the synthesis of the desired compounds can be possible in medicinal plant cell suspension cultures, which involve culturing of single cells or aggregates of cells of varying sizes in the agitated liquid medium after induction of an undifferentiated mass of plant cells, that is, plant callus, usually in the specific phytohormone- (mainly auxins and cytokinins) supplemented artificial culture medium.

Withania somnifera is one of the highly reputed traditional medicinal plants of India owing to their steroidal lactone phytochemicals with antitumor, antioxidant, antiaging, antiserotogenic, and antistress activities. Withanolides are biosynthesized by guiding the metabolic flow away from the isoprenoid pathway toward the biosynthesis of phytosterols and withanolides by generating a linear C30 compound, squalene, through the activity of the key enzyme squalene synthase (SQS). Several metabolic engineering approaches targeted this regulatory branching step of the isoprenoid pathway in *W. somnifera* for improving the yield of withanolides (Grover et al. 2013). The upregulation of the *SQS* gene in the CSCs of *W. somnifera* was able to increase 2.5-times withanolide A production and de novo accumulation of withaferin A in the transformed CSCs than the control non-transformed CSCs (Grover et al. 2013).

Transgenic tobacco callus lines overexpressing a flavonol-specific transcription factor, *MYB12*, obtained from *A. thaliana*, increased rutin production, indicating the biotechnological possibility of AtMYB12-expressing callus cultures for rutin synthesis (Pandey et al. 2012).

Van Moerkercke et al. (2015) identified a jasmonate-regulated basic helix-loop-helix (bHLH) iridoid synthesis 1 (*BIS1*) transcription factor from *C. roseus*, which transactivate the expression of all of the genes encoding the enzyme upstream of loganic acid in a complementary manner to the ethylene response factor ORCA3. Moreover, overexpression of *BIS1* sufficiently enhanced the accumulation of medicinally important high-value iridoids and MIAs in CSCs of *C. roseus*.

In comparison with control, a substantial amount of qualitative and quantitative (threefold and 12-fold) variations in anthraquinone and its derivatives was observed in *Rubia cordifolia* callus lines overexpressing native (threefold) or mutant (12-fold) forms of calcium-dependent protein kinase gene of *A. thaliana* (*AtCPK1*) via enhancement of expression of genes involved in anthraquinone metabolism or their regulatory mechanism (Shkryl et al. 2016).

Hidalgo et al. (2017) reported the potential of metabolically modified CSCs of *Silybum marianum* for trans-resveratrol production by stable ectopic expression of *stilbene synthase 3* gene isolated from *Vitis vinifera*. Further, elicitation of these metabolically modified CSCs of *S. marianum* with MJ and methylated β -cyclodextrins elicitors led to extracellular trans-resveratrol accumulation at the level of milligrams per liter (Hidalgo et al. 2017).

In a similar study, ectopic expression of a constitutively active, Ca^{2+} -independent-CPK1 gene of *Arabidopsis thaliana* (AtCPK1-Ca) in transformed callus cultures of *V. amurensis* showed enhancement of maximum 90-fold resveratrol content along with increase biomass accumulation, which represents overall 137 times enhanced resveratrol accumulation relative to wild-type calli via overexpression of important enzyme of resveratrol biosynthetic pathway like 4-coumarate-CoA ligase enzyme (Veremeichik et al. 2017).

Yamada et al. (2017) demonstrated that the ectopic expression of the *WRKY1* transcription factor of *Coptis japonica* enhanced the production of sanguinarine, chelerythrine, chelirubine, protopine, allocryptopine, and 10-hydroxychelerythrine in culture medium via a transcriptional activator of many genes that encode BIA biosynthetic enzymes in *Eschscholzia californica*.

Sharma et al. (2018) incorporated and upregulated two *TDC* and *STR* genes that encode two enzymes, tryptophan decarboxylase and strictosidine synthase, respectively, in callus and leaf of *C. roseus*. These efforts were able to shift TIA pathway metabolic flow in the direction of dimeric alkaloid vinblastine and vincristine biosynthesis and resulted in nine times enhancement of vindoline and catharanthine yield and five times enhancement of vinblastine yield (Sharma et al. 2018). The callus transformation reported a maximum of 0.027% DW vindoline and 0.053% DW catharanthine production, whereas the transiently transformed leaves reported a maximum of 0.30% DW vindoline, 0.10% catharanthine, and 0.0027% DW vinblastine content (Sharma et al. 2018).

Transgenic callus cultures of *Vitis amurensis*, overexpressing three stilbene synthase (*STS1a*, *STS2*, and *STS3* genes) genes from *Picea jezoensis* showing a comparatively low level of homology with the native *STS* genes of *V. amurensis* resulted in a significant increase of the total stilbenes content in 3.6- to sixfold, 2.5- to 2.9-fold, and 4.1- to 16.1-fold, respectively, in comparison with the control calli (Suprun et al. 2020).

Modification of silymarin biosynthetic pathway of silymarin in transformed CSCs of *Silybum marianum* through the heterologous expression of either *MYB12* transcription factor of *Arabidopsis thaliana* (AtMYB12) gene or chalcone synthase gene of *Cicer arietinum* (*CaCHS*) showed alteration in glycoside derivatives of chlorogenic acid and flavanone naringenin accumulation (Villar et al. 2020). The simultaneous ectopic expression of AtMYB12 and *CaCHS* in same CSC of *Silybum marianum* yielded a reduced amount of chlorogenic derivatives but the same amount of Ng glycosides in comparison with the *CaCHS* lines (Villar et al. 2020).

WRKY44-transgenic *Taraxacum antungense* calli that overexpressed *T. antungense*-derived *WRKY44* transcription factor enhanced luteolin production via upregulation of several genes in the luteolin biosynthesis pathway (Li et al. 2021).

Interestingly, artificial microRNA-mediated inhibition of *JAZ9* gene of *V. vinifera* showed ten times and sevenfold enhancement in resveratrol quantity and productivity, respectively, in *V. vinifera* CSCs with slightly reduced growth than in wild-type culture (Makhazen et al. 2021).

Salutaridinol 7-*o*-acetyltransferase (*SalAT*) is a key gene in thebaine biogenesis. According to Qaderi et al. (2021), *SalAT*-overexpressing transgenic CSCs of *Papaver bracteatum* established via transformation with LBA4404 strain of *A. tumefaciens* that harbor *SalAT* gene of *P. bracteatum* under the CaMV35S promoter in pBI121 expression vector showed 1.7-fold (0.58 mg g^{-1}) increased amounts of thebaine in transformed culture than in non-transformed CSC (0.34 mg g^{-1}).

Aleynova et al. (2022) recently developed 16 transgenic cell lines of *V. amurensis* transformed with the five calmodulin-like protein (CML) genes under double CaMV 35S promoter, which showed 3.8- to 23.7-fold enhanced stilbene production compared to NT callus line without effecting on biomass accumulation (Aleynova et al. 2022).

28.7 Secondary Metabolite Production in Metabolic Engineered Transgenic Medicinal Plants

28.7.1 Homologous Expression of Single or Multiple Gene(s) of SM Biosynthetic Pathway

Transient overexpression of homologous mitogen-activated protein kinase (*MPK3*) gene in *C. roseus* leaf tissue is reported to cause overexpression of several MIA biosynthesis pathway genes, such as *TDC*, *STR*, *D4H*, and *DAT* as well as enhance production of specific MIAs (Raina et al. 2012).

Patel et al. (2015) reported enhancement of 1.5-fold to twofold total withanolide production and fourfold to 4.5-fold Withaferin A and Withanolide A production in leaf tissue in engineered *W. somnifera* plants that overexpressed *SQS* gene of *W. somnifera*.

Artemisinic aldehyde $\Delta 11$ (13) reductase (*DBR2*) is a checkpoint enzyme that is responsible for the conversion of artemisinic aldehyde to dihydro artemisinic aldehyde in artemisinin biosynthetic pathway (Yuan et al. 2015). Seven independent transgenic *A. annua* plant lines overexpressing the *DBR2* gene under the CaMV 35S promoter showed significant enhanced accumulation of artemisinin and its direct precursor dihydroartemisinic acid along with stimulation of arteannuin B and artemisinic acid production (Yuan et al. 2015).

Approximately 1.4-fold increase in ginsenoside content and upregulation of ginsenoside biosynthesis-related genes, such as *squalene synthase 1*, *squalene epoxidase 1*, and *dammarenediol synthase*, was reported in transgenic *P. ginseng* roots overexpressing 13-lipoxygenase (*LOX6*) gene (Rahimi et al. 2016).

A. annua is a medicinal plant well recognized for the biosynthesis of antimalarial artemisinin, a sesquiterpene lactone with a unique endoperoxide bridge. The overexpression of 4-hydroxy-3-methylbut-2-enyl diphosphate reductase 1 (*HDR1*) gene catalyzes the final step of the MEP pathway to synthesize isopentenyl

pyrophosphate (IPP), and dimethylallyl diphosphate (DMAPP), isolated from leaves of *A. annua* increased accumulation of artemisinin, arteannuin B, and other sesquiterpenes, along with multiple monoterpenes in *A. annua* (Ma et al. 2017a). They also demonstrated that the suppression of *HDR* gene by antisense led to reduced accumulation of artemisinin.

Metabolic modification of benzyloisoquinoline alkaloid biosynthesis in *M. cordata* was reported by overexpression of berberine bridge enzyme-encoding gene isolated from *M. cordata* and *P. somniferum* (Huang et al. 2018). These transgenic plants showed higher levels of benzyloisoquinoline alkaloids (sanguinarine, protopine, and berberine) than empty vector or control treatments. However, all the transgenic lines demonstrated slightly reduced sanguinarine and chelerythrine yield compared to control lines, which may be due to feedback inhibition resulting from overexpression of *BBE* gene (Huang et al. 2018).

The genetic modification of steviol glycoside biosynthetic pathway by homologous overexpression of *DXS1* and kaurenoic acid hydroxylase (*KAH*) genes showed the potential of metabolic engineering to boost steviol glycoside production in leaves of transgenic *Stevia rebaudiana* plants without affecting their growth and development (Zheng et al. 2019). The drastic increase in Reb A to stevioside ratio, i.e., from 0.30 to 1.55, was observed in leaves of *S. rebaudiana* transgenic plant lines that up-regulating *UDP-glycosyltransferase 76G1* gene compared to the leaves of non-transformed plants without any significant changes in total steviol glycosides content (Kim et al. 2019).

Trachyspermum ammi is a well-known pharmaceutical plant in Iran with several medicinal properties, including antidiarrhoeal, anti-spasm, antimicrobial, antiviral, and relieving rheumatic pains. Overexpression of a key enzyme involved in the thymol synthesis pathway, that is, terpene synthase 2 (*TPS2*) gene in *T. ammi* via *A. tumefaciens*-mediated transformation enhanced thymol production compared to the non-transgenic plants (Nomani et al. 2019).

Deacetylvindoline 4-*O*-acetyltransferase (*DAT*) is a crucial enzyme of the TIA biosynthesis pathway in *C. roseus* that generates vinblastine and vincristine in the last step of vindoline biosynthesis (Pham et al. 2019). Overexpression of *DAT* transgene in *C. roseus* by using *Agrobacterium*-mediated transformation showed an increase in vincristine accumulation (Pham et al. 2019). Furthermore, the vincristine concentration of four transgenic lines in T1 generation increased in a range of 1.63- to 2.48-fold than the NT plants (Pham et al. 2019).

Zhang et al. (2021) established two homozygous transgenic plant lines of *A. belladonna* (*T2GH03* and *T2GH06*) with glyphosate resistance and high-yield scopolamine than wild-type plants using metabolic engineering technology. The scopolamine contents of homozygous transgenic lines harboring *G2-EPSPS* and *HnH6H* increased around tenfold in leaves and fivefold in roots of *A. belladonna* (Zhang et al. (2021).

Engineering of tropane alkaloid biosynthesis pathway by overexpression of ornithine decarboxylase (*ODC*) genes from *Atropa belladonna* in transgenic *A. belladonna* plants demonstrated a significantly increased accumulation of hyoscyamine and anisodamine in comparison to non-transformed plants (Zhao et al.

2022). Homologous overexpression of the *ODC* gene in transgenic *A. belladonna* plants drastically enhanced the accumulation of putrescine in leaves and roots and *N*-methyl putrescine in the roots compared with the control (Zhao et al. 2022).

28.7.2 Heterologous Expression of Single or Multiple Gene(s) of SM Biosynthetic Pathway

The metabolic engineering of *A. belladonna* by overexpression of the *h6h* gene from *Hyoscyamus niger* that encodes the rate-limiting enzyme in the scopolamine biosynthetic pathway able to break the downstream rate-limiting steps resulted in a more metabolic flow toward the biosynthesis of scopolamine and enhanced scopolamine synthesis (4.2-fold) along with the improvement in several agronomic characteristics in transgenic plants (Li et al. 2013).

Heterologous expression of linalool synthase (*LIS*) gene from *Clarkia breweri*, which encodes the LIS enzyme that catalyzes linalool synthesis, caused up to 1000% increase of linalool content in the youngest leaves of *Lavandula latifolia* plants compared to control (Mendoza-Poudereux et al. 2014). Interestingly, both essential oil yield and linalool content were lower in double DXS-LIS transgenic plants than their parentals, which may be due to co-suppression effects related to the structures of the constructs used (Mendoza-Poudereux et al. 2014).

Heterologous expression of the IPP isomerase (*Iso*) gene of hybrid poplar and the limonene synthase (*Limo*) gene from *Citrus* in *Mentha spicata* via *A. tumefaciens*-mediated transformation influence qualitative and quantitative variation in terpenoid biosynthesis (Kang et al. 2015). Genetically modified MS*Iso* plants showed the presence of 1,8-cineole, linalool, camphor, terpinene, limonene, borneol, safranal, geraniol, thymol, 1- α -terpineol, and methyl eugenol, while transgenic MS*Limo* showed the presence of linalool, menthone, terpinene, limonene, menthol-isomer, thymol, and piperitone (Kang et al. 2015).

The economic value of *A. belladonna* can be increased by enhancing the conversion rate of hyoscyamine into high-value scopolamine by metabolic engineering (Quan et al. 2016; Xia et al. 2016). Xia et al. (2016) established metabolically modified *A. belladonna* that harbor the putrescine *N*-methyltransferase (*PMT*) gene from *N. tabacum* and *h6h* gene from *H. niger* to investigate TA biosynthesis in different plant parts. T1 progeny of transgenic *A. belladonna* demonstrated enhanced scopolamine accumulation (2.94–5.13 mg g⁻¹) in in vivo conditions (Xia et al. 2016). In a similar study, scopolamine concentration in T1 offspring of transgenic *A. belladonna* plant overexpressing *PMT* gene of *N. tabacum* and *h6h* gene of *H. niger* varies from 15- to 36-fold and 37- to 108-fold greater than in wild-type leaves and stems, respectively (Quan et al. 2016).

All nine transgenic *A. annua* plants that overexpressed the *GA20ox* gene from *Torenia fournieri* demonstrated significantly higher plant height, number of branches, branch length, and artemisinin content compared to wild-type plants

(Inthima et al. 2017). Among the nine transgenic plants, maximum artemisinin accumulation and yield were noted in *TfGA20ox2*-overexpressing plant, which was twofold greater than the wild type. Interestingly, relative to the wild type, the number and size of glandular trichomes in transgenic leaves were about 30 and 35% higher, respectively. In addition, two monoterpenes (eucalyptol and borneol), four sesquiterpenes (α -caryophyllene, β -guaiene, δ -cadinene, and β -cubebene) and one triterpene (isomultiflorenone) were detected only in transgenic extract, whereas D- α -tocopherol, a diterpenoid compound, was found only in wild type but not in the transgenic plant (Inthima et al. 2017).

Heterologous overexpression of a major isoprenoid biosynthesis pathway gene, *DXS*, obtained from *Pelargonium graveolens*, in the well-known medicinal plant *W. somnifera* resulted in a 2.8-fold to fivefold increase in total withanolide content in various transgenic lines (Jadaun et al. 2017). Interestingly, there was a significant correlation between the degree of overexpression of the *GrDXS* gene and the withanolide accumulation, indicating the relevance of this gene in the withanolide production pathway (Jadaun et al. 2017).

Transient overexpression of the 3-hydroxy-3-methylglutaryl Co-A reductase (HMGR) gene of *Ocimum kilimandscharicum* in *Ocimum* spp., *Withania somnifera*, and *Artemisia annua* showed tissue- and plant-specific enhancement effects on total withanolide content in *W. somnifera* (up to 44%), artemisinin content in *A. annua* (7.4%), and sesquiterpenoids in *Ocimum* spp. (Bansal et al. 2018).

The heterologous overexpression of caffeic acid 3-*O*-methyltransferase (COMT) gene of *Azadirachta indica* resulted in a 1.5- to 2.5-fold increased ferulic acid production in leaf tissues of genetically modified *W. somnifera*, *O. sanctum*, *O. basilicum*, *O. kilimandscharicum*, and *O. gratissimum* as compared to control leaf tissues (Narnoliya et al. 2021). This discovery broadens the scope of metabolic engineering methods for the manufacture of potentially therapeutically relevant phytomolecules in heterologous systems.

Transgenic *O. rugosa* plants overexpressing the *STR* gene derived from *N. foetida* revealed approximately twofold enhanced camptothecin production compared to the NT plants (Singh et al. 2020). Interestingly, the development of metabolic engineered variety of *A. belladonna* plants with significant enhanced hyoscyamine yield and without anisodamine and scopolamine was achieved via CRISPR/Cas9-mediated native hyoscyamine 6 β -hydroxylase (*AbH6H*) gene silencing (Zeng et al. 2021).

28.7.3 Over- or Downregulation of Transcription Factors

Homologous overexpression of *ORCA3* (octadecanoid-derivative responsive catharanthus AP2-domain) transcription factor and *G10H* genes alone in *C. roseus* plants drastically enhanced the accumulation of strictosidine, vindoline, catharanthine, and ajmalicine, whereas it showed insignificant impacts on anhydrovinblastine and vinblastine productivity (Pan et al. 2012). Moreover,

co-expression of *ORCA3* and *G10H* genes not only increased the accumulation of the midstream metabolite, ajmalicine and catharanthine, but also the downstream vindoline in *C. roseus* plants (Pan et al. 2012).

Virus-induced gene silencing (VIGS) of a novel negative regulator AP2/ERF transcription factor *Catharanthus roseus 1 (CR1)* resulted in the upregulation of seven genes (*G10H*, *SLS*, *TDC*, *STR*, *SGD*, *DAT*, and *PRX*) and enhanced vindoline and serpentine production, without significant effect on catharanthine in the leaves of *CR1*-silenced plants (Liu et al. 2017a).

Liu et al. (2017b) reported overexpression of enhanced drought tolerance 1 (*EDT1*) transcription factor from *A. thaliana* enhanced root elongation and salvianolic acids (rosmarinic acid, lithospermic acid, salvianolic acid B) production in *S. miltiorrhiza* plants by upregulation of salvianolic acid biosynthetic genes, as well as a decrease in tanshinone synthesis.

Overexpression of the *S. miltiorrhiza MYC2* transcription factor significantly improved phenolic acid accumulation in the roots of *S. miltiorrhiza* plant by simultaneous activation of both primary and secondary metabolic pathways (Yang et al. 2017). The roots of *SmMYC2* overexpressing plant line 12 (OEM-12) showed 1.88-fold higher salvianolic acid B production compared to the empty vector control plant (Yang et al. 2017).

Schweizer et al. (2018) reported boosted monoterpenoid indole alkaloid (MIA) synthesis in metabolic-engineered *C. roseus* by a combinatorial overexpression of the transcription factors (*BIS1*, *ORCA3*, and *MYC2a*). The MIA pathway undergoes a significant remodeling upon expression of a modified de-repressed *MYC2a*, ultimately resulting in a substantially enhanced production of more than 23 MIAs (Schweizer et al. 2018). Transient homologous overexpression of *ERF5* transcription factor in *C. roseus* petals resulted in drastic stimulation in the expression of vital regulatory genes of the MIA pathway leading to strong enhancement in anhydrovinblastine, vinblastine, ajmalicine, vindoline, and catharanthine contents (Pan et al. 2019). When *ERF5* gene of *C. roseus* is repressed, these bisindole alkaloids and MIAs reduce in transgenic plants (Pan et al. 2019).

In *C. roseus* seedlings, a Leu-Leu-Met domain *GATA* transcription factor (*GATA1*) was identified, which regulates light-induced vindoline production (Liu et al. 2019b). Transient overexpression of their *GATA1* transcription factor significantly increased vindoline accumulation by trans-activation of vindoline biosynthesis pathway genes, such as *T16H2*, *T3O*, *T3R*, *D4H*, and *DAT* in *C. roseus*, whereas a VIGS-mediated gene silencing of endogenous *GATA1* gene decreased vindoline accumulation by significant repression of key vindoline pathway genes in young *C. roseus* leaves (Liu et al. 2019b). Furthermore, transient overexpression or VIGS of *C. roseus* phytochrome interacting factor 1 (*PIF1*) repressed or upregulated the *CrGATA1* gene and vindoline biosynthesis in *C. roseus* seedlings under the dark condition (Liu et al. 2019b).

The homologous overexpression of *WRKY1* transcription factor under the CaMV35S promoter or the trichome-specific CYP71AV1 promoter in stably transformed *A. annua* plants, resulted in >100-fold and 40-fold increased expression at the transcription level, respectively (Han et al. 2021). Trichome-specific

overexpression of *AaWRKY1* gene is more effective than the constitutive overexpression of *AaWRKY1* in artemisinin production (Han et al. 2021).

28.7.4 Shift in Metabolic Flux by Gene Silencing

Silencing of endogenous phytoene synthase (*PSY*) gene that competes with taxadiene synthase for geranylgeranyl diphosphate showed an approximately two-fold increase in taxadiene accumulation ($48 \mu\text{g g}^{-1}$ DW) in *N. benthamiana* plants (Hasan et al. 2014).

Simultaneous silencing of *SQS* and sesquiterpene (*5-epi-aristolochene*) synthase genes resulted in a 2.8-fold increase in sesquiterpene (+)-valencene by enhancing the availability of substrate of heterologous valencene synthase, that is, farnesyl diphosphate in *N. benthamiana* via inhibition of competing pathway (Cankar et al. 2015).

In *Papaver somniferum*, the 3'-hydroxyl-*N*-methylcoclaurine 4'-*O*-methyltransferase (4'*OMT2*) enzyme controls the biosynthesis of benzyloisoquinoline alkaloids. The knockout of the 4'*OMT2* gene using the type II CRISPR/SpCas9 system resulted in a considerable decrease in BIA biosynthesis (e.g., morphine, thebaine) as well as the formation of unique uncharacterized alkaloid in transgenic plants (Alagoz et al. 2016).

Lowering in sanguinarine and noscapine production and increasing in morphine production were achieved by virus-induced gene silencing of the endogenous *BBE1* gene using a *pTRV2-BBE* construct in genetically modified *P. somniferum* plants (Sohrabi et al. 2018). *P. somniferum* plants harboring *pTRV2-COM* construct that simultaneously silenced both *T6ODM* and *CODM* genes showed a reduction in morphine and codeine content and a significant rise in thebaine and papaverine accumulation. Moreover, simultaneous silencing of *T6ODM*, *CODM*, and *BBE1* genes in this plant showed increased accumulation of thebaine and papaverine and decreased morphine, codeine, sanguinarine, and noscapine (Sohrabi et al. 2018). Interestingly, simultaneous silencing of *T6ODM*, *CODM*, and *BBE1* genes and overexpression of 4'*OMT2* gene in the ACS-4'*OMT2* plant of *P. somniferum* showed a decrease in codeine, sanguinarine, and noscapine content and enhancement in morphine, thebaine, and papaverine (Sohrabi et al. 2018).

28.8 Conclusion

The potential of prokaryotic microbe-based heterologous secondary metabolite biosynthesis platforms, such as *S. cerevisiae* and *E. coli*, are suffering from very low productivity, the requirement of multiple gene incorporation, comprehensive genetic background optimization, and codon biasness-related issues, which makes the entire process expensive and time-consuming and requires extensive knowledge to achieve the desired goal. The introduction of multiple heterologous enzyme

coding genes from the plant biosynthesis pathway into metabolically engineered microorganisms frequently results in metabolic burdening-mediated low productivity due to flux imbalance, harmful intermediate accumulation, and genetic instability.

Apart from microbial metabolic engineering, several types of research have been undertaken over the last ten years in the field of metabolic engineering involving plant-based systems, such as cell suspension culture, callus culture, hairy root culture, and whole regenerated plants. Similar to the microbial system, medicinal plant-based metabolic engineering is also showing a promising role in the production of therapeutic phytochemicals to combat with high global demand for these compounds and reduce overexploitation pressure on natural medicinal plants. Plant-based heterologous SM biosynthesis offers simple, scalable, cost-effective, and safe platforms with potentially higher amounts of product and rapid growth-mediated biomass production as they already have evolutionary evolved endogenous cellular, genetic and biochemical frameworks similar to native plants. Thus, minimal gene insertions and optimization is required for the de novo production of SMs.

In recent times, *N. tabacum*, *N. benthamiana*, and *P. patens* have emerged as new attractive photosynthetic chassis for stable heterologous biopharming of commercial products by heterologous expression of reconstructed plant natural product pathways. Apart from these plants, several higher plants were metabolically manipulated in metabolic engineering to improve native in planta metabolite synthesis or de novo in planta SM production as they have tissue and cellular differentiation, native genetic and biochemical background, compartmentalization by manipulation of only desirable key steps, and fewer optimization steps. However, the need for reproducible regeneration protocol, understanding of complicated metabolic cross-talk, a huge cultivation area, difficulties in the purification of desired phytochemicals, the requirement of numerous trials, and approval are major problems with this system. To improve metabolic flux toward desired secondary metabolite application of several strategies, such as overexpression of bottleneck rate-limiting enzymes, inhibition of the competitive pathway, modulation of expression of the transcription factor, precursor channelling, enzyme fusion, etc., have been possible in more effective ways than the earlier due to the current progress and advances in genetic and metabolic engineering, biotechnology, omics techniques, genome or gene editing tools, and pathway optimization bioinformatic tools. Advancements in large-scale bioreactor techniques and downstream processes in recent times ensure the optimum yield, productivity, and quality of desired molecules.

These technical advances, without a doubt, increase the applicability of metabolic engineering in broader aspects from the microbial system to various transgenic plant cultures for the production of diverse SMs. However, the achievements are too small in contrast to their potential and our goal. In addition to many success stories, many unexpected results were also noted, which are not fully understandable in the present scenario. More understanding of complicated plant metabolic pathways, their regulatory mechanism at transcription, translation, metabolic levels, and cross-talk interaction is essential to further improvements. Many challenges need to be addressed to explore the full potential of plant-based systems of metabolic engineering. These

challenges may be overcome in the near future by more extensive background information, effective genetic tools and engineering methodologies, efficient enzymes, high-throughput computational engineering models with the support of artificial intelligence, and advances in synthetic biology tools. Advancement in large-scale bioreactor techniques through the involvement of computational artificial regulatory circuits or fluid dynamics ensures the optimum yield, productivity, and quality of desired molecules. Several advancements in the downstream process of secondary metabolite production also may be game changers in cutting the overall commercial production cost of the therapeutic phytomolecules.

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Chapter 29

Stationary, Agitated, and Bioreactor Cultures of *Verbena officinalis* L. (Common Vervain): A Potential Rich Source of Bioactive Phenolic Compounds for Pharmacy, Health Food Industry, and Cosmetology



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Abstract *Verbena officinalis* L. is a medicinal plant widespread in the wild throughout almost of all continents. The medicinal raw material obtained from this species is the herb. Its main constituents include iridoids (e.g., verbenalin, hastatoside), different groups of polyphenols (e.g., phenylpropanoid glycosides, simple phenolic acids and depsides, and flavonoids), triterpenoids (e.g., ursolic acid), phytosterols, essential oil, and bioelements. This species has an important position both in traditional European and North American medicine as well as in Traditional Chinese Medicine (TCM). In 2008, *Verbenae herba* was introduced into official European medicine. In the United States, this species has long been known and used in official allopathic and homeopathic medicine. The vervain herb also appears in the Chinese Pharmacopoeia (2005). This article presents basic information on the morphology, ecology, and distribution of natural habitats of this species worldwide and reviews the main groups of its chemical components occurring in the herb and possible therapeutic uses of this raw material. The important position of this species in cosmetology was also underlined. In addition, biotechnological studies maintained by our research teams on the biosynthetic potential of cells cultured in vitro were presented. These studies aimed to optimize conditions for in vitro

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cultures in order to facilitate the accumulation of bioactive phenolic compounds. The optimization was performed by testing a wide range of plant growth regulators, light conditions (white fluorescent light, darkness, monochromatic LED lights), and culture types differing in the degree of organogenesis, that is, undifferentiated callus cultures and microshoot cultures (stationary, agitated, and bioreactor cultures). The results of the biotechnological studies have indicated that the biosynthetic potential of cells in *in vitro* cultures is specifically oriented toward the production of phenylpropanoid glycosides: verbascoside and isoverbascoside. The maximum contents of these compounds reaching ca. 9 g% of verbascoside (stirred tank bioreactor cultures) and ca. 0.6 g% of isoverbascoside (agitated cultures) obtained in undifferentiated cultures are interesting from practical perspective. On the other hand, microshoot cultures of this species produce essential oil at quantities comparable to its contents in the soil-grown plants (ca. 8 g/kg DW in a Plantform bioreactor) but differing in chemical composition, which is also the result offering the potential for application.

Keywords Common vervain · Botanical and chemical characteristics · Biological activities · Therapeutic values · Significance in cosmetology · Undifferentiated *in vitro* cultures · Microshoot cultures · Production of phenolic compounds · Production of essential oil

Abbreviations

2iP	6-(γ,γ -Dimetyloalliloamino)purine
BA	6-Benzyladenine
BB	Balloon bioreactor
BZD	Benzodiazepine
DW	Dry weight
GABA _A	Ionotropic receptor for γ -aminobutyric acid, class A
GC/FID	Gas chromatography/flame ionization detection
GC/MS	Gas chromatography/mass spectrometry
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
JNK	c-Jun N-terminal kinases
KIN	Kinetin
LED	Light-emitting diode
MAO	Monoamine oxidase
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
NREM	A phase of sleep characterized by non-rapid eye movement
PGRs	Plant growth regulators

PKR	Protein kinase R
STB	Stirred-tank bioreactor
TDZ	Thidiazuron
TLC	Thin-layer chromatography
UHPLC-hr-q-TOF/MS	Ultrahigh-performance liquid chromatography with ultrahigh-resolution time-of-flight mass spectrometry

29.1 Introduction

Verbena officinalis L. (common vervain) is a perennial plant of the family Verbenaceae (subfamily Verbeneae). It is a cosmopolitan species widespread throughout almost all continents. The natural habitats of this plant exist in Europe, Asia, North America, South America, and Africa.

The herb of this plant species (aerial parts collected during flowering) is a raw material significant for medicinal applications. This raw material contains as the main group of bioactive compounds—iridoids represented by, for example, verbenalin, hastatoside, and aucubin. This group is accompanied by different types of phenolic compounds, which include the known antioxidants: phenylpropanoid glycosides (verbascoside, isoverbascoside, eucovoside), simple phenolic acids, depsides, and flavonoids (as well common in the plant kingdom compounds as rare, e.g., pedalitin, scutellarein). Other important components are triterpenoids (e.g., ursolic acid), phytosterols, essential oil, and bioelements.

The common vervain herb shows antioxidant, antimicrobial (antibacterial and antifungal), anti-inflammatory, and local analgesic properties and produces a wide range of actions on the central nervous system function (anxiolytic, sedative, sleep quality improving, anticonvulsant, neuroprotective, and antidepressant) and gastroprotective activity. Extracts of *V. officinalis* herb accelerate wound healing. Scientific studies have proved that extracts exhibited also anticancer activity.

The vervain herb is also used in homeopathy, mainly as a sedative and mood-enhancing agent, which ensures a restful sleep and relieves fatigue. It is also helpful in constipation and shows analgesic and antipyretic properties (Adaptogeny n.d.).

The phenomenon of chemical diversity common in the nature progressing of environmental pollution and climate changes have boosted interest in biotechnological solutions to make high-quality raw materials available for therapeutic and/or health-promoting and/or cosmetic applications.

Until now, vervain was a subject of only few biotechnological studies aimed at developing micropropagation protocols. The objects of interest of our research teams focused for the first time on the biosynthetic potential of *V. officinalis* cells cultured in vitro. These studies included a wild optimization of in vitro culture conditions aimed at obtaining a high content of biologically active compounds. The best conditions for the cultivation of undifferentiated callus cultures and differentiated

microshoot cultures starting from stationary cultures through agitated cultures to bioreactor cultures were optimized.

These studies were considered important and interesting from practical point of view. Moreover, it was expected to obtain application results that would allow proposing *V. officinalis* as an alternative to plants growing *in vivo* as a rich, biotechnological source of bioactive compounds for pharmaceutical and/or health-promoting and/or cosmetic purposes.

29.2 Botanical Characteristic

29.2.1 *Synonyms and Common Names in Different Languages*

Verbena officinalis is known under the synonymous Latin names: *V. sororia* D.Don and *V. spuria* L. Its common names in different languages are as follows: vervain, common vervain, common verbena, European vervain (English), Verbene, Sagenkraut, Eisenkraut, Katzenblutkraut, Wunschkraut (German), verveine officinale, and verveine sauvage (French) (Anioł et al. 1993; Strzelecka and Kowalski 2000; U.S. National Plant Germplasm System 2019).

29.2.2 *Species Morphology*

V. officinalis is a perennial plant 70 cm to 1 m tall. It has erected, branched in the upper part, woody, quadrangular stems covered with scabrous hairs. The upper leaves are sessile with crenate margins, and the lower leaves are petiolate, deeply pinnately lobed, while the middle leaves are tripartite with a larger leaflet at the apex. The leaves are oppositely arranged on the stem. The plant develops spike inflorescences with light lilac flowers and with a delicate scent. The flowers are bisexual. The corolla is slender almost two-lipped, and it has four to five petals forming a slightly banded tube. The fruit is oblong reticulate schizocarp. The plant growing in the temperate climate zone blooms from June to September (Fig. 29.1) (Van Wyk and Wink 2004; Miraj and Kiani 2016).

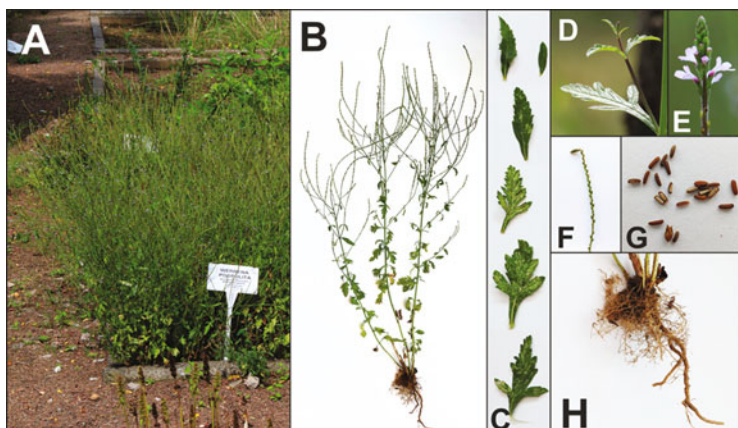


Fig. 29.1 *Verbena officinalis* L.: (a) plants growing in vivo, (b) the appearance of a single plant, (c) leaves, (d) young floral shoot, (e) top of the inflorescence, (f) inflorescence at the end of flowering, (g) seeds, (h) roots

29.2.3 Distribution of Natural Habitats and Ecological Requirements

V. officinalis is a cosmopolitan species common in most climatic zones on different continents in Europe, Asia, North America, South America, and in Africa (Wichtl 2004; Van Wyk and Wink 2008).

In Poland, natural localities of common vervain are distributed mostly in the Carpathian Foothills and lower parts of the mountains. A few localities have also been found in the lowlands in southern Poland (Macku and Krejca 1989; Strzelecka and Kowalski 2000; Broda and Mowszowicz 2001; Lai et al. 2006; Van Wyk and Wink 2008; Dildar et al. 2012; Abebe et al. 2017).

V. officinalis is treated by some authors as a ruderal species. *V. officinalis* is a xerothermic species, growing well on fertile humus soils of medium humidity but avoiding acidified soils. It grows best in sunny locations. Vervain occurs at sites such as croplands, pastures, ditches, rocky areas, dry mountain slopes, roadsides, and wastelands. This species can be encountered in the vicinity of water bodies and at altitudes up to 1200 m a.s.l. (Anioł et al. 1993; Khan et al. 2015, 2016; Abebe et al. 2017).

29.3 Chemical Composition

The vervain herb, that is, aerial parts collected during flowering, is medicinal raw material. The chemical composition of this raw material is presented in Table 29.1.

Table 29.1 The chemical composition of vervain herb

Group of metabolites	Compounds	References
Iridoids	Verbenalin, hastatoside, aucubin, verbeofflin, verbenoside A and B	Van Wyk and Wink (2004), Xu et al. (2010), Schönlichler et al. (2013), Shu et al. (2014), Khan et al. (2016)
Phenylpropanoid glycosides	Verbascoside, isoverbascoside, eukovoside, campenoside II, isocampenoside II, betonyoside A	Van Wyk and Wink (2004), Bilia et al. (2008), Zhang et al. (2011), Schönlichler et al. (2013), Encalada et al. (2015), Khan et al. (2016)
Flavonoids	Kaempferol, luteolin, apigenin, isorhamnetin, quercetol, pedalitin, scutellarein	Calvo et al. (1997), Duan et al. (2011), Rehecho et al. (2011), Verma and Siddiqui (2011), Zhang et al. (2011), Khan et al. (2016), Sun et al. (2017)
Phenolic acids	Chlorogenic acid, ferulic acid, protocatechuic acid, rosmarinic acid	Kubica et al. (2017), Sun et al. (2017), Zhang and Pan (2017)
Terpenoids	Diterpenoids: carnosol, carnosolic acid, rosmanol, isorosmanol	Sun et al. (2017)
	Triterpenoids: ursolic acid and its derivatives, oleanolic acid and its derivatives	Deepak and Handa (2000a), Zhang et al. (2011), Shu et al. (2013), Kaur et al. (2014), Khan et al. (2016)
Carbohydrates	Arabinose, galactose, galacturonic acid, glucose, mannose, rhamnose, xylose	Lai et al. (2006)
Sterols	Daucosterol, α -sitosterol, β -sitosterol	Zhang et al. (2011), Khan et al. (2016)
Fatty acids	Oleic acid	Deepak and Handa (2000a), Zhang et al. (2011), Khan et al. (2016)

The main components of this herb include iridoid glycosides and phenylpropanoid glycosides. The first group of glycosides is represented mainly by verbenalin (verbenalloside), hastatoside, and aucubin (aucuboside). On the other hand, verbascoside (acteoside), isoverbascoside (isoacteoside), and eucovoside are representatives of phenylpropanoid glycosides (Verma and Siddiqui 2011; Kubica et al. 2017).

The herb also contains simple phenolic acids, such as ferulic acid and protocatechuic acid and depsides: chlorogenic acid and rosmarinic acid and in addition, dicaffeoylquinic acids (Kubica et al. 2017).

The raw material is also rich in flavonoids, as well those common in the plant kingdom: kaempferol, luteolin, and apigenin together with their glycosides, as rare compounds, like scutellarein and pedalitin and their glycosides (Rehecho et al. 2011).

The presence of a triterpenoid compound, ursolic acid and its derivatives, and phytosterols, α -sitosterol, β -sitosterol, and daucosterol, is also important (Verma and Siddiqui 2011; Shu et al. 2013).

Moreover, essential oil occurring in the herb is a mixture of ca. 40 compounds in which monoterpenoids are the dominating group. The chemical composition of the essential oil is very variable and depends mostly on the origin of raw material. The main compounds of the essential oil include: anethole, cyneol, citral, eucalyptol, carvone, hexadecanoic acid, limonene, linalool, isobornyl formate, spathulenol, and caryophyllene oxide (de Martino et al. 2008, 2009).

The herb is also composed of a number of bioelements: potassium, sodium, iron, magnesium, calcium, phosphorus, copper, zinc, and manganese (Lai et al. 2006; Rehecho et al. 2011; Dildar et al. 2012).

The chemical composition of other parts of the plant is poorly understood. One paper comparing the contents of four compounds (ursolic acid, verbenalin, hastatoside, and verbascoside) in different plant organs documented high contents of hastatoside and verbascoside in the roots. Research by other authors has proven the presence of many bioelements both in the leaves and roots (Deepak and Handa 2000b).

29.4 Medicinal Properties: Traditional Medicine and Official Phytotherapy

V. officinalis herb is a medicinal raw material long used in traditional European and North American medicine as well as in Traditional Chinese Medicine (TCM).

The herb was introduced into official European therapy in 2008. The monograph of *Verbenae herba* has appeared unchanged in the European Pharmacopoeia since Ph. Eur. 6th Edition to the currently valid Ph. Eur. 11th Edition. The translations of this monograph into national languages are listed in the pharmacopoeias of the countries belonging to the European Union. The translation has also appeared in the Polish Pharmacopoeia since Ph. Pol. 8th Edition till the currently valid 12th Edition of this document.

The monograph of the *Verbenae herba* can be found in the Chinese Pharmacopoeia (8th Edition of 2005), whereas the British Pharmacopoeia and French Pharmacopoeia have listed *Verbenae herba* monograph even earlier. In the United States, this species is long known and used both in allopathy and homeopathy. A description of this plant and its different formulations can be found in Pharmacopoeia of the American Institute of Homeopathy of 1897.

In the European Union countries, pharmacopoeial raw material, *Verbenae herba*, is standardized for the content of verbenalin, which should not be lower than 1.5%.

In traditional medicine, vervain is recommended as a component of stimulating and memory-improving preparations. It plays an important role in alleviating gynecological problems and dysmenorrhea. It stimulates lactation. Due to its choleric effect, it is recommended as a remedy in gastrointestinal disturbances. In addition, it shows diuretic effect. The herb extracts lower body temperature in the common cold. It is used as an adjunctive medication in the treatment of malaria and rheumatism.

Table 29.2 Biological activity of extracts from *V. officinalis* herb and essential oil used in cosmetology and medicine, an overview of studies

Biological activity	Mechanism of action	Compounds responsible for biological activity	References
Antioxidant action	• High antiradical activity	Flavonoids, caffeic acid derivatives and phenylpropanoid glycosides	Speroni et al. (2007), Casanova et al. (2008), Rehecho et al. (2011), Abebe et al. (2017)
	• High reductive potential		
	• Inhibition of xanthine oxidase, an enzyme inducing the formation of reactive oxygen species.		
Antibacterial action	• Gram-positive bacteria, suppressing effect on <i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i> , methicillin-resistant <i>S. aureus</i> , <i>Streptococcus pneumoniae</i> , <i>Streptococcus pyogenes</i>	Not established	Dildar et al. (2012), Abebe et al. (2017)
	• Inhibitory action on Gram-negative bacteria: <i>Acinetobacter baumannii</i> , <i>Escherichia coli</i> , <i>Proteus vulgaris</i> , <i>Salmonella typhi</i>		
Antifungal action	• Inhibitory effect on <i>Penicillium expansum</i> and <i>Rhizopus stolonifer</i>	Caffeic acid derivatives/ phenylpropanoid glycosides	Casanova et al. (2008)
Anti-inflammatory action	• Soothing effect in the treatment of chronic gingivitis	Iridoids, caffeic acid derivatives, flavonoids, phenylpropanoid glycosides	Calvo (2006), Speroni et al. (2007), Grawish et al. (2016)
	• Inhibition of reactive radical production		
	• Antioedematous effect		

Furthermore, vervain is recommended in dermatological problems as an anti-inflammatory, analgesic, antibacterial, and skin softening agent (Van Wyk and Wink 2004; Kou et al. 2013; Shu et al. 2014; Khan et al. 2016; Abebe et al. 2017).

Current professional scientific researches have confirmed some of the abovementioned traditional therapeutic applications and documented a number of other valuable properties of herb extracts, such as antioxidant, antibacterial, antifungal, anti-inflammatory, locally analgesic, anticonvulsant, sleep quality-improving, neuroprotective, antidepressant, anxiolytic, sedative, and gastroprotective effects. Moreover, the anticancer effect of the extracts has been proven. The herb extracts also accelerate wound healing. Some selected directions of biological activity of vervain extracts and the mechanism of these actions are presented in Tables 29.2 and 29.3.

Verbenae herba has an important position in homeopathy. It is recommended mostly as a sedative, mood-enhancing agent assuring restful sleep and relieving

Table 29.3 Medically important biological activities of extracts from *V. officinalis* herb and essential oil, an overview of studies

Biological activity	Mechanism of action	Compounds responsible for biological activity	References
Analgesic action	• Analgesic effect probably due to suppressive action on the central nervous system	Iridoids, caffeic acid derivatives, flavonoids, phenylpropanoid glycosides	Calvo (2006)
Antiseizure action	• Probably via GABA_A receptor activation • Possible enhancement of GABAergic transmission caused by agonistic action at BZD and KOP receptors	Flavonoids (for example scutellarein) verbascoside and phenolic acids	Khan et al. (2016), Rashidian et al. (2017)
Sleep quality-improving action	• Significantly increased periods of NREM sleep	Iridoids and phenylpropanoid glycosides	Makino et al. (2009)
Neuroprotective action	• Suppressive effect on kinases PKR and JNK activated by amyloid-β • Protection of neurons against oxidative stress • Possible inhibition of neuronal apoptosis	Not established	Lai et al. (2006)
Antidepressant action	• Elevation of serotonin, noradrenaline and dopamine concentrations in nerve endings (elevated levels of these neurotransmitters can result from the inhibition of monoamine oxidase (MAO) activity in the brain)	Not established	Jawaid et al. (2015)
Anticancer effect	• Proapoptotic effect may be linked with caspase-3 activation	Essential oil, phenylpropanoid glycosides	de Martino et al. (2008), De Martino et al. (2011), Kou et al. (2013), Encalada et al. (2015)
Gastroprotective action	• The action connected with antioxidant properties because free radicals are formed in pathological changes in the gastric mucosa	Phenylpropanoid glycosides, flavonoids	Speroni et al. (2007)
Wound healing accelerating effect	• Anti-inflammatory action in the injured tissue • Antibacterial effect	Phenylpropanoid glycosides, flavonoids	Speroni et al. (2007)

fatigue. It is effective as an analgesic and antipyretic medication and eliminates constipation (Calvo 2006; Khan et al. 2016).

In allopathic medicine, *V. officinalis* herb is used in the treatment of infections of the upper airways. On the European market, *V. officinalis* extract is an ingredient of a very popular medicinal preparation Sinupret (manufactured by Bionorica Company, Germany). This raw material is also a component of many dietary supplements with a similar profile of activity, including those produced by Polish manufacturers.

In 2010, the European Food Safety Authority (EFSA) issued an opinion about *Verbenae herba*, which is valid to this day. This opinion confirms that *Verbenae herba* may protect cells and tissues against oxidative stress-induced damage and may increase physiological defense mechanisms of the body (EFSA 2011).

29.5 Significance in Cosmetology

V. officinalis occupies an important position in the cosmetics industry. According to the CosIng database (Cosmetic Ingredient Database), compiled by the European Commission, different raw materials from *V. officinalis* (in the number 11) were authorized for use in the production of cosmetics; they include lysate from callus cells, herb and root extract, extract from flowers, aqueous solution from flowers obtained by distillation, extract from flowers/leaves, aqueous solution from flowers/leaves obtained by distillation, absolute obtained from leaves, leaf extract, rectified essential oil from leaves, aqueous solution from leaves/stems obtained by distillation, and aqueous solution from stems obtained by distillation (Table 29.4) (CosIng 2021).

Among the listed raw materials approved for use in the production of cosmetics, the use of lysate from callus cells—undifferentiated biomass growing under in vitro conditions—deserves particular attention.

Table 29.4 Different raw materials from *Verbena officinalis* propose for cosmetic product in Cosmetic Ingredient Database

Raw materials	Functions
<i>Verbena officinalis</i> callus lysate	Antioxidant
<i>Verbena officinalis</i> extract	Skin conditioning, emollient
<i>Verbena officinalis</i> flower extract	Skin conditioning
<i>Verbena officinalis</i> flower water	Skin conditioning
<i>Verbena officinalis</i> flower/leaf extract	Fragrance, skin conditioning
<i>Verbena officinalis</i> flower/leaf water	Flavoring, fragrance, perfuming, skin conditioning
<i>Verbena officinalis</i> leaf absolute	Perfuming
<i>Verbena officinalis</i> leaf extract	Fragrance, skin conditioning
<i>Verbena officinalis</i> leaf rectified oil	Perfuming
<i>Verbena officinalis</i> leaf/stem water	Skin conditioning
<i>Verbena officinalis</i> water	Skin conditioning

The proposed raw materials can be used not only as components of fragrance compositions or skin conditioners but also as antimicrobial, antiaging, regenerative, and epidermis softening ingredients.

29.6 Biotechnological Studies

V. officinalis was earlier the subject of single biotechnological studies. They were concerned on the development of micropropagation protocols (Türker et al. 2010; Kumar et al. 2019). The initiation of the studies connected with endogenous production of bioactive compounds in different types of in vitro cultures belongs to our research teams.

29.6.1 Micropropagation Protocols

The Turkish research team (Türker et al. 2010) developed a micropropagation protocol based on shoot regeneration from stem fragments (internodes) and leaves of seedlings growing on different variants of Murashige and Skoog (MS) medium. The highest propagation efficiency was achieved on the MS medium variant containing 0.23 $\mu\text{M/L}$ TDZ (thidiazuron) and 0.57 $\mu\text{M/L}$ IAA (indole-3-acetic acid). MS medium supplemented with 5.71 $\mu\text{M/L}$ IAA proved to be the best rooting medium for microshoots (Kubica et al. 2018, 2020a).

Moreover, the team from India (Kumar et al. 2019) developed a micropropagation protocol using in vitro cultures established from shoot fragments (nodes). The obtained shoot cultures were propagated on MS medium with the addition of various concentrations of plant growth and development regulators (PGRs). The best result of microshoot propagation was achieved with 1.5 mg/L of kinetin. Next, the microshoots were rooted on half-strength MS medium (1/2 MS) supplemented with different PGRs. The most efficient rooting was obtained with 2 mg/L IBA. The studies attempted also to stimulate shoot development from the obtained callus cultures.

29.6.2 Endogenous Production of Bioactive Phenolic Compounds

Intensive studies on the biosynthetic potential of *V. officinalis* cells cultured in vitro were initiated by our research teams representing the Chair and Department of Pharmaceutical Botany, Medical College of the Jagiellonian University in Krakow

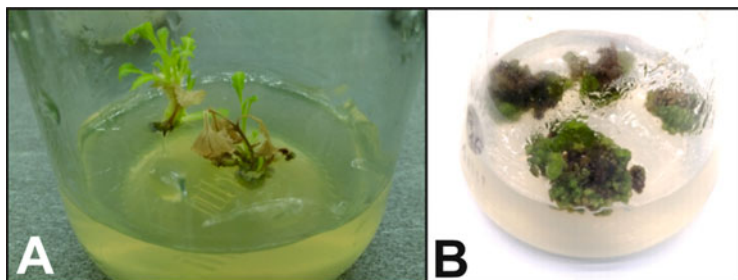


Fig. 29.2 *Verbena officinalis* callus cultures: (a) initiation of cultures from shoot fragments, (b) obtained callus culture (MS medium with the addition of 1 mg/L BA and 0.5 mg/L NAA)

(Poland) (Fig. 29.2) and the Chair and Department of Pharmacognosy with Medicinal Plant Garden of the Medical University of Gdansk (Poland).

The research aimed to establish optimal conditions for maintaining *in vitro* cultures to achieve best biomass increments and high accumulation of bioactive compounds. The optimization involved testing of different basic composition of culture medium, choice of PGRs and their concentrations, degree of organogenesis of the cultured biomass, culture type (agar, stationary liquid, agitated, and bioreactor cultures), and duration of growth cycles. Also, light conditions in which *in vitro* cultures were maintained (white fluorescent light, darkness and monochromatic lights emitted by electroluminescent diodes) were tested. Compounds were analyzed in methanolic extracts by different HPLC methods. The contents of verbascoside were estimated in the first step of experiments with HPLC method by Ellnain-Wojtaszek and Zgóřka (1999). In the next step, the quantification of verbascoside, isoverbascoside, verbenalin, and hastatoside was made using HPLC method by Schönlichler et al. (2013). The phenolic acids and flavonoid contents were estimated by Ellnain-Wojtaszek and Zgóřka (1999).

29.6.2.1 Undifferentiated *In Vitro* Cultures

The Effect of PGRs and Light Conditions (White Fluorescent Light and Darkness)

Callus cultures were established on MS medium with addition of 1 mg/L BA and 0.5 mg/L NAA from shoot fragments of parent plants.

In the first step of our researches, callus cultures were maintained on 12 variants of MS medium containing different combinations of a wide range of PGRs, that is, cytokinins and auxins, BA and NAA, KIN and NAA, BA and IBA (indole-3-butyric acid), and KIN and IBA. Each of these cytokinin-auxin combinations was tested in the following concentrations: [mg/L]: 0.5 and 3; 1 and 1; 3 and 1, respectively. The cultures were maintained under continuous artificial white light emitted by fluorescent lamps of 4 W/m² light intensity and in the darkness for 4 weeks. The effect of

the abovementioned conditions on biomass increments and accumulation of bioactive compounds, verbascoside and phenolic acids (free and bound), were examined (Kubica et al. 2017).

The dry biomasses increments on the tested MS variants differed from 1.7- to 5.4-fold. The best “growth medium” was MS variant containing 1 mg/L BA and 1 mg/L IBA.

The contents of verbascoside and free and bound phenolic acids were determined in methanolic extracts from the biomass and herb of the parent plant. The analysis of verbascoside and free phenolic acids was conducted directly in methanolic extracts. Analysis of bound phenolic acids was performed after prior hydrolysis (2 M HCl for 2 h).

The experiment revealed a clear impact of cytokinin and auxin type and concentrations in culture media on the content of verbascoside. The amounts of this compound in cultures growing in the light varied in a wide range from 0.45 to 2.45 g/100 g DW. The highest verbascoside concentrations were documented on two MS medium variants containing 1 mg/L BA and 1 mg/L IBA (2.45 g/100 g DW) and 3 mg/L KIN and 0.5 mg/L IBA (2.32 g/100 g DW). The contents of verbascoside in biomass extracts obtained from the remaining medium variants did not exceed 1.58 g/100 g DW.

The verbascoside accumulation in *V. officinalis* cultures growing in the darkness was proven to depend also on the composition of the tested MS medium variants. Its contents were very diverse from 0.78 to 2.14 g/100 g DW. The highest contents were determined in the extracts of biomass cultivated on two MS medium variants enriched in 1 mg/L KIN and 1 mg/L IBA (2.14 g/100 g DW) and 1 mg/L BA and 1 mg/L IBA (2.13 g/100 g DW). Only slightly lower amounts were documented in biomass collected from two other medium variants containing 0.5 mg/L KIN and 3 mg/L NAA, and 0.5 mg/L KIN and 3 mg/L IBA (1.99 g/100 g DW and 1.93 g/100 g DW, respectively). Extracts from biomass growing on the remaining tested MS medium variants showed verbascoside contents lower than 1.52 g/100 g DW.

On the majority of the tested MS medium variants, higher amounts of this metabolite were seen in cultures maintained in the presence of light.

The verbascoside contents in extracts of the *V. officinalis* herb (aerial parts harvested in spring) quantified in this work for comparison, amounted to 0.75 g/100 g DW. Therefore, the maximum content of verbascoside obtained in biomass cultured in vitro was 3.28-fold higher (2.45 g/100 g DW) than in the herb of parent plant.

Five phenolic acids of 23 compounds assayed were confirmed to be present in methanolic extracts under study, namely, protocatechuic acid, ferulic acid, *o*-coumaric acid, *m*-coumaric acid, and rosmarinic acid. The contents of ferulic acid were very diversified. In extracts from biomass growing in the light, they ranged from 2.10 to 23.19 mg/100 g DW, while in extracts of biomass cultivated in the darkness, from 1.99 to 10.51 mg/100 g DW. *o*-Coumaric acid was also present in a wide range of concentrations from 0.84 to 19.22 mg/100 g DW (light) and from 1.12 to 22.68 mg/100 g DW (darkness). For both of these compounds, the impact of PGRs present in the tested MS medium variants on their accumulation was very

clear. The contents of the remaining acids, namely, protocatechuic acid, *m*-coumaric acid, and rosmarinic acid, were low (they did not exceed 10 mg/100 g DW in cultures grown in the light and 7.5 mg/100 g DW in the darkness).

The total contents of free phenolic acids in extracts from cultures cultivated in the light varied within a very wide range from 10.74 to 46.02 mg/100 g DW. Their maximum content was confirmed in extracts from biomass cultured on the MS medium variant containing 1 mg/L KIN and 1 mg/L NAA.

On the other hand, in extracts from *V. officinalis* cultures growing in the darkness, the total contents of phenolic acids amounted from 10.86 to 34.12 mg/100 g DW. The highest total contents of free phenolic acids (above 30 mg/100 g DW) were determined in extracts from biomass cultured on two medium variants, namely, those supplemented with 3 mg/L BA and 0.5 mg/L NAA, and 3 mg/L BA and 0.5 mg/L IBA.

Extracts of the herb of parent plant were found to contain only two free phenolic acids: ferulic acid (12.10 mg/100 g DW) and protocatechuic acid (1.35 mg/100 g DW).

After the acid hydrolysis, the tested *V. officinalis* biomass extracts were found to contain vanillic acid, caffeic acid, syringic acid, and isoferulic acid. The caffeic acid was the quantitatively dominating compound. Its amounts ranged from 19.16 to 101.15 mg/100 g DW (in the light) and from 33.99 to 91.46 mg/100 g DW (in the darkness). The highest amount of this compound was accumulated in biomass cultivated in the light on the medium enriched in 3 mg/L KIN and 0.5 mg/L IBA. The maximum contents of the remaining acids did not exceed 16 mg/100 g DW in the light and 19 mg/100 g DW in the darkness.

After acid hydrolysis, the total content of phenolic acids in extracts from biomass growing in the light was 37.77–141.05 mg/100 g DW and in the biomass growing in the darkness from 53.19 to 135.30 mg/100 g DW. The highest amounts were obtained in biomass cultivated on MS medium with addition of 3 mg/L KIN and 0.5 mg/L IBA (light) and 0.5 mg/L KIN and 3 mg/L NAA (darkness).

Analysis of the *V. officinalis* herb (parent plant) after acid hydrolysis revealed the presence of four phenolic acids with two dominating compounds: vanillic acid (36.93 mg/100 g DW) and caffeic acid (28.40 mg/100 g DW). Isoferulic acid and syringic acid occurred at lower quantities (8.01 mg/100 g DW and 3.12 mg/100 g DW, respectively). The total content of phenolic acids in the herb following acid hydrolysis was 66.46 mg/100 g DW.

The results of this research provided the encouragement to extend the studies to evaluate the effect of other light conditions (monochromatic LED lights) on biomass increments and dynamics of accumulation of the compounds under study. In addition, it was decided to extend phytochemical analyzes to quantify isoverbascoside.

The applied hydrolysis conditions led to degradation of free phenolic acids, which was detected before hydrolysis (after hydrolysis the lack of ferulic acid, *o*-coumaric, *m*-coumaric, and rosmarinic acids was documented), and it is because we decided in the next step of experiments to estimate only free phenolic acids.

The Effect of Light Conditions (Monochromatic LED Lights, White Fluorescent Light and Darkness)

Experimental agar callus cultures were cultivated under three LED lights: red light, blue light, and the combination of red and blue light (70%/30%) and in the darkness. White fluorescent light (of 40 $\mu\text{mol}/\text{m}^2/\text{s}$ intensity) was used as control conditions (Fig. 29.3). The cultures were maintained on the MS medium variant containing 1 mg/L BA and 1 mg/L IBA (selected on the basis of first step of experiments as the optimal medium for growth and production of phenylpropanoid glycosides), under 16/8 h photoperiod for a duration of 2-, 3-, and 4-week growth cycles. The studies were conducted in the biotechnological laboratory of the Department of Ornamental Plants and Garden Art, Faculty of Biotechnology and Horticulture, Hugon Kołłątaj University of Agriculture in Kraków (Poland) (Kubica et al. 2020c).

The growth increment of the biomass under tested light conditions valid from 9.22- to 19.19-fold. The best conditions for biomass growth were blue light and 3-week growth period.

Both light conditions and duration of growth cycles significantly affected the contents of verbascoside and isoverbascoside in extracts. The contents of verbascoside varied in a wide range from 2.28 to 6.72 g/100 g DW. Under blue light, verbascoside content increased from 4.06 g/100 g DW (2 weeks) through 6.03 g/100 g DW (3 weeks) to the maximum value of 6.72 g/100 g DW (4 weeks). In the biomass grown under the combination of red and blue light (70%/30%), verbascoside concentrations ranged from 4.00 g/100 g DW (2 weeks) through 5.81 g/100 g DW (3 weeks) to 6.02 g/100 g DW (4 weeks). In these light conditions, the maximum verbascoside content was noted also after 4-week growth cycles. The dynamics of accumulation of this compound under red light was different. The amounts of verbascoside were determined to be markedly reduced amounting to from 2.28 g/100 g DW (2 weeks) through 5.20 g/100 g DW (3 weeks) to 5.17 g/100 g DW (4 weeks). Approximately 2.3-fold increase in the content of this compound was noted between the second and third week of growth cycles, and this high level was retained for the fourth week of culture. In cultures cultivated in the darkness, verbascoside contents changed from 3.07 g/100 g DW (2 weeks) through 5.06 g/100 g DW (3 weeks) to 4.69 g/100 g DW (4 weeks). The maximum contents were confirmed after 3-week growth cycles, like in the cultures grown

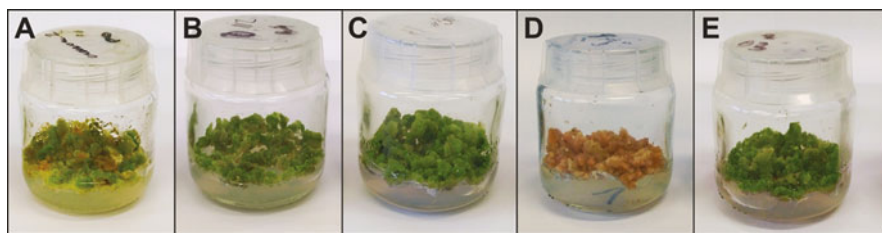


Fig. 29.3 *V. officinalis* callus cultures maintained under different light conditions: (a) red light, (b) blue light, (c) red and blue light (70%/30%), (d) darkness, (e) control (white fluorescent light)

under red light, while in the fourth week, it gradually decreased. In control cultures, verbascoside content increased from 3.68 g/100 g DW (2 weeks) through a maximum of 5.61 g/100 g DW (3 weeks), and then it clearly decreased the value of 3.66 g/100 g DW (4 weeks).

The isoverbascoside contents determined in this study were of another order of magnitude and varied in a very wide range from 57.04 to 382.51 mg/100 g DW. Under blue light, the amounts of this compound grew from 60.10 mg/100 g DW (2 weeks), through 128.80 mg/100 g DW (3 weeks) to a maximum value equal to 333.91 mg/100 g DW (4 weeks). Likewise, isoverbascoside contents in biomass growing under the combination of red and blue light (70%/30%) increased from 101.75 mg/100 g DW (2 weeks) through 206.60 mg/100 g DW (3 weeks) up to the maximum content of 378.90 mg/100 g DW (4 weeks). Under red light, the isoverbascoside accumulation was significantly lower and changed in a wide range from 57.04 mg/100 g DW (2 weeks), through 140.69 mg/100 g DW (3 weeks) to 162.63 mg/100 g DW after 4-week growth cycles. The amounts of isoverbascoside and its dynamics of accumulation in the darkness were similar to those observed in the biomass growing under combination of red and blue light (70%/30%). These amounts changed from 95.42 mg/100 g DW (2 weeks) through 263.57 mg/100 g DW (3 weeks) to 378.47 mg/100 g DW (4 weeks). In control conditions, isoverbascoside contents increased from 135.00 mg/100 g DW (2 weeks), through 263.57 mg/100 g DW (3 weeks) to 382.51 mg/100 g DW (4 weeks).

The total content of verbascoside and isoverbascoside varied widely from 2.33 to 7.05 g/100 g DW. The maximum content of both compounds was obtained in biomass cultivated under blue light in cultures maintained for 4 weeks. It was also high (6.02 g/100 g DW) in extracts from biomass grown under the combination of red and blue light (70%/30%) for 4 weeks. Under the remaining light conditions, the maximum contents of both compounds were noted after 3-week growth: under red light, 5.34 g/100 g DW; in the darkness, 5.32 g/100 g DW; and in the control conditions, 5.85 g/100 g DW.

The conducted research proved a beneficial effect of blue light and the combination of red and blue light on the accumulation of both compounds and brought to the attention the dynamics of accumulation of these compounds during growth cycles.

Under all tested light conditions, the presence of five free phenolic acids (protocatechuic, ferulic, *o*-coumaric, *m*-coumaric, and rosmarinic acids) was confirmed.

Under the light conditions tested, the total contents of phenolic acids (free compounds) were moderately diverse and ranged from 53.64 to 65.09 mg/100 g DW, depending on the LED lights quality and on duration of growth cycles. The contents were higher than those obtained under control conditions (max. 52.17 mg/100 g DW). In the darkness, the total contents showed a wide variability from 38.46 mg/100 g DW (2 weeks) through 36.32 mg/100 g DW (3 weeks) to 63.95 mg/100 g DW (4 weeks). The results of quantitative analyses evidenced that the combination of red and blue light (70%/30%) and darkness contributed to the accumulation of phenolic acids (with the maximum contents reaching 65.09 mg/100 g DW and 63.95 mg/100 g DW, respectively). In the presence of blue light, the

maximum content of phenolic acids was 58.61 mg/100 g DW (2 weeks) and under red light 61.54 mg/100 g DW (3 weeks). The amounts of phenolic acids determined in all tested extracts were relatively low.

Interesting results of the studies on optimization of both, culture media composition and light conditions to promote biomass growth and production of the tested compounds in agar callus cultures of *V. officinalis* became an inspiration to continue for further stages of biotechnological studies. It was attempted to establish suspension culture and bioreactor cultures of this species for the first time.

The Effect of Different Types of In Vitro Cultures

Agar callus cultures and successfully established suspension and bioreactor cultures (Fig. 29.4) were maintained for 2-week growth cycles on the MS medium variant supplemented with 1 mg/L BA and 1 mg/L IBA (selected on the basis of earlier studies as the optimal medium for growth and production of phenylpropanoid glycosides) (Kubica et al. 2017, 2020b). The first aim of the studies was to optimize the proportion of inoculum to the volume of the medium in agar callus cultures and suspension cultures.

The next task was to scale up the agar cultures through suspension cultures to bioreactor cultures. Bioreactor cultures were maintained in two types of bioreactors designed for suspension cultures, namely, in a balloon bioreactor (BB) and in a stirred tank bioreactor (STB) (Kubica et al. 2020b). STB is a commercially available bioreactor with a stirrer (Bellco Glass, Vineland, NJ, USA), while BB was constructed by an employee of the Department of Pharmacognosy of the Medical University of Gdansk (Poland), Dr. Adam Kokotkiewicz. All abovementioned culture types were maintained in the laboratory of biotechnology of this department.

These studies involved the testing of different weights of inoculum: 0.3, 0.6, and 0.9 g of fresh biomass for callus cultures and 1.5, 3.0, and 4.5 g of fresh biomass for suspension cultures. The medium volume for both types of cultures was 30 mL.

The best proportion of inoculum to medium volume was established as 0.3 g/30 mL of medium for agar callus cultures and 1.5 g/30 mL of medium for suspension cultures. In both types of bioreactors (Fig. 29.4c, d), the proportion of inoculum to

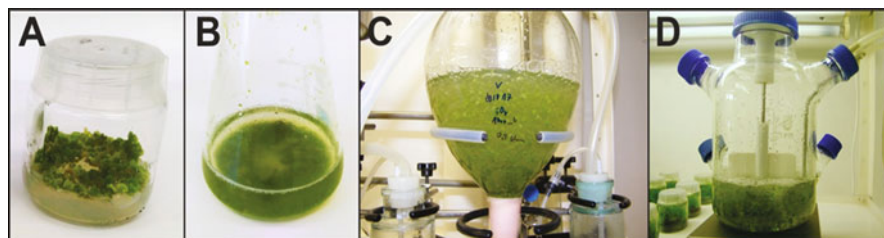


Fig. 29.4 Different types of *Verbena officinalis* in vitro cultures: (a) agar cultures, (b) agitated callus cultures, (c) cultures grown in balloon bioreactor (BB), (d) cultures grown in stirred-tank bioreactor (STB)

medium volume was identical and amounted to 50 g of fresh biomass per 1000 mL of medium.

In the tested types of undifferentiated cultures after 2 weeks, growth cycles (on MS medium containing 1 mg/L BA and 1 mg/L IBA) and growth increments (Gi, relative increases of biomasses $\times 100\%$) were comparable in suspension cultures and bioreactor cultures (in two types of bioreactors, BB and STB), 335%, 360%, and 367%, respectively. On the other hand, in agar callus cultures, growth increments were few folds higher (1561%).

Extracts of biomass from agar callus cultures were found to contain very high contents of verbascoside. Depending on the tested inoculum, they changed only slightly from 7.15 g/100 g DW (0.3 g inoculum) through 7.18 g/100 g DW (0.6 g) to the maximum amount of 7.25 g/100 g DW (0.9 g).

The amounts of isoverbascoside determined in the biomass were of another order of magnitude. They varied in wider limits from 442.81 mg/100 g DW (0.3 g) through 528.17 mg/100 g DW (0.6 g) to 609.26 mg/100 g DW (0.9 g). The best accumulation of both studied compounds was achieved at inoculum weight of 0.9 g. The highest total content of both compounds was 7.86 g/100 g DW.

The extracts of biomass from suspension cultures were also demonstrated to contain very high levels of verbascoside. Depending on the inoculum, they ranged from 6.85 g/100 g DW (1.5 g) through 7.06 g/100 g DW (3.0 g) to the value of 6.29 g/100 g DW (4.5 g). Like in the callus cultures, also in this culture type, isoverbascoside amounts were of lower order of magnitude. They depended on the tested inoculum weight and changed from 264.36 mg/100 g DW (1.5 g) through 387.19 mg/100 g DW (3.0 g) to 477.25 mg/100 g DW (4.5 g). The maximum total content of both phenylpropanoid glycosides under study (7.45 g/100 g DW) was noted for the inoculum weight of 3.0 g.

The amounts of verbascoside in extracts of biomass from both tested bioreactor types were higher in comparison with the maximum content of this compound in both agar callus culture and suspension culture extracts. They reached 7.69 g/100 g DW (BB) and as much as 9.18 g/100 g DW (STB).

The opposite was found for isoverbascoside. Its amounts in extracts from the cultures grown in both types of bioreactors were decidedly lower (306.40 mg/100 g DW in BB and 339.91 mg/100 g DW in STB) than its maximum amounts determined both in agar callus cultures and suspension cultures. The total content of both phenylpropanoid glycosides was considerably higher in cultures maintained in STB bioreactor (9.52 g/100 g DW) than in BB bioreactor (8.00 g/100 g DW).

Among all analyzed phenolic acids, extracts of biomass from all tested culture types contained six compounds: protocatechuic acid, chlorogenic acid, vanillic acid, caffeic acid, ferulic acid, and rosmarinic acid. The contents of this group of metabolites in the extracts under study were estimated to be of different order of magnitude compared with those of phenylpropanoid glycosides. In the extracts from agar callus cultures, the total contents of phenolic acids differed depending on the inoculum tested. Protocatechuic acid and ferulic acid were the quantitatively dominant compounds. Their contents ranged from 6.91 mg/100 g DW to 9.58 mg/100 g DW and from 4.91 mg/100 g DW to 7.35 mg/100 g DW, respectively. The amounts of the

remaining compounds, namely, caffeic acid and rosmarinic acid, did not exceed 3.6 and 3.8 mg/100 g DW, while those of chlorogenic and vanillic acid were at most 1.8 and 1.3 mg/100 g DW. The total content of phenolic acids varied from 19.42 mg/100 g DW (0.3 g inoculum) through 26.90 mg/100 g DW (0.6 g) to 25.00 mg/100 g DW (0.9 g).

In the examined biomass extracts from suspension cultures, the accumulated amounts of phenolic acids were also clearly dependent on the inoculum tested. The main compounds were three phenolic acids: protocatechuic acid, ferulic acid, and rosmarinic acid. With increasing inoculum, the contents of these acids changed from 2.09 mg/100 g DW to 10.64 mg/100 g DW, from 6.78 mg/100 g DW to 10.44 mg/100 g DW and from 13.39 mg/100 g DW to 26.34 mg/100 g DW, respectively. The maximum contents of the other compounds were low and amounted to the following: for caffeic acid 4.80 mg/100 g DW and for chlorogenic acid and vanillic acid only 1.25 mg/100 g DW and 1.43 mg/100 g DW, respectively. The total content of phenolic acids rose with the increase in inoculum reaching from 28.09 mg/100 g DW (1.5 g inoculum) through 49.46 mg/100 g DW (3.0 g inoculum) to 50.72 mg/100 g DW (4.5 g inoculum).

In extracts of biomass from both bioreactor types, the same three compounds as in suspension cultures quantitatively dominated, namely, protocatechuic acid, ferulic acid, and rosmarinic acid. Their contents were confirmed to amount to 5.07, 2.12, and 10.22 mg/100 g DW (in BB bioreactor) and 9.68, 7.10, and 14.87 mg/100 g DW (in STB bioreactor). The levels of caffeic acid were estimated at 1.61 mg/100 g DW (in BB bioreactor) and 3.68 mg/100 g DW (in STB bioreactor), whereas the amounts of the remaining phenolic acid were not higher than 1 mg/100 g DW. The total contents of phenolic acids in the tested bioreactor types were diverse; in BB, they reached 19.88 mg/100 g DW, while it was doubled in STB (36.78 mg/100 g DW). The total content of phenolic acids in extracts from biomass growing in STB was clearly higher than their maximum levels extracted from agar callus cultures (26.90 mg/100 g DW), but it was below the maximum values obtained for extracts from suspension cultures (50.72 mg/100 g DW).

Analysis of methanolic extracts of the soil-grown *V. officinalis* herb revealed the presence of two phenylpropanoid glycosides: verbascoside (1.73 g%) and isoverbascoside (78.34 mg/100 g DW), and three phenolic acids: ferulic acid (29.76 mg/100 g DW), protocatechuic acid (25.75 mg/100 g DW), and rosmarinic acid (2.53 mg/100 g DW). The herb extracts were shown to contain also four compounds, which were not confirmed in the extracts from in vitro cultures; these were two iridoids: verbenalin (4.21 g/100 g DW) and hastatoside (398.87 mg/100 g DW), and two flavonoids: scutellarin (172.13 mg/100 g DW) and apigenin-7-glucuronide (41.77 mg/100 g DW).

A comparison of the results of phytochemical analyses of biomass extracts from the tested undifferentiated *V. officinalis* in vitro cultures (agar callus cultures, suspension cultures, and bioreactor cultures) with the results of phytochemical analyses obtained for the herb extracts showed clear differences in the qualitative composition of compounds. Plants growing in vivo have biosynthetic potential to produce mostly verbenalin, verbascoside, and hastatoside, whereas cells from

biomass cultivated in vitro synthesize verbascoside and isoverbascoside. The maximum contents of these two compounds in STB bioreactor were 5.3-fold and 7.8-fold higher, respectively, than in the herb extracts.

29.6.2.2 Microshoot Cultures

Microshoot cultures were established on the “initiation” medium (MS medium, BA—1 mg/L, IBA—1 mg/L) from the shoot fragments of parent plant. In the next steps of biotechnological investigation, we tried to establish different types of microshoot cultures of *V. officinalis* (Kubica et al. 2022). All types of microshoot cultures were maintained on Schenk and Hildebrandt (SH) medium with addition of 2 mg/L 2iP and 0.22 mg/L TDZ.

Experimental microshoot cultures (Fig. 29.5) were initiated from 1 g of biomass per 30 mL of medium (agar, stationary liquid, and agitated). Agar and stationary liquid cultures were grown in Magenta® vessels (6 cm × 6.5 cm × 10 cm), while agitated cultures were cultivated in Erlenmeyer flasks (100 mL). Cultures in commercially available temporary immersion bioreactors dedicated for microshoot cultures in RITA® (Vitropic, Saint-Mathieu-de-Trévières, France) were maintained with the same proportions of biomass in relation to medium (6.7 g inoculum per 200 mL medium) (Fig. 29.6). Bioreactor cycles comprised the immersion of biomass in medium for 5 and 85 min of rest (16 cycles/24 h). Growth cycles lasted 28 days



Fig. 29.5 Microshoot agar cultures of *Verbena officinalis*



Fig. 29.6 Microshoot cultures of *Verbena officinalis*: (a) agitated culture, (b) stationary liquid culture, (c) culture maintained in RITA® bioreactor

for agar, stationary liquid, and agitated cultures and 21 days for bioreactor cultures. Biomass collected from all tested types of in vitro cultures was lyophilized.

In the tested types of microshoot cultures (agar, stationary liquid, and agitated cultures) after 4-week growth cycles (on SH medium with 2 mg/L 2iP and 0.22 mg/L TDZ) (Gi) of biomasses changed, respectively, 688%, 670%, and 1027%. In RITA, bioreactors after 3-week growth cycles (Gi) reached 461% and were satisfactory enough.

Methanolic extracts of biomass from agar microshoot cultures and the soil-grown plants were analyzed using UHPLC-hr-q-TOF/MS for qualitative evaluation of their chemical composition. At the next stage, methanolic extracts of biomass from microshoot cultures (agar, stationary liquid, agitated, and bioreactor) were subjected to qualitative and quantitative analysis using of HPLC method, which entailed the analysis of phenylpropanoid glycosides, phenolic acids, iridoids, and flavonoids. The *V. officinalis* herb collected during the whole flowering period in 2017 was used as a comparative plant material.

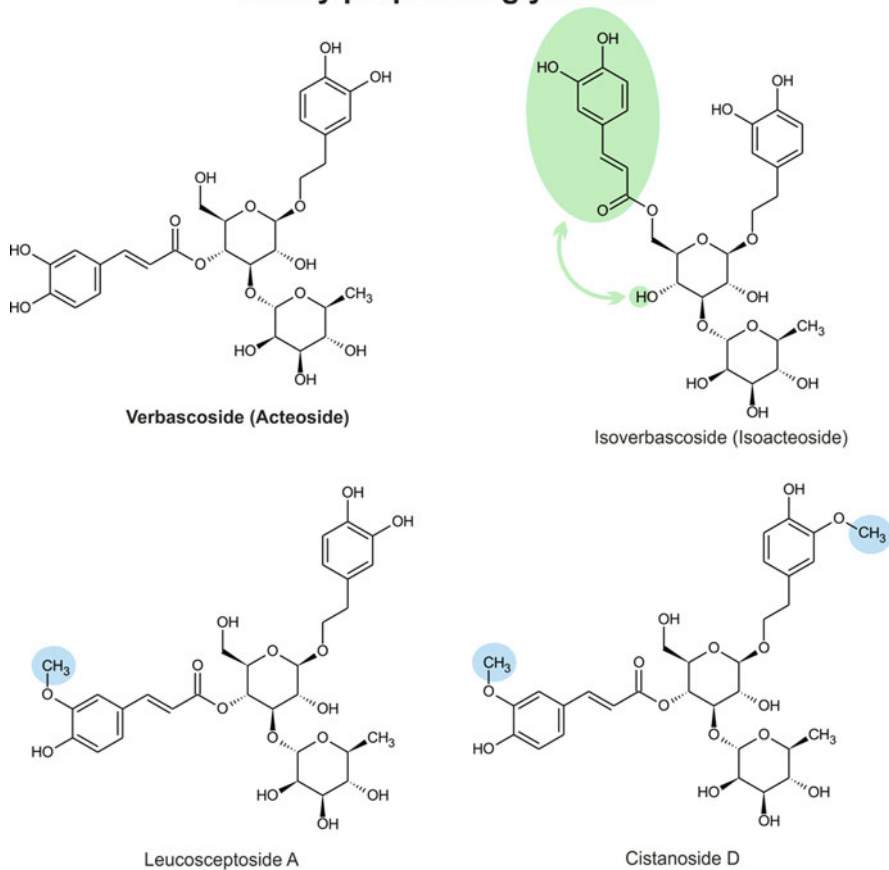
The UHPLC-hr-q-TOF/MS analysis showed that phenylpropanoid glycosides are the main compounds in the tested extracts. Extracts from agar microshoot cultures and the soil-grown plants were demonstrated to contain: verbascoside, isoverbascoside, leucoseptoside A and its two isomers and cistanoside D and its isomer. Additionally in plant extracts, the presence of 3,4-dihydroverbenalin was confirmed; however, this compound wasn't quantified (Fig. 29.7). The UHPLC-hr-q-TOF/MS analyses were conducted in the specialized research laboratory, Drug Screening Institute and Centrum für Chemie und Biomedizin in Innsbruck (Austria).

Quantitative analyses of the contents of verbascoside and isoverbascoside performed with HPLC method indicated that the amounts of phenylpropanoid glycosides differed slightly depending on the culture type. Verbascode was the quantitatively dominant compound in biomass extracts from all culture types. Its contents ranged from 4.72 g/100 g DW (bioreactor RITA[®]) through 4.78 g/100 g DW (stationary liquid cultures) and 4.82 g/100 g DW (agar cultures) to 4.88 g/100 g DW (agitated cultures). The amounts of isoverbascoside were more diversified but also of another order of magnitude, and they changed from 264.90 mg/100 g DW (stationary liquid cultures) through 266.50 mg/100 g DW (bioreactor RITA[®]) and 330.56 mg/100 g DW (agar cultures) to 451.80 mg/100 g DW (agitated cultures). The greatest total content of both compounds (5.33 g/100 g DW) was documented in extracts of biomass from agitated cultures. In microshoot cultures maintained in a RITA[®] bioreactor, the total content of both phenylpropanoid glycosides was 4.99 g/100 g DW. The analysis documented the absence of phenylpropanoid glycosides in extracts of lyophilized media from the tested culture types.

In the herb extracts, the content of verbascoside was 1.73 g% and isoverbascoside 78.34 mg/100 g DW. The levels of both phenylpropanoid glycosides in all extracts from in vitro cultures were larger than in the extracts of plant material. The amount of verbascoside accumulated in in vitro cultures was from 2.73- to 2.82-fold higher and that of isoverbascoside from 3.38- to 5.77-fold higher than in extract of the herb.

Among the tested phenolic acids, ferulic acid and protocatechuic acid were identified in all biomass extracts from *V. officinalis* microshoot cultures. However,

Phenylpropanoid glycosides



Iridoids

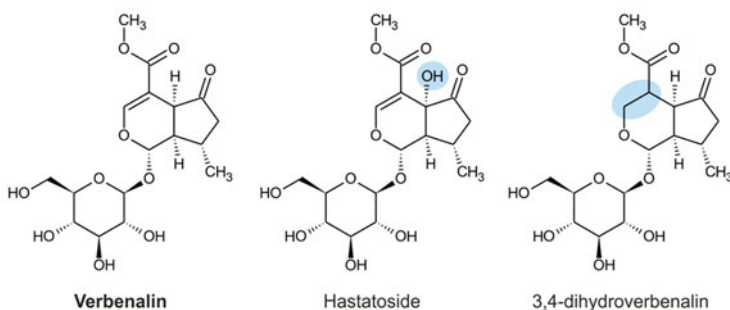


Fig. 29.7 Chemical structures of compounds confirmed using UHPLC-hr-q-TOF/MS in methanolic extracts from soil-grown plants. In biomass extract, only phenylpropanoid glycosides were documented

the levels of these compounds were low, namely, the content of ferulic acid did not exceed 24 mg/100 g DW, and that of protocatechuic acid was below 8 mg/100 g DW. The highest contents of phenolic acids were noted in extracts from agitated cultures. The herb extract was found to contain three phenolic acids: ferulic acid, protocatechuic acid, and rosmarinic acid (29.8 mg/100 g DW, 25.8 mg/100 g DW, and 2.5 mg/100 g DW, respectively).

The extracts of microshoot cultures were not confirmed to contain four compounds, which were identified in the herb extracts by our studies (Kubica et al. 2020b). These were two iridoids: verbenalin (4.21 g/100 g DW) and hastatoside (398.87 mg/100 g DW), and two flavonoids: scutellarin (172.1 mg/100 g DW) and apigenin-7-glucuronide (41.77 mg/100 g DW).

The establishment of different types of *V. officinalis* microshoot cultures (agar, stationary liquid, agitated, and bioreactor) showing good growth characteristics prompted us to check whether some types of microshoot cultures were capable of efficient production of essential oil, to examine its chemical composition and to compare essential oil production efficiency and chemical composition with the oil produced by soil-grown plants.

29.6.3 The Production of Essential Oil

The first stage of research involved maintaining the *V. officinalis* microshoot cultures for 2 years. They were cultivated on an agar-solidified SH medium supplemented with 2iP (2 mg/L) and TDZ (0.22 mg/L) and passaged in 4-week intervals. The cultures were periodically tested for the contents of volatile components using thin-layer liquid chromatography (TLC). The profile of compounds has not changed throughout 2 years from culture establishment. The accumulation of volatile compounds in biomass from in vitro cultures was remained at a stable level.

The experimental cultures were maintained in two types of commercially available temporary immersion bioreactors: RITA[®] and Plantform (Plant Form AB, Lomma, Sweden). Microshoot cultures grew on the liquid SH medium supplemented with 1 mg/L IBA. The inoculum/medium ratio depended on bioreactor type and amounted to 7 g/200 mL (RITA[®] bioreactor) or 17.5 g/500 mL (Plantform bioreactor). The immersion time was 5 min, and the rest time was 85 min. Growth cycles in RITA[®] bioreactors lasted 5 weeks. After that time, almost whole bioreactor vessel was filled with shoots, and first signs of necrosis (drying of shoot tips, discoloration of leaves) started to appear. In Plantform bioreactors, after a 5-week periods, a large volume of bioreactor vessel remained unused. Therefore, the studies were expanded by performing further experiments in which the growth cycle was extended by additional 3 weeks, while the fresh medium was supplemented after 5 weeks. The shoots elongated and proliferated filling a greater part of the bioreactor volume; however, the biomass growing for 56 days also exhibited visible signs of necrosis. The tested culture types were maintained in the laboratory of biotechnology, Department of Pharmacognosy, Medical University of Gdansk (Poland).

Phytochemical studies were performed on the biomass from agar microshoot cultures cultivated for 28 days, in RITA[®] bioreactors for 35 days, biomass from cultures grown in Plantform bioreactors for 35 and 56 days, and the herb of *V. officinalis* collected in the M. Koczwara Garden of Medicinal Plants, Jagiellonian University Medical College in Kraków (Poland). In order to obtain volatile fractions, the biomass was harvested from in vitro cultures, and the herb was dried and subjected to simultaneous hydrodistillation and extraction with dichloromethane using a Likens-Nickerson apparatus.

Qualitative analyses of essential oil were conducted with the use of gas chromatography with mass spectrometry (GC/MS), while gas chromatography with a flame-ionization detector (GC/FID) was used for quantitative analysis of the components. The analyses were performed in the Department of Analytical Chemistry of the Gdansk University of Technology (Poland).

The highest content of the oil of ca. 8 g/kg DW was obtained in biomass cultivated in Plantform bioreactors after 56 days and in the *V. officinalis* herb. The contents of the oil in shoots from agar cultures and in shoots grown in RITA[®] bioreactors were significantly lower. It was demonstrated that extension of 35-day growth cycle in Plantform bioreactors by another 3 weeks had a positive effect on the amount of essential oil produced.

The main compounds identified in the oil from biomass from *V. officinalis* microshoot cultures included: 1-octen-3-ol (4.7–57.7%), benzyl alcohol (6.0–8.5%), phenylethyl alcohol (5.9–11.4%), coumaran (5.0–19.3%), and hexadecanoic acid (0–22.4%). The type of in vitro culture had the greatest influence on 1-octen-3-ol, coumaran, and hexadecanoic acid contents. The oil from microshoots growing in both RITA[®] and Plantform bioreactors for 35 days contained 1-octen-3-ol as the dominating compound. In contrast, coumaran dominated in agar cultures, while hexadecanoic acid was present only in agar cultures and in Plantform bioreactor-grown cultures for 56 days. The extension of the experiment time in Plantform bioreactors up till 8 weeks resulted in reduction contents of all main constituents except from hexadecanoic acid, the concentration of which, increased by over 20%.

The composition of essential oil obtained from the *V. officinalis* herb significantly differed compared with the oil distilled/extracted from biomass cultivated under in vitro conditions. The main components of volatile fraction of *V. officinalis* herb included hexadecanoic acid (>20%) and in sequence linalool (>8%), anethole (>5%) and carvone (>3%), camphor, estragole, coumaran, α -terpineol, phytone, and isomentone (>2% each).

The results of analyses proved for the first time the presence of benzyl alcohol, phenylethyl alcohol, and coumaran among components of oil from the *V. officinalis* herb. The studies documented qualitative differences in the composition of the oil from microshoots cultured in vitro compared with *V. officinalis* herb under study. Moreover, the investigations documented that *V. officinalis* bioreactor cultures maintained in Plantform bioreactors for 8 weeks could produce high levels of essential oil, comparable with its content in the herb (ca. 8 g/kg DW).

The cultivation of plant material in bioreactor *in vitro* cultures can guarantee the possibility to the production of essential oil with constant controlled chemical composition. The main compounds of the oil, namely, 1-octen-3-ol, benzyl alcohol, phenylethyl alcohol, coumaran, and hexadecanoic acid, are known to possess biological activities, including antimicrobial (benzyl alcohol, phenylethyl alcohol), attracting insects (1-octen-3-ol), and insecticidal (benzyl alcohol and coumaran). Therefore, *V. officinalis* microshoot cultures conducted in Plantform bioreactors can be proposed as an alternative source for the extraction of essential oil (Kokotkiewicz et al. 2021).

29.7 Summary: Evaluation of Our Biotechnological Studies

29.7.1 Undifferentiated *In Vitro* Cultures

- *V. officinalis* callus cultures were successfully established from fragments of shoots of parent plant on the “initiation medium” (MS medium, BA—1 mg/L, NAA—1 mg/L).
- In agar callus cultures maintained under white fluorescent light and darkness, 12 variants of MS medium supplemented with BA + NAA, BA + IBA, Kin + NAA, and Kin + IBA were tested. The MS medium variant containing 1 mg/L BA and 1 mg/L IBA was selected as the optimal “growth” medium.
- The type and concentrations of PGRs tested in MS media variants and light conditions (white fluorescent light and darkness) influenced in agar callus cultures the contents of the investigation compounds: verbascoside and phenolic acids (free and bound). The maximum obtained contents of verbascoside reached 2.46 g% and 2.14 g% (white fluorescent light and darkness, respectively). The maximum contents of phenolic acids were many times lower—maximum 46 mg % (free acids) and 141 mg% (bound acids).
- In agar callus cultures, the maximum content of verbascoside was 3.3-fold higher than in the herb of the parent plant and that of free phenolic acids was 3.2-fold higher and bound phenolic acids 2.1-fold higher versus the herb.
- In agar callus cultures maintained on MS variant (1 mg/L BA and 1 mg/L IBA), the tested light conditions (monochromatic LED lights, white fluorescent light (control), and darkness) significantly affected the contents of two phenylpropanoid glycosides under study: verbascoside and isoverbascoside and phenolic acids (free acids). Under LED lights, the highest content of verbascoside (6.72 g%) was documented in biomass growing in the presence of blue light while that of isoverbascoside (378.90 mg/100 g DW), under combination of red and blue light (70%/30%). The highest total content of phenolic acids (65.09 mg/100 g DW) was also seen in extracts of biomass growing in the presence of the combination of red and blue light.
- The maximum amount of verbascoside obtained in biomass extracts from the studied agar callus cultures maintained under LED lights was 3.8-fold higher and

that of isoverbascoside 4.9-fold higher than in extracts from the herb of soil-grown plants. The maximum content of phenolic acids in biomass extracts from *in vitro* cultures was almost identical as in the herb extracts (ca. 65 mg/100 g DW).

- In agar callus cultures maintained under monochromatic LED lights, the investigation on the optimal duration of growth cycles (2-, 3-, and 4-week cycles) indicated that the accumulation of verbascoside, isoverbascoside, and phenolic acids changed in accordance with light conditions and differed among compounds. The maximum amount of verbascoside (6.72 g%) was obtained in the presence of blue light after 4-week growth cycles. High contents of this compound (6.02 g%) were also obtained after 4 weeks under combination of red and blue light. The maximum content of isoverbascoside (382.51 mg/100 g DW) was noted in control conditions (white fluorescent light) also after 4-week growth cycles. Similar values (378.90 and 378.47 mg/100 g DW) were found in the presence of combination of red and blue lights and in the darkness also after 4-week growth cycles. The dynamics of accumulation of phenolic acids was different. Their highest contents amounting to 65.09 mg/100 g DW and 63.95 mg/100 g DW were documented, respectively, in extracts from biomass growing under combination of red and blue lights after 2-week growth cycles and in the darkness after 4-week growth cycles.
- Suspension cultures and bioreactor cultures of undifferentiated biomass in two types of bioreactors (balloon bioreactor (BB) and stirred tank bioreactor (STB)) were successfully established for the first time.
- The contents of verbascoside, isoverbascoside, and phenolic acids were determined in extracts from biomass from agar callus cultures, suspension cultures, and bioreactor cultures (BB and STB bioreactors). Extracts of biomass from the abovementioned culture types contained very different contents of verbascoside (from 7.06 g% to 9.18 g%) and isoverbascoside (from 306.40 to 609.26 mg/100 g DW). The extracts were confirmed to contain six phenolic acids (protocatechuic, chlorogenic, vanillic, caffeic, ferulic, and rosmarinic acids). Two acids quantitatively dominated: ferulic acid and protocatechuic acid. The total content of phenolic acids was low and ranged from 19.88 to 54.90 mg/100 g DW (Tables 29.5 and 29.6).
- The highest contents of verbascoside were obtained in commercially available STB bioreactor, isoverbascoside, in agar callus cultures and the highest total amounts of phenolic acids in suspension cultures (Tables 29.5 and 29.6).
- In the extracts of the herb of soil-grown plants, the presence of verbascoside (1.73 g%), isoverbascoside (78.34 mg/100 g DW), and three phenolic acids: ferulic acid (29.76 mg/100 g DW), protocatechuic acid (25.75 mg/100 g DW), and rosmarinic acid (2.53 mg/100 g DW), was confirmed (Tables 29.5 and 29.6). Extracts of *in vitro* cultures were found to contain additionally chlorogenic, vanillic, and caffeic acids. Whereas the presence of four other compounds was documented in the herb extracts, which were not present in extracts of biomass from *in vitro* cultures, namely, two iridoids, verbenalin (4.21 g%) and hastatoside

Table 29.5 Maximal contents of individual phenylpropanoid glycosides and their highest total contents [mg/100 g dry weight ± OS] in extracts from biomasses of all studied types of in vitro cultures and from the herb of *V. officinalis*

Type of culture	Verbascoside	Isoverbascoside	Total content of investigated compounds
Undifferentiated cultures	Agar	609.26 ± 22.48	7857.92 ± 276.24
	Suspension	477.25 ± 11.52	7536.63 ± 46.13
	BB bioreactor	306.4 ± 25.2	7991.4 ± 525.59
	STB bioreactor	339.91 ± 41.9	9516.79 ± 214.24
Microshoot cultures	Agar	330.56 ± 0.17	5148.78 ± 80.06
	Stationary liquid	264.9 ± 1.33	5042.01 ± 6.56
	Agitated	451.8 ± 2.85	5333.42 ± 102.55
	RITA® bioreactor	266.5 ± 2.32	4988.7 ± 145.06
Herb	1728.97 ± 29.78	78.34 ± 3.28	1807.3 ± 33.05

Table 29.6 Maximal contents of individual phenolic acids and their highest total contents [mg/100 g dry weight $\pm$ OS] in extracts from biomasses of all studied types of in vitro cultures and from the herb of *V. officinalis*

Type of culture	Ferulic acid	Protocatechuic acid	Rosmarinic acid	Chlorogenic Acid	Vanillic acid	Caffeic acid	Total content of investigated compounds
Undifferentiated cultures	Agar	7.35 $\pm$ 0.03	9.58 $\pm$ 0.01	3.79 $\pm$ 0.55	1.77 $\pm$ 0.03	1.28 $\pm$ 0.01	27.29 $\pm$ 0.64
	Suspension	10.44 $\pm$ 0.03	10.64 $\pm$ 0.06	26.34 $\pm$ 1.22	1.25 $\pm$ 0.04	1.43 $\pm$ 0.04	54.9 $\pm$ 1.45
	BB bioreactor	2.12 $\pm$ 0.67	5.07 $\pm$ 1.12	10.22 $\pm$ 2.47	0.28 $\pm$ 0.1	0.59 $\pm$ 0.36	19.88 $\pm$ 5.51
	STB bioreactor	7.1 $\pm$ 0.85	9.68 $\pm$ 0.9	14.87 $\pm$ 2.74	0.73 $\pm$ 0.09	0.73 $\pm$ 0.15	36.78 $\pm$ 4.9
Microshoot cultures	Agar	8.35 $\pm$ 0.54	3.76 $\pm$ 0.07	nd ^a	nd	nd	12.11 $\pm$ 0.6
	Stationary liquid	17.19 $\pm$ 0.48	5.3 $\pm$ 0.19	nd	nd	nd	22.5 $\pm$ 0.66
	Agitated	23.69 $\pm$ 0.21	7.59 $\pm$ 0.48	nd	nd	nd	31.27 $\pm$ 0.69
	RTA [®] bioreactor	16.66 $\pm$ 0.75	3.51 $\pm$ 0.08	nd	nd	nd	20.17 $\pm$ 0.83
Herb	29.76 $\pm$ 2.51	25.75 $\pm$ 1.65	2.53 $\pm$ 0.11	nd	nd	nd	58.04 $\pm$ 4.17

^a nd not detected

(398.87 mg/100 g DW), and two flavonoids: scutellarin (172.13 mg/100 g DW) and apigenin-7-glucuronide (41.77 mg/100 g DW) (Table 29.6).

- The maximum content of verbascoside obtained in extracts of biomass from the tested types of in vitro cultures was 5.3-fold higher and isoverbascoside 7.8-fold higher than in the herb of soil-grown plants.
- The contents of the tested phenylpropanoid glycosides in extracts of biomass from all types of undifferentiated in vitro cultures were higher than in extracts of the herb of soil-grown plants. The level of verbascoside was from 4.1-fold (agar callus cultures) to 5.3-fold (bioreactor cultures, STB) higher and isoverbascoside from 3.9-fold (bioreactor cultures, BB) to 7.8-fold higher (agar callus cultures). The total content of phenolic acids was comparable (in suspension cultures) or from 1.6-fold (STB) to 2.9-fold (BB) lower than in the herb extracts.
- Based on the conducted biotechnological studies, undifferentiated cultures of *V. officinalis* can be proposed as a very rich source of verbascoside, especially, the cultures maintained in the commercially available STB bioreactor in which an extremely high content of verbascoside (9.18 g%) and a satisfactory amount of isoverbascoside (339.91 mg/100 g DW) was obtained (Table 29.5).

29.7.2 Microshoot Cultures

- *V. officinalis* microshoot cultures have been successfully established on the “initiation” medium (MS medium; BA—1 mg/L, IBA—mg/L) from the shoot fragments of parent plant.
- For microshoot cultures, SH medium with the addition of two cytokinins: 2 mg/L 2iP and 0.22 mg/L TDZ, was selected as the optimal growth culture medium that allowed for obtaining high biomass increments and satisfactory morphological features of microshoots.
- These cultures were also subjected to optimization of culture conditions in the aim scaling up the process from agar cultures through stationary liquid and agitated cultures to bioreactor cultures in commercially available temporary immersion bioreactors (RITA[®]).
- Extracts from microshoot cultures (agar cultures) and the herb from the soil-grown plants were confirmed to contain verbascoside and isoverbascoside using UHPLC-hr-q-TOF/MS method. Moreover, other phenylpropanoid glycosides, leucoseptoside A and its two isomers and cistanoside D and its isomer, were confirmed in the abovementioned extracts using this method.
- Extracts from all types of microshoot cultures and extract of the herb from soil-grown plants were tested for the contents of verbascoside, isoverbascoside, and phenolic acids using HPLC method. The amounts of verbascoside in all culture types were similar and fluctuated in a narrow range from 4.72 g% to 4.88 g%. The level of isoverbascoside was lower and more variable from 264.90 to 451.80 mg/100 g. Phenolic acids were accumulated in small amounts. Their total content ranged from 12.11 to 31.27 mg/100 g. Among all tested microshoot culture types,

the highest accumulation of both verbascoside and isoverbascoside, and phenolic acids was obtained in agitated cultures (Tables 29.5 and 29.6).

- Volatile compounds in essential oil obtained from biomass of microshoots cultivated in two temporary immersion bioreactors (RITA[®] and Plantform) and in the herb of soil-grown plants were determined, and the highest, almost the same amounts (ca. 8 g/kg DW) were documented in biomass cultured in Plantform bioreactors for 56 days and in the herb.
- The dominating compounds present in essential oil from biomass of microshoot cultures included: 1-octen-3-ol (max. 57.7%), benzyl alcohol (max. 8.5%), phenylethyl alcohol (max. 11.4%), coumaran (max. 19.3%), and hexadecanoic acid (max. 22.4%). The composition of essential oil from *V. officinalis* herb significantly differed compared with the essential oil obtained from the biomass grown in vitro. Its main components were hexadecanoic acid (22.92%), linalool (8.60%), anethole (5.50%), and carvone (3.39%).
- Based on the conducted biotechnological studies of microshoot cultures, bioreactor microshoot cultures maintained in bioreactors RITA[®] can be proposed as a rich source of verbascoside (4.72 g%) and isoverbascoside (266.50 mg/100 g DW). Despite the fact that higher contents of both abovementioned compounds were obtained in agitated microshoot cultures (4.88 g% and 451.8 mg/100 g DW), the bioreactor cultures, due to the possibility of parallel connection of many bioreactors at the same time, can be proposed as potential rich source of these compounds.
- Furthermore, microshoot cultures maintained in commercially available Plantform bioreactors can be proposed as a rich source of essential oil with stable chemical composition, which components however are different than those of the essential oil from *V. officinalis* herb.

29.8 Conclusions and Prospects

- The established well-growing *V. officinalis* in vitro cultures of different degrees of differentiation can be proposed as a good model for further biotechnological studies on the possibility of increasing production of the compounds under study, that is, verbascoside and isoverbascoside, by implementation of known strategies in plant biotechnology, such as precursor feeding and elicitation. The use of these strategies can be expected both to increase the accumulation of verbascoside, isoverbascoside, and phenolic acids and to direct biosynthetic pathways within in vitro cultured cells to the production other bioactive compounds characteristic of soil-grown plants, for example, iridoids or flavonoids.
- It would also be essential to quantify other phenylpropanoid glycosides in the biomass cultivated in vitro, leucoseptoside A and its isomers and cistanoside D and its isomer, the presence of which was confirmed by UHPLC-hr-q-TOF/MS method, and to define the effect of the mentioned strategies (e.g., precursor feeding and elicitation) on the production of these compounds.

- The investigated *V. officinalis* cultures did not show an ability to produce iridoids, which are the main compounds in the vervain herb. However, the advantage of in vitro cultures, especially those maintained in the commercially available bioreactor, STB, consists in a very high content of verbascoside (over 9 g%). So high contents of this compound and lower amounts of accompanying other compounds may make its isolation and purification easier. It is a significant advantage that supports the proposal to use *V. officinalis* in vitro cultures as a rich source of verbascoside for pharmaceutical and/or health food and/or cosmetic industry.
- This main product possess a lot of valuable biological activities, for example, antioxidant, anti-inflammatory, cytoprotective, antibacterial, antiviral, and antiandrogen activities, which are important in therapy health-promoting and cosmetology (Fu et al. 2008; Alipieva et al. 2014; Xue and Yang 2016).
- The maximum contents of verbascoside obtained in *V. officinalis* bioreactor cultures (STB bioreactor—9.2 g%) are among the highest contents obtained in different types of in vitro cultures of many plant species conducted so far (Table 29.7). This content can be even further enhanced by, for example, feeding with biosynthetic precursors or elicitation.
- Essential oil can be obtained from bioreactor microshoot cultures of *V. officinalis* (Plantform bioreactor) in comparable quantities as those present in the herb (ca. 8 g/kg DW). However, its composition is different compared with the essential oil obtained from the *V. officinalis* herb. It would be of interest to perform further studies in order to examine its biological activity and compare their biological activities. The possibility of high accumulation of essential oil in bioreactor cultures could potentially increase the scale of its production. It is possible to connect multiple Plantform bioreactors in parallel and conduct cultures at the same time in many number of bioreactors.

Table 29.7 The highest verbascoside production in in vitro cultures of various plant species, a review of scientific research

Verbascoside (maximal content)	Species	Type of culture	References
11.6 g/100 g DW	<i>Buddleja cordata</i>	Suspension cultures	Estrada-Zúñiga et al. (2009)
9.5 g/100 g DW	<i>Paulownia tomentosa</i>	Hairy root cultures	Wysokińska and Rózga (1998)
9.3 g/100 g DW	<i>Plantago media</i>	Microshoot cultures	Budzianowska et al. (2019)
9.18 g/100 g DW	<i>Verbena officinalis</i>	Bioreactor cultures (STB)	Kubica et al. (2020b)
8.6 g/100 g DW	<i>Buddleja cordata</i>	Callus cultures	Estrada-Zúñiga et al. (2009)
2.2–6.0 g/100 g DW	<i>Rehmannia glutinosa</i>	Hairy root cultures	Grąbkowska et al. (2010), Piąteczak et al. (2015, 2016), Rahmat et al. (2021)
4.88 g/100 g DW	<i>Verbena officinalis</i>	Agitated microshoot cultures	Kubica et al. (2022)
4.5 g/100 g DW	<i>Verbascum thapsus</i>	Callus cultures	Temporiti et al. (2020)
4.0 g/100 g DW	<i>Verbascum thapsus</i>	Microshoot cultures	Temporiti et al. (2020)
3.8 g/100 g DW	<i>Cistanche deserticola</i>	Suspension cultures (with addition of elicitors)	Chen Chun-Ming Xu et al. (2007)
3.2 g/100 g DW	<i>Verbascum thapsus</i>	Suspension cultures	Temporiti et al. (2020)
1.1 g/100 g DW	<i>Rehmannia glutinosa</i>	Microshoot cultures	Piāteczak et al. (2015)
165 mg/L/day	<i>Harpagophytum procumbens</i>	Bioreactor cultures	Georgiev et al. (2011)
689 mg/L	<i>Cistanche salsa</i>	Suspension cultures (with addition of precursors)	Liu et al. (2007)

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