

Ravindra H. Patil · Mohini P. Patil ·  
Vijay L. Maheshwari

# Apocynaceae Plants

Ethnobotany, Phytochemistry,  
Bioactivity and Biotechnological  
Advances

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Ravindra H. Patil  
Department of Microbiology  
and Biotechnology  
R. C. Patel Arts, Commerce and Science  
College, Shirpur  
Dhule, Maharashtra, India

Mohini P. Patil  
Department of Microbiology  
and Biotechnology  
R. C. Patel Arts, Commerce and Science  
College, Shirpur  
Dhule, Maharashtra, India

Vijay L. Maheshwari  
Department of Biochemistry,  
School of Life Sciences  
KBC North Maharashtra University  
Jalgaon, Maharashtra, India

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*To,  
Our student, late Dr. Bhushan*

# Preface

Plant biodiversity on Earth is incredibly diverse, with an estimated 391,000 plant species known to science as of 2021. This includes a wide variety of plants, ranging from tiny algae to towering trees, and from mosses to flowering plants. Angiosperm Phylogeny Group (APG) recognizes approximately 416 plant families, while other taxonomic systems may recognize a different number of families. Plant biodiversity is not evenly distributed across the planet. The tropics, particularly rainforests, are the most diverse regions, containing the highest numbers of plant species per unit area. Other areas with high plant biodiversity include Mediterranean-type ecosystems, temperate forests, and grasslands. Plants play a vital role in the environment, contributing to its health and sustainability in several ways. Conservation efforts are therefore crucial for maintaining plant biodiversity. These efforts may include protecting natural habitats, implementing sustainable land-use practices, promoting the use of native plant species in landscaping, and establishing seed banks and botanical gardens to preserve threatened species. By preserving plant biodiversity, we can help ensure the continued health and productivity of our planet and its ecosystems. Despite their ecological and economic importance, many species in the Apocynaceae family are threatened by habitat destruction, climate change, and other human activities. Conservation efforts, such as the establishment of protected areas and the cultivation of threatened species, are essential for preserving the biodiversity and ecological functions of this important plant family. Apocynaceae is one of the largest families in the plant kingdom. Robert Brown separated Asclepiadoideae from Apocynaceae for a practical reason. According to this updated classification, the family Apocynaceae consists of 424 genera with more than 4600 species distributed in five subfamilies: Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae, and Asclepiadoideae. Most of the members of this family are poisonous. Plants in the Apocynaceae family are diverse in appearance and habitat. Many are woody shrubs or small trees, while others are climbers or epiphytes as well as herbaceous. One of the most well-known genera in the Apocynaceae family is the genus *Vinca*, which includes the common garden plant, *Vinca minor*. Other familiar genera include *Apocynum*, which is also known as

dogbane, and Nerium, which includes the ornamental plant, oleander. Chapter 1 provides an outline of the morphology, ecology, taxonomy, diversity, habitat, and geographical distribution of the Apocynaceae family. Applications of medicinal plants and their products for health management are an imperative part of tribal culture. Indian tribal people have a tremendous passion and knowledge of medicinal plants, and they use them for a wide range of health-related applications. Traditionally, local tribal communities have explored the medicinal uses of hundreds of plants found locally in their ecosystem. Today, all over the world, there is a growing appreciation of the value of traditional knowledge, and therefore, there is renewed interest among plant scientists to explore the heritage of traditional knowledge. It is believed that it can make a significant contribution to sustainable development. Most indigenous and local communities are situated in areas where the vast majority of the world's plant genetic resources are found. Many of them have been cultivated and used in a sustainable way for thousands of years. Chapter 2 highlights the traditional knowledge, ethnomedicinal importance, and practical uses of Apocynaceae members. Secondary metabolites are produced by plants through subsidiary pathways that are not directly involved in basic cellular processes and therefore are not essential for the host. However, they are responsible for mediating environmental interactions and are crucial for survival and reproduction. Plants are known to produce more than 50,000 different secondary metabolites. Secondary plant metabolites are the basis for the therapeutic effects of many modern medications and medicinal plants. The discovery of novel secondary metabolites is a continuous process that intends to discover a novel molecule with better therapeutic value. Chapter 3 highlights the role of the plant secondary metabolites as a source of new drugs. Many species in the Apocynaceae family contain chemical compounds with medicinal properties, including cardiac glycosides, alkaloids, and terpenoids. Chapter 4 describes the principle, methodology of extraction, and isolation of secondary metabolites from Apocynaceae plants.

The phytochemical tests are usually performed to determine the presence or absence of major classes of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, and phenolic compounds. These tests are often performed using simple colorimetric tests or specific reagents that react with the target compounds. Chapter 5 provides details about phytochemical screening and chemical characterization of the bioactive metabolites. A large-scale separation and purification of metabolites can be carried out using modern characterization techniques such as ultraviolet spectroscopy (UV), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), and mass spectrometry (MS) that are commonly used for the complete identification of the compound. Chapter 6 highlights the identification of bioactive secondary metabolites of Apocynaceae members using these modern spectroscopic and chromatographic techniques. Apocynaceae plants are rich in cardiac glycosides, terpenoids, flavonoids, tannins, steroids, proanthocyanidins, pigments, and other bioactive metabolites, which are widely used for various medicinal applications. Chapter 7 presents a detailed outlook of phytochemistry aspects of Apocynaceae members. Ethnomedicinal uses and traditional knowledge of Apocynaceae plants have resulted in the extensive evaluation of the

pharmacological properties of many members of this family. Crude extracts as well as isolated compounds are known to possess a wide range of bioactivities, such as antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, and cytotoxic activities. Chapter 8 provides a thorough update on the biological and pharmacological properties of Apocynaceae members. Agricultural insects and pests cause damage to crops, livestock, or stored products. Biopesticides are a type of pesticide derived from natural sources such as plants, microbes, and other naturally occurring substances. Apocynaceae plants have been widely used as a source of insecticidal metabolites. Chapter 9 highlights the agricultural importance of Apocynaceae members with emphasis on their pesticidal and insecticidal properties. Plant tissue culture has been used as a major platform for obtaining medicinally and industrially important secondary metabolites. Apocynaceae plants have been widely explored for micropropagation, *in vitro* propagation, and hairy root culture using tools and techniques of plant tissue culture. Chapter 10 highlights the plant tissue culture-based approaches for the production of pharmaceutically important bioactive compounds from Apocynaceae members. Enzymes and enzyme inhibitors have been used in various industries for many centuries. Chapter 11 explains the enzymes and enzyme inhibitors from Apocynaceae plants with reference to their industrial and biomedical applications. Endophytes are the microbes that inhabit the internal parts of plants regardless of the outcome of the association. Apocynaceae plants are known to harbor diverse endophytic microorganisms. Chapter 12 highlights the endophytes of Apocynaceae plants with reference to their biodiversity, bioactivities, and biotechnological significance. Due to overexploitation, many species of the family Apocynaceae are on the verge of extinction, and conservation efforts are urgently needed. Micropropagation is considered the best solution for the conservation of elite genotypes of plants. Chapter 13 explains the micropropagation-based conservation strategies for Apocynaceae plants. Nanotechnology is a branch of applied science and technology whose basis includes the control and synthesis of matter on a molecular level in the range of 1–100 nm. It involves the synthesis, characterization, and application of nanomaterial in various fields. Plant-based synthesis of nanoparticles using Apocynaceae plants is a green synthesis approach that utilizes Apocynaceae plants and their extracts to produce nanoparticles. This method is considered eco-friendly because it uses natural, renewable, and biodegradable materials and avoids the use of toxic chemicals and solvents. Chapter 14 thoroughly describes the principle, methodology, and practical applications of Apocynaceae plants in the field of nanotechnology.

We hope that the book will be of great interest to plant biologists, medicinal chemists, and natural product chemists. Moreover, this book offers a vast panorama of structures and bioactivities of natural products from the plants of the Apocynaceae family. It is hoped that the present book will be received with the same enthusiasm and interest as our previous work on different topics.

I would like to express my sincere thanks to Mr. Fumiko Yamaguchi, Editor, Books, Biomedical & Life Sciences, Springer Nature Singapore Pvt. Ltd., Singapore, for coordinating the initial review process of this book and giving us the opportunity to work on this project. We are grateful to the assistance of



Ms. Michelle Harding, Project Coordinator for Springer Nature books. We hope that the contents of the book will serve as a useful resource for all those working in the area and will be received with enthusiasm and interest.

We are thankful to Hon. Amrishbhai Patel, Hon. Bhupeshbhai Patel, and Hon. Rajgopal Bhandari and Principal of the college for their continued support, encouragement and for providing the necessary facilities to undertake research and write the books.' The support of my family members especially Utkarsh and Shreya, friends, and well-wishers is lovingly acknowledged.

Shirpur, Maharashtra, India  
Shirpur, Maharashtra, India  
Jalgaon, Maharashtra, India  
May 2023

Ravindra H. Patil  
Mohini P. Patil  
Vijay L. Maheshwari

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## About the Authors

**Ravindra H. Patil** is working as Professor in the Department of Microbiology and Biotechnology at R. C. Patel Arts, Commerce and Science College, Shirpur, MS, India. His areas of research are in microbial and plant secondary metabolites and endophyte derived natural products. He has over eighteen years of teaching experience in microbiology and biotechnology for graduate students. Six students have been awarded with PhD degrees under his guidance and four are currently working. He has published over fifty research and review articles in national and international peer-reviewed journals. He has authored three textbooks for undergraduate students. He has also served as an editor for two books published by Springer Nature Singapore Pvt. Ltd., Singapore. Dr. Patil has received the Young Scientist award from the Govt. of India, Dept. of Science & Technology, in the form of a research grant. He has successfully handled several research projects funded by various federal funding agencies.

**Mohini P. Patil** is working as Asst. Professor in the Department of Microbiology and Biotechnology at R. C. Patel Arts, Commerce and Science College, Shirpur, MS, India. She has over ten years of teaching and research experience in microbiology and biotechnology. Dr. Patil has received Junior Research Fellowship from the Department of Science and Technology (DST, New Delhi), Government of India, in 2013. She has completed her doctoral research on endophytic microorganisms and their bioactive metabolites. Dr. Patil was also awarded a postdoctoral fellowship from the Indian Council of Medical Research (ICMR, New Delhi), Government of India, for her work on novel enzyme inhibitors of biomedical importance. Her areas of research are endophytic fungi and plant secondary metabolites of biological and pharmacological significance. She has guided over twenty students for their postgraduate dissertations. She has published over 15 research and review articles in national and international peer-reviewed journals. She has coauthored one textbook for undergraduate students.



**Vijay L. Maheshwari** is currently serving as the Vice-Chancellor at Kavayitri Bahinabai Chaudhari North Maharashtra University, Jalgaon, MS, India. He has teaching experience of over 30 years. Prof. Maheshwari has received the “Young Scientist” award by IUBMB, India, in 1994. He is also a recipient of the BOYSCAST fellowship of Govt. of India, Dept. of Science & Technology for postdoctoral research at the University of Virginia, USA. He was Norman E. Borlaug fellow of US Department of Agriculture, USA, and undertook advanced training in Risk Assessment in Genetically Modified Food. He has served as the Regional Editor for the journal *Physiology and Molecular Biology of Plants*. He is a honorary Fellow of the Society of Science and Environment (FSSE) and the Maharashtra Academy of Sciences. Prof. Maheshwari has received the Best Teacher Award (2009–2010) from the Government of Maharashtra for his outstanding contribution in teaching and research. He has developed and transferred one technology to a local industry on the isolation of a pesticidal principle from seeds of *Annona squamosa* and provided consultancy to industries. He has attended more than 20 national and international conferences. He has published around one hundred research papers and review articles in journals of national and international repute. He has also served as editor for two books published by Springer Nature Singapore Pvt. Ltd., Singapore, and authored three textbooks. He has successfully handled several research projects and supervised 22 students for doctoral degrees.

His areas of research/expertise include protein biochemistry, plant tissue culture, and secondary metabolites and their biological activities. (A detailed profile is available on [www.nmu.ac.in](http://www.nmu.ac.in).)

# Chapter 1

## Morphology, Ecology, Taxonomy, Diversity, Habitat and Geographical Distribution of the Apocynaceae Family

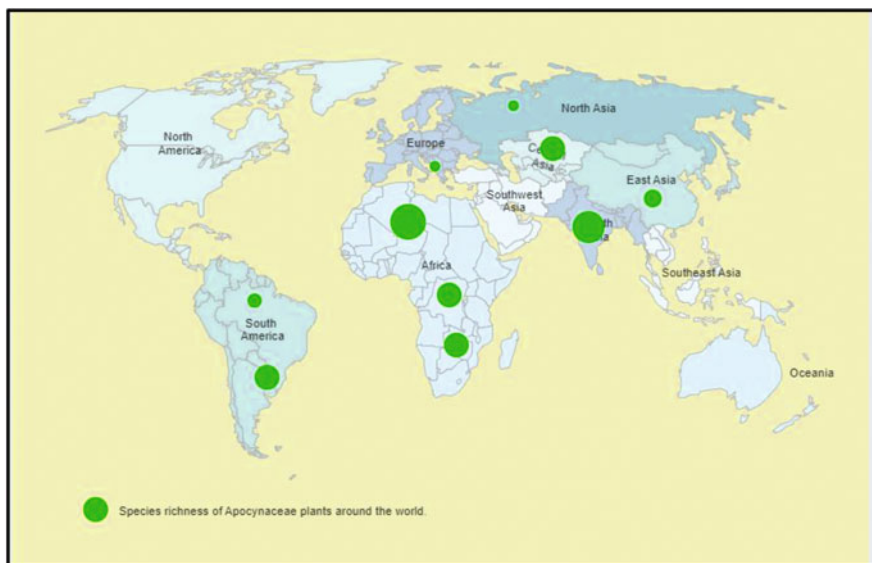


### Introduction

Apocynaceae is an important family in angiosperms, also known as the dogbane family of flowering plants. It is a large family comprising approximately 5000 species and 415 genera according to updated classification (Endress et al., 2014). The family is widely distributed across tropical and subtropical regions of the world, although some species can also be found in temperate regions. Robert Brown (1810) separated the family Asclepiadiaceae from Apocynaceae for easier understanding of these two large families. Apocynaceae consists of five subfamilies: Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae, and Asclepiadoideae. Most of the members of this family are poisonous. Apocynaceae plants are rich in cardiac glycosides and various alkaloids and therefore are widely used for various medicinal applications.

### Geographical Distribution

Plants of the family Apocynaceae are native throughout India, Pakistan, China, Bangladesh, and Sri Lanka (Mahmood et al., 2011). Several members of this family are abundantly found in the tropics and subtropics (Fig. 1.1). Members of the Apocynaceae family are trees and shrubs or herbaceous plants with milky latex. The majority of the plants in this family are abundant in alkaloids and have significant therapeutic value. Additionally, many species are planted for decorative purposes. People in rural regions utilise some Apocynaceae species as food and some of them as poisons (Aiyambo, 2010). Due to the widespread use of Apocynaceae plants for medicinal purposes, members of this family are found in many different traditional medical practices, including Indian, Chinese, and Thai medicine (Van Beek et al., 1984; Bhat et al., 2012). The Indian subcontinent has a



**Fig. 1.1** Species richness of Apocynaceae plants around the world

dominant presence of Apocynaceae plants. Approximately 84 species belonging to 30 genera are present in India.

## Habitat

Apocynaceae plants show universal distribution. Birds and anthropological activities were found to be responsible for their spread in many parts of the world. This family's members can be found in temperate, subtropical, and tropical regions. The majority of the vegetation in this family is trees and shrubs, while subshrubs and herbs are very infrequently encountered. According to Oze et al. (2007), *Alstonia scholaris* (R. Br.) is found all over the tropics and rainforests of West and Central Africa. According to Motwani et al. (2012), *Carissa carandas* (Linn.) is a natural and widespread plant in India, Sri Lanka, Java, Malaysia, Myanmar, and Pakistan. Originating from Madagascar Island, *Catharanthus roseus* (Linn.) has been widely farmed for hundreds of years and may now be found growing wild in most warm climates across the world (Aslam et al., 2010). *Holarrhena antidysentrica* (Wall) is another important member of this family distributed across India (Shah et al., 2010). *Nerium oleander* is a beautiful ornamental tree 10–12 ft. in height grown alongside roads and highways due to its aesthetic appearance and beautiful flowers. It is native to North Africa and Southeast Asia (Dolgova & Ignateva, 1963). *Rauvolfia serpentina* (Benth. ex Kurtz) is native to Asia and found in Thailand and Burma. In India, it is abundantly present in Punjab, Haryana. The Himachal Pradesh, Sikkim

and Himalayan regions include Bhutan (Vakil, 1955). According to Muzafar and Daulatabadi (1986), *Tabernaemontana divaricata* (Linn.) can be found in North India up to an elevation of 3000 feet in the Konkan, Western Ghats in Malabar, and other regions of India. In tropical and subtropical areas, *Thevetia peruviana*, sometimes known as yellow oleander, is found (Langford & Boor, 1996). *Wrightia tinctoria* (R. Br.) is particularly common in India. In southern India, it is widely referred to as Indrajav or a tree that treats jaundice (Ramchandra et al., 1993).

## General Characteristics of the Apocynaceae Family

The members of the family Apocynaceae are mostly herbs, and some are trees and shrubs bearing white and milky latex. Leaves are simple, alternate, opposite, or whorled, and the margin is entire. Flowers are actinomorphic to hermaphrodite and hypogynous. The calyx is either free or united. The corolla is gamopetalous, funnel-shaped, valvate, or twisted. The androecium is epipetalous and introrse, whereas the gynoecium is bicarpellary and syncarpous. The ovary is superior and united by the styles only. The fruits are of a follicle or berry type. The seeds usually have a crown of hairs.

### *Vegetative Characteristics*

**Root System:** Highly complex roots with an extensively branched tap root system.

**Stem:** Erect, herbaceous, erect, solid and woody, solid, branched to unbranched, succulent with latex.

**Leaves:** Simple, alternate, opposite, or whorled, the margin is entire.

### Floral Characteristics

#### **Inflorescence:**

Cymose is the most common inflorescence among Apocynaceae plants, terminal or axillary, rarely cyme (Genus: *Carissa*) or umbellate cyme (Genus: *Rauwolfia*).

#### **Flower:**

Ebracteate or Bracteate, complete, pedicellate, hermaphrodite, tetra- or pentamorous actinomorphic.

#### **Calyx:**

Sepals 5, the calyx is of gamo- or polysepalous type, small, often granular, deeply lobed, imbricate, or valvate.

#### **Corolla:**

Gamopetalous, with five petals and a corona present (hairy scales or protrusion), usually twisted, occasionally imbricate, and infrequently valvate.

**Androecium:**

Stamens 4–5, alternipetalous, epipetalous, filament short, free, ditheous, connate to stigma, dehiscing longitudinally, introrse.

**Gynoecium:**

Ovaries are free, superior style is short, bicarpellary, syncarpous, and contained in a tube-shaped corolla. Distally, the stigma is thickened. With marginal placentation, the ovary is unilocular; however, when the ovaries are united, axile placentation takes place.

**Fruit:**

The fruits are of a follicle or berry type. A drupe (Thevetia), berry (Carissa), and follicle (Nerium, Amsonia) are also reported among the Apocynaceae members.

**Seed:**

The seeds usually have a crown of hairs. Endospermic, hairy or winged.

**Pollination:**

Entomophilous (through insects).

**General Outline of the Classification of the Family Apocynaceae**

For practical considerations, Robert Brown (1810) separated the asclepiads from Jussieu's (1789) Apocynaceae and designated them as a new family, Asclepiadaceae. Traditionally, these two are recognised as separate but highly similar families for nearly 200 years. Based on morphological and molecular characteristics, Endress and Bruyns (2000) reunited the two families and accepted five subfamilies, namely, Periplocoideae, Rauvolfioideae, Asclepiadoideae, Secamonoideae, and Apocynoideae. Endress et al. (2014) presented an updated suprageneric classification for Apocynaceae based on recent morphological, molecular, and mainly phylogenetic-based results. According to this classification, Apocynaceae includes a total of 366 genera placed within five subfamilies, 25 tribes, and 49 subtribes (Fig. 1.2).

According to *The Plant List*, Apocynaceae contains 410 plant genera. The Plant List database includes scientific names of over 18,268 species for this family. Of these, 5556 are accepted species names. The Plant List includes a further 1996 scientific plant names of intraspecific rank for the family Apocynaceae. These are primarily included because species rank names are synonyms of accepted intraspecific names. Out of 18,268, 168 are recorded as invalid, 414 are illegitimate and 13 are found to be spelling variants.

(<http://www.theplantlist.org/Apocynaceae/>)

**Major Genera and Species**

Over 300 genera of Apocynaceae are placed across the five subfamilies (Table 1.1). A complete list of Apocynaceae is provided in Table 1.1 (<http://www.theplantlist>.

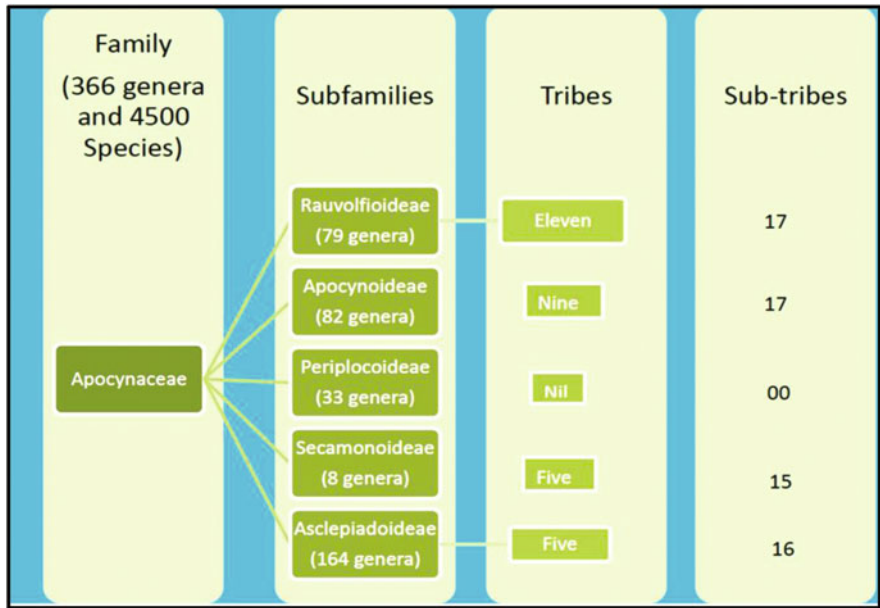


Fig. 1.2 General outline of the classification of the Apocynaceae family

[org/1.1/browse/A/Apocynaceae/](#)). *Carissa carandas* (*H. Karaunda*) is a small spiny shrub. The fruits of *C. carandas* are eaten as wild raw fruits. They are also used as vegetables and pickles. *Catharanthus roseus* is the most popular ornamental plant worldwide. Another important member of this family is *Rauwolfia serpentina*. It is abundant in sub-Himalayan tracts. The roots of *R. serpentina* are hypnotic, sedative and used in managing blood pressure. *Thevetia peruviana* is an evergreen shrub with highly poisonous milky juice and bitter bark (Fig. 1.3). It is popular as an arrow poison. *Plumeria alba* is widely grown in India as an ornamental tree because of its pleasant smell. *Wrightia tomentosa*, a small deciduous tree, is popular for the antidote property of the bark of stem and roots. *Baissea multiflora* is a climber with beautifully scented flowers and ornamental value. *Nerium indicum* is widely cultivated as an ornamental shrub worldwide (Fig. 1.3). Many members of the *Trachelospermum* genus are lovely woody vines, including the star jasmine, *T. jasminoides*, *Mandevilla*, and *Allamanda*. *Hoodia*, *Huernia*, and [carrion flower](#) (*Stapelia*) produce unique smells that attract pollinating insects and flies. Arrow poisons are obtained from several members of this family. The poisonous alkaloids present in the genera [Strophanthus](#) and [Rauwolfia](#) are used in medicines due to their biological activities. Pollination in Apocynaceae generally takes place through insects.

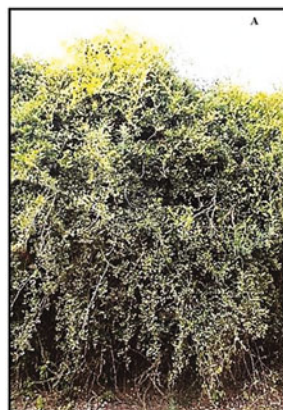
**Table 1.1** Genera in Apocynaceae

<i>Absolmisia</i>	<i>Anechites</i>	<i>Baeolepis</i>	<i>Calathostelma</i>	<i>Dactylostelma</i>	<i>Ecdysanthera</i>	<i>Fammia</i>	<i>Gonocryphia</i>	<i>Huerniopsis</i>
<i>Acerates</i>	<i>Angadenia</i>	<i>Baharua</i>	<i>Callichilia</i>	<i>Daemia</i>	<i>Echidnopsis</i>	<i>Furquharia</i>	<i>Gonolobus</i>	<i>Hunteria</i>
<i>Acokanthera</i>	<i>Angolluma</i>	<i>Bahiaella</i>	<i>Calocrauer</i>	<i>Dalzielia</i>	<i>Echites</i>	<i>Fernaldia</i>	<i>Gonostemon</i>	<i>Hybanthera</i>
<i>Acrocoryne</i>	<i>Anisopus</i>	<i>Baissea</i>	<i>Calostigma</i>	<i>Decabelone</i>	<i>Ecliptostelma</i>	<i>Finlaysonia</i>	<i>Gothofreda</i>	<i>Hylaea</i>
<i>Acustelma</i>	<i>Anisotoma</i>	<i>Ballyanthus</i>	<i>Calotropis</i>	<i>Decaceras</i>	<i>Ectadiopsis</i>	<i>Fischeria</i>	<i>Goydera</i>	<i>Hypobolus</i>
<i>Adelostemma</i>	<i>Anodendron</i>	<i>Barjonia</i>	<i>Calyptranthera</i>	<i>Decalepis</i>	<i>Ectadium</i>	<i>Fockea</i>	<i>Graphistemma</i>	<i>Ibatia</i>
<i>Adenium</i>	<i>Anomolassa</i>	<i>Baroniella</i>	<i>Cameraria</i>	<i>Decanema</i>	<i>Ecua</i>	<i>Folisia</i>	<i>Gummesia</i>	<i>Ichmocarpus</i>
<i>Aganoneurion</i>	<i>Anthonotis</i>	<i>Baseonema</i>	<i>Campestigma</i>	<i>Decanemopsis</i>	<i>Edithcolea</i>	<i>Fontellaea</i>	<i>Gymnanthera</i>	<i>Irmischia</i>
<i>Aganosma</i>	<i>Anthiostelma</i>	<i>Basistelma</i>	<i>Cantocarpus</i>	<i>Decastelma</i>	<i>Elcomarhiza</i>	<i>Forsteronia</i>	<i>Gymnema</i>	<i>Ischnolepis</i>
<i>Ahouai</i>	<i>Anitoxicum</i>	<i>Batesanthus</i>	<i>Caralluma</i>	<i>Desmidorchis</i>	<i>Elytropus</i>	<i>Freerea</i>	<i>Gymnemopsis</i>	<i>Ischnostemma</i>
<i>Alafia</i>	<i>Anura</i>	<i>Baynesia</i>	<i>Carissa</i>	<i>Dewevrella</i>	<i>Emicocarpus</i>	<i>Funastrum</i>	<i>Gymnolaema</i>	<i>Isonema</i>
<i>Alexitoxicon</i>	<i>Aphanostelma</i>	<i>Beaumontia</i>	<i>Carpodinus</i>	<i>Dichaelia</i>	<i>Emplectanthus</i>	<i>Funtumia</i>	<i>Haemadictyon</i>	<i>Ixodonerium</i>
<i>Allamanda</i>	<i>Aphanostylis</i>	<i>Belostemma</i>	<i>Caruthersia</i>	<i>Dicranthus</i>	<i>Ephippiocarpa</i>	<i>Fammia</i>	<i>Haemax</i>	<i>Jasminanthus</i>
<i>Allomarkgrafia</i>	<i>Apocynum</i>	<i>Bidaria</i>	<i>Caruncularia</i>	<i>Dicyophleba</i>	<i>Epicton</i>	<i>Farquharia</i>	<i>Hancornia</i>	<i>Jobinia</i>
<i>Allowoodsonia</i>	<i>Araujia</i>	<i>Biondia</i>	<i>Carvalhoa</i>	<i>Dipladenia</i>	<i>Epigynum</i>	<i>Fernaldia</i>	<i>Hanghomia</i>	<i>Kametia</i>
<i>Alstonia</i>	<i>Argelia</i>	<i>Blastotrophe</i>	<i>Cascabela</i>	<i>Diplocyatha</i>	<i>Epistemma</i>	<i>Finlaysonia</i>	<i>Haplophyton</i>	<i>Kanahia</i>
<i>Alycia</i>	<i>Aritia</i>	<i>Blepharanthura</i>	<i>Catharanthus</i>	<i>Diplolepis</i>	<i>Eriostemma</i>	<i>Fischeria</i>	<i>Haplostemma</i>	<i>Kasailo</i>
<i>Amalocalyx</i>	<i>Asclepias</i>	<i>Blepharodon</i>	<i>Centrostemma</i>	<i>Diplorhynchus</i>	<i>Ervatamia</i>	<i>Fockea</i>	<i>Hazunia</i>	<i>Kerbera</i>
<i>Ambelantia</i>	<i>Asketanthera</i>	<i>Blyntia</i>	<i>Cerbera</i>	<i>Diplostigma</i>	<i>Eucorymbia</i>	<i>Folisia</i>	<i>Heligme</i>	<i>Kibatalia</i>
<i>Amblyoglossum</i>	<i>Aspidoglossum</i>	<i>Bonifousia</i>	<i>Cerberiopsis</i>	<i>Dischidanthus</i>	<i>Eustegia</i>	<i>Fontellaea</i>	<i>Helioestemma</i>	<i>Kopsia</i>
<i>Amblyopetalum</i>	<i>Aspidonepsis</i>	<i>Boucerosia</i>	<i>Ceropegia</i>	<i>Dischidia</i>	<i>Excavatia</i>	<i>Forsteronia</i>	<i>Hemidesmus</i>	<i>Krebsia</i>
<i>Amblystigma</i>	<i>Aspidosperma</i>	<i>Boustignia</i>	<i>Chamaecaciliandra</i>	<i>Dischidopsis</i>	<i>Ecdysanthera</i>	<i>Freerea</i>	<i>Hemipogon</i>	<i>Lucmellea</i>
<i>Ampelamus</i>	<i>Asstelma</i>	<i>Brachystelma</i>	<i>Chilocarpus</i>	<i>Ditassa</i>	<i>Echidnopsis</i>	<i>Funastrum</i>	<i>Heterostemma</i>	<i>Lagarinthus</i>
<i>Amphidetes</i>	<i>Asstephanus</i>	<i>Buckollia</i>	<i>Chlorocarythus</i>	<i>Ditoceras</i>	<i>Echites</i>	<i>Funtumia</i>	<i>Heynella</i>	<i>Landolphia</i>
<i>Amphineurion</i>	<i>Asterostemma</i>	<i>Baeolepis</i>	<i>Chonemorpha</i>	<i>Dolichopetalum</i>	<i>Ecliptostelma</i>	<i>Glossonema</i>	<i>Hiepia</i>	<i>Larryleachia</i>
<i>Amphistelma</i>	<i>Atherandra</i>	<i>Baharua</i>	<i>Chumamalia</i>	<i>Drakebrockmania</i>	<i>Ectadiopsis</i>	<i>Glossostelma</i>	<i>Himantanthus</i>	<i>Lastostelma</i>
<i>Ansonia</i>	<i>Atherolepis</i>	<i>Bahiella</i>	<i>Cibirhiza</i>	<i>Dregea</i>	<i>Ectadium</i>	<i>Glossostephanus</i>	<i>Holarrhena</i>	<i>Lauberia</i>
<i>Anabatta</i>	<i>Australuma</i>	<i>Baissea</i>	<i>Cionura</i>	<i>Duvalia</i>	<i>Ecua</i>	<i>Gomphocarpus</i>	<i>Holostemma</i>	<i>Lavrania</i>
<i>Anantherix</i>	<i>Anechites</i>	<i>Ballyanthus</i>	<i>Cleghornia</i>	<i>Duvaliandra</i>	<i>Edithcolea</i>	<i>Gongronema</i>	<i>Hoodia</i>	<i>Laxoplumeria</i>
<i>Leachiella</i>	<i>Maclaudia</i>	<i>Microstemma</i>	<i>Oitsonema</i>	<i>Pentanura</i>	<i>Plocostemma</i>	<i>Rhinolobium</i>	<i>Taberna</i>	<i>Urceola</i>

<i>Leichardtia</i>	<i>Macoubea</i>	<i>Minaria</i>	<i>Onthalogonus</i>	<i>Pentarrhium</i>	<i>Plumeria</i>	<i>Rhombonema</i>	<i>Tabernaemontana</i>	<i>Urechites</i>
<i>Lepinia</i>	<i>Macroditassa</i>	<i>Miraglossum</i>	<i>Oncinema</i>	<i>Pentasachme</i>	<i>Poacynum</i>	<i>Rhyncharrhena</i>	<i>Tabernanthe</i>	<i>Urostelma</i>
<i>Lepiniopsis</i>	<i>Macropelma</i>	<i>Mitolepis</i>	<i>Oncinotis</i>	<i>Pentascame</i>	<i>Podanthes</i>	<i>Rhynchostigma</i>	<i>Tacazea</i>	<i>Vahadenia</i>
<i>Leptadenia</i>	<i>Macropetalum</i>	<i>Mitostigma</i>	<i>Oncostemma</i>	<i>Pentastelma</i>	<i>Podostelma</i>	<i>Rhysosolobium</i>	<i>Tanulepis</i>	<i>Vallariopsis</i>
<i>Leuconotis</i>	<i>Macropharynx</i>	<i>Molongum</i>	<i>Ophionella</i>	<i>Pentatropis</i>	<i>Poecilopsis</i>	<i>Rhyssostelma</i>	<i>Tapeinostelma</i>	<i>Vallaris</i>
<i>Lhotzkyella</i>	<i>Macroscelis</i>	<i>Mondia</i>	<i>Orbea</i>	<i>Pentopetia</i>	<i>Polystemma</i>	<i>Rhytidocaulon</i>	<i>Tassadia</i>	<i>Vallesia</i>
<i>Lobostephanus</i>	<i>Madarsosperma</i>	<i>Monolluma</i>	<i>Orbeanthus</i>	<i>Peplonia</i>	<i>Pootia</i>	<i>Riocrexia</i>	<i>Tavarexia</i>	<i>Vienamia</i>
<i>Lochnera</i>	<i>Mahawoa</i>	<i>Monostemma</i>	<i>Orbeopsis</i>	<i>Pergalaria</i>	<i>Pottisia</i>	<i>Robbia</i>	<i>Telecladium</i>	<i>Vinca</i>
<i>Luckhoffia</i>	<i>Malouetia</i>	<i>Monostylaceum</i>	<i>Oreosparte</i>	<i>Periglossum</i>	<i>Prestonia</i>	<i>Rojasia</i>	<i>Telminostelma</i>	<i>Vincetoxicopsis</i>
<i>Lygsma</i>	<i>Mandevilla</i>	<i>Morrenia</i>	<i>Orphanthera</i>	<i>Periploca</i>	<i>Pseudobatia</i>	<i>Ruelhsia</i>	<i>Telosma</i>	<i>Vincetoxicum</i>
<i>Lyonsia</i>	<i>Mangenotia</i>	<i>Mortoniella</i>	<i>Orthopichonia</i>	<i>Pervillaea</i>	<i>Pseudolithos</i>	<i>Saba</i>	<i>Tenuadenia</i>	<i>Virchowia</i>
<i>Leachella</i>	<i>Mananthrix</i>	<i>Motandra</i>	<i>Orthosia</i>	<i>Pervinca</i>	<i>Pseudopectinaria</i>	<i>Sacleuxia</i>	<i>Tenaris</i>	<i>Voacanga</i>
<i>Leichardtia</i>	<i>Margaretta</i>	<i>Mucua</i>	<i>Oxyptalum</i>	<i>Petalostelma</i>	<i>Pseudosarcobolus</i>	<i>Sarcobolus</i>	<i>Tetragonocarpus</i>	<i>Widgrenia</i>
<i>Lepinia</i>	<i>Marsdenia</i>	<i>Myriopterion</i>	<i>Oxystelma</i>	<i>Petchia</i>	<i>Pseusmagenetus</i>	<i>Sarcophagophilus</i>	<i>Tetraphysa</i>	<i>Willughbeta</i>
<i>Lepiniopsis</i>	<i>Mascarenhasia</i>	<i>Nanostelma</i>	<i>Pachycarpus</i>	<i>Petopentia</i>	<i>Pteralyxia</i>	<i>Sarcorrhiza</i>	<i>Thenardia</i>	<i>Winchia</i>
<i>Leptadenia</i>	<i>Matelea</i>	<i>Nautonia</i>	<i>Pachycymbium</i>	<i>Peziscarpus</i>	<i>Pycnanthera</i>	<i>Sarcostemma</i>	<i>Thevetia</i>	<i>Woodia</i>
<i>Leuconotis</i>	<i>Metaderma</i>	<i>Neisosperma</i>	<i>Pachypodium</i>	<i>Phaeostenma</i>	<i>Pulvinaria</i>	<i>Sarmasikia</i>	<i>Thoreauca</i>	<i>Wrightia</i>
<i>Lhotzkyella</i>	<i>Melinia</i>	<i>Neobraccia</i>	<i>Pacouria</i>	<i>Phorotrichis</i>	<i>Pycnobotrya</i>	<i>Sattadia</i>	<i>Thozetia</i>	<i>Xysmalobium</i>
<i>Lobostephanus</i>	<i>Mellichampia</i>	<i>Neocouma</i>	<i>Pala</i>	<i>Philibertia</i>	<i>Pycnobraegma</i>	<i>Schistogyne</i>	<i>Thysanthella</i>	<i>Zschokkea</i>
<i>Lochnera</i>	<i>Melodinus</i>	<i>Neoschumannia</i>	<i>Papuachites</i>	<i>Philibertia</i>	<i>Pycnorhachis</i>	<i>Schistonema</i>	<i>Thysanthus</i>	<i>Zygostelma</i>
<i>Luckhoffia</i>	<i>Merrillanthus</i>	<i>Nephrodia</i>	<i>Parabarium</i>	<i>Phyllanthera</i>	<i>Pycnostelma</i>	<i>Schizoglossum</i>	<i>Tintinnabularia</i>	<i>Urceola</i>
<i>Lygsma</i>	<i>Mesechites</i>	<i>Nerium</i>	<i>Paralancornia</i>	<i>Physanthus</i>	<i>Quaqua</i>	<i>Schizonotus</i>	<i>Tondulia</i>	<i>Urechites</i>
<i>Lyonsia</i>	<i>Metalepis</i>	<i>Notechidnopsis</i>	<i>Parameria</i>	<i>Physanthus</i>	<i>Quatania</i>	<i>Schizostenma</i>	<i>Toxocarpus</i>	<i>Urostelma</i>
<i>Leachella</i>	<i>Metaplexis</i>	<i>Notonarium</i>	<i>Parapodium</i>	<i>Piaranthus</i>	<i>Quisumbingia</i>	<i>Schizostephanus</i>	<i>Trachelospermum</i>	<i>Vahadenia</i>
<i>Leichardtia</i>	<i>Metastelma</i>	<i>Obesia</i>	<i>Parepignum</i>	<i>Picralima</i>	<i>Raphionacme</i>	<i>Schizozygia</i>	<i>Trachomitum</i>	<i>Vallariopsis</i>
<i>Lepinia</i>	<i>Micholitzia</i>	<i>Ochrosia</i>	<i>Parsonia</i>	<i>Pinochia</i>	<i>Raphistemma</i>	<i>Schlechterella</i>	<i>Traunia</i>	<i>Vallaris</i>
<i>Lepiniopsis</i>	<i>Microchites</i>	<i>Odontadenia</i>	<i>Pectinaria</i>	<i>Plectanicia</i>	<i>Rauwolfia</i>	<i>Schubertia</i>	<i>Treutlera</i>	<i>Vallesia</i>
<i>Leptadenia</i>	<i>Microdacylon</i>	<i>Odontanthera</i>	<i>Peltastes</i>	<i>Pleocarpa</i>	<i>Rhabdadenia</i>	<i>Secamone</i>	<i>Trichocaulon</i>	<i>Vienamia</i>
<i>Leuconotis</i>	<i>Microcloma</i>	<i>Odontostephana</i>	<i>Pentacypus</i>	<i>Pleoceras</i>	<i>Rhazya</i>	<i>Secamonopsis</i>	<i>Trichosacme</i>	<i>Vinca</i>
<i>Lhotzkyella</i>	<i>Microplumeria</i>	<i>Oianthus</i>	<i>Pentalinon</i>	<i>Pleurostelma</i>	<i>Rhigospira</i>	<i>Secondatia</i>	<i>Trichosandra</i>	<i>Vincetoxicopsis</i>

(Source: <http://www.theplantlist.org/1.1/browse/A/Apocynaceae>)



*Catharanthus roseus**Tabernaemontana divaricata**Wrightia tinctoria*(A) *C. carandas* plant, (B) Leaves (C) Stem of *C. carandas**Nereum oleander**Rauvolfia serpentina**Plumeria alba**Allamanda schottii***Fig. 1.3** Common members of the Apocynaceae family found in India and Southeast Asia

## Economic Importance

Apocynaceae or the ‘Dogbane’ family has attracted attention because of many life-saving natural medicines and novel phytochemicals. Many plants of Apocynaceae have potential applications in the rubber and pesticide industry. The pulpy fruits of *Carissa carandus* are edible and used for making pickles. These wild fruits are widely used by tribal people in India. Apocynaceae members such as *Hancora*, *Dyera*, and *Urceola* are used as inexpensive and economic sources of rubber. Thevetine, a poisonous glycoside, is obtained from the seeds of *Thevetia*. Acokanthera latex is employed in the poisoning of arrows. Rat control is practiced by using a poison found in the bark and wood of *Nerium indicum*. The seeds of *Wrightia tinctoria* are used to obtain the dye. On the other hand, *nerium*, *Catharanthus* (syn. Vinca), *Thevetia*, *Wrightia*, and *Allamanda* are cultivated as ornamental plants in gardens.

## Ethnomedical Considerations

Plants from the Apocynaceae family are known all over the world for their vast ethnomedicinal uses. Bhadane et al. (2018) provided a detailed account of the traditional knowledge and ethnomedicinal applications of several members of this family. It highlighted the uses of *C. roseus*, *A. scholaris*, *R. serpentina*, *H. antidysentriaca*, *N. indicum* and *T. peruviana*. The ethnomedicinal properties of these members have been documented in classical Indian knowledge systems such as Ayurveda, Siddha, Unani and Homeopathy.

*C. carandas* produces nut-sized fruits that are used for their stomachic, antidiarrheal and antihelminthic properties. The stem of *C. carandas* is used to enhance the tendons; fruits are used for skin care, and leaves are used as antipyretic agents (Kirtikar & Basu, 1998). Traditionally, *T. divaricata* has been recommended for the management of fever, pain, and dysentery (Boonyaratanakornkit & Supawita, 2005). Its applications in the treatment of diarrhea, epilepsy, asthma, fever, fractures, headache, inflammation, leprosy, rheumatic pain, ulceration, and vomiting are well documented (Nadkarni, 1996). *A. boonei* stem bark is used in the treatment of febrile illness, renal infections, rheumatism and liver ailments (Asuzu & Anaga, 1991; Ojewole, 1984; Majekodunmi et al., 2008; Bello et al., 2009; Majekodunmi & Odeku, 2009). It is also used to control intestinal helminths (Olajide et al., 2000). *C. roseus* (Linn.) is used for the treatment of diabetes, fever, malaria, throat infections, and chest complaints. It is also used for the regulation of menstrual cycles (Santhi et al., 2016). The parts of *H. antidysentriaca* have been used in the treatment of dysentery and diarrhea. In the Ayurvedic and Unani systems of medicine, it is used as an anthelmintic and antidiarrheal agent and is also used to treat skin diseases (Shah et al., 2010). According to traditional beliefs, *N. oleander* offers therapeutic properties for a number of ailments, including kushtha (leprosy), valipalita (ageing),

upadamsha (syphilis), indralupta (alopecia), and conjunctivitis (Chakara dutta) (Banerjee et al., 2011).

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## Chapter 2

# Traditional Knowledge, Ethnomedicinal Importance and Practical Uses of Apocynaceae Members



### Introduction

Indian traditional systems of medicine, i.e., Ayurveda, Siddha and Unani, are practiced all over world. Moreover, it is known for its vast reservoir of living traditions of plant-based systems of medicine. The rural population of India, who has limited access to modern health services, mostly relies on traditional knowledge, home remedies and formulae being practiced for generations. Traditionally, Indian people and ethnomedicine systems relied on plant-derived medicines over medicines of animal origin due to the presence of abundant natural flora and the practice of vegetarian diets. The idea of disease prevention and treatment is well documented in ancient scripts such as *Ayurveda*. The emphasis on the maintenance of positive health or *Swastha Vrutta* is a distinguishing feature of *Ayurveda* (Lele, 2001). *Ayurveda* believes that any patient can be cured with the help of herbs present in the environment. The main approaches to illness in *Ayurveda* are preventive and curative (Upadhyay, 1997). Ayurveda advocates the harmonious balance between the three humors of the body, viz. 'Vata', 'Pitta' and 'Kafa', which enhance positive health. The imbalance of these factors may cause disease(s) (Lakshmi et al., 2004).

Applications of medicinal plants and their products for health management are an imperative part of tribal culture. Indian tribal people employ medicinal plants for a variety of health-related purposes and have great interest and knowledge for them. Traditionally, local tribal communities have explored the medicinal uses of hundreds of plants found locally in their ecosystem. They have their own management system for health-related problems that is inherited from one generation to another. Although the process of creating traditional knowledge is not well documented scientifically, it has been practiced for hundreds of years in tribal India. There is a rising understanding of the importance of traditional knowledge, and as a result, plant biologists are once again interested in learning more about the history of traditional knowledge. It is anticipated that it will significantly advance sustainable development. The vast majority of the world's plant genetic resources are found in

regions where the bulk of indigenous and local communities are located. Since ancient times, many of them have been grown and used sustainably. However, indigenous and local populations go well beyond their responsibilities as managers of natural resources to contribute to the preservation and sustainable use of biological variety. Their knowledge and methods serve as a vital resource for the whole community and thus serve as an important model for setting biodiversity goals.

## Plant Metabolites in Modern Medicine

Recently, plants have been used as fine chemical factories to produce recombinant proteins of pharmaceutical interest. Plant-derived natural chemicals have also served as leads for the synthesis of novel drugs (Rupesh & Gautaml, 2013). Digitoxin isolated from *Digitalis purpurea* (Plantaginaceae) is used as a cardiotonic agent. 'Taxol', the world's first multibillion dollar anticancer compound, has been extracted from *Taxus brevifolia* (Taxaceae). The antimalarial drugs quinine and artemisinin were isolated from *Cinchona officinalis* (Rubiaceae) and *Artemisia annua* (Asteraceae), respectively. Other plant-assisted drug discoveries with bioactivities for hypertension and amoebiasis include *Rauwolfia serpentina* and *Holarrhena antidysenterica* (Apocynaceae), respectively. Curcumin from *Curcuma longa* (Zingiberaceae) is an anti-inflammatory agent, baccosides from *Bacopa monnieri* (Scrophulariaceae) for memory enhancement and many other steroidal lactones as immunomodulators have been widely explored (Patwardhan, 2000). Apart from their medicinal applications, there is also an increase in demand for medicinal plants for the purpose of food supplements and cosmetics preparations (Ramawat & Goyal, 2008). In the USA, the food drug and cosmetic act characterizes herbal plants on the basis of their intended use as food (dietary supplement), drug (biological drugs) and cosmetics. According to the Food Drug and Cosmetics Act, in sect. 201 (ff), dietary supplementation means that a product (other than tobacco) containing various dietary ingredients, including herbs or another botanical substance, an amino acid, a mineral, and vitamins, is used by people to increase their total dietary intake (Chang, 1998). Many pharma and biotech companies have shown their interest in herbal preparations that can be marketed as nutraceuticals or health foods (Kamboj, 2000). Pigments such as anthocyanins, betalains, and carotenoids present in herbal plants are used as coloring agents in food preparations, and they also have bioactivities (Aberoumand, 2011). Currently, food industries are turning their attention to natural food colours from plant origin because certain artificial colours in food have been demonstrated to cause adverse effects on consumption (Bridle & Timberlake, 1997).



## Ethnomedical Uses of Some Members of the Family Apocynaceae

There is widespread knowledge that members of the Apocynaceae family have a variety of ethnomedical applications. The ethnomedicinal significance of numerous species in the Apocynaceae family is compiled in Table 2.1. Ayurveda, Siddha, Unani, and homoeopathy offer descriptions of some significant members of this family, including *C. roseus*, *A. scholaris*, *R. serpentina*, *H. antidysentriaca*, *N. indicum*, and *T. peruviana*. Indigenous systems of medicine use plants and their constituent parts to treat a variety of illnesses and ailments.

In African countries such as Ghana, Uganda, Nigeria and Senegal, Apocynaceae members such as *T. crassa*, *V. africana* and *Rauvolfia vomitoria* are commonly used for similar ethnomedicinal uses. The leaf, stem, root and flower extracts of these plants have been widely used for anti-arthritic activity, in the treatment of [rheumatism](#), parasitic skin infections including [leprosy](#), used as [diuretic](#), insecticidal as well as in the management of fever, [wound dressing](#), ringworm and as a [local anaesthetic](#) in bones (Siddiqui et al., 1995; Agwu & Akah, 1990).

In the Indian subcontinent, the Himalayan ranges of the southernmost mountains at altitudes of 600–1200 m above sea level are richly colonized by Apocynaceae plants. Western states of India are geographically and culturally rich and diverse and are widely exploring plant diversity for various ethnomedicinal purposes.

The Western Ghats of India are a biodiversity hotspot and rich in medicinal plants that harbour over 3000 species of medicinal importance (Venkatachalapathi et al., 2018). The tribals of Western Ghats are using these plants for their primary health care. Due to the diverse ecosystems in this area, the Western Ghats and Nilgiri districts of Tamilnadu have a significant diversity of medicinal plants (Ramachandran, 2007).

Another member of the Apocynaceae family, *Caralluma europaea*, is indigenous to Morocco, Algeria, Tunisia, Libya, Egypt, Jordan, Spain, and Italy. External parts of these plants have been utilized for centuries to treat a variety of conditions, including diabetes mellitus, kidney stones, cysts, kidney infections, cancer, respiratory infections, digestive disorders, urogenital infections, metabolic disorders, and cardiovascular issues (Ouassou et al., 2021).

**Table 2.1** Ethnomedicinal uses of selected members of Apocynaceae (Reproduced with permission from: Bhadane et al., 2018)

Ailment	Plant species	Plant parts used	Remedy	References
Stomach problems	<i>Wrightia tinctoria</i>	Seeds	Oral use of seed juice	Muthu et al. (2006)
	<i>Carrisa edulis</i>	Root and stem bark	Decoctions administered orally	Gakuya et al. (2013)
Diarrhoea/ loose motions	<i>Holarrhena antidysenterica</i>	Fruits, leaves		Kaul and Atal (1983)
	<i>Alstonia scholaris</i>	Bark extract	Bark extracts used in chronic diarrhea	Wiat (2006)
Fever	<i>Wrightia tinctoria</i>	Fruits	Raw fruits are eaten	Muthu et al. (2006)
	<i>Thevetia neriifolia</i> <i>Voacanga Africana</i>	Leaves	Raw leaves are eaten to control the fever	Ayoola et al. (2008)
	<i>Tabernaemontana divaricata</i>	Leaves	Decoctions administered orally	Rahmatullah et al. (2009)
	<i>Rauvolfia serpentina</i> (L.)	Leaf extract	Decoctions administered orally	Roy et al. (2022)
Skin care and skin infections	<i>Wrightia tinctoria</i>	Oil and latex	Liquid latex used in dandruff control	Muthu et al. (2006)
	<i>Rauvolfia tetraphylla</i>	Leaf and bark extract	Bark powder and castor oil is applied on skin	Kirtikar and Basu (2003)
	<i>Geissospermum sericeum</i>	Stem bark.	Water extract applied on skin	Steele et al. (2002)
	<i>Nerium indicum</i>	Leaves	Crushed leaves applied on skin	Rahmatullah et al. (2009)
		Leaves and roots	Paste of leaves and root used against ringworm	Upadhyay et al. (2007)
	<i>Rauvolfia serpentina</i> (L.)	Leaf extract	Paste of leaves and root used against ringworm	Roy et al. (2022)
	<i>Voacanga africana</i>	Leaf, stem and root extract	Crushed leaves applied on skin	Agwu and Akah (1990)
	<i>Tabernaemontana crassa</i>	Leaf and root extract	Crushed leaves applied on skin	Siddiqui et al. (1995)
	<i>Rauvolfia vomitoria</i> Azfel	Leaf, stem and root extract	Crushed leaves applied on skin	Agwu and Akah (1990)
Ameboic dysentery	<i>Holarrhena antidysenterica</i>	Leaves	Leaves boiled in water and the juice is taken orally	Rahmatullah et al. (2009)
Tooth and oral problems	<i>Voacanga Africana</i>	Stem	Bark used in toothache	Ayoola et al. (2008)
	<i>Alstonia scholaris</i>	Latex	Used for relief from toothache	Wiat (2006)

(continued)



**Table 2.1** (continued)

Ailment	Plant species	Plant parts used	Remedy	References
Wound healing	<i>Acokanthera schimperi</i>	Fresh leaves	Crushed fresh leaves applied on wound	Teklehaymanot and Giday (2007)
	<i>Holarrhena antidysenterica</i>	Latex	The liquid is applied on wound	Kaul and Atal (1983)
	<i>Rauvolfia serpentina</i> (L.)	Leaf extract	Applied on skin	Roy et al. (2022)
	<i>Rauvolfia vomitoria</i> Azfel	Leaf, stem and root extract	Applied on skin	Agwu and Akaha (1990)
	<i>Tabernaemontana crassa</i>	Leaf and root extract	Applied on skin	Siddiqui et al. (1995)
Malaria	<i>Carrisa edulis</i>	Root and stem bark preparation	Applied on wound	Gakuya et al. (2013)
Rheumatism	<i>Holarrhena antidysenterica</i>	Stem bark	Bark is used as plaster	Kaul and Atal (1983)
	<i>Carissa carandas</i>	Root and root bark	Root and root bark extracts are used in rheumatism and joint pain.	Upadhyay et al. (2007)
	<i>Tabernaemontana crassa</i>	Leaf and root extract	Used in joint pain	Siddiqui et al. (1995)
Allergy/ asthma	<i>Alstonia scholaris</i>	Flowers used in respiratory trouble.	Decoctions administered orally	Guha Bakshi et al. (1999)
Contraceptive/ birth control	<i>Nerium indicum</i>	Roots	Roots extract is used for abortion	Upadhyay et al. (2007)
	<i>Tabernaemontana crassa</i>	Leaf and root extract	Roots extract is used for abortion	Siddiqui et al. (1995)
Women health	<i>Holarrhena pubescens</i>	Bark	Bark powder is used to control vaginal discharge.	Hossan et al. (2010)
	<i>Alstonia scholaris</i>	Leaves and stem bark.	Preparation is used in menstrual problems.	Kala (2005)
Lactation	<i>Alstonia scholaris</i>	Leaves	Decoctions administered orally	Wiar (2006)
		Fresh bark	Decoctions in milk administered orally	Sharma and Sharma (2010)
Pediatric care	<i>Holarrhena antidysenterica</i>	Seeds	Seed preparation given to young children	Kaul and Atal (1983)
Snake bite	<i>Carissa carandas</i>	Leaf extract	Extract given orally.	Desai (1975)
	<i>Rauvolfia serpentina</i>	Roots	Squeezed roots tied on bitten area.	Prakasha et al. (2010)

(continued)

**Table 2.1** (continued)

Ailment	Plant species	Plant parts used	Remedy	References
	<i>Alstonia scholaris</i>	Stem bark	Oral chewing of the bark	Prakasha et al. (2010)
	<i>Tabernaemontana divaricata</i>	Roots	Root paste given orally in water of buttermilk	Prakasha et al. (2010)
Urinary tract infection	<i>Hemidesmus indicus</i>	Leaves	Cosumed orally.	Hossan et al. (2010)
Premature ejaculation	<i>Carissa spinarum</i>	Unripened	Infusion is used	Belayneh and Bussa (2014)
	<i>Tabernaemontana crassa</i>	Leaf and root extract	Cosumed orally	Siddiqui et al. (1995)
Mouth, throat and coughing problems	<i>Acokanthera schimperi</i>	Stem and leaves	Gargling is effective in tonsillitis	Belayneh and Bussa (2014)
	<i>Alstonia scholaris</i>	Leaves paste	Taken orally with honey	Rahmatullah et al. (2009)
Cardiac disorders	<i>Thevetia neriifolia</i> <i>Voacanga Africana</i> <i>Stropanthus hispidus</i>	Stem bark and root bark	Used as cardi tonic	Ayoola et al. (2008)
Measles	<i>Thevetia neriifolia</i>	Leaves	Applied on skin	Ayoola et al. (2008)
Diabetes	<i>Catharanthus roseus</i>	Whole plant	Whole plant powder taken orally	Muthu et al. (2006)
		Leaves	Boiled leaves infusion taken orally	Erasto et al. (2005)
	<i>Plumeria obtuse</i>	Leaves	Boiled leaves infusion taken orally	Siddiqui et al. (2004)
Ear pain	<i>Nerium oleander</i>	Leaves and roots	Boiled leaves infusion taken orally	Muthu et al. (2006)
Sexually transmitted infections	<i>Catharanthus roseus</i>	Roots	Boiled roots administered anally in Gonorrhoea infection	Pereira et al. (2010)
	<i>Alstonia scholaris</i>	Stem bark powder	Dried stem bark powder taken orally to control Gonorrhoea infection	Sahu (2011)
	<i>Rauvolfia vomitoria</i> Azfel	Leaf, stem and root extract	Dried stem bark powder taken orally to control Gonorrhoea infection	Agwu and Akah (1990)
Appetizer	<i>Carissa carandas</i>	Ripened fruits	Ripened fruits used as an appetizer	Rahmatullah et al. (2009)
Insecticidal	<i>Tabernaemontana crassa</i>	Leaf and root extract	Water extracts are used as larvecidal	Siddiqui et al. (1995)
	<i>Voacanga africana</i>	Leaf, stem and root extract	Water extracts are used as larvecidal	Agwu and Akah (1990)

(continued)

**Table 2.1** (continued)

Ailment	Plant species	Plant parts used	Remedy	References
Local anaesthetic	<i>Tabernaemontana crassa</i>	Leaf and root extract	Applied on external tissues	Siddiqui et al. (1995)
	<i>Rauvolfia vomitoria</i> Azfel	Leaf, stem and root extract	Applied on external tissues	Agwu and Akah (1990)
Eye care	<i>Rauvolfia serpentina</i> (L.)	Leaf extract	Used for better sight and in eye infections	Roy et al. (2022)

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# Chapter 3

## Plant Secondary Metabolites as a Source of New Drugs



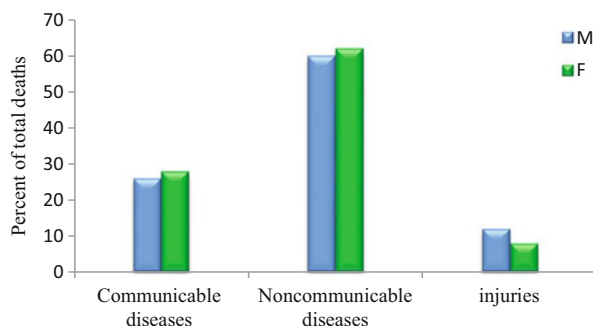
### Introduction

An increasing number of new pathogens, the emergence of multidrug drug-resistant (MDR) and extremely drug-resistant (XDR) bacterial and fungal pathogens, diseases associated with unhealthy diets and insufficient physical activity have been major problems concerning human health globally. Despite continuous progress in new drug discovery, the battle against many life-threatening diseases is still far from over. The recent emergence of MDR and XDR *Mycobacterium*, *Pseudomonas*, and *Candida spp.* led to thousands of new infections and deaths. Furthermore, infections by common human and animal opportunistic pathogens such as *Aspergillus*, *Cryptococcus*, and *Candida* in immune-compromised patients are increasing at an alarming rate. Therefore, new drugs from natural sources can certainly benefit society from infectious and noninfectious diseases.

### Global Outlook of Communicable and Noncommunicable Diseases

The development of antibiotic-resistant infections and the appearance of novel diseases appear to be significant risks in human history, causing an unfathomable number of illnesses and fatalities. Recently, the global burden of various diseases, such as tuberculosis, HIV, H1N1, SARS, malaria, cancer, and cardiovascular disease, has been increasing. The WHO predicts that between 2004 and 2030, communicable disease fatalities in low-income countries will decrease by approximately half, but deaths from noncommunicable diseases (NCDs) are projected to increase (World Health Organization, 2008). Over 56 million deaths were reported worldwide in 2012, of which 38 million were due to NCDs. According to the World

**Fig. 3.1** Global deaths according to cause and sex (2008) as per the World Health Organization (WHO)



Health Organization, approximately 60 million deaths occurred due to various communicable as well as noncommunicable diseases (Fig. 3.1).

## Communicable Diseases

Communicable diseases refer to diseases that can be transmitted through infective agents such as bacteria, fungi, viruses, parasites, and protozoa. The main communicable diseases in India with significant morbidity and mortality are diarrhea, HIV-AIDS, influenza, dengue and acute respiratory infections. Nearly one-fifth of all fatalities worldwide occur in children under the age of five, and diarrheal diseases are the second-greatest cause of infectious disease, accounting for almost 1000 million episodes and over two million deaths per year (Brewer, 2000). Similarly, TB bacilli are present in approximately one-third of the world's population. Over 33 million people have been identified to have HIV, and over two million have died in the past 10 years (Brewer, 2000). Malaria, a vector-borne disease, flourishes in poor tropical nations, leading to over one million deaths each year, and the majority of the deaths involve young children. *Nevertheless, a remarkable decline has been noticed in the morbidity and mortality rate of infectious scourges because of wider access to effective antimicrobial treatments and the dramatic reduction in their prices* (Concepcion et al., 2001).

## Noncommunicable Diseases

According to the World Health Organization, NCDs prematurely take 16 million lives annually. Cardiovascular diseases are the most important cause of mortality worldwide, with over 17.5 million deaths per year, which is 46% of total deaths caused by NCDs. Other diseases, such as cancers (8.2 million deaths), respiratory diseases (4.0 million deaths) and diabetes (1.5 million deaths), are among the main killers. Thus, these four major NCDs were responsible for 82% of total deaths due to

NCDs. According to the world economic forum, the economic losses due to NCDs in low- and mid-income countries during 2011–2030 are estimated at \$47 trillion (Bloom et al., 2011).

The mortality rate due to diabetes and heart disease is gradually increasing worldwide. The global prevalence of diabetes in 2014 increased by 7%. According to projections from the WHO. The number of deaths due to cancer will rise from 8.2 million to 12.6 million annually, while the number of deaths due to cardiovascular diseases is expected to increase from 17.5 million in 2012 to 22.2 million in 2030 (World Health Organization, 2014).

## Need for New and Effective Drugs

Despite the ever-increasing economic burden, the treatment and prevention of communicable and noncommunicable diseases has not attracted global attention. The global load of chronic noncommunicable diseases cannot be reduced without development and appropriate access to effective medicines. Recent data on over 40 countries show that generic medicines for the treatment of acute diseases were available in 56.15% and 65.6% of the public and private sectors, respectively. For NCDs, medicine availability was only 36% and 54.7%, respectively (Gelders, 2006; Kotwani, 2009). For over 280 million people suffering from diabetes mellitus worldwide, the cost of basic oral treatment is unaffordable, especially in low- and middle-income countries. Access to insulin remains poor in many regions of the world due to high prices. Similarly, approximately 5.5 million cancer patients in developing countries are suffering without adequate treatment (Abegunde, 2007).

The development of effective medicines has played a pivotal role in the treatment of cardiovascular diseases, diabetes, chronic respiratory diseases, and different types of cancers, mental and neurological disorders. Nonetheless, due to the prevalence and global burden of these disorders, a large number of people with such problems do not receive proper treatment and care (Kohn, 2004; Meyer, 2010). Thus, the development of new, safe and affordable medicines is essential for the primary and secondary prevention of communicable and noncommunicable diseases, especially in the developing world.

According to WHO estimates, over 80% of people in underdeveloped countries rely on traditional medicines, the majority of which are made from plants, for their most basic medical requirements. The United States Food and Drug Administration (USFDA) has registered more than 49% of novel medications produced from natural materials or their derivatives. From 2000 to 2011, more than 63% of authorized anticancer medications were from natural sources, up from more than 60% of approved drug candidates during the years 1989–1995. The WHO has listed over 21,000 medicinal plants, most of which are temperate species. Over 2500 plant species that are utilized in traditional medicines can be found in abundance throughout the Indian subcontinent. Tribal culture is fundamentally based on the control of health through the use of medicinal plants. These people obviously possess



extensive traditional knowledge of medicinal herbs and employ them for a variety of purposes relating to health. Numerous flora and plant-based substances have been used medicinally by indigenous tribes in the area according to long-standing customs. The people of tribal villages have systems for health-related problems and management that are generally transmitted from one generation to another.

## Natural Products as a Potential Source for New Metabolites

Ever since the first antibiotic was discovered in 1929, there has been a continuous increase in the demand for new and safe substances for the management of human illnesses. Bioactive compounds to treat various diseases can be obtained by chemical synthesis as well as from natural sources such as plants, animals, and microorganisms. A report by Bérdy (2012) described over ten million molecules with pharmaceutical potential that are mainly obtained by chemical synthesis (Table 3.1).

The approximate number of medicinal metabolites derived from natural sources is only approximately one-tenth that of compounds derived from synthetic sources (Table 3.1), and natural compounds, particularly those derived from microbes, typically have significantly higher bioactivity than synthetic compounds. Moreover, the structural and functional diversity of natural products is immense, and their use is safe and environmentally benign. Hence, after the discovery of penicillin, a metabolite from *Penicillium notatum*, several novel molecules were isolated and studied from new natural sources together with semisynthetic metabolites by the pharmaceutical industry, and hundreds of new antibiotics were discovered (Table 3.1). However, the trend of discovering new antibiotics has turned downwards in the last decade. However, the proportion of novel compounds to resistant infections has become unbalanced due to the increase in the number of resistant bacteria. Considering the pace of new molecule discovery, it is imperative to refocus our efforts on searching for new metabolites from natural sources.

## Secondary Metabolites from Natural Sources

Secondary metabolites are naturally derived substances and/or byproducts from plants, animals and microorganisms (Baker et al., 2000).

**Table 3.1** Approximate numbers of known synthetic compounds, natural products, and microbial products (Bérdy, 2012)

	Synthetic chemical compounds	Natural product	Microbial products
Numbers	8–10 million	~500,000	~70,000
Drugs	2000–2500	1200–1300	450–500
Percentage	0.005	0.6	1.6

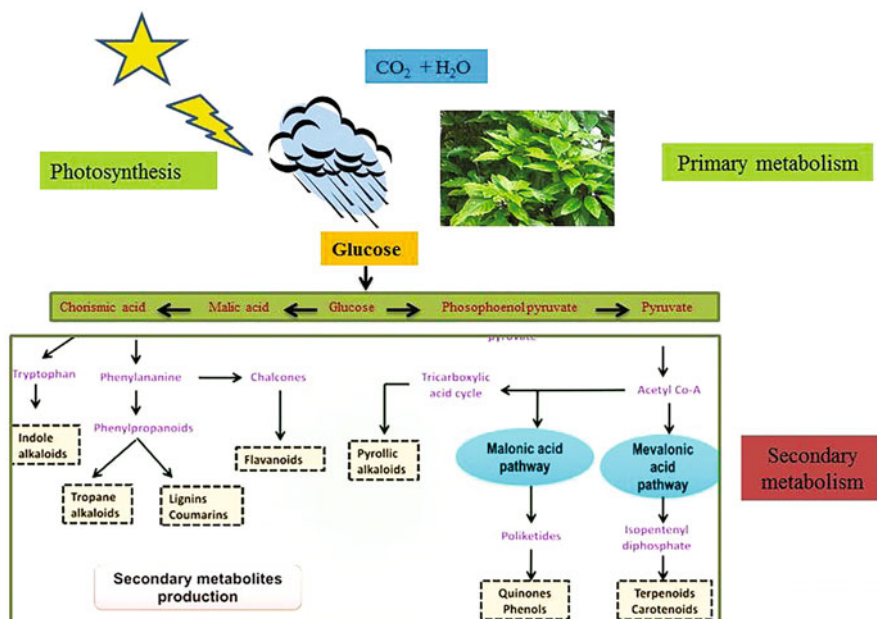


Fig. 3.2 Primary and secondary metabolism in plants

Secondary metabolites are produced by plants through subsidiary pathways that are not directly involved in basic cellular processes and therefore are not essential for the host. However, they are responsible for mediating environmental interactions and are crucial for survival and reproduction. Plants are known to produce more than 50,000 different secondary metabolites. Plant secondary metabolites are considered the basis for the biological effects of several modern molecules and medicinal plants. The discovery of novel secondary metabolites is a continuous process that intends to discover a novel molecule with better therapeutic value. During evolution, plants have developed effective countermeasures to withstand herbivorous pests/diseases. These metabolites are species-specific and exert a wide range of effects on the plant itself and other living organisms. Living cells share primary metabolites such as carbohydrates, lipids, nucleotides, and peptides, which are essential to life activities. On the other hand, secondary metabolites, which are produced from primary metabolites (Fig. 3.2), serve to protect plants from external and environmental stresses such as disease, pests, and herbivores.

A number of plants produce secondary metabolites, such as alkaloids, phenolics and oils, which have been used for insect and pest control for a long time. There are several types of secondary plant metabolites with insecticidal/pesticidal properties, viz., repellents, antifeedants, phagostimulants and toxins (Krishnayya & Rao, 1996; Nivsarkar et al., 2001). Plant proteins and other secondary metabolites have been shown to have insecticidal and pesticidal properties and hence were proposed as possible candidates to develop insecticidal molecules (Simmonds et al., 1989; Kim

Alkaloids	Terpenoids and steroids	Phenolic compounds	Sulfur-containing compounds
<ul style="list-style-type: none"> <li>•Cyanogenic glucosides</li> <li>•Non protein amino acids (Ex. Taxol)</li> </ul>	<ul style="list-style-type: none"> <li>• Hemiterpenes (single isoprene unit)</li> <li>• Monoterpenes (two isoprene units)</li> <li>• Sesquiterpenes (three isoprene units)</li> <li>• Diterpenes (four isoprene units)</li> <li>• Sesterterpenes (five isoprene units)</li> <li>• Triterpenes (six isoprene units)</li> <li>• Carotenoids (eight isoprene units)</li> </ul>	<ul style="list-style-type: none"> <li>• Lignin</li> <li>• Flavonoids</li> <li>• Isoflavonoids</li> <li>• Tanins</li> </ul>	<ul style="list-style-type: none"> <li>• Glutathione</li> <li>• Defensins</li> <li>• Alenin</li> </ul>

**Fig. 3.3** Major classes of plant secondary metabolites

et al., 2003). In contrast to synthetic pesticides, several of these chemicals have less severe effects on insects and exhibit chronic rather than acute toxicity (Estruch et al., 1997; Park et al., 2003). Secondary metabolites are classified into four major groups: alkaloids, terpenoids, phenolic compounds and sulfur-containing compounds (Fig. 3.3).

After the ‘wonders medication’ penicillin was discovered, along with Pasteur’s work on fermentation for the culture of microbial cells, the scientific community began to seriously look into microbes as a potential source of secondary metabolites. The early age (1940–1974) of microbial metabolite production was mainly focused on *Streptomyces*, while bacterial and fungal sources were less explored. The role of fungi as secondary metabolite producers was recognized by the early 1980s, and today, fungal metabolites account for the same percentage (45%) of metabolites as those from *Streptomyces* and *actinobacteria*.

Over the years, the discovery of bioactive secondary metabolites was centered on microorganisms that were largely obtained from soil. Selecting the ecological habitat for isolating prospective microbes is an important step in drug discovery. Thus, it is imperative to carefully select the biological source before proceeding instead of a random approach in the selection of biological sources. It is known that organisms are subjected to constant metabolic and abiotic stress that could produce novel secondary metabolites (Schutz, 2001). Several natural sources, such as soil, animals, plants and marine environments, are found to harbor microbial communities with ‘novel chemistry’.

## ***Soil***

The unanticipated discovery of penicillin from the contaminant fungus *Penicillium notatum* isolated from soil has prompted intensive investigation of soil as well as other natural habitats in the search for microorganisms with novel properties. Other novel compounds, such as cephalosporin (from *Cephalosporium cryptosporium*), tetracycline and other polyketides (from *Actinomycetes*), originate in soil-dwelling fungi. With the advent of new sophisticated isolation techniques and high-throughput screening methods, the isolation of microorganisms from soil has now become rapid, economical and more productive. However, it is estimated that less than 1% of soil microbes have only been cultured and characterized.

It appears that pharmaceutical companies have screened almost the entire world's soil in search of beneficial microbial communities. Therefore, it is necessary and interesting to explore and exploit alternate sources for microbes that could produce new compounds of therapeutic potential. Additionally, novel natural products and their producers offer better opportunities in the discovery of new drugs and agrochemicals. Thus, challenges to finding novel microbes remain.

## ***Marine Habitats***

Due to extreme temperature, pressure and salinity, ocean water has been the source of microbiota that could produce natural compounds of therapeutic potential. However, it is difficult to grow marine bacteria in the laboratory and coax them to produce their full repertoire of therapeutic molecules. Methicillin-resistant *Staphylococcus aureus* (MRSA), a gram-positive bacterium, is an emerging challenge, particularly for public health, due to the rapid development of resistance towards all major antibiotics. Macrolactin, Pseudonocardins A, and Kandenol A, compounds reported for antibacterial activities against MRSA, were isolated from *Pseudomonas* species, a marine bacterium (Sung-Hwan et al., 2013). Halichondrine B, an anticancer drug active against lung cancer, was isolated from *Halichondria okadai*, a marine-derived sponge. Some of the most potent cytotoxic substances ever identified are produced by aquatic organisms, but their yields are often low, making it unlikely that natural sources will supply enough material for drug development investigations.

## ***Insects***

Insects are the most diverse form of life present on Earth. Their extraordinary diversity in terms of numbers and morphological forms has led to the isolation of microorganisms that could produce natural compounds of therapeutic potential. The

insect gut is the reservoir of complex microbial communities that can produce a variety of bioactive substances. Grasshopper gut-associated bacteria were screened for antagonistic activity against common human pathogens (Chellaram et al., 2012). A number of bioactive substances have been produced by insect pathogenic fungi (Masahiko et al., 2005).

### ***Plants: A Potential Source of Microbial Communities***

Plant-based medicines have sparked renewed interest because they are relatively safe and rarely cause negative effects. Humans have been using plants and medications derived from plants to cure a wide range of diseases throughout history. However, widespread use of the world's plant diversity has led to the extinction of numerous species of medicinally important plants. Discovering other resources and methods to produce metabolites that are similar to those derived from plants is therefore essential. There are 3,00,000 nearby plant species on Earth, and each species, in turn, is hosted by a unique microbial community. The huge biodiversity of plants can be explored for isolating novel microbes for chemical and functional diversity.

### **Secondary Metabolites of Commercial Importance**

Secondary metabolites from plants have played a vital role in the treatment of many diseases and serve as building blocks for the creation of more potent medications. The oldest of the substances often employed in the therapy of cardiac disorders is digitaloxigenin, a steroid glycoside derived from *Digitalis purpurea*. In addition to being used to treat congestive heart failure, it is also useful in the treatment of arrhythmia. An acceptable antiarrhythmic drug is quinidine, a stereoisomer of the antimalarial quinine found in cinchona bark. Atropine, a metabolite of *Atropa belladonna*, is used to treat slow heartbeat (bradycardia). Paclitaxel (Taxol), the first anticancer medication to reach the \$1 billion mark, is a natural substance obtained from the yew plant *Taxus brevifolia* (Wani et al., 1971).

Artemisinin is an antimalarial sesquiterpene from the Chinese medicinal herb *Artemisia annua* that has been used in herbal remedies since ancient times (Arvigo & Balick, 1993; Farnsworth et al. 1985). Other bioactive constituents, such as aspirin and quinine, have also been isolated from medicinal plants.

Table 3.2 summarizes a few important secondary metabolites used as drugs, their sources and medical/pharmaceutical applications.

**Table 3.2** Important secondary metabolites used as drugs, their source and medical/pharmaceutical applications

Sr. no.	Secondary metabolites (drugs)	Plant source	Medical/pharmaceutical application
1.	Ajmalicine, solasodine, $\alpha$ -solanine, serpentine and reserpine, $\beta$ -carboline, alstonine, reserpiline	<i>Rauwolfia</i> species	Insomnia, epilepsy, schizophrenia, antidote against snake bite
2.	Oleanane triterpenoids, cinnamic acid, coumarins, flavonoids	<i>Baccharis megapotamica</i>	Invigorating blood circulation, antihelmintic, anti-inflammatory, anti-neoplastic, antioxidant, antiviral
3.	Berberine, coptisine, jatrorrhizine	<i>Coptis</i> species	Anti-inflammatory, neuroprotective, antioxidant
4.	Betalains, apigenin flavonoids like vitexin, vitexin-2-O-rhamnoside,	<i>Beta vulgaris</i>	Antihypertensive, hypoglycaemic, antioxidant, anticancer agent.
5.	Borneol, $\alpha$ -terpineol, carvacrol, thymol, geraniol, carotenoids, rosmarinic acid	<i>Thymus</i> species	Anti-inflammatory, hypoglycemic, antihyperlipidemic activity, antioxidative capacity, flavouring agent
6.	Camptothecin, 10-hydroxycamptothecin, 10-methoxycamptothecin	<i>Camptotheca acuminata</i>	Anti-cancer, cytotoxic
7.	Cucurbitacins	<i>Trichosanthes</i> , <i>Cucurbita</i> , <i>Cucumis</i> and <i>Citrullus</i>	Anti-inflammatory, anti-diabetic activity, antitumour
8.	Diosgenin	<i>Dioscorea doryphora</i>	Cancer therapy, hypercholesterolemia,
9.	Diosgenin,, allantoin, phytic acid, coumaric acid, myricetin	<i>Dioscorea</i> species	Antitumour, anorexiant, antifungal, antimutagenic, hypoglycemic, immunomodulatory, antidotes.
10.	Ephedrine alkaloids (1-ephedrine, <i>d</i> -pseudoephedrine, vicenin-1 and vicenin-2), proanthocyanidines	<i>Ephedra</i>	Appetite suppression, psychostimulant and hypotension caused by spinal anaesthesia and urinary incontinence
11.	Ginsenosides (dammarane and oleanane)	<i>Panax ginseng</i>	Diabetes, anti-stress agents, cardioprotective, antioxidant, anxiolytic, anticancer, aphrodisiac
12.	Hypericin, hyperforin	<i>Hypericum perforatum</i>	Antidepressant, anti-inflammatory, antiviral, anticancer, antioxidant
13.	Indole alkaloids, catharanthine	<i>Catharanthus roseus</i>	Cancer therapy
14.	Kaempferol, isorhamnetin, quercetin, bilobalide, ginkgolide A, B, C, ginsenoside	<i>Ginkgo biloba</i>	Anti-inflammatory, cardiovascular diseases, cancer therapy

(continued)

**Table 3.2** (continued)

Sr. no.	Secondary metabolites (drugs)	Plant source	Medical/pharmaceutical application
15.	Odorobioside G, verodoxin, glucodogifucoside, strosposide, digitoxin, digoxin, ouabain, oleandrin, proscillaridin	<i>Digitalis lanata</i>	Treatment of congestive heart failure, cytotoxic, antidiabetic, neuro, cardioprotective properties
16.	Paclitaxel, essential oils, flavonoids, Taxol, baccatin III, lignans	<i>Taxus</i> species	Antiepileptic, anti-inflammatory, anticancer, antipyretic, analgesic, immunomodulatory, antimicrobial activities.
17.	Pelletierine, ellagic acid, phenol,	<i>Punica granatum</i>	Anticancer, treat dental problems immune modulatory activity
18.	Phenolics steroids, polysaccharides, quinochalcone C-glycosides, quinone-containing chalcones	<i>Carthamus tinctorius</i>	Cardiovascular diseases, cerebrovascular diseases anticoagulant, gastrointestinal, antioxidant,
19.	Phloroglucinols like aspidin, deaspidin, filixic acid	<i>Dryopteris filix-mas</i>	Anti-inflammatory, treating rheumatoid arthritis, wounds, ulcers
20.	Prenylated isoflavones (scandenone and auriculasin)	<i>Maclura pomifera</i>	Astringent, antioxidant and fungicidal agents, cardiovascular activity, insect repellent, natural dye
21.	Quinolone alkaloids, furanocoumarins, acridone, flavonoids	<i>Ruta</i> species	Contraceptive preparations, anti-inflammatory, antipyretic, antioxidant, analgesic, antiviral,
22.	Quinolozidine, alkaloids, nesodine, vertine, demethylsubine I & II	<i>Heimia salicifolia</i>	Antipyretic, laxative and diuretic activity, agents to reduce psychodysleptic activity
23.	$\beta$ -Sitosterol, forskolin, apigenin, chavicol, cavacrol, eugenol, quercetin	<i>Coleus blumei</i>	Cardiotonic, nervous system depressant, vasodilator, immunosuppressant, antioxidant
24.	Scopolamine, hyoscyamine	<i>Scopolia parviflora</i>	Anticholinergic drug, antispasmodic drug hallucinogenic agent
25.	Ajmalicine, vinblastine, vincristine, tabersonine, vindoline, vinceine	<i>Catharanthus roseus</i>	Hodgkin's disease, antineoplastic drug
26.	Phytosterols, triterpenoids, lignans, phenolics, gallic acid, syringin, chlorogenic acid, ethyl gallat, rutin, isoquercitrin	<i>Saussurea medusa</i>	Spasmolytic, antimicrobial, CNS depressant, antioxidant
27.	Shikonin	<i>Arnebia euchroma</i>	Antimicrobial, anticancer, antipyretic, anti-inflammatory
28.	Stevioside, steviol rebaudioside, essential oils	<i>Stevia rebaudiana</i>	Cancer, renal disease, obesity, treating inflammatory bowel disease, dental caries
29.	Kaempferol, syringetin, isorhamnetin, epicatechin, flavonols, vineferin	<i>Vitis vinifera</i>	Laxative, purgative, diuretic, antioxidative, anti-inflammatory, antithrombotic, neuroprotective agents, antianxiotic, anticancer agents

(continued)

**Table 3.2** (continued)

Sr. no.	Secondary metabolites (drugs)	Plant source	Medical/pharmaceutical application
30.	Tropane alkaloids (atropine, hyoscyamine and scopolamine)	<i>Atropa belladonna</i>	Motion sickness, inflammation, antidote for snake bites
31.	Atropine and scopolamine)	<i>Datura stramonium</i>	Treat epilepsy and depression, powerful mind altering drug, anti-inflammatory, antimicrobial
32.	Tropane alkaloids(anisodamine, anisodine and hyoscyamine), piperidine, pyridine alkaloid	<i>Anisodus luridus</i>	Anticholinergic, treatment of septic shock
33.	Phytosterols, saponins, tannins, phenolic compounds, anthraquinone glycosides, triterpenoids	<i>Rubia tinctorum</i>	Treatment of skin disease, spleen disorders, astringent, febrifuge, antithrombotic, antioxidants
34.	Visnagin, khellin, $\gamma$ -pyrones, pyranocoumarins, apigenin, luteolin, chrysoeriol, essential oils	<i>Ammi visnaga</i>	Treatment of renal colic, abdominal cramps, mild anginal symptoms
35.	Syringetin, isorhamnetin	<i>Wrightia tinctoria</i>	Stomach aches
36.	Syringetin, epigenin	<i>Carrisa edulis</i>	Stomach aches
37.	Sterols, alkaloids	<i>Holarrhena antidysenterica</i>	Diarrhea, stomach aches
38.	Rutin, quercetin	<i>Alstonia scholaris</i>	Diarrhea, diuretic
39.	Periconicin A	<i>Taxus cuspidata.</i>	Skin infection, antibacterial, antifungal
40.	Periconicin B	<i>Taxus cuspidata.</i>	Skin infection, antibacterial, antifungal
41.	Methyl triacetic lactone	<i>Polygonum senegalense</i>	Skin infection, antibacterial, antifungal
42.	Altersolanol A	<i>Urospermum picroides</i>	Motion sickness, inflammation, antidote for snake bites
43.	Pestacin	<i>Ephedra fasciculata</i>	Spasmolytic, antimicrobial, CNS depressant, antioxidant
44.	Isopestacin	<i>Ephedra fasciculata</i>	Spasmolytic, antimicrobial, CNS depressant, antioxidant
45.	Pyrrocidine A and B	<i>Zea mays</i>	Antioxidant, antimicrobial
46.	Phomol	<i>Erythrina crista-galli</i>	Antioxidant, antimicrobial, urinary tract infections
47.	Guanacastepene A to L	<i>Daphnopsis Americana</i>	Antioxidant, antimicrobial, sour throat
48.	Xylariaquinone A	<i>Sandoricum Koetjape,</i>	Cytotoxic, anticancer, antifungal
49.	Penicillenols	<i>Aegiceras corniculatum</i>	Cytotoxic, anticancer, antifungal
50.	Nigerasperone	<i>Colpomenia sinuosa</i>	Cytotoxic, anticancer, antifungal
51.	Phomopsin A	<i>E. agallocha</i>	Cytotoxic, anticancer, antifungal

(continued)



**Table 3.2** (continued)

Sr. no.	Secondary metabolites (drugs)	Plant source	Medical/pharmaceutical application
52.	Brefeldin A and B	<i>Taxus mairei</i>	Cytotoxic, anticancer, antifungal
53.	Vincristine	<i>Catharanthus roseus</i>	Anticancer, antifungal
54.	Vinblastine	<i>Catharanthus roseus</i>	Anticancer, antifungal
55.	Paclitaxel (Taxol)	<i>T. brevifolia</i>	Anticancer, antifungal
56.	Torreyanic acid,	<i>T. taxifolia</i>	Anticancer, antifungal

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## Chapter 4

# Extraction and Isolation of Secondary Metabolites from Apocynaceae Plants



### Introduction

Secondary metabolites are compounds produced by plants that are not directly involved in growth and development but instead are involved in various ecological functions, such as defense against predators, attraction of pollinators, and communication with other organisms. Plants produce a wide range of secondary metabolites, including alkaloids, terpenoids, flavonoids, phenolic compounds, and glucosinolates. These compounds can have a variety of biological activities, such as antimicrobial, antifungal, antiviral, antioxidant, anti-inflammatory, and cytotoxic effects. Common examples of plant secondary metabolites include alkaloids (caffeine in coffee, nicotine in tobacco, morphine in poppies), terpenoids (menthol in peppermint, carotenoids in carrots, cannabinoids in cannabis), flavonoids (quercetin in apples, anthocyanins in blueberries, catechins in green tea), polyphenols (resveratrol in grapes, curcumin in turmeric, ellagic acid in berries), glucosinolates (sulforaphane in broccoli, isothiocyanates in mustard, indole-3-carbinol in cruciferous vegetables). The isolation of secondary metabolites from plants is a long and laborious process that involves a series of steps that can vary depending on the type of metabolite being isolated and the plant source. However, some general steps in the isolation process include the following:

**Extraction:** This involves the removal of secondary metabolites from the plant material using a suitable solvent. Different solvents can be used depending on the polarity of the compounds of interest. For example, polar solvents such as methanol or ethanol are often used for flavonoid extraction, while nonpolar solvents such as hexane or chloroform are used for terpenoid extraction.

**Filtration:** After extraction, the solvent is filtered to remove any solid particles or impurities. **Concentration:** The extracted solution is then concentrated to reduce the volume of solvent and increase the concentration of secondary metabolites.

**Purification:** Depending on the complexity of the mixture, further purification steps may be necessary to isolate specific metabolites. This can include techniques

such as column chromatography, thin-layer chromatography, or high-performance liquid chromatography (HPLC).

## **Collection of Plant Material**

The collection of plant material for secondary metabolite isolation should be done carefully to ensure the purity and quality of the compounds obtained. Plant collections are important because they serve as voucher specimens in taxonomic studies. Additionally, they aid in classifying the family, genus, and species. A herbarium is essentially a collection of botanical specimens that have been organized according to a recognized classification system and are available for use in scientific research and other applications. Plants including herbaceous (nonwoody), roots, bulbs, rhizomes, etc., should be gently dug up, and dirt should be removed with caution. Cut one or two small branches when gathering shrubs and trees. For each specimen, enough material should be collected for identification as well as for preparing the herbarium sheet. Plants that are too large to fit on a single sheet can be separated and then pressed as multiple sheets. Having duplicates is a good idea while collecting. This will enable the researcher to create multiple herbarium specimens, and it will be helpful in the event that one specimen is destroyed or lost. It should be kept in mind that the specimens should be representative of the population and should include the range of variation of the plants. The underground parts, such as roots, bulbs, and rhizomes, should be carefully removed. The specimen must include fruits, flowers, etc., for identification purposes. Standard procedures need to be followed for collecting plant material from healthy plants. Leaves and stem bark of healthy plants should be collected in a sterile polythene bag and brought safely to the laboratory. The plant parts should be adequately numbered or labelled in envelopes or packets with the main specimen. The bushy twigs should be pruned to have a flatter specimen so that it can be preserved properly. Thorny plants may first be placed under a board before pressing to prevent tearing the paper. After collection, the surface of plant materials is cleaned by washing under running tap water and then shade dried before extraction. Moreover, local regulations and laws regarding the collection of plant material should be followed to ensure ethical and sustainable collection practices. Additionally, proper documentation and record-keeping should be maintained to ensure the traceability of the plant material.

## **Extraction of Plant Material**

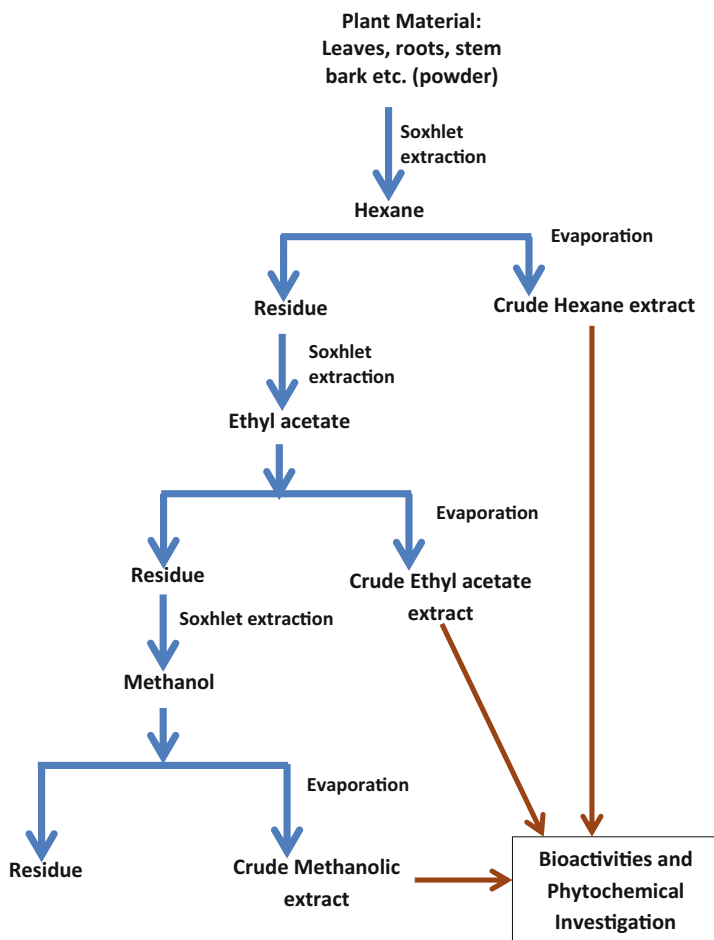
Extraction and preparation of plant extracts are the first and most important steps in medicinal plant research. It is important to note that extraction is a critical step in the isolation of secondary metabolites, and optimization of the extraction process can significantly impact the yield and purity of the final product. Plant material collected

as per scientific methods is authenticated by an expert taxonomist and then subjected to extraction, fractionation, and isolation of bioactive constituents. Extraction of plant material involves the separation of bioactive secondary metabolites such as flavonoids, alkaloids, terpenoids, saponins, steroids, and glycosides using an appropriate solvent and standard extraction principles.

Several techniques are available for the extraction and fractionation of plant material. It includes cold and hot maceration, infusion, decoction, percolation, digestion and Soxhlet extraction, superficial extraction, ultrasound-assisted extraction, and microwave-assisted extraction. The choice of extraction method depends on the nature and type of the plant material, extraction solvent to be utilized, pH and polarity of the solvent, extraction temperature and solvent-to-sample ratio. The extracted secondary metabolites are further purified using partition or column chromatographic techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), paper chromatography (PC), and gas chromatography (GC).

In brief, the plant material to be extracted, such as the leaves, roots and stem bark, etc., is dried under a shade and ground to obtain a fine powder. Successive extraction of plant material is carried out using Soxhlet extraction (Redfern et al., 2014). The fine powder of leaves and stem bark is extracted by the Soxhlet apparatus (Fig. 4.1) using organic solvents with increasing polarity, such as hexane, ethyl acetate, and methanol.

The selection of the extraction solvent depends on the type of plant material to be extracted, the nature of the phytoconstituents in the plant, and the availability of solvent. Generally, polar solvents such as water, ethanol, ethyl acetate, and methanol are preferred for the extraction of polar constituents, whereas hexane and dichloromethane are used in the extraction of nonpolar compounds (Pandey & Tripathi, 2014). Liquid–liquid extraction involves two miscible solvents: water–dichloromethane, water–ether, and water–hexane. Water is used at varying proportions due to its high polarity and miscibility with other organic solvents. The phytoconstituent of interest is extracted using liquid–liquid extraction and should be soluble in organic solvents but not in water for easier separation. To ease separation (Majekodunmi, 2015). The solvents used for the extraction of phytochemicals are selected based on their polarity as per the polarity and miscibility chart (Table 4.1). For example, *n*-hexane, which is the least polar compared to water. Water is the most polar or universal solvent which is commonly used for the extraction of phytoconstituents. The general strategy for successive extraction is depicted in Fig. 4.1. Initially, plant material is extracted using hexane for 48–72 h. The phytochemicals extracted in hexane are (hexane extract) collected in a separate container, and the residue remaining is further subjected to extraction using ethyl acetate and later on with methanol for 48–72 h. After each extraction step, the solvent of the collected extract is evaporated and concentrated under reduced pressure using a rotary vacuum evaporator (Fig. 4.1) to afford the solid/semisolid residue. The test extracts obtained after evaporation from both plants are properly labelled with different codes, usually with the name of the solvents, and the extracts are stored in a refrigerator at 4 °C until use. Overall, solvent extraction is a popular and



**Fig. 4.1** General strategy for the extraction of phytoconstituents from plant material

versatile method for the extraction of secondary metabolites from plants. However, it is important to consider the potential drawbacks, including safety concerns, environmental impacts, and the need for appropriate quality control measures. The extraction process depends on factors such as the choice of solvent, extraction time and temperature, and the type of plant material used. Optimization of these factors can significantly impact the yield and purity of the extracted compounds. The applications, advantages, and drawbacks of some commonly used solvents for the extraction of phytochemicals are summarized in Table 4.2.

Several procedures are commonly used for the extraction of medicinal materials (Ingle et al., 2017). Many methods are used with modifications as per the requirements of the researchers. Nonetheless, the choice of an appropriate extraction method is very important. This section highlights the principles and applications

**Table 4.1** Solvent polarity chart showing formula, boiling point, melting point and solubility in water

Solvent	Formula	Boiling point (°C)	Melting point (°C)	Density (g/mL)	Solubility in H <sub>2</sub> O <sup>1</sup> (g/100 g)	Relative polarity
Cyclohexane	C <sub>6</sub> H <sub>12</sub>	80.7	−6.6	0.779	0.005	0.006
Pentane	C <sub>5</sub> H <sub>12</sub>	36.1	−129.7	0.626	0.0039	0.009
Hexane	C <sub>6</sub> H <sub>14</sub>	69	−95	0.655	0.0014	0.009
Heptane	C <sub>7</sub> H <sub>16</sub>	98	−90.6	0.684	0.0003	0.012
Carbon tetrachloride	CCl <sub>4</sub>	76.7	−22.4	1.594	0.08	0.052
Carbon disulfide	CS <sub>2</sub>	46.3	−111.6	1.263	0.2	0.065
<i>p</i> -xylene	C <sub>8</sub> H <sub>10</sub>	138.3	13.3	0.861	0.02	0.074
Toluene	C <sub>7</sub> H <sub>8</sub>	110.6	−93	0.867	0.05	0.099
Benzene	C <sub>6</sub> H <sub>6</sub>	80.1	5.5	0.879	0.18	0.111
Ether	C <sub>4</sub> H <sub>10</sub> O	34.6	−116.3	0.713	7.5	0.117
Methyl <i>t</i> -butyl ether (MTBE)	C <sub>5</sub> H <sub>12</sub> O	55.2	−109	0.741	4.8	0.124
Diethylamine	C <sub>4</sub> H <sub>11</sub> N	56.3	−48	0.706	M	0.145
Dioxane	C <sub>4</sub> H <sub>8</sub> O <sub>2</sub>	101.1	11.8	1.033	M	0.164
N,N-dimethylaniline	C <sub>8</sub> H <sub>11</sub> N	194.2	2.4	0.956	0.14	0.179
Chlorobenzene	C <sub>6</sub> H <sub>5</sub> Cl	132	−45.6	1.106	0.05	0.188
Anisole	C <sub>7</sub> H <sub>8</sub> O	153.7	−37.5	0.996	0.10	0.198
Tetrahydrofuran (THF)	C <sub>4</sub> H <sub>8</sub> O	66	−108.4	0.886	30	0.207
Ethyl acetate	C <sub>4</sub> H <sub>8</sub> O <sub>2</sub>	77	−83.6	0.894	8.7	0.228
Ethyl benzoate	C <sub>9</sub> H <sub>10</sub> O <sub>2</sub>	213	−34.6	1.047	0.07	0.228
Dimethoxyethane (glyme)	C <sub>4</sub> H <sub>10</sub> O <sub>2</sub>	85	−58	0.868	M	0.231
Diglyme	C <sub>6</sub> H <sub>14</sub> O <sub>3</sub>	162	−64	0.945	M	0.244
Methyl acetate	C <sub>3</sub> H <sub>6</sub> O <sub>2</sub>	56.9	−98.1	0.933	24.4	0.253
Chloroform	CHCl <sub>3</sub>	61.2	−63.5	1.498	0.8	0.259
3-pentanone	C <sub>5</sub> H <sub>12</sub> O	101.7	−39.8	0.814	3.4	0.265
1,1-Dichloroethane	C <sub>2</sub> H <sub>4</sub> Cl <sub>2</sub>	57.3	−97.0	1.176	0.5	0.269
di- <i>n</i> -butyl phthalate	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	340	−35	1.049	0.0011	0.272
Cyclohexanone	C <sub>6</sub> H <sub>10</sub> O	155.6	−16.4	0.948	2.3	0.281
Pyridine	C <sub>5</sub> H <sub>5</sub> N	115.5	−42	0.982	M	0.302
Dimethylphthalate	C <sub>10</sub> H <sub>10</sub> O <sub>4</sub>	283.8	1	1.190	0.43	0.309
Methylene chloride	CH <sub>2</sub> Cl <sub>2</sub>	39.8	−96.7	1.326	1.32	0.309
1-octanol	C <sub>8</sub> H <sub>18</sub> O	194.4	−15	0.827	0.096	0.537
Pentane	C <sub>5</sub> H <sub>12</sub>	36.1	−129.7	0.626	0.004	0.009
1-pentanol	C <sub>5</sub> H <sub>12</sub> O	138.0	−78.2	0.814	2.2	0.568

(continued)

**Table 4.1** (continued)

Solvent	Formula	Boiling point (°C)	Melting point (°C)	Density (g/mL)	Solubility in H <sub>2</sub> O <sup>1</sup> (g/100 g)	Relative polarity
2-pentanol	C <sub>5</sub> H <sub>12</sub> O	119.0	−50	0.810	4.5	0.488
3-pentanol	C <sub>5</sub> H <sub>12</sub> O	115.3	−8	0.821	5.1	0.463
2-pentanone	C <sub>5</sub> H <sub>10</sub> O	102.3	−76.9	0.809	4.3	0.321
3-pentanone	C <sub>5</sub> H <sub>12</sub> O	101.7	−39.8	0.814	3.4	0.265
1-propanol	C <sub>3</sub> H <sub>8</sub> O	97	−126	0.803	M	0.617
Pyridine	C <sub>5</sub> H <sub>5</sub> N	115.5	−42	0.982	M	0.302
Tetrahydrofuran (THF)	C <sub>4</sub> H <sub>8</sub> O	66	−108.4	0.886	30	0.207
Toluene	C <sub>7</sub> H <sub>8</sub>	110.6	−93	0.867	0.05	0.099
<i>p</i> -xylene	C <sub>8</sub> H <sub>10</sub>	138.3	13.3	0.861	0.02	0.074
Water	H <sub>2</sub> O	100.00	0.00	0.998	M	1.000

M miscible. Source: Christian Reichardt, *Solvents and Solvent Effects in Organic Chemistry*, Wiley-VCH Publishers, 3rd ed., 2003

**Table 4.2** Applications, advantages, and drawbacks of some commonly used solvents for the extraction of phytochemicals (Reichardt, 2003)

Sr. no.	Solvent	Extraction applications	Advantages	Drawbacks
1.	Water	One of the most polar solvents. Used for extraction of a wide range of polar compounds	Highly polar, suitable for a wide range of phytoconstituents, cheap, nontoxic, and nonflammable	Causes hydrolysis and very high heat energy is required to concentrate the extract
2.	Alcohol	Polar in nature, miscible with water, and could extract polar secondary metabolites	Nontoxic at low concentrations, less heat energy is required to concentrate the extract	Cannot be used for fats, gums, and wax; it is flammable and volatile.
3.	Chloroform	A nonpolar used for the extraction of terpenoids, flavonoids, fats, and oils	Colorless, has a sweet smell, absorbed by living tissues	Known for its sedative and carcinogenic properties
4.	Petroleum ether	A nonpolar used for the extraction of alkaloids, terpenoids, coumarins, and fatty acids	Testless, miscible with water, has a low boiling point	Highly volatile and flammable
5.	<i>Ionic liquid</i>	Highly polar and extremely heat stable	Highly polar, suitable for microwave-assisted extraction, nonflammable, useful for liquid–liquid extraction	Not ideal for the preparation of tinctures.

of commonly used extraction methods. A number of factors need to be considered before selecting the extraction method. For example, the heat sensitivity of the plant material, biochemical properties of the solvent, intended use of the drug/metabolite,



cost of the product, duration of extraction, etc., must be considered when selecting the extraction method. Commonly used extraction methods, their principles and applications are summarized in Table 4.3.

## Activity-Guided Fractionation for Isolation of Bioactive Secondary Metabolites

The use of medicinal plants in the form of crude extracts involves several challenges. The concentration of bioactive phytoconstituent(s) in a given plant may vary depending on the geographical region, season and time of harvesting, age of plant, etc. Many plant bioactive compounds can be potent poisons if consumed in large amounts. On the other hand, the suboptimal dosage may not show the desired bioactivity if the plant extract has a lower concentration of bioactive compound(s). The medicinal potential of the plant also deteriorates during prolonged and inadequate storage conditions. Therefore, it is necessary to separate and isolate the bioactive compounds in plant extracts. It is possible to increase the efficacy and reduce the adverse effects of crude extracts through structural alteration of extracted and discovered bioactive chemicals from plant extracts. Research on phytochemical aspects in the pharmaceutical industry is concentrated on the development of new medications, novel/indigenous methods for existing medications, and the development of medications derived from plants (Patwardhan et al., 2004). The use of modern technologies has significantly increased the success rate of discovering new leads using plant-derived molecules. Several molecules of plant origin, such as quinine from *Cinchona* bark, morphine and codeine from the opium poppy, digoxin from *Digitalis purpurea* and podophyllotoxin from the rhizome of *Podophyllum*, are in clinical use, and several others are used as lead compounds for the synthesis of new bioactive products (Bhadane et al., 2018). Activity-guided fractionation of plant extracts in combination with chromatographic separation techniques results in better and more effective extraction of biologically active compounds. The development of techniques for the fractionation of plant extracts based on biological activity rather than a specific class of the component is a result of the recent interest in photochemistry-based drug discovery. The majority of naturally occurring bioactive substances are secondary metabolites or species-specific chemical molecules that fall under a variety of categories. The isolated fraction/compound is simultaneously subjected to biological and/or chemical analysis (Phillipson, 2001). The recently discovered natural products such as paclitaxel, camptothecin, etoposide, mevastatin, and artemisinin as well as older ones such as quinine and morphine are the outcome of activity-guided fractionation techniques.

Bioassay-guided fractionation involves the sequential separation of extracted components based on differences in their physicochemical properties and evaluation of their biological activity. It is a widely used strategy to isolate a pure chemical

**Table 4.3** Commonly used extraction methods with their working principle, advantages and drawbacks

Sr. No.	Extraction method	Brief description of the method	Application/ advantages	Drawbacks	Reference
1.	Soxhlet extraction (continuous hot extraction)	Completely dried and finely powdered plant material is placed inside a porous cloth bag or Whatman filter paper. The solvent is poured into the bottom flask which is kept in the heating mantle. As the heating starts, the solvent evaporates and passes through the condenser where it condenses and flows down to the extraction chamber and extracts the drug from the plant material. When the level of solvent in the extraction chamber reaches the top of the siphon, the solvent and the extracted plant material flow back to the flask. The process is repeated for 48–72 h or until the drug is completely extracted. Impurities	Suitable for high-volume extraction. Needs a lesser amount of solvent. The solvent can be reused. No filtration is needed, and a high amount of heat could be applied	Lack of regular shaking and hence extraction takes a longer time. The method is not suitable for thermolabile materials	Ingle et al. (2017), Azwanida (2015), Pandey and Tripathi (2014), Majekodunmi (2015)
2.	Maceration	The coarsely powdered plant material is placed inside a container; the menstruum is poured on top and then closed and kept for at least 3 days. The mixture is stirred periodically. At the	Maceration is suitable for thermo labile plant material. Can be carried out at room temperature. Compounds can be extracted depending on	Consume a large amount of solvent	Doughari (2012)

(continued)

**Table 4.3** (continued)

Sr. No.	Extraction method	Brief description of the method	Application/ advantages	Drawbacks	Reference
		end of extraction, the micelle is separated by filtration or decantation, and the micelle is then separated from the menstruum by evaporation	the polarity of the solvent		
3.	Digestion	Digestion uses moderate heat for. The extraction solvent is poured into a clean container and then the powdered drug material is added. The mixture is placed over a water bath/ oven at approximately 50 °C. the heat is applied throughout the extraction process decreases the viscosity of the extraction solvent and separates the secondary metabolites	This method is suitable for soluble plant materials		Ingale et al. (2017), Pandey and Tripathi (2014), Majekodunmi (2015)
4.	Percolation	It is carried out using a percolator. The coarsely powdered plant material is moistened with the extraction solvent and kept for 4–6 h. the extraction solvent is poured to saturate the plant material. The liquid was allowed to drip slowly from the lower part of the percolator. At the same, the solvent is added continuously as the extraction	Carried out at room temperature, occasionally under heat	Consume a large amount of solvent	Pandey and Tripathi (2014), Majekodunmi (2015)

(continued)

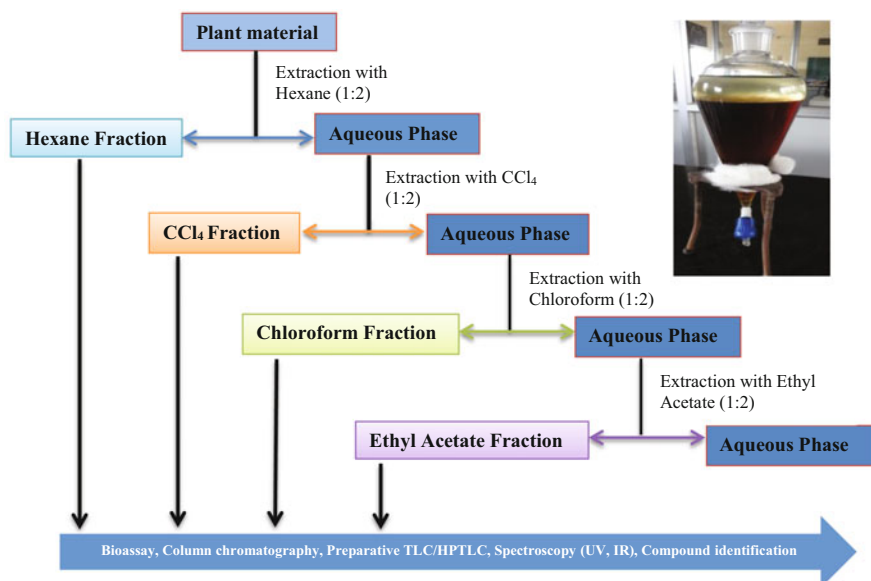
**Table 4.3** (continued)

Sr. No.	Extraction method	Brief description of the method	Application/ advantages	Drawbacks	Reference
		continues. The extract is separated by filtration or centrifugation			
5.	Microwave-assisted extraction	Microwave-assisted extraction works on the principle of dipole rotation and ionic transfer by displacement of charged ions in plant material and solvent. The electromagnetic radiation in frequencies between 300 MHz and 300 GHz and wavelength between 1 cm and 1 m are bombarded onto the extraction material that produces heat which is transferred in the plant material leading to the extraction of phytoconstituents. Used	Uses little quantity of solvent and has less time of extraction as well as the increase in the outcome	It is suitable only for phenolics and flavonoids. Other compounds such as tannins and anthocyanins may be degraded because of high heating	Pandey and Tripathi (2014), Majekodunmi (2015), Altemimi (2017)
6.	Ultrasound-assisted extraction	This method uses sound energy at a very high frequency (greater than 20 KHz) that disrupts the cells and membranes of plant cells increasing the drug surface area for solvent penetration which leads to the release of secondary metabolites. The coarsely powdered plant material is moistened with the extraction	It applies to small samples. Extraction time is less. Uses lesser amounts of solvents and maximizes the yield	Lesser reproducibility. The high amount of energy applied may degrade the phytochemicals	Pandey and Tripathi (2014), Majekodunmi (2015), Azwanida (2015)

(continued)

**Table 4.3** (continued)

Sr. No.	Extraction method	Brief description of the method	Application/ advantages	Drawbacks	Reference
		solvent in an ultra-sonic extractor. The high-frequency sound waves enhance the extraction process by reducing the heat			
7.	Supercritical fluid extraction	It uses supercritical fluids that can dissolve a wide variety of natural products. Supercritical fluids change their solvating properties due to changes in pressure and temperature. Supercritical carbon dioxide (S-CO <sub>2</sub> ) is widely used in supercritical fluid extraction because of its low critical temperature (31 °C), inertness, low cost; nontoxicity as well as it can extract thermolabile secondary metabolites	The low polarity of S-CO <sub>2</sub> makes it ideal for the extraction of nonpolar compounds such as lipid and volatile oils		Zhang et al. (2018)
8.	Enzyme assisted extraction	Enzyme-assisted extraction makes use of enzymes such as Cellulases, $\alpha$ -amylase, proteases and pectinase. The hydrolytic action of these enzymes on plant cell components such as the cell wall and membranes, natural products inside the cell are released	Higher extraction yield. No use of toxic solvents	Enzymes are costlier as compared to solvents	Zhang et al. (2018)



**Fig. 4.2** Liquid-liquid extraction scheme for the isolation of bioactive principles from plant extracts

agent from complex natural sources. The strategy for bioassay-guided fractionation is presented in Fig. 4.2.

The partial purification and separation of phytochemicals are carried out according to the polarity of crude plant material (Eloff, 1998). The crude plant extract is suspended in distilled water in a separating funnel and successively partitioned with hexane, chloroform, ethyl acetate, and water in this order (in increasing polarity). These solvents with increasing polarities will selectively extract different phytoconstituents that are soluble in a particular solvent. It will yield four to five fractions of active plant extract. The resulting fractions are further evaluated for intended biological activities (*in vitro* or *in vivo*). The fractions showing potent activities are used for the isolation of active principles by column chromatography or preparative HPTLC/TLC methods. The isolation of the bioactive phytochemicals from selected fractions of active medicinal plants can be carried out by column chromatography followed by preparative HPTLC/TLC. These separation techniques provide valuable information about phytoconstituents present in the crude extracts and are also invariably handsome techniques for the isolation of desired compounds from the crude plant material based on differences in polarity. Thus, bioassay-guided fractionation and purification techniques are essential for the successful discovery of bioactive natural products from medicinal plants.

## Advantages of Activity-Guided Fractionation

Activity-guided fractionation enables the identification of the specific bioactive compounds responsible for the observed biological activity. This allows for the isolation and purification of these compounds, which can be used for further studies or potential drug development. Activity-guided fractionation allows for the efficient use of resources, including time, effort, and materials. By targeting the most active fractions, the isolation and purification process can be streamlined, reducing the need for extensive separation and purification steps. Plant extracts can contain hundreds or thousands of different compounds, making it difficult to identify the specific compounds responsible for the observed activity. Activity-guided fractionation reduces the complexity of the extract by focusing on the most active fractions, making it easier to identify and isolate the target compounds. It enables the isolation and purification of the bioactive compounds, resulting in higher purity and quality of the final product. Activity-guided fractionation can lead to the discovery of new bioactive compounds with potential therapeutic applications. By identifying the specific compounds responsible for the observed biological activity, new drug candidates can be developed and tested.

In summary, activity-guided fractionation is an effective method for the isolation and purification of bioactive secondary metabolites from plant extracts. It allows for the identification of the specific compounds responsible for the observed activity, leading to higher purity and quality of the final product and the potential for drug discovery.

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# Chapter 5

## Phytochemical Screening and Chemical Characterization of the Bioactive Metabolites



### Introduction

Preliminary phytochemical analysis of the crude extract is carried out for the detection of primary as well as secondary metabolites present in the crude extracts. The phytochemicals such as alkaloids, flavonoids glycoside, steroids and triterpenoids, saponins, tannins etc. are detected by various chemical methods. This proximate chemical analysis of the crude extract gives an idea about the presence or absence of certain metabolites in the extract which helps to decide the purification strategy for the compound of interest. In addition to revealing the components of plant extracts and which one predominates over the others, phytochemical screening is effective for finding bioactive compounds that can be used in the production of therapeutic pharmaceuticals.

In general phytochemical screening involves preliminary and specific tests. Preliminary tests are usually performed to determine the presence or absence of major classes of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, and phenolic compounds. These tests are often performed using simple colorimetric tests or specific reagents that react with the target compounds. Once the presence of a particular class of compound is confirmed using a preliminary test, specific tests can be performed to identify the individual compounds within that class. These tests can include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), and mass spectrometry (MS). After identifying the compounds present in the extract, quantitative analysis can be performed to determine the amount of each compound. This can be done using various analytical techniques such as UV-visible spectroscopy, HPLC, or GC. In the end, the results of the phytochemical screening can be analyzed to determine the potential medicinal or industrial uses of the plant extract. The compounds identified may have antibacterial, anti-inflammatory, antioxidant, or other biological activities.

It is important to note that phytochemical screening is just one step in the process of evaluating the biological activity of plant extracts. Further studies, including in vitro and in vivo assays, are often needed to fully understand the potential medicinal or industrial applications of the compounds identified.

## **Phytochemical Screening Methods**

### ***Tests for Proteins***

#### **Biuret Test**

Three millilitre of aqueous tuber extract is added with a few drops of NaOH (4%) and CuSO<sub>4</sub> solution. The appearance of violet or pink color indicates the presence of proteins.

#### **Million's Test**

Three millilitre of aqueous tuber extract is mixed with 5 mL of Million's reagent. The white precipitate is obtained. After warming the obtained precipitate, the development of a brick-red color indicates the presence of proteins.

#### **Ninhydrin Test**

In the 3 mL of aqueous tuber extract, three drops of ninhydrin solution (5%) are added. The mixed solution is heated in a boiling water bath for 10 min formation of the purple or bluish color indicates the presence of proteins.

#### **Xanthoproteic Test**

One millilitre of the extract was taken and placed into a test tube. Then few drops of nitric acid were added and shaken. The emergence of yellow color indicates the presence of protein.

## ***Test for Carbohydrates***

### **Molish Test**

To 2–3 mL of aqueous tuber extract, a few drops of the alcoholic alpha-naphthol solution is added. The reaction mixture is shaken and the solution is allowed to stabilize. Afterward, the concentrated sulphuric acid from the sides of the test tube is added. The violet ring formed at the junction of two liquids confirms the presence of carbohydrates.

### **Fehling Test**

One millilitre of Fehling's solution A and 1 mL of Fehling's solution B are mixed and boiled for 1 min. An equal volume of aqueous tuber extract is added to it and heated in a boiling water bath for 5–10 min. The formation of a yellow, then brick-red precipitate indicates the presence of carbohydrates.

### **Benedict's Test**

One millilitre of aqueous tuber extract is mixed with 1 mL of benedict's reagent. The mixed solution is heated in a boiling water bath for 5 min. The solution appeared green, yellow, or red depending on the amount of reducing sugar present in the test solution indicating the presence of carbohydrates.

### **The Tannic Acid Test for Starch**

Formation of precipitate after adding a few drops of tannic acid solution (20%) to tuber extract indicated the presence of starch.

## ***Test for Alkaloids***

### **Mayer's Test**

One millilitre of test extract is treated with 1–2 drops of Mayer's reagent. White or creamy precipitate in the reaction mixture indicates the presence of alkaloids (Kokate, 1994; Khandelwal, 2006).

**Wagner's Test**

One millilitre of test extract is treated with 1–2 drops of Wagner's reagent. Reddish brown precipitate in the reaction mixture indicates the presence of alkaloids.

**Dragendorff's Test**

One millilitre of test extract is treated with 1–2 drops of Dragendorff's reagent. Reddish brown precipitate in the reaction mixture indicates the presence of alkaloids.

**Hager's Test**

One millilitre of test extract is treated with 1–2 drops of Hager's reagent. Yellow precipitate in the reaction mixture indicates the presence of alkaloids.

***Test for Flavonoids*****Shinoda Test**

To the crude extract (2 mL), pieces of magnesium ribbons were added and simultaneously concentrated  $\text{H}_2\text{SO}_4$  is added by the side wall of the test tube. Pink scarlet or crimson red color in the reaction mixture indicates the presence of flavonoids.

**Zinc Hydrochloride Test**

To the test extract (2 mL), a pinch of zinc dust is added, and then concentrated  $\text{H}_2\text{SO}_4$  is added by the side wall of the test tube. Formation of red color after few min. Indicates the presence of flavonoids.

***Test for Glycosides*****Borntrager's Test**

To the crude extract (2 mL), 3 mL chloroform is added and shaken for a few min. A few drops of 10% ammonia solution were added to the reaction mixture. The pink color in the reaction mixture indicates the presence of glycosides.

**Keller-Killiani Test**

Test extract (2 mL) is mixed with an equal volume of glacial acetic acid. One drop of 5%  $\text{FeCl}_3$  is added to the mixture. A few drops of concentrated  $\text{H}_2\text{SO}_4$  are added to the side wall of the test tube. The bluish-green colour in the reaction mixture indicates the presence of glycosides.

**Legals Test**

One millilitre of an extract is taken, and then an equal volume of sodium nitroprusside is added followed by few quantity of sodium hydroxide solution and shaken. The formation of pink-to-blood-red precipitate signifies the existence of cardiac glycoside.

***Test for Steroids and Triterpenoids*****Salkowski Test**

Two millilitre extract is mixed with an equal volume of chloroform and treated with a few drops of concentrated  $\text{H}_2\text{SO}_4$  and the mixture is vigorously shaken. The red color ring at the bottom of the test tube indicates the presence of steroids and the yellow colored lower layer indicates the presence of triterpenoids.

**Libermann Burchard's Test**

This method is utilized for an alcoholic extract. The extract needs to dry out first through evaporation, then be extracted again with chloroform. Add a few drops of acetic anhydride followed by sulfuric acid from the side of the test tube. The formation of violet-to-blue-colored rings at the junction of the two liquids indicates the presence of steroids.

***Test for Saponins*****Froth Formation Test**

The crude extract is mixed with an equal volume of water and shaken vigorously for a few min. and left it to stand. The stable froth (foam) formation indicates the presence of saponins.

## ***Test for Tannins***

### **Ferric Chloride Test**

A few drops of 5%  $\text{FeCl}_3$  solution are added to the 2 mL test extract. The formation of blue color indicates the presence of hydrolyzable tannins and the formation of green color indicates the presence of condensed tannins.

### **Skin Test for Gold Beater**

Ox skin is used to make a Gold Beater's Skin. After being submerged in 2% hydrochloric acid, the skin of the Gold Beater is rinsed with distilled water. Following a 5-min soak in an extract solution, it is rinsed with distilled water. It is then dipped into a solution of 1% ferrous sulfate. Tannins are present if the Gold Beater's Skin turned brown or black (Pandey & Tripathi, 2014).

### **Gelatin's Test**

A test tube is filled with 1 mL of the extract. After adding and shaking 1% gelatin solution containing sodium chloride. The presence of tannins is indicated by the presence of white precipitate (Pandey & Tripathi, 2014).

## ***Total Phenolics and Flavonoids***

The total phenolic contents of the plant extracts such as leaves, stem bark, and roots can be determined by spectrophotometric methods as described by various researchers (Singleton & Rossi, 1965). One millilitre of 10% Folin-ciocalteu's reagent is added to 1 mL crude extract (1000  $\mu\text{g/mL}$ ) and mixed thoroughly. To the mixture, 4 mL sodium carbonate (7.5%) and 10 mL of distilled water are added and thoroughly mixed. The mixture is allowed to stand for 2 h at room temperature and the absorbance can be read at 760 nm. The tests are performed in three replicates for better accuracy. A standard curve is also obtained using various concentrations of gallic acid (10–100  $\mu\text{g/mL}$ ) and the total phenols are expressed as mg of GAE gallic acid equivalent/g of extract. Total flavonoid contents in plant extracts such as leaves, stem bark, and roots can be determined by spectrophotometric methods developed by Christel et al. (2000). The crude extract (1 mL) is mixed with an equal volume of 2% methanolic solution of Aluminum chloride. Quercetin equivalents at different concentrations are employed to prepare a standard curve. All the mixtures are incubated at room temperature for 15 min and absorbance is recorded at 430 nm.

Each test is performed in three replicates. Total flavonoids content can be expressed in mg/mL of quercetin equivalent.

**Preliminary Phytochemical Characters of Different Extracts of Some Members of the Family Apocynaceae (*C. carandas* and *T. divaricate*, *C. procerra*)**

The phytochemical analysis of various extracts extract is carried out for the screening of major phytoconstituents. These methods are widely published with their advantages and drawbacks (Kokate, 1994). The phytochemicals present in the extracts of Apocynaceae plants are widely studied. Apocynaceae members such as *C. carandas*, *T. divaricata* and *C. procerra* have been investigated for the presence of phytochemicals like alkaloids, flavonoids glycoside, steroids and triterpenoids, saponins, tannins, etc.

Preliminary phytochemical investigation different extracts of *T. divaricata* revealed that alkaloids and tannins were present in higher concentrations, tannins and glycosides were in moderate amounts and flavonoids were found present in fewer amounts. The ethyl acetate extract of this plant showed the presence of a wider group of phytochemicals in it. Table 5.1 represents the results of the phytochemical

**Table 5.1** Phytochemical analysis of different extracts of *T. divaricata*

Phytochemicals	Test	<i>T. divaricata</i> extracts				
		Td (L)E	Td (L)M	Td (S)E	Td (S)M	Td Callus
Alkaloids	Mayer’s test	+++	+++	+++	+++	++
	Wagner’s test	++	++	++	++	+
	Dragendorff’s test	+++	+++	+++	+++	++
	Hager’s test	++	++	++	++	+
Flavonoids	Shinoda test	+	–	–	–	–
	Zinc HCl test	+	–	–	–	–
Glycosides	Keller-Killiani test	++	–	+	+	–
	Borntrager’s test	+	–	–	–	–
Steroids and triterpenoids	Salkowski test	+++	+++	+++	+++	++
Tannins	Ferric chloride test	++	++	++	++	++
	Gelatin test	++	++	++	++	++
Saponins	Froth formation test	–	+	–	–	+

(–) Not detected; (+) present in low concentration; (++) present in moderate concentration; (+++) present in high concentration. **Extract codes:** (Td(L)H *T. divaricata* leaves hexane extract, Td(L)E *T. divaricata* leaves ethyl acetate extract, Td(L)M *T. divaricata* leaves methanol extract, Td(S)H *T. divaricata* stem bark hexane extract, Td(S)E *T. divaricata* stem bark ethyl acetate extract, Td (S)M, *T. divaricata* stem bark methanol extract)

**Table 5.2** Phytochemical analysis of different extracts of *C. carandas*

Phytochemicals	Test	<i>C. carandas</i> extracts				
		Cc (L)E	Cc (L)M	Cc (S)E	Cc (S)M	Cc Callus
Alkaloids	Mayer's test	++	+++	+++	+++	++
	Wagner's test	++	++	++	++	+
	Dragendorff's test	+++	+++	+++	+++	++
	Hager's test	++	++	++	++	+
Flavonoids	Shinoda test	+	+	+	+	—
	Zinc HCl test	+	+	+	+	—
Glycosides	Keller-Killiani test	+	—	—	—	—
	Borntrager's test	+	—	—	—	—
Steroids and triterpenoids	Salkowski test	+++	+++	+++	++	++
Tannins	Ferric chloride test	+++	+++	++	++	++
	Gelatin test	++	++	++	++	++
Saponins	Froth formation test	—	+++	—	++	+

(—) Not detected; (+) present in low concentration; (++) present in moderate concentration; (+++) present in high concentration. **Extract codes:** Cc(L)H *C. carandas* leaves hexane extract, Cc(L)E *C. carandas* leaves ethyl acetate extract, Cc(L)M *C. carandas* leaves methanol extract; Cc(S)H *C. carandas* stem bark hexane extract; Cc(S)E *C. carandas* stem bark ethyl acetate extract; Cc(S)M *C. carandas* stem bark methanol extract)

analysis. The cells of *T. divaricata* retain their ability to synthesize phytochemicals in an *in vitro* condition also when cultivated using plant tissue culture techniques (Bhadane et al., 2018b). The total phenolic content (TPC) of Td(L)E extract was found to be 97.67 mg GAE/g of extract. The number of phenolics was comparatively less (64.50 mg GAE/g of extract) in Td(C) extract.

Preliminary phytochemical investigation of *C. carandas* is also widely studied and well documented. The phytochemical screening revealed that alkaloids and steroids were present in higher concentrations in Cc(L)E and Cc(L)M. Tannins were present in moderate amounts and flavonoids as well as glycosides were reported at least concentration. However, saponins were dominantly present in Cc (L)M and Cc(S)M only. The maximum number of phytochemicals is found to be present in ethyl acetate and methanol extract of leaves of this plant. Table 5.2 represents the results of the phytochemical analysis. Callus extract showed a moderate presence of the same phytochemicals except for flavonoids and glycosides in comparison with wild plant extracts. It indicates that cells of *C. carandas* retain their ability to synthesize phytochemicals in an *in vitro* condition also.

The TPC of different extracts of *C. carandas*. The TPC of Cc(L)E was found to be 106.67 mg GAE/g of extract. The amount of phenolics was comparatively less (72.50 mg GAE/g of extract) in Cc(S)M.

*C. procera*, another member of this family is screened for the phytochemical investigation (Patil et al., 2015). The total ash was found to be 27.10% whereas, the



water and acid-soluble ash was found to be 2.95% and 1.85%, respectively. Preliminary phytochemical screening of acetone and methanol crude extract revealed the presence of alkaloids, triterpenoids, phenols, flavonoids, tannins and saponins (Mainasara et al., 2012). Phytochemical investigation showed dominant presence of triterpenoids in both the methanol and acetone extract. The methanolic and acetone crude extracts showed the presence of polyphenols at 582.94 and 823.20 µg GAE/g of extracts, respectively (Patil et al., 2015).

## **Chromatographic Separation of Compounds from a Mixture or Extracts**

The fractionation process carried out using solvents of increasing polarity separates the plant extracts into various fractions. Fractionation techniques are classified into physical or chemical methods. The physical fractionation is carried out using various chromatographic techniques until a pure compound is obtained. Chromatographic techniques are used for separating compounds based on size, shape, and charge from complex biological mixtures. The idea of chromatography involves the use of a stationary phase, such as silica gel or Sephadex combined with calcium sulfate as a binder, and a mobile phase, which is the extraction solvent (Sasidharan et al., 2011). The macromolecules such as amino acids, carbohydrates, fatty acids, lipids, and alkaloids can be separated using silica gel. Proteins and amino acids can be isolated using Sephadex. Aluminum is helpful in the separation of phenols, steroids, alkaloids, vitamins, and carotenes. Separating amino acids, food colors, and alkaloids all employ cellulose powder. Steroids and organic cations can be separated using celite as a matrix. Chromatographic separation of the biomolecules is based on adsorption, partition, affinity, ion exchange, or size exclusion (Sasidharan et al., 2011; Ingle et al., 2017). The chromatographic techniques include paper chromatography (PC), Thin layer chromatography (TLC), column chromatography (CC), liquid chromatography (LC), GC, and HPLC (Smith, 2013). These techniques work on various mechanisms that are responsible for the separation of phytoconstituents (Table 5.3).

## **Application of TLC and HPTLC in the Separation of Plant Extracts**

TLC uses an adsorption mechanism for the separation of phytoconstituents from crude extracts. Separation is based on the interaction between the compounds in a mixture and stationary phase (usually an inert material. TLC is used for the separation of compounds with low molecular weight (Ingle et al., 2017). The silica gel is dissolved in distilled water to make slurry. The slurry of gel is then poured into a glass/aluminum plate with dimensions 20 cm × 20 cm to produce a uniform layer of

**Table 5.3** Separation mechanisms in different chromatographic techniques

Sr. no.	Chromatography	Mechanism of separation	Reference
1.	Partition chromatography	Separation occurs by the addition of two or more immiscible solvents into the extract. The compound partitions itself based on its solubility. The immiscible liquids are separated using a separating funnel to afford the individual compounds	Ingle et al. (2017)
2.	Adsorption chromatography	Separation occurs based on the interaction between compounds to be separated and the stationary phase. It involves noncovalent interactions such as hydrophobic, non-covalent Van der Waals forces. The compound that interacts loosely with the stationary phase gets eluted early	Smith (2013)
3.	Affinity chromatography	It uses a ligand positioned in a separating column as a stationary phase. The mobile phase separates compounds that have low or no affinity for the stationary phase therefore the compounds with high affinity for the stationary phase get attracted and separated	Smith (2013)
4.	Size exclusion chromatography.	Separation takes place based on the molecular size of the compounds. It is also known as molecular sieving or gel filtration. A small, medium and larger pores size mesh is used as a stationary phase	Sasidharan et al. (2011)
5.	Ion exchange chromatography	It is used for the separation of polar compounds. Separation occurs based on the electrical charge present in the biomolecules. The charged substances are attracted to each other and get separated	Sasidharan et al. (2011)

gel having a thickness of 1.5 mm. It is then kept for 1 h at 105 °C to get activated. The TLC analysis of bioactive metabolites from different crude extracts can also be performed on precoated 20 × 20 cm silica gel 60 F254 (0.25 mm thick) thin-layer chromatography plates (Merck, Germany). The TLC plates of desired size are cut and approximately 10–15 µL extracts are spotted on the TLC plates using the capillary tube, needle, or by using an automatic sample applicator (Fig. 5.1). The spots are air-dried and plates developed at room temperature in ascending manner. Chamber saturation time is usually 20 min or it may vary depending on the mobile phase composition. The plate development is carried out in a vertical chromatographic chamber (Fig. 5.1) with suitable dimensions. Different combinations of mobile phases can be used for separation. Table 5.4 represents mobile phases used for TLC separation. Wagner and Bladt (1996) described the separation of major phytochemicals like alkaloids, terpenoids, flavonoids, glycosides, and saponins.

After 2/3 development, the plates are removed from the chamber and the mobile phase associated with the plates is evaporated using heat treatment in a hot air oven at 80 °C for 1–2 min or by using the hair drier. The detection of the separated phytoconstituents can be carried out by observing the plates under UV light at 254 nm and 365 nm wavelengths. Derivatization can be carried out using different reagents



**Fig. 5.1** HPTLC automatic sample applicator, Chromatography chambers and documentation system for TLC visualization and analysis

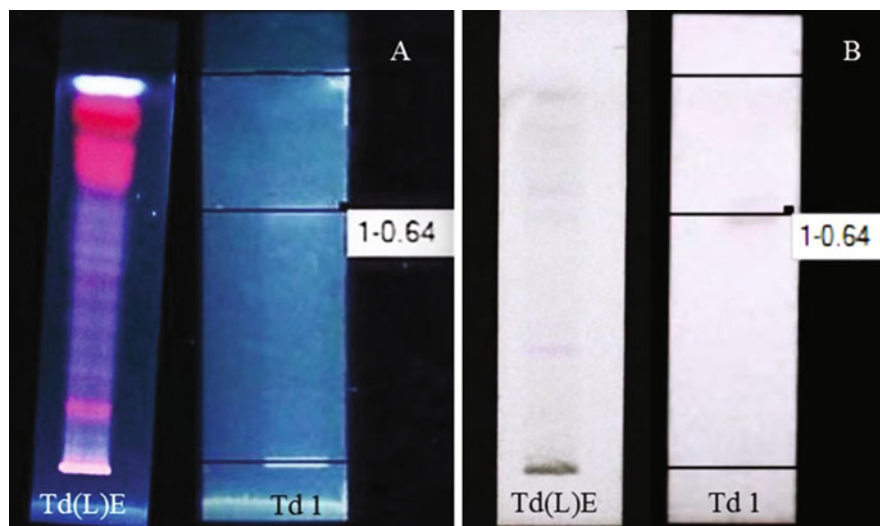
**Table 5.4** Composition of mobile phases (v/v) used for separation of bioactive compounds of plant extracts

Sr. no.	Mobile phase	Combinations
1.	Chloroform:Methanol:NH <sub>4</sub> OH:Water	70:20:2:2
2.	Ethyl acetate:Methanol:Water	77:15:8
3.	Hexane:Ethyl acetate	60:40, 80:20, 50:50
4.	Toluene:Methanol	80:20, 90:10
5.	Chloroform:Ethyl acetate	70:30
6.	Ethyl acetate:Methanol	100:12
7.	Toluene:Ethyl acetate	70:10, 60:20
8.	Toluene:Ethyl acetate:Diethylamine	70:20:10
9.	Dichloromethane:Ethyl acetate	90:10
10.	Toluene:Ethyl acetate:Acetic acid	70:15:5
11.	Ethyl acetate:Formic acid:Glacial acetic acid:Water	100:11:11:26
12.	Chloroform:Glacial acetic acid:Methanol:Water	65:32:12:8
13.	Toluene:Chloroform:Ethanol	40:40:10

like 10% ethanolic sulfuric acid or anisaldehyde sulphuric acid to visualize the colored bands. The imaging and  $R_f$  (Relative flow) value of separated compounds is determined using an automatic luminous documentation system and software.  $R_f$  values of observed compounds were also determined by given formulae.

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

The separation of metabolites in crude extract using HPTLC is more or less similar to that of TLC. The application of the extract on the TLC/HPTLC plate is carried out using an automatic sample applicator that applies a specific quantity of sample using a thin glass syringe with the help of pressured nitrogen gas (Fig. 5.2).



**Fig. 5.2** TLC profile of ethanol extract of *Tabernamontana divaricate* leaves and the purified fraction showing the  $R_f$  0.64 under UV 365 nm; (b) and in visible light (Bhadane et al., 2018a)

## Column Chromatography

Purification of the bioactive principles can also be carried out using column chromatography. The column of varying dimensions is used for his purpose. The glass columns of 30 cm in length and 1.8 cm internal diameter equipped with the sintered disc are normally preferred used for separation (Fig. 5.3). The selection of the mobile phase for separation is generally based on the separation results achieved in TLC/HPTLC. The solvents that gave better separation in TLC are used for the elution of the column. For column preparation, Silica gel of 60–120 mesh size is poured into a clean beaker and mixed with mobile phase (1:1) to make a slurry. Before preparation of the column bed, the empty column is filled with elution solvent. Silica gel slurry is then poured gradually into the column by tapping on the outer wall of the column. For better column packing, the outer side of the column is usually tapped using silicon tubing and fingers. During column packing care should be taken that air bubbles and gaps are not formed in the column. After column packing, the mobile phase is poured on the top of the column bed and eluted several times, and the column is left to wet overnight in the mobile phase for uniform packing and saturation.

The plant extract to be separated is loaded by the dry loading method. Samples are mixed with a small amount of silica 60–120 mesh and mobile phase. The mobile phase is allowed to evaporate in rota vapor to get dry silica powder containing plant extract. The resulting sample containing plant extract is then subjected to column chromatographic separation using mobile phases as an eluent for the respective plant

**Fig. 5.3** Silica gel column used for activity-guided separation

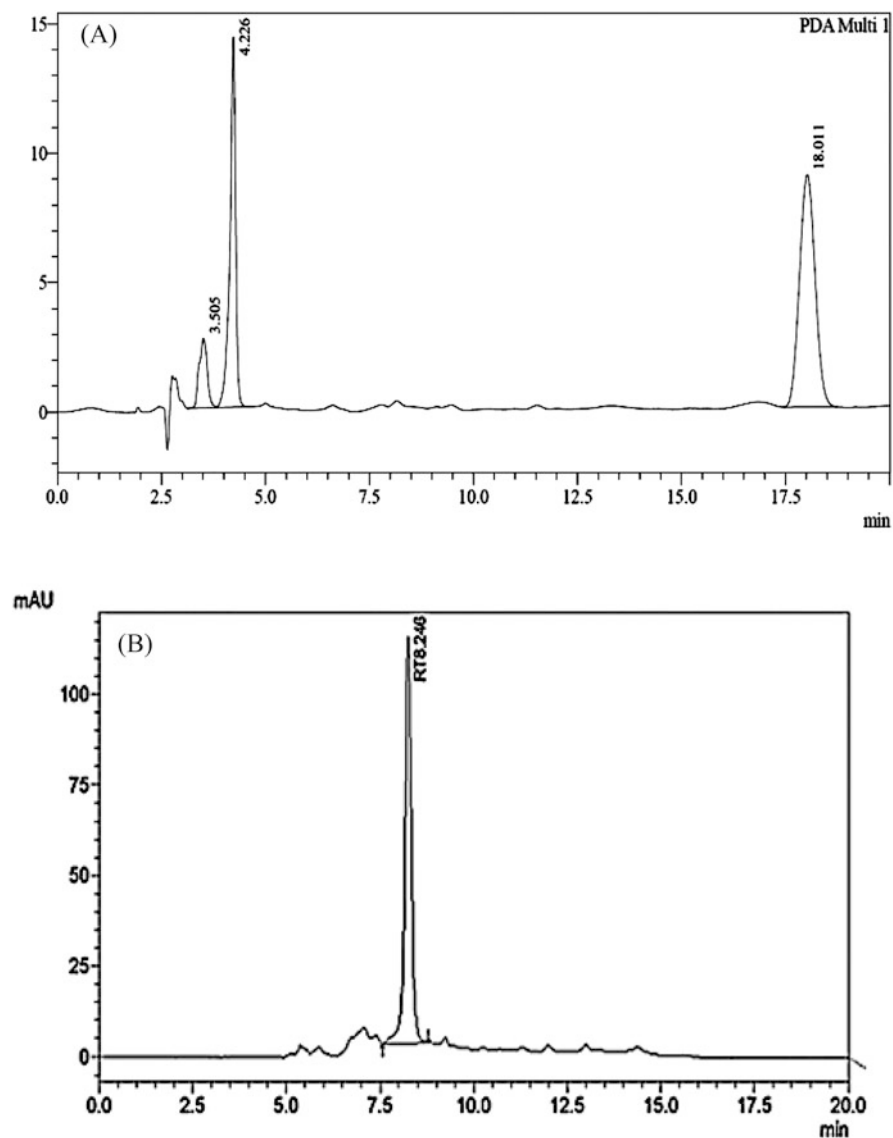


extract. The sample is poured carefully on the top of the column bed to get an even layer. Simultaneously, the mobile phase is added using a Pasteur pipette to wet the sample with care so that the pipette does not touch to column bed. After loading the sample, the mobile phase is poured through the side wall of the column and fractions are collected in 5 mL quantity. During elution, the constant flow rate of eluent is maintained for all fractions. Simultaneously, TLC of the eluted fraction can be carried out to check the presence of a single compound. The fractions that show the same pattern on the TLC plate with the same  $R_f$  value are mixed and used for further analysis (Patil et al., 2015).

## High-Performance Liquid Chromatography (HPLC)

HPLC is widely used for the isolation and characterization of natural products. It is a flexible, reliable, and preferred method. HPLC is a chromatographic method that can separate a mixture of compounds and is used in phytochemistry to identify, measure, and purify the individual components of the mixture (Cannell, 1998). Being the preferred method for fingerprinting research for the quality monitoring of herbal plants, this methodology is currently widely used among other analytical techniques (Fan et al., 2006). In order to completely describe a natural product's qualities, it is usually separated after a biological assay evaluates a relatively crude extract. Such multi-component samples can be processed quickly on an analytical and preparative scale because of the HPLC's resolution power. Due to its wide range of applications, reversed-phase chromatography is the separation method employed most frequently in HPLC. According to estimates, the reversed-phase mode is used for more than 65% of all HPLC separations. This is due to the ease of use, adaptability, and range of the reversed-phase approach, which can handle molecules with various polarities such as secondary plant metabolites. Several reports describe HPLC-based isolation and characterization of secondary metabolites of Apocynaceae plants, mainly phenol compounds, terpenoids, saponins, steroids, flavonoids, and alkaloids (Patil et al., 2015).

The HPLC characterization protocols vary for different metabolites. The partially purified extracts or the bioactive fractions eluted from the silica gel column or the HPTLC eluted bands can be subjected to HPLC analysis. HPLC is carried out using a number of chromatography systems of varying configurations of pumps, detectors, columns etc. For example, LC 20 Ad liquid chromatography system (Shimadzu, Japan) equipped with SPD-M20A PDA detector and C18 120 (250 × 4.60) mm column with 5 µm internal diameter. For loading the sample, the partially purified fractions are filtered using 0.45 µ filter and sonicate prior to analysis. 20–30 µL sample is injected and the chromatogram is allowed to develop using various mobile phases in different combinations. Mobile phases commonly used for plant secondary metabolites consist of acetonitrile: water (70:30, 85:15), methanol: water (80:20, 60:40, 50:50), and 90% acetonitrile: ammonium acetate (100 mM, pH 4.2). The flow rate is in the range of 0.5–1 mL/min. Detection of eluting compounds can be monitored in the range of 280–350 nm (Dubber & Kanfer, 2004; Hwang et al., 2011). A chromatogram (Fig. 5.4) obtained from Shimadzu LC solution software is indicated absorbance intensities and retention time of the eluted compound. Usually, the number of cycles is repeated with the same parameters to check the reproducibility in analysis.



**Fig. 5.4** A typical chromatogram of partially purified extract of leaf of *T. divaricate* (a). Chromatogram of pure compound (b)

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# Chapter 6

## Identification of Bioactive Secondary Metabolites of Apocynaceae Members



### Introduction

The partially purified extracts or the isolated fractions containing the single compound obtained using chromatographic procedures are subjected to compound identification. Chromatographic techniques can be used for the separation as well as for the identification of the active components in a mixture. Large-scale separation and purification of metabolites can be carried out using these techniques. Several methods were used for the identification of isolated compounds from Apocynaceae members. These methods are based on the detection of functional groups, the presence of multiple bonds and rings, hydrogen and carbon arrangement and full structural elucidation. Modern techniques such as ultraviolet spectroscopy (UV), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), and mass spectrometry (MS) are commonly used for complete identification of the compound.

### UV–Visible Spectroscopy

Spectroscopy deals with the study of the interaction between electromagnetic radiation and matter. UV–vis spectrophotometry is one of the most commonly employed techniques in phytochemical and pharmaceutical analysis. UV–vis visible spectrophotometry measures the amount of UV or visible light absorbed by an absorbing substance in a medium. Ultraviolet–visible spectrophotometers measure the ratio of the intensity of two beams of light in the UV–vis visible region. The principle of ultraviolet–visible spectrophotometers is based on the Beer–Lambert law, which states that when a beam of parallel monochromatic light is passed through an absorbing material, the intensity of light may decrease. The absorbance is proportional to the path length of the cell through the sample and the concentration

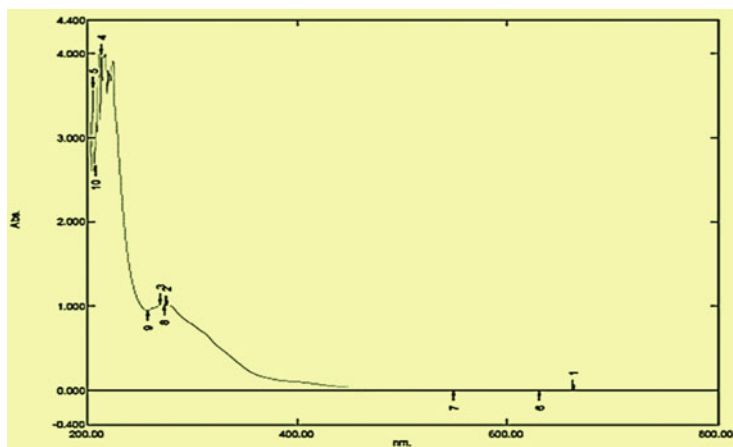
**Table 6.1** Wavelength ranges representing specific secondary metabolites

Sr. no.	Absorption wavelength (nm)	Secondary metabolites
1.	234–676	Flavonoids, alkaloids, phenolic compounds
2.	230–285	Flavonoids and their derivatives
3.	230–290	Flavonoids
4.	300–350	Derivatives of flavonoids
5.	350–500	Tannins, terpenes, saponins
6.	400–450	Carotenoids
7.	400–550	Terpenoids
8.	600–700	Chlorophyll

of the absorbing material. In UV analysis, a sample is placed between a light source and a photodetector, and the intensity of a beam of light is measured before and after passing through the sample. Each spectrum is background corrected using a “blank”—a cuvette filled with only the dispersing medium to guarantee that spectral features from the solvent are not included in the sample extinction spectrum (Markham, 1982). UV–visible spectroscopy can be performed for qualitative analysis and for the identification of diverse chemical compounds in both pure and biological mixtures. For UV analysis, the crude portion of the partially purified extract is centrifuged at 5000 rpm for 15 minutes to remove debris and impurities. The supernatant liquid is diluted to 1:10 when the crude extract is to be measured. The extract is scanned/spectrum is measured in wavelengths ranging from 200 to 800 nm in a spectrophotometer. The distinctive peaks of the UV–Vis can be seen at multiple wavelengths. Typical values of wavelength ranges representing specific secondary metabolites are shown in Table 6.1.

The UV spectrophotometric analysis of *Tabarnamontana divaricta* revealed maximum absorption at 280 nm when scanning was performed in the range of 200–800 nm. Some additional peaks were also noted in this range (Fig. 6.1).

Flavones and flavonols are closely related in their structures, but they can be differentiated on the basis of their UV–vis visible spectrum. Flavones show two absorption peaks: peak I between 330–350 nm and peak II between 250–270 nm. On the other hand, flavonols show peak I between 350–390 nm and peak II between 250–270 nm. The disadvantage of UV–Vis spectroscopy is that it has limitations in assigning peaks to some constituents present in the extract. Therefore, the results of UV–Vis must be supplemented with other analytical techniques, such as GC–MS or LC–MS, for exact phytochemical profiling of the sample and identification of the compound.

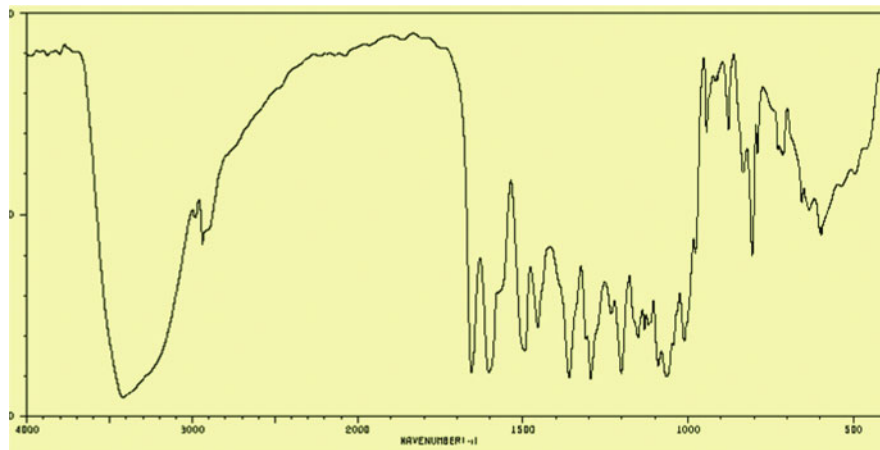


**Fig. 6.1** The UV spectrophotometric analysis of *Tabarnamontana divaricata*

## Fourier Transform Infrared (FT-IR) Spectroscopy

Fourier transform infrared spectroscopy (FT-IR) is a physicochemical analytical technique that provides a clear picture of the functional groups of the active components based on the peaks observed in the infrared region. FT-IR is widely used to elucidate the structure of unknown metabolites and the intensity of absorption spectra based on the molecular composition of the specific chemical functional groups present in the molecule (Bobby et al., 2012). Using FTIR, it has been possible to identify the complicated structures of plant secondary metabolites as well as the characterization of bacterial, fungal and plant species (Hori & Sugiyama, 2003; Yang & Yen, 2002). The dried powdered plant material or the column eluted purified fractions or the callus tissue extract, etc., is encapsulated in a 100-mg potassium bromide (KBr) pellet to prepare the sample for analysis. Usually, a thin film is prepared by applying pressure. The FT-IR scanning of the sample is carried out in the spectral range of  $4000\text{--}400\text{ cm}^{-1}$  using various models/makes of IR spectrometers (e.g., Shimadzu 8202 PC, Kyoto, Japan, IFS 25, Bruker, Germany), and the FT-IR spectrum was analysed (Liu et al., 2014; Zohdi et al., 2015).

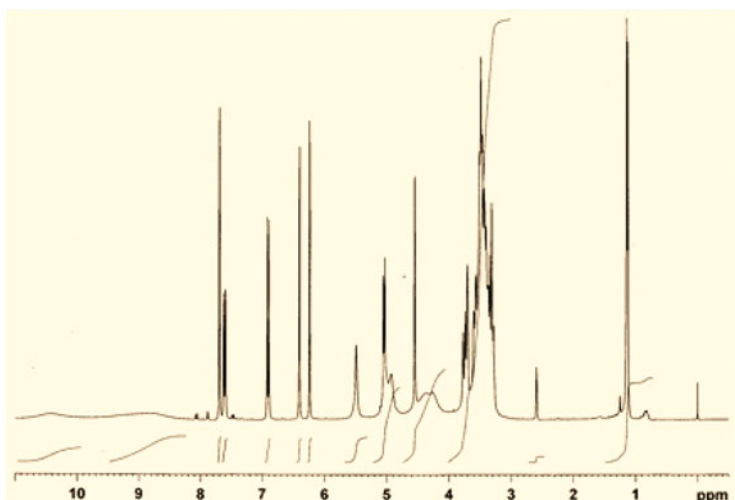
The FT-IR spectrophotometric analysis of *Tabarnamontana divaricata* leaf extract revealed the presence of common bonds (Fig. 6.2) in the functional group region; stretching vibrations at  $3336\text{ cm}^{-1}$  represented the presence of OH alcohol or NH amine, and stretching vibration of  $\text{CH}_2$  was revealed by two bands at  $2916$  and  $2848\text{ cm}^{-1}$ . Presence of C–H stretch vibrations in the fingerprint region at  $1390$  and  $1107\text{ cm}^{-1}$ . Another band analysed at  $1268\text{--}1037\text{ cm}^{-1}$  in the fingerprint region arises from  $\text{CH}_2$  wagging and twisting vibrations.



**Fig. 6.2** FT-IR spectrum of partially purified leaf extract of *Tabernaemontana divaricata* (Bhadane, 2017)

## Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance spectroscopy is a nondestructive analytical method for identifying and locating carbon and hydrogen atoms in biomolecules. It studies the radiofrequency of radiation absorption by nuclei in a magnetic field. Nuclear magnetic resonance techniques often fall into one of two categories:  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR. In NMR, the interaction between radiofrequency electromagnetic radiation and atom nuclei is studied. Nuclear spin exists in nuclei with odd numbers of protons or neutrons, such as  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ , and  $^{19}\text{F}$ , and such nuclei can only be studied by NMR. It pays more attention to the physical properties of the bioactive molecule, such as the number and array of carbon atoms and the presence of isotopes of carbon, hydrogen atoms, and protons. The principle of NMR is based on the fact that all nuclei have electrical charges and therefore exhibit spin. NMR spectroscopy is based on electromagnetic radiation's ability to absorb energy between the radiofrequency range of 3 kHz and 300 GHz. As a result of their electrical charge and spin, atom nuclei produce magnetic fields. Atomic nuclei will align themselves in one of two directions when an external magnetic field is present: either in the direction of the external magnetic field or in the opposite direction. Energy is transferred from the ground state to the excited state when an external magnetic field is present. When an electron transitions from an excited state to the ground state, it occurs at a wavelength that coincides with radio frequencies, and when it does, it generates a radio wave with the same frequency. The NMR spectrum is provided by this radio frequency. The intensity of the applied external magnetic field directly relates to the radiofrequency that is being radiated (Doughari, 2012; Ingale et al., 2017).



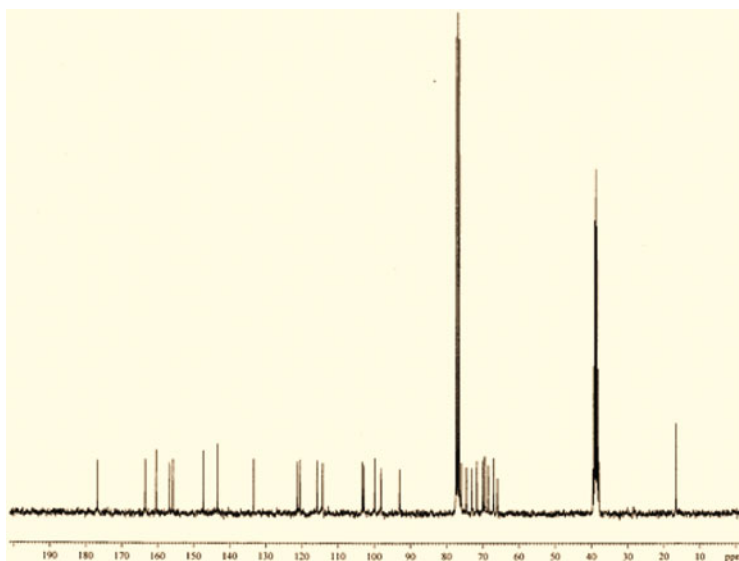
**Fig. 6.3**  $^1\text{H}$  NMR spectrum of a flavonoid isolated from *C. procera*

**Table 6.2** The assigned functional groups to various signals in  $^1\text{H}$  – NMR spectrometry ( $\text{CDCl}_3$ ,  $\delta$  ppm) of the isolated flavonoid from *C. procera*

$\delta$ (ppm)	Protons
1.12, d, 3H	$\text{H6}^{\text{iii}}$
3.25–3.74, m, 4H	$\text{H5}^{\text{ii}}$ and $\text{H5}^{\text{iii}}$
4.20–4.60, m, 6H	Sugar - OH
4.80–5.20, m, 8H	$\text{H2}^{\text{ii}}$ $\text{H2}^{\text{iii}}$ , $\text{H3}^{\text{ii}}$ $\text{H3}^{\text{iii}}$ , Ar - OH
5.50, s, 2H	$\text{H1}^{\text{ii}}$ and $\text{H1}^{\text{iii}}$
6.41, d, 1H	$\text{H8}$
6.92, d, 1H	$\text{H5}^{\text{i}}$
7.60, d, 1H	$\text{H6}^{\text{i}}$
7.70, s, 1H	$\text{H2}^{\text{i}}$

A number of flavonoids have been identified in Apocynaceae plants using NMR spectroscopy. Tatsuzawa et al. (2021) isolated and successfully characterized [anthocyanins](#), [flavonols](#), and [chlorogenic acid](#) from the flowers of [Adenium obesum](#), [Mandevilla sanderi](#), and [Nerium oleander](#) using nuclear magnetic resonance (NMR) and [mass spectrometry](#) (MS). [Cyanidin 3-O-\[6-O-\(rhamnosyl\)-galactoside\]](#) and [cyanidin 3-O-\(galactoside\)](#) were identified as the major and minor anthocyanins, respectively, in three *A. obesum* seedlings that had red and red–purple flowers (Tatsuzawa et al., 2021).

The  $^1\text{H}$  NMR spectrum (Fig. 6.3) of the flavonoids isolated from *C. procera* showed the presence of two doublets at  $\delta$  6.92 and  $\delta$  7.60, evidencing a *p*-hydroxylated B ring, and two broad singlets at  $\delta$  5.50 and  $\delta$  6.24 for the 5,7,9-trioxygenated A ring of a flavonoid (Table 6.2). One doublet at  $\delta$  1.12 of a methyl group, one doublet at  $\delta$  5.20, and signals at  $\delta$  3–4 suggested the presence of a rhamnosyl moiety linked to the aglycone.



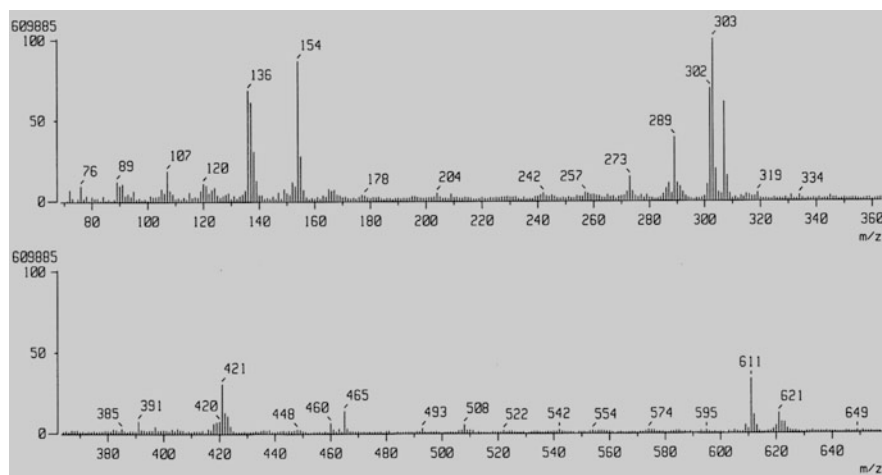
**Fig. 6.4**  $^{13}\text{C}$  NMR spectrum of a flavonoid isolated from *C. procera*

The  $^{13}\text{C}$  NMR spectrum (Fig. 6.4) showed signals at  $\delta$  69.51, 70.11, 73.09 and 74.60 for hydroxymethine carbons, one signal at  $\delta$  38.80 for one methyl carbon and at  $\delta$  102.93 for anomeric carbon, which confirmed the presence of a rhamnosyl unit linked to the aglycone. The  $^{13}\text{C}$  NMR data of the compound were as follows: 176 (C4), 163.4 (C8a), 160.44 (C4a), 156.71, 155.80 (d, C1<sup>ii</sup>, C1<sup>iii</sup>), 147 (C3<sup>i</sup>), 143.45 (C4<sup>i</sup>), 133.35 (C5), 121.27 (d, C5<sup>ii</sup>), 115.83 (C7), 103.35, 102.93 (d, C2<sup>ii</sup>, C2<sup>iii</sup>), 99.91 (C2), 98.20 (C1<sup>i</sup>, C5<sup>i</sup>), 93.02 (C6), 76.56, 77.42 (t, C6<sup>ii</sup>), 74.60, 73.0, (C3<sup>ii</sup>, C3<sup>iii</sup>), 70.11, 69.51 (C4<sup>ii</sup>, C4<sup>iii</sup>), 38.80 (q, C6<sup>ii</sup>).

This spectrum also shows signals at  $\delta$  176, 155.80 and 133.35, suggesting the presence of an  $\alpha,\beta$ -unsaturated ketone moiety of a flavonol. Comparison with literature data for flavonol aglycones evidenced a downfield shift for C-2 and C-4 and an upfield shift for C-3 for the compound, suggesting the location of the sugar moiety at C-31. The remaining signals of this spectrum confirmed the presence of and oxygenation pattern of the A and B rings (Salunke, 2006).

## Mass Spectrometry

Mass spectrometry is the method of choice for the identification of compounds based on their chemical structure and molecular mass. It aims to sequence and identify the constituents in the complex mixture. Mass spectrometry is mainly used to identify oligonucleotides and peptides (Doughari, 2012; Ingale et al., 2017). The mass spectrometry reaction involves bombarding an organic molecule with a high-energy

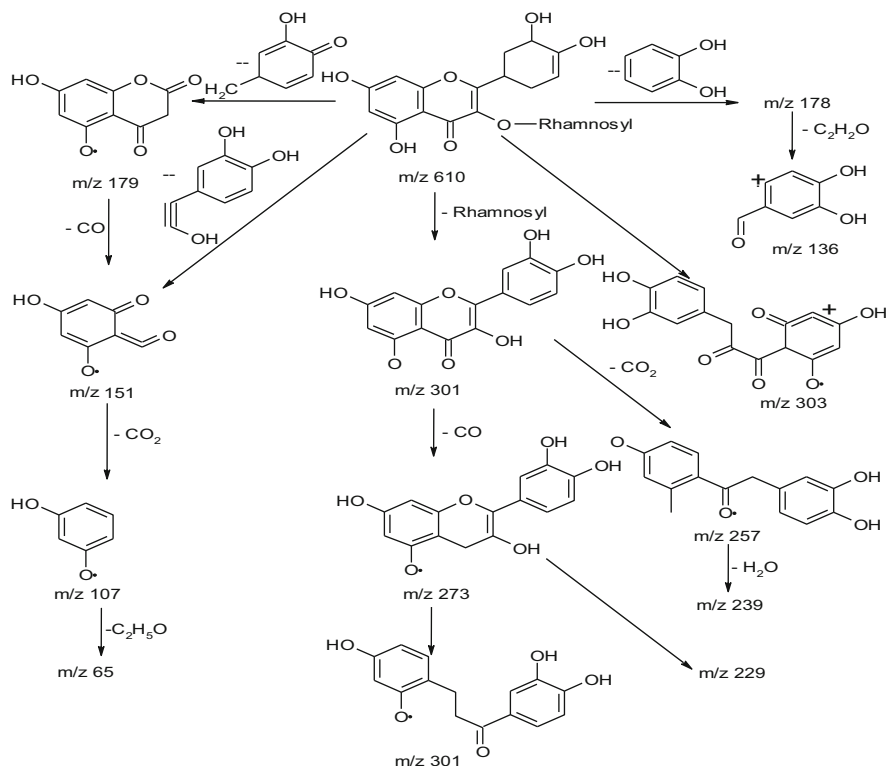


**Fig. 6.5** Mass spectrum of flavonoid (rutin) isolated from *C. procera* (Salunke, 2006)

electron that converts it into high-energy charged ions. The signals are then detected using electron ionization energy of 70 eV, and the spectra of the compound are detected and recorded as percentage peaks. The identification of the unknown constituent is based on their relative molecular mass and molecular weight. It is interpreted by plotting the mass of the fragmented ions against the charges of individual ions (Doughari, 2012; Ingale et al., 2017; Makita et al., 2016). The advantage of mass spectroscopic data is that they provide abundant and meaningful information on organic molecules. Therefore, one of the standard procedures for identifying metabolites from medicinal plants is the combination of HPLC–MS (high-performance liquid chromatography–mass spectrometry) or GC–MS (gas chromatography–mass spectrometry), which are also popular hyphenated techniques.

The fast-atom bombardment mass spectrum of several metabolites of Apocynaceae plants is well documented (Fig. 6.5) (Salunke, 2006).

The bioflavonoid rutin exhibits peaks at ( $m/z$ ) 611, 421, 303 (base peak), 302, 289, 273, 257, 178, 154, and 136 as per the fragmentation pattern in MS (Fig. 6.6). Mass spectral data displayed the fragmentation pattern typical of glycosyl derivatives (losing the terminal sugars), with ions at  $m/z$  465 (M-H-Rha) and  $m/z$  302 (M-H-Rha-Glu)<sup>−</sup>; this latter ion again corresponds to the (M-H)<sup>−</sup> ion of aglycon quercetin or rutin.



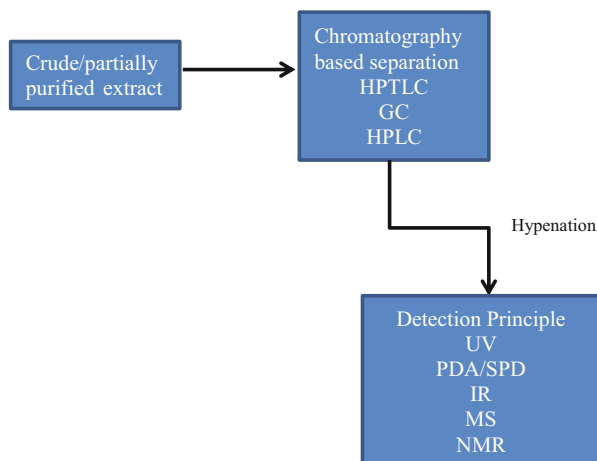
**Fig. 6.6** Mass fragmentation pattern of rutin (Salunke, 2006)

## Hyphenated Techniques in Phytoconstituent Identification

Hirschfield first introduced the term “hyphenation”, which refers to the online combination of a separation technique and spectroscopic detection system. The hyphenated techniques involve a combination or coupling of a separation technique and an online modern spectroscopy-based detection technology. In the first step, chromatography produces single or nearly single compound fractions in a mixture. Spectroscopy produces selective information for identification using standards or searchable library spectra (Fig. 6.7). The continuous improvements in hyphenated techniques in the last few years have significantly increased their applications in the discovery of phytoconstituents and natural products. A number of hyphenated techniques, such as GC–MS, HPLC–MS, LC–PDA, LC–MS, LC–FTIR, LC–NMR, LC–NMR–MS, ESI–MS, and DESI–MS, have been widely used for the analysis of crude and partially purified extracts, fractions from plants and fermentation extracts. Particular emphasis is given to the hyphenated techniques that involve gas chromatography as the separation tool.



**Fig. 6.7** Block diagram of the hyphenated technique involving the separation and detection system



## Gas Chromatography–Mass Spectrometry (GC–MS)

GC/MS is a robust and very widely used technique that combines high sensitivity and specificity for a diverse group of compounds. GC–MS makes use of single- and triple-quadrupole, ion trap and time-of-flight (TOF) mass spectrometers as a detection system. It was developed by coupling GC and MS and was the first of its kind that became most useful for the analysis of volatile phytochemicals and pharmaceuticals. Mass spectra obtained by GC–MS provide more structural information based on the interpretation of the pattern of fragmentation. The fragment ions generated after ionization has different relative abundances, which can be compared with the standard compounds using the library spectra. Phytochemicals that are volatile, of low molecular weight, and sufficiently stable at high temperatures in GC conditions can be easily analysed by GC–MS. Polar compounds, especially those with a number of hydroxyl groups, are usually derivatized for GC–MS analysis. The common derivatization process involves the conversion of the metabolite to its trimethylsilyl derivative. In GC–MS, a sample is injected into the injection port of the GC device, vaporized, separated in the GC column, and analysed by an MS detector, and the spectra are recorded.

The analysis provides the “retention time” (RT), which is the time elapsed between injection and elution. GC–MS consists of an injection port at one end of a metal column packed with a sand-like material, a stationary phase, to promote maximum separation and a mass detector at the other end of the column. A number of carrier gases, such as argon, helium, nitrogen, hydrogen, etc., are used as mobile phases to propel the samples down the column. The separation of the components occurs based on their affinity to the stationary and mobile phases. The eluted compounds are ionized in a mass spectrometer, and the detector provides information that aids in the structural identification of each component (Tang et al., 2020).

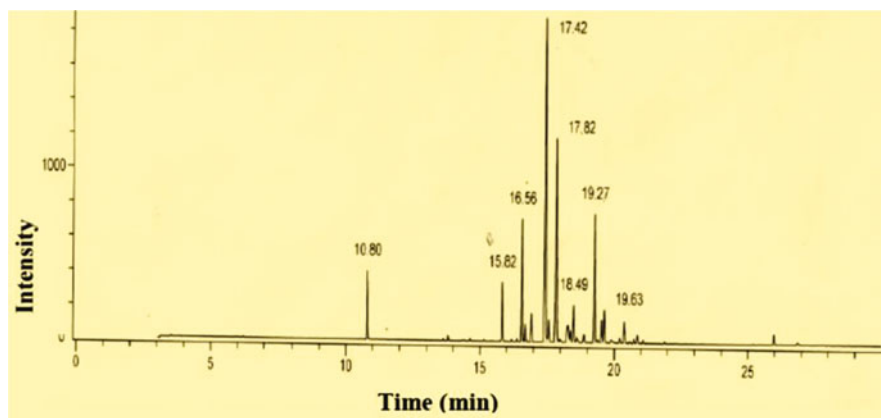
**Table 6.3** Commercially available standard databases for GC–MS analysis (Halket et al., 2005)

Online database	Class	Spectra	Website
Ehrenstorfer	Pesticides, agrochemicals	1450	<a href="http://www.ehrenstorfer.com">www.ehrenstorfer.com</a>
NIST20	Organic compounds/general	123,434	<a href="http://www.hdsience.com">www.hdsience.com</a>
PMW	Toxins, drugs	6300	Instrument manufacturers
Stan	Pesticides, agrochemicals	300	<a href="http://www.chem.agilent.com">www.chem.agilent.com</a>
Wiley	Organic compounds/general	444,000	<a href="http://www.hdsience.com">www.hdsience.com</a>
Wiley/de Leeuw	Minerals	1100	<a href="http://www.hdsience.com">www.hdsience.com</a>
Wiley/Hennerberg	Organic compounds/general	40,000	<a href="http://www.hdsience.com">www.hdsience.com</a>
Wiley/Makin	Steroids, saponins	2500	<a href="http://www.hdsience.com">www.hdsience.com</a>
Wiley/Roesner	Designer drugs	1700	<a href="http://www.hdsience.com">www.hdsience.com</a>
Wiley/Yarkov	Organic compounds	37,055	<a href="http://www.hdsience.com">www.hdsience.com</a>
Wiley/Zeist	Volatile compounds from food and plants	1620	<a href="http://www.hdsience.com">www.hdsience.com</a>

Wedge et al. (2009) described a typical GC-HRMS analysis method for volatile metabolites of *Tabarnamontana divaricata*. The partially purified and column-eluted fractions were separated using an Agilent 7890–5 GC (Santa Clara, CA, USA) fused with an HP-5 silica capillary column (30 m × 0.20 mm, phase thickness 0.25 µm) coupled with a mass spectrometer (Jeol Accu TOF GCV) and FID detector. The inlet temperature was 250 °C. The injection volume was 10 µL. The system was carried out in split mode with a ratio of 20:80, and the flow rate of the carrier gas was maintained at 1 ml per minute. The identification of compounds present in column-eluted fractions as well as callus extracts is carried out using the NIST 05 library search database. A number of integrated online MS databases are available with several thousand reference compounds that are digitally searchable and hence offer spectral matches for the identification of separated components. Table 6.3 provides the details of some commercially available databases for a different group of organic compounds, pesticides, volatiles, etc. (Halket et al., 2005).

### ***Practical Approaches for GC-HRMS Characterization of Extracts of C. procera***

The partially purified extracts of *C. procera* have been fully characterized by GC-HRMS (Patil et al., 2015). The GC-HRMS spectra and the bioactive metabolites present in the purified fraction are represented in Fig. 6.8 and Table 6.4. The spectra revealed six identified compounds in order of their elution from the column: phenol, 2,4-bis (1,1-dimethylethyl) ester; disisobutyl phthalate; 1,2-benzenedenedicarboxylic acid, bis (2-methylpropyl) ester; dibutyl phthalate (two peaks) and phthalic acid pentyl 2-pentylester. These findings indicate that the purified fractions of *C. procera*



**Fig. 6.8** Gas chromatogram of the purified fraction from *C. procera* (Bhadane, 2017)

**Table 6.4** The GC-HRMS profile of the compounds present in the bioactive fraction of *C. procera*

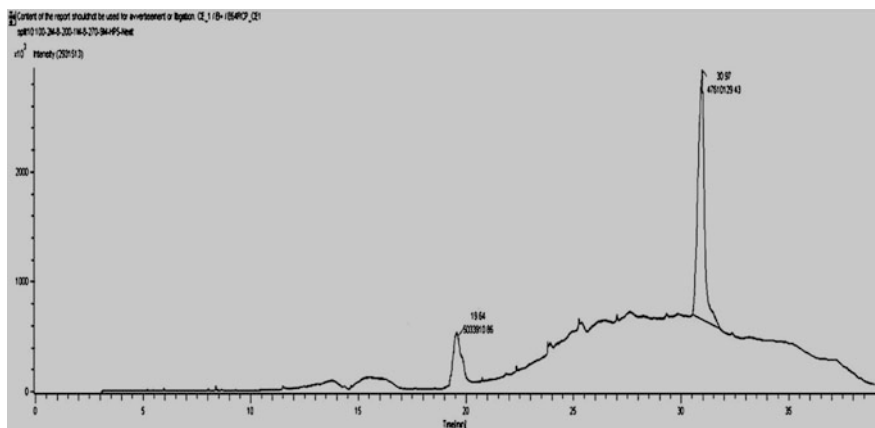
Retention time	Molecular weight	Molecular formula	Compound name as per NIST library
10.80	206	C <sub>14</sub> H <sub>22</sub> O	Phenol, 2, 4 –bis (1,1-dimethylethyl) ester.
15.82	278	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	Disisobutyl phthalate
16.56	223	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	1,2 benzenedicarboxylic acid, bis (2-methylpropyl) ester
17.42	278	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	Dibutyl phthalate
17.82	278	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	Dibutyl phthalate
19.63	306	C <sub>18</sub> H <sub>26</sub> O <sub>4</sub>	Phthalic acid pentyl 2- pentyl ester

contain diterpenes as major phytoconstituents, in agreement with a previous report (Velmurugan et al., 2012).

Other cyclic di-terpenoids, such as 1,2-benzenedicarboxylic acid bismethyl propyl ester (C<sub>16</sub>H<sub>22</sub>O<sub>4</sub>), were identified as major (57.95%) compounds. The other compound identified is pentyl-2-pentyl ester (C<sub>18</sub>H<sub>26</sub>O<sub>4</sub>) with a molecular weight of 306 using GC-HRMS.

### ***Practical Approaches for GC-HRMS Characterization of Extracts of C. Carandas***

*Carissa carandas* is another member of the family Apocynaceae that is chemically characterized by GC-HRMS. Bhadane et al. (2018) revealed a major peak with a retention time of 30.97 min and a minor peak with a retention time of 19.54 min (Fig. 6.9). The chromatogram revealed the presence of two compounds, identified as benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-octadecyl ester



**Fig. 6.9** Gas chromatogram of purified fraction from *Carissa carandas* (Bhadane, 2017)

( $C_{35}H_{62}O_3$ ) with molecular mass 530 and 4-tert-butyl calix [4] arene ( $C_{44}H_{56}O_4$ ) with molecular mass 648.

Among the compounds identified by GC-HRMS analysis, a purified fraction of *Carissa carandas* revealed the presence of benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-octadecyl ester as a major compound. This compound belongs to a class of saturated fatty acids (Orole, 2016) and was previously reported in *Catharanthus pusillus* (Subbaiyan et al., 2014). More than 30 different compounds have been reported in various parts of *Catharanthus pusillus*, and benzenepropanoic acid ester was reported for its antioxidant activity. A derivative of benzenepropanoic acid ester with hypocholesterolaemic and anti-atherosclerotic effects was isolated and characterized from the methanolic leaf extract of *Amaranthus viridis* (Salvamani et al., 2016). Another compound, 4-tert-butyl calix arene, with a minor peak shown in the GC-MS spectra, was previously reported by Vanitha (2011) in an ethanolic extract of *Annona squamosa* leaves. Nutan and Baikar (2013) and Devi et al. (2015) reported 4-tert-butyl calix arene in *Psoralea corylifolia* stem extract and in *Zanthoxylum acanthopodium* leaf extract, respectively.

The metabolites present in the callus extract of *C. carandas* (mainly fatty acids and steroids) were identical to metabolites present in the crude extract as well as in the purified fractions. Therefore, it can be concluded that the biological activities shown by the callus extract may be attributed to the phytochemicals present in it.

The profile of phytoconstituents present in *C. carandas* calli with respect to their RT, name, molecular formula, and molecular weight is presented in Table 6.5.

Androstane-11,17-dione, 3[trimethylsilyl 10 oxy]-17-O-phenylmethyl (RT 35.73) was previously reported in *Anacardium occidentale* with antiproliferative and antiandrogenic activity (Fadeyi et al., 2015). Steroidal saponin (RT 35.18) was also previously reported in the extracts of *Calophyllum inophyllum* and *Terminalia arjuna* with antimicrobial, antioxidant and anti-inflammatory activity (Malarvizhi & Ramakrishnan, 2011; Chaudhari & Mahajan, 2015).

**Table 6.5** Phytoconstituents identified by GC-HRMS in *C. carandas* callus extract

Sr. no.	RT	Compound name	Molecular formula	M. W
1.	5.18	6-acetyl-β-d-mannose	C <sub>8</sub> H <sub>14</sub> O <sub>2</sub>	222
2	7.49	Dihydrojasnone	C <sub>11</sub> H <sub>18</sub> O	166
3.	10.36	Desulphosinigrin	C <sub>10</sub> H <sub>17</sub> NO <sub>6</sub> S	279
4.	13.05	2-propen-1-ol, 3-(2,6,6-trimethyl-2-cyclohexen-1-yl)-	C <sub>12</sub> H <sub>20</sub> O	180
5.	17.43	9,12-Octadecadienoyl chloride,(z,z)-	C <sub>18</sub> H <sub>31</sub> ClO	298
6.	20.93	9,12,15 Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[(trimethylsilyl)oxy)methyl] ethyl ester, (z,z,z)-	C <sub>27</sub> H <sub>52</sub> O <sub>4</sub> Si <sub>2</sub>	496
7.	23.29	Benzoxazol, 2,3 dihydro-2-thioxo	C <sub>14</sub> H <sub>16</sub> N <sub>2</sub> OS	260
8.	23.78	10 Undecenoic acid	C <sub>12</sub> H <sub>22</sub> O <sub>2</sub>	198
9.	35.18	Spirost-8-en-11-one, 3-hydroxy-, (3β,5α,14β,20β,22β,25R)	C <sub>27</sub> H <sub>40</sub> O <sub>4</sub>	428
10.	35.73	Androstane-11,17-dione,3[(trimethylsilyl)oxy]-17-[O9phenylmethyloxime],[3α,5α]	C <sub>29</sub> H <sub>43</sub> NO <sub>3</sub> Si	481
11.	36.72	1-Monolinoleoylglycerol trimethylsilyl ether	C <sub>27</sub> H <sub>54</sub> O <sub>4</sub> Si <sub>2</sub>	498

Glucosinolate-desulfogirin at RT 10.36 min. is reported in *Brassica oleracea* leaf extract for antioxidant activity (Fernandez-Leon et al., 2014). The alkaloid benzoxazole (RT 23.29 min) was isolated from the sea whip *Pseudopterogorgia elisabethae* (Rodriguez et al., 1999) and reported to have antituberculosis and antioxidant activity (Padalkar et al., 2011). Among the fatty acids, 10-undecenoic acid is previously isolated from the seed oil of *Cassia sophora* and reported to have insecticidal, antifungal and antitumour activities (Mostafa et al., 2007). The other fatty acids present in the extract were previously reported in the extracts of *Cynodon dactylon* (Jananie et al., 2011), *Andrographis paniculata* (Kalaivani et al., 2012) and *Cassia italica* (Sermakkani & Thangapandian, 2012).

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# Chapter 7

## Phytochemistry of Apocynaceae Members



### Introduction

Secondary metabolites are substances produced by living cells either in response to environmental stress or as biproducts of metabolism. The cells of plants, animals and microorganisms are known to produce secondary metabolites at varying concentrations (Baker & Sardari, 2021). Apocynaceae plants are reported to be inexpensive and sources of bioactive secondary metabolites through subsidiary pathways. These metabolites are responsible for mediating environmental interactions and are crucial for survival and reproduction. Plants are known to produce more than 50,000 secondary metabolites of medicinal and therapeutic importance. The discovery of novel secondary metabolites is a continuous process intended to discover novel molecules with better therapeutic value. Plants have evolved strong defences against herbivorous pests and other phytopathogens over the course of evolution. These species-specific metabolites have diverse effects on both plants and other living things. All living things share the basic metabolic building blocks of carbohydrates, lipids, nucleotides, and peptides, which are essential to all life processes. In contrast, secondary metabolites, which are formed from primary metabolites, serve as the plant's defense against pathogens, pests, herbivores, environmental stress, etc.

Apocynaceae is the largest family of the plant kingdom, with over 400 genera and more than 4600 species. It also has five subfamilies: Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae, and Asclepiadoideae. Most of the members of this family are poisonous. Apocynaceae plants are rich in cardiac glycosides, terpenoids, alkaloids and tannins and therefore are widely used for various medicinal applications. A number of plants from this family are **cultivated** for ornamental purposes. Apocynaceae plants are abundantly distributed in India, Pakistan, China, Bangladesh and Sri Lanka (Mahmood et al., 2011). Members of the Apocynaceae family are trees and shrubs or herbaceous plants with milky latex. The majority of the plants in this family are abundant in alkaloids and have significant therapeutic value. Additionally, many species are planted for decorative purposes. People in



rural regions utilize some Apocynaceae species as food and some of them as poisons (Aiyambo, 2010). Due to the widespread use of Apocynaceae plants for medicinal purposes, members of this family are found in many different traditional medical practices, including Indian, Chinese, and Thai medicine (Van Beek et al., 1984; Bhat et al., 2012). The Indian subcontinent has a dominant presence of Apocynaceae plants. Approximately 84 species or 30 genera are present in India. Our previous report (Bhadane et al., 2018) provided a detailed account of the phytochemistry and pharmacological properties of members from this family. The important medicinal plants from this family include *C. roseus*, *A. scholaris*, *R. serpentina*, *H. antidysentriaca*, *N. indicum* and *T. Peruvian*. The plants and their parts described in this section are used in indigenous systems of medicine for the treatment of various diseases and disorders.

## Phytochemistry

Phytochemistry involves the study of chemicals derived from medicinal plants. Apocynaceae plants have been used for centuries in traditional medicine systems worldwide, and their therapeutic properties and ethnomedicinal applications are often attributed to the presence of various bioactive compounds. Phytochemical analysis involves the identification, isolation, and characterization of these chemical constituents present in plants.

Some common classes of phytochemicals found in Apocynaceae plants include alkaloids, glycosides, tannins, terpene lactones, proteins, steroids, proanthocyanidins, pigments, ecdysones, and saponins. Based on the research carried out in our laboratory and the extensive literature search on ethnomedicinal uses, recent updates on the phytochemistry of members of the family Apocynaceae are presented in the ensuing sections. Table 7.1 summarizes the phytoconstituents isolated from various parts of Apocynaceae plants with necessary details.

## Alkaloids

Alkaloids are a diverse group of nitrogen-containing compounds. They often possess strong physiological effects and are commonly found in Apocynaceae plants with medicinal properties. Examples of alkaloids include morphine from opium poppy (*Papaver somniferum*), caffeine from coffee (*Coffea* spp.), and quinine from cinchona bark (*Cinchona* spp.).

The common alkaloids isolated from Apocynaceae plants are indole, Iboga and vinca alkaloids (Fig. 7.1). Vincristine, vinblastine and tabernaemontanine (2–4) are alkaloids derived from the Madagascar periwinkle plant, *Catharanthus roseus* and *T. divaricate*. Kam et al. (1993) isolated conophylline and conophyllidine ibogan (Conolabine A and B) (15) and vallesamine alkaloid derivatives. Irofoline B was

**Table 7.1** List of phytochemicals isolated from selected members of Apocynaceae family (Reproduced with permission: Bhadane, 2017; Bhadane et al., 2018).

Phytochemical class/ subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
<b>1. Alkaloids</b> Indole alkaloid	1	Voacangine	73,255	<i>T. divaricata</i>	Flowers	Arambewela and Ranatunge (1991)
	2	Voacristine	196,982	<i>T. divaricata</i>	Leaves, flowers	Arambewela and Ranatunge (1991)
	3	Vobasine	5,374,722	<i>T. divaricata</i>	Stem bark	Arambewela and Ranatunge (1991)
	4	Tabernaemontanine	45,268,651	<i>T. divaricata</i>	Stem bark	Arambewela and Ranatunge (1991)
	5	Conophylline	15,226,696	<i>T. divaricata</i>	Leaves	Kam et al. (1993)
	6	Conophyllidine	44,566,831	<i>T. divaricata</i>	Leaves	Kam et al. (1993)
	7	Lirofolines	46,184,733	<i>T. divaricata</i>	Stem bark	Low et al. (2010)
	8	Vinblastine	241,903	<i>C. roseus</i>	Whole plant	Van der Heijden et al. (2004)
Pentacyclic indole alkaloid	9	Serpentine	73,391	<i>C. roseus</i>	Leaves, roots	Van der Heijden et al. (2004)
	10	Vincristine	5978	<i>C. roseus</i>	Whole plant, leaf	Van der Heijden et al. (2004)
	11	Ajmalicine	441,975	<i>C. roseus</i>	Whole plant, leaf, flower, roots	Van der Heijden et al. (2004)
	12	3',4'-anhydrovinblastine	443,324	<i>C. roseus</i>	Leaves, shoot	Van der Heijden et al. (2004)
	13	Picrinine	46,229,104	<i>A. scholaris</i>	Leaves	Xiang-Hai et al. (2008)
Terpenoidal indole alkaloid	14	E and Z- alstoscholarine	16,215,339 and 16,215,340	<i>A. scholaris</i>	Leaves	Xiang-Hai et al. (2008)
	15	Conolabine A and B	–	<i>T. divaricata</i>	Stem bark	Kam et al. (1993)
	16	Vallesamine	13,783,712	<i>T. divaricata</i>	Stem bark	Kam et al. (1993)
Vinca alkaloids	17	Leurosine	442,111	<i>C. roseus</i>	Whole plant	Svoboda et al. (1959)

(continued)

Table 7.1 (continued)

Phytochemical class/ subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
Steroidal alkaloid	18	Vincal leukoblastine	241,902	<i>C. roseus</i>	Whole plant	Svoboda et al. (1959)
	19	Regholarhenine	101,529,337	<i>H. antidysenterica</i>	Stem bark	Bhutani et al. (1988)
	20	Holadysenterine	16,742,955	<i>H. antidysenterica</i>	Seeds	Kumar et al. (2007)
	21	Conessine	441,082	<i>H. antidysenterica</i>	Seeds	Kumar et al. (2007)
	22	Isoconessimine	11,772,257	<i>H. antidysenterica</i>	Seeds	Kumar et al. (2007)
2. Flavonoids	23	Kurchessine	442,979	<i>H. antidysenterica</i>	Seeds	Kumar et al. (2007)
	24	Rutin	5,280,805	<i>W. tinctoria</i> , <i>W. tomentosa</i> , <i>W. coccinea</i>	Fresh leaves	Muruganandam et al. (2000)
	25	Epicatechin	72,276	<i>C. carandas</i>	Fruits	Patil et al. (2012)
	26	Quercetin	5,280,343	<i>C. carandas</i>	Fruits	Patil et al. (2012)
				<i>A. scholaris</i>	Leaves	Hui et al. (2009)
				<i>T. divaricata</i>	Leaves	Jain et al. (2013)
	27	Kaemferol	5,483,905	<i>C. carandas</i>	Fruits	Patil et al. (2012)
				<i>A. scholaris</i>	Leaves	Hui et al. (2009)
	28	Isohammetin	5,281,654	<i>A. scholaris</i>	Leaves	Hui et al. (2009)
	29	Quercetin 3-O-beta-D-galactopyranoside	–	<i>A. scholaris</i>	Leaves	Hui et al. (2009)
	30	Kaemferol 3-O-beta-D-galactopyranoside	–	<i>A. scholaris</i>	Leaves	Hui et al. (2009)
	31	Isohammetin 3-O-beta-D-galactopyranoside	–	<i>A. scholaris</i>	Leaves	Hui et al. (2009)
	32	Calycosin	5,280,448	<i>T. divaricata</i>	Root and stem	Liang et al. (2006)
	33	Formononetin	5,280,378	<i>T. divaricata</i>	Root and stem	Liang et al. (2006)

	34	Farnisin	5,378,220	<i>T. divaricata</i>	Root and stem	Liang et al. (2006)
	35	Ellagic acid	5,281,855	<i>T. divaricata</i>	Leaves	Jain et al. (2013)
				<i>C. carandas</i>	Fruits	Patil et al. (2012)
	36	Hyperoside	5,281,643	<i>T. divaricata</i>	Leaves	Jain et al. (2013)
<b>3. Terpenes</b>	37	3-carene	26,049	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
Monoterpenes						
	38	Camphene	6616	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
	39	Menthol	16,666	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
	40	p-Cymene	7463	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
	41	$\alpha$ -Terpineol	17,100	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
	42	Piperitone	6987	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
	43	Neryl acetate	1,549,025	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
	44	Geranyl acetate	1,549,026	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
	45	$\beta$ -Ionone	638,014	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
Sesquiterpenes	46	Carindone	101,316,738	<i>C. carandas</i>	Root	Singh and Rastogi (1972)
	47	(+)-Carrisone	5,086,419	<i>C. carandas</i>	Root	Singh and Rastogi (1972)
	48	Nerolidol	5,284,507	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
	49	Farnesol	445,070	<i>C. carandas</i>	Flowers	Zaki et al. (1981)
Triterpenes	50	Lupeol	259,846	<i>C. carandas</i>	Roots	Pakrashi et al. (1968)
				<i>H. antidysenterica</i>	Seeds	Bhattacharya et al. (2009)
	51	Carrisic acid	–	<i>C. carandas</i>	Leaves	Naim et al. (1988)
	52	Carrisol	–	<i>C. carandas</i>	Fruits	Naim et al. (1985)
	53	Betulnic acid	64,971	<i>C. carandas</i>	Leaves	Begum et al. (2013)
				<i>N. oleander</i>	Leaves	Siddiqui et al. (1986), Fu et al. (2005)
				<i>H. antidysenterica</i>	Seeds	Bhattacharya et al. (2009)
	54	16 $\beta$ -hydroxybetulinic acid	–	<i>C. carandas</i>	Root	Singh and Uppal (2015)
	55	Ursolic acid	64,945	<i>C. carandas</i>	Roots	Pakrashi et al. (1968)

(continued)

Table 7.1 (continued)

Phytochemical class/ subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
				<i>N. oleander</i>	Leaves	Siddiqui et al. (1986), Fu et al. (2005)
	56	$\alpha$ -Amyrin	73,170	<i>C. carandas</i>	Root	Singh and Uppal (2015)
				<i>T. divaricata</i>	Root and stem	Liang et al. (2006)
	57	$\alpha$ -Amyrin acetate	92,842	<i>T. divaricata</i>	Root and stem	Liang et al. (2006)
	58	$\beta$ -Amyrin	73,145	<i>W. tinctoria</i>	Seed pods	Ramchandra et al. (1993)
	59	Betulin	72,326	<i>N. oleander</i>	Leaves	Siddiqui et al. (1986), Fu et al. (2005)
	60	Oleanderol	–	<i>N. oleander</i>	Leaves	Siddiqui et al. (1986)
	61	Oleandric acid	–	<i>N. oleander</i>	Leaves	Fu et al. (2005)
	62	Oleanerolide	11,113,483	<i>N. oleander</i>	Leaves	Fu et al. (2005)
	63	Epoxydammarane 3 $\beta$ , 25-diol	–	<i>N. oleander</i>	Leaves	Fu et al. (2005)
	64	Wrightial	–	<i>W. tinctoria</i>	Seed pods	Ramchandra et al. (1993)
	65	Taraxasterol acetate	13,889,352	<i>T. divaricata</i>	Root and stem	Liang et al. (2006)
	66	Alstonic acid A and B	91,895,416 and 91,895,417	<i>A. scholaris</i>	Leaves	Wang et al. (2009)
	67	2,3-Secofermane	–	<i>A. scholaris</i>	Leaves	Wang et al. (2009)
	68	<i>Cis</i> and <i>trans</i> Karenin	6,440,661 and 101,921,671	<i>N. oleander</i>	Leaves	Siddiqui et al. (1995)
	69	3 $\beta$ ,27-dihydroxy-urs- 18-en-13,28-olid	–	<i>N. oleander</i>	Uncrushed leaves	Begum et al. (1999)
	70	3 $\beta$ ,22 $\alpha$ ,28-trihydroxy-25- nor-	–	<i>N. oleander</i>	Uncrushed leaves	Begum et al. (1999)

		Lup-1(10),20(29)-dien-2-one					
	71	Oleanolic acid	10,494	<i>C. carandas</i>	Root	Itankar et al. (2011)	
				<i>N. oleander</i>	Leaves	Siddiqui et al. (1986)	
	72	Betulinolaldehyde	99,615	<i>H. anti dysenterica</i>	Seeds	Bhattacharya et al. (2009)	
Isopropene triterpenoid	73	Caradinol	102,202,376	<i>C. carandas</i>	Leaves	Begum et al. (2013)	
<b>4. Glycosides</b>	74	Kaneroside	—	<i>N. oleander</i>	Leaves	Siddiqui et al. (1986)	
Cardiac glycosides	75	Neriumoside	—	<i>N. oleander</i>	Leaves	Siddiqui et al. (1986)	
	76	Oleandrin	11,541,511	<i>N. indicum</i>	Fresh leaves	Dat et al. (2011)	
	77	Nerianthin	12,313,293	<i>N. indicum</i>	Fresh leaves	Dat et al. (2011)	
	78	Adynerin	441,840	<i>N. indicum</i>	Fresh leaves	Dat et al. (2011)	
	79	Deacetylloeandrin	37,401	<i>N. indicum</i>	Fresh leaves	Dat et al. (2011)	
	80	Odoroside-H	205,840	<i>C. carandas</i>	Roots	Hegde and Joshi (2009)	
				<i>N. oleander</i>	Leaves	Begum et al. (1999)	
	81	Rhamnoside	—	<i>C. carandas</i>	Roots	Hegde and Joshi (2009)	
	82	Rhamnoglycoside	—	<i>C. carandas</i>	Roots	Hegde and Joshi (2009)	
	83	Evomoside	—	<i>C. carandas</i>	Roots	Hegde and Joshi (2009)	
	84	Thevetin	159,331	<i>T. peruviana</i>	Seeds	Kohls et al. (2012)	
	85	Acetylthevetin	90,479,481	<i>T. peruviana</i>	Seeds	Kohls et al. (2012)	
Cardenolides	86	Neridiginoside	—	<i>N. oleander</i>	Leaves	Begum et al. (1999)	
	87	Nerizoside	10,816,404	<i>N. oleander</i>	Leaves	Begum et al. (1999)	
	88	Neritaloside	44,566,654	<i>N. oleander</i>	Leaves	Begum et al. (1999)	
	89	Nerifolin	441,867	<i>T. peruviana</i>	Stem bark	Miyagawa et al. (2009)	
	90	Peruvoside	12,314,120	<i>T. peruviana</i>	Stem bark	Miyagawa et al. (2009)	
	91	Thevefolin	44,537,896	<i>T. peruviana</i>	Stem bark	Miyagawa et al. (2009)	
	92		—	<i>T. peruviana</i>	Stem bark	Miyagawa et al. (2009)	

(continued)

Table 7.1 (continued)

Phytochemical class/ subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
		(20S)-18,20 epoxydigitoxinigenin R-L-thvetoside				
Terpenoids glycosides (Sesquiterpene glucoside)	93	Carandoside	44,179,851	<i>C. carandas</i>	Stem	Wangteeraprasert and Likhitwitayawuid (2009)
(Anthocynidin glycosides)	94	7-O-Methylcyanidin 3-robinobioside	–	<i>C. roseus</i>	Flowers	Toki et al. (2008)
	95	Rosinidin 3-robinobioside	–	<i>C. roseus</i>	Flowers	Toki et al. (2008)
(Secoiridoid glycoside)	96	Alstonoside	–	<i>A. scholaris</i>	Stem	Thomas et al. (2008)
Flavonoid glycosides (Isoflavone glycosides)	97	Formononetin 7-O- $\beta$ -D- apiofuranosyl -(1-6)- $\beta$ -D-glucopyranoside	–	<i>A. scholaris</i>	Stem	Thomas et al. (2008)
	98	Biochanin A 7-O- $\beta$ -D- apiofuranosyl 1-(1-6)- $\beta$ -D- glucopyranoside	–	<i>A. scholaris</i>	Stem	Thomas et al. (2008))
(Flavonol glycosides)	99	Syringetin-3-O- robinobioside	–	<i>C. roseus</i>	Stem	Brun et al. (1999)
	100	Quercetin-3-O- (2,6-di-O-rhamnosyl- galactoside)	–	<i>C. roseus</i>	Stem, leaves, seed, petals	Ferreres et al. (2008)
	101	Quercetin –3-O- (2,6-di-O-rhamnosyl- glucoside)	–	<i>C. roseus</i>	Petals	Ferreres et al. (2008)
	102	Kaempferol-3-O- (2,6-di-O-rhamnosyl- galactoside)	–	<i>C. roseus</i>	Stem, leaves, seed, petals	Ferreres et al. (2008)

	103	Kaempferol-3- <i>O</i> -(2,6-di- <i>O</i> -rhamnosylglucoside)	–	<i>C. roseus</i>	Stem, seed, petals	Ferreres et al. (2008)
	104	Isorhamnetin-3- <i>O</i> -(2,6-di- <i>O</i> -rhamnosylgalactoside)	–	<i>C. roseus</i>	Stem, leaves	Ferreres et al. (2008)
	105	Isorhamnetin-3- <i>O</i> -(2,6-di- <i>O</i> -rhamnosylglucoside)	–	<i>C. roseus</i>	Seeds	Ferreres et al. (2008)
	106	Quercetin 3- <i>O</i> -{ $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)-[ $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)]- $\beta$ -D-galactopyranoside}	–	<i>T. peruviana</i>	Leaves	Tewtrakul et al. (2002)
(flavanone glycosides)	107	(2 <i>S</i> )-5- <i>O</i> - $\beta$ -D-glucopyranosyl-7,4'-dihydroxy-3',5'-dimethoxyflavanone	–	<i>T. peruviana</i>	Leaves	Tewtrakul et al. (2002)
	108	(2 <i>R</i> )-5- <i>O</i> - $\beta$ -D-glucopyranosyl-7,4'-dihydroxy-3',5'-dimethoxyflavanone	–	<i>T. peruviana</i>	Leaves	Tewtrakul et al. (2002)
<b>5. Sterols</b>	109	Stigmasterol	5,280,794	<i>A. scholaris</i> <i>W. tinctoria</i>	Root, root bark Stem	Adotey et al. (2012) Ramchandra et al. (1993), Jain and Bari (2010)
				<i>C. carandas</i> <i>A. scholaris</i>	Roots Stem	Galipalli et al. (2015) Adotey et al. (2012)
	110	$\beta$ -Sitosterol	222,284	<i>W. tinctoria</i> <i>C. carandas</i>	Stem Roots	Ramchandra et al. (1993) Pakrashi et al. (1968), Bhaskar and Balakrishnan (2015)

(continued)



Table 7.1 (continued)

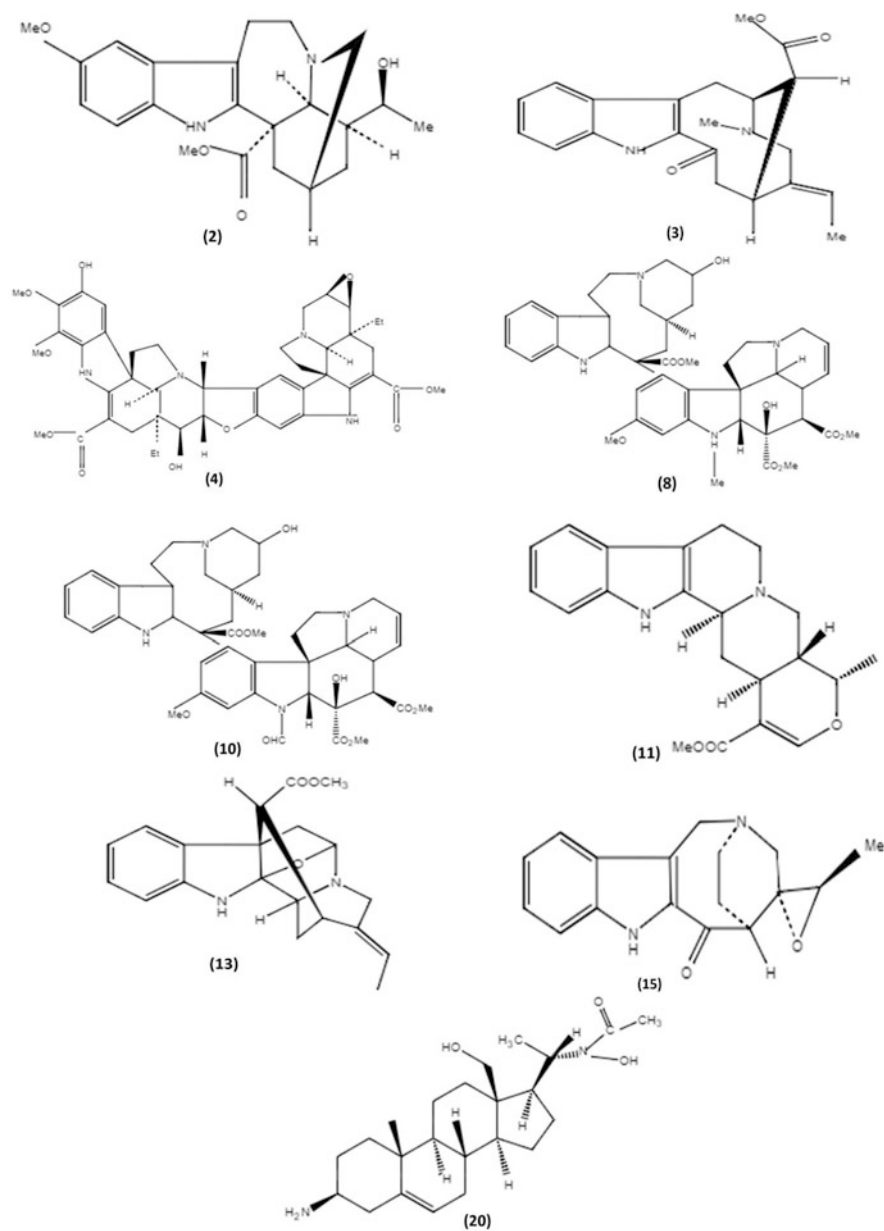
Phytochemical class/ subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
	111	Campesterol	173,183	<i>A. scholaris</i>	Root, root bark	Adotey et al. (2012)
				<i>W. tinctoria</i>	Stem	Ramchandra et al. (1993), Jain and Bari (2010)
	112	14 $\alpha$ -methylzymosterol	–	<i>W. tinctoria</i>	Seeds	Akhisa et al. (1988)
	113	Desmosterol	439,577	<i>W. tinctoria</i>	Seeds	Akhisa et al. (1988)
	114	Clerosterol	5,283,638	<i>W. tinctoria</i>	Seeds	Akhisa et al. (1988)
	115	24-methylene-25-methylcholesterol	5,283,645	<i>W. tinctoria</i>	Seeds	Akhisa et al. (1988)
	116	24-dehydropollinastanol	–	<i>W. tinctoria</i>	Seeds	Akhisa et al. (1988)
	117	7-dihydrositosterol	5,283,634	<i>R. serpentine</i>	Roots	Karmakar and Chakraborty (1983)
	118	Sitosterol glucoside	70,699,351	<i>C. carandas</i>	Roots	Hegde et al. (2012)
	119	Cholest-5-en-3- $\beta$ -ol	–	<i>C. carandas</i>	Roots	Balakrishnan et al. (2011)
	120	20-Hydroxypregnan-18-oic acid	–	<i>C. carandas</i>	Leaves	Bhadane and Patil (2017)
	121	Sitosta-5,23-dien-3 $\beta$ -ol	–	<i>H. antidysenterica</i>	Stem bark	Usmani (1995)
<b>6. Simple phenolic compounds</b>	122	Syringic acid	10,742	<i>C. carandas</i>	Fruits	Patil et al. (2012)
	123	Vanillic acid	8468	<i>C. carandas</i>	Fruits	Patil et al. (2012)
	124	p-Coumaric acid	637,542	<i>C. roseus</i>	Leaves	Proestos et al. (2005)
	125	Caffeic acid	689,043	<i>C. carandas</i>	Fruits	Patil et al. (2012)
	126	Chlorogenic acid	1,794,427	<i>C. carandas</i>	Fruits	Patil et al. (2012)
				<i>C. roseus</i>	Leaves	Proestos et al. (2005)
	127	Scopoletin	5,280,460	<i>C. carandas</i>	Root	Galipalli et al. (2015)

	128	Gallic acid	370	<i>C. roseus</i>	Leaves	Proestos et al. (2005)
	129	Hydroxytyrosol	82,755	<i>C. roseus</i>	Leaves	Proestos et al. (2005)
	130	Ferulic acid	445,858	<i>C. roseus</i>	Leaves	Choi et al. (2004)
	131	3-O-Caffeoylquinic acid (3-CQA)	–	<i>C. roseus</i>	Leaves	Ferres et al. (2011), Wong et al. (2014)
	132	4-O-Caffeoylquinic acid (4-CQA)	–	<i>N. oleander</i>	Leaves	Wong et al. (2014)
	133	5-O-Caffeoylquinic acid (5-CQA)	–	<i>C. roseus</i>	Leaves	Ferres et al. (2011), Wong et al. (2014)
	134	Carinol	–	<i>N. oleander</i>	Leaves	Wong et al. (2014)
<b>7. Lignans</b>	135	(6R,7S,8S)-7a-[(b-glucopyranosyl) oxy] lyoniresinol	–	<i>C. carandas</i>	Roots	Pal et al. (1975)
	136	Carissanol	340,931	<i>C. carandas</i>	Stem	Wangteeraprasert and Likhitwitayawuid (2009)
	137	(–)-nortrachelogenin	394,846	<i>C. carandas</i>	Stem	Wangteeraprasert and Likhitwitayawuid (2009)
	138	Syringaresinol 4-O-b-glucopyranoside		<i>V. major</i>	Leaves	Sohretoglu et al. (2013)
<b>8. Others</b>	139	Eicosanoic acid	10,467	<i>C. carandas</i>	Seeds	Shrivastava and Bakodia (1979)
	140	Hexadecanoic acid, 15-methyl ester	985	<i>T. divaricata</i>	Flower	Ali Khan et al. (2013)
				<i>C. carandas</i>	Seeds	Shrivastava and Bakodia (1979)
				<i>W. tinctoria</i>	Flower	Ramalakshmi et al. (2012)
				<i>C. roseus</i>	Leaves	Brun et al. (2001)

(continued)

Table 7.1 (continued)

Phytochemical class/ subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
				<i>T. divaricata</i>	Flower	Ali Khan et al. (2013)
				<i>H. antidysenterica</i>	Flower	Brun et al. (2001)
	141	Octadecanoic acid, 9Z,12Z-octadecadienoic acid	–	<i>C. carandas</i>	Seeds	Shrivastava and Bakodia (1979)
	142	9Z-octadecenoic acid	445,639	<i>T. divaricata</i>	Flower	Ali Khan et al. (2013)
	143	Pentadecanoic acid ester	38,762	<i>C. carandas</i>	Seeds	Shrivastava and Bakodia (1979)
	144	Caproic acid	8892	<i>W. tinctoria</i>	Flower	Ramalakshmi et al. (2012)
	145	Caprylic acid	–	<i>C. roseus</i>	Leaves	Brun et al. (2001)
	146	Caprylic acid	379	<i>C. roseus</i>	Leaves	Brun et al. (2001)
	147	Nonanoic acid	8158	<i>C. roseus</i>	Leaves	Brun et al. (2001)
	148	Tridecanoic acid	12,530	<i>C. roseus</i>	Leaves	Brun et al. (2001)
	149	Arabinogalactun	24,847,856	<i>N. oleander</i>	Leaves	Dong and Fang (2001)
	150	L-ascorbic acid	54,670,067	<i>C. carandas</i>	Fruits	Zaki et al. (1981), Patil et al. (2012)
	151	Dihydrojasnone	62,378	<i>C. carandas</i>	Flowers	Rastogi et al. (1966)
	152	6-Decaprenylphenol	–	<i>C. carandas</i>	Roots	Zaki et al. (1981), Patil et al. (2012)
	153	4-Amino-1-(4-amino-2-oxo- 1(2H) -pyrimidinyl)-1,4-dideoxy -b-D-glucopyranuronic acid	–	<i>C. carandas</i>	Roots	Zaki et al. (1981), Patil et al. (2012)
	154	Vitamin E	14,985	<i>T. divaricata</i>	Flower	Ali Khan et al. (2013)



**Fig. 7.1** Alkaloids isolated from Apocynaceae plants (Bhadane, 2017)

reported from *T. divaricata* (Low et al., 2010). Indole alkaloids (vinblastine, serpentine, vincristine ajmalicine, and 3',4'-anhydrovinblastine) (8,10,11) were reported in *C. roseus* (Van der Heijden et al., 2004). Svoboda et al. (1959) reported

leurosine and vincaleukoblastine. Picrinine (**13**) and alstoscholarine were isolated from *A. scholaris* (Xiang-Hai et al., 2008; Jagetia). Bhutani et al. (1988) isolated regholarrhenine from *H. antidysenterica*. Holadysenterine, conessine, isoconessimine and kurchessine (**20**) were extracted from *H. antidysenterica* (Kumar et al., 2007).

These are just a few examples of alkaloids found in plants of the Apocynaceae family. Each alkaloid has unique properties and potential medicinal applications. However, it is important to note that some alkaloids can be highly toxic or have significant side effects, so their use should be strictly controlled and monitored by healthcare professionals.

## Flavonoids

Flavonoids are a class of polyphenolic compounds with diverse biological activities, including antioxidant, anti-inflammatory, and anticancer properties. Apocynaceae plants are not typically known for their high flavonoid content compared to other plant families; some species within this family do contain flavonoids. Figure 7.2 shows isolated flavonoids from Apocynaceae plants. Rutin was from fresh leaves of the genus *Wrightia* (Muruganandam et al., 2000). Other flavonoids, such as rutin, epicatechin, quercetin and kaempferol (**24**), were isolated from *C. carandas* fruits (Patil et al., 2012). Similarly, Hui et al. (2009) isolated quercetin, kaempferol and isorhamnetin from the leaves of *A. scholaris*. *T. divaricata* root and stem extracts showed the presence of novel flavonoids, namely, calycosin (**32**), formononetin (**33**), and farnisin (Liang et al., 2006; Jain et al., 2013).

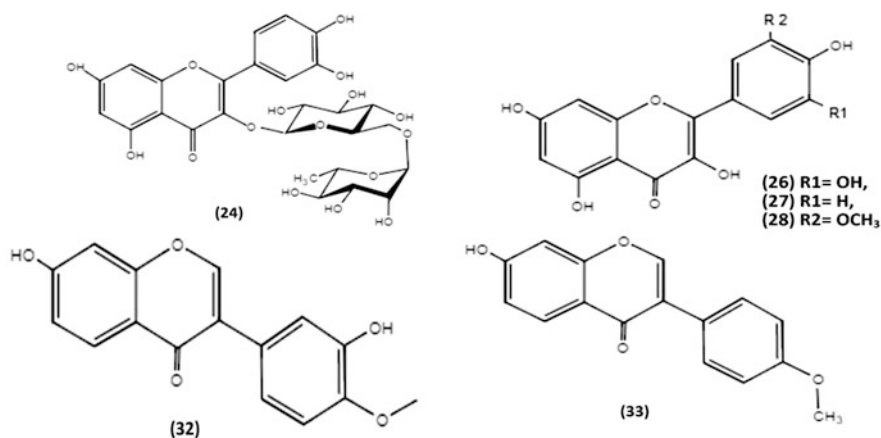


Fig. 7.2 Some examples of flavonoids isolated from Apocynaceae plants (Bhadane, 2017)

## Terpenes

Apocynaceae plants are rich sources of terpenes, which are a large and diverse class of natural compounds derived from the isoprene unit. Terpenes are responsible for the aromatic and medicinal properties of many plants. Terpenes are largely present in plants from the family Asteraceae (such as daisies and sunflowers). Apocynaceae members generally contain all four classes of terpenes.

**Monoterpenes:** Monoterpenes are composed of two isoprene units and are known for their pleasant aromas. Some Apocynaceae plants may contain monoterpenes such as limonene, which is responsible for the citrusy aroma found in citrus fruits and some species of Apocynaceae.

**Diterpenes:** Diterpenes consist of four isoprene units and are commonly found in various plant families, including Apocynaceae. One well-known diterpene from Apocynaceae is Taxol (paclitaxel), which is derived from the bark of the Pacific yew tree (*Taxus brevifolia*). Taxol is used as a chemotherapy agent for various cancers.

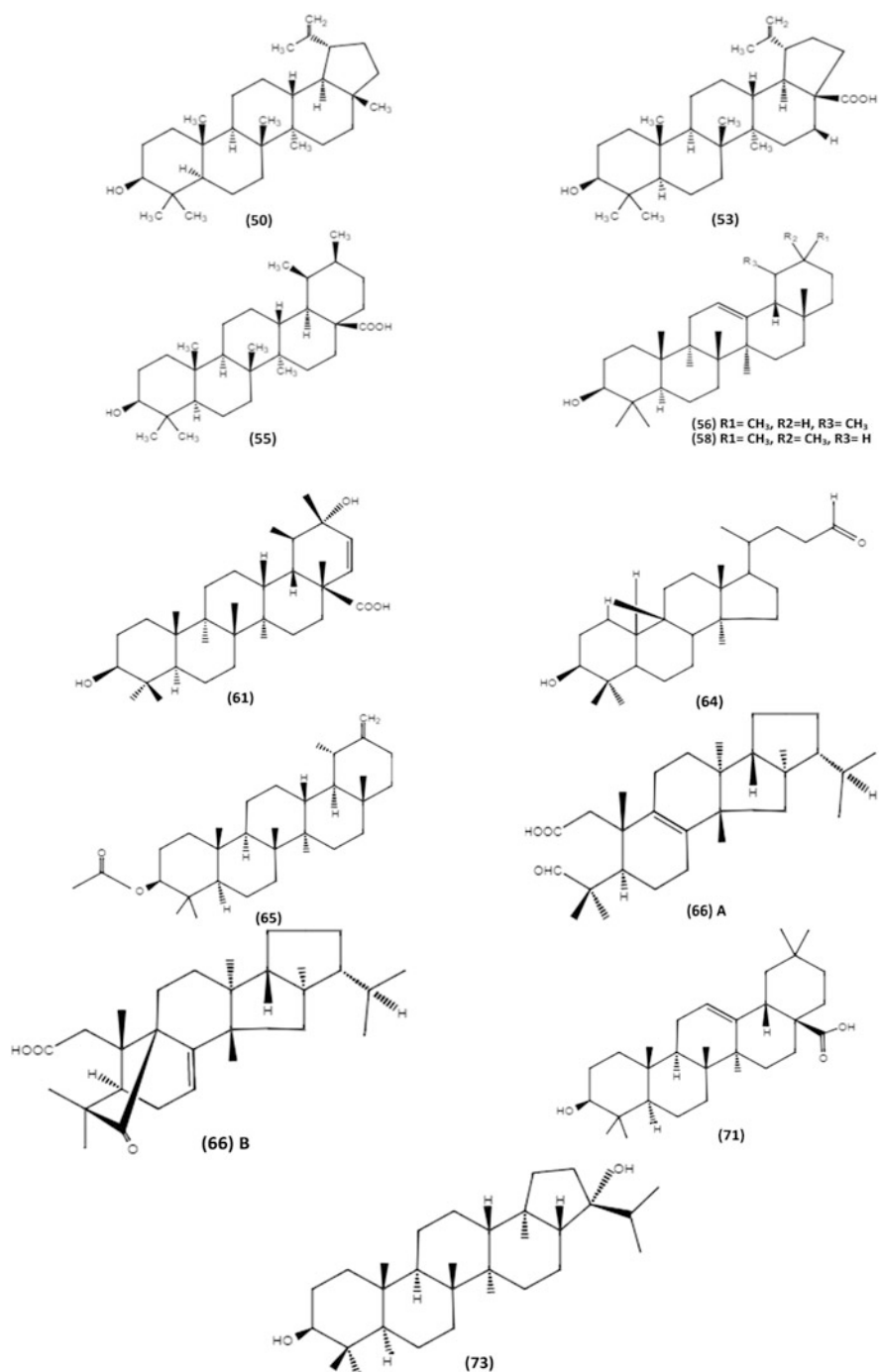
**Triterpenes:** Triterpenes are composed of six isoprene units and are widespread in the plant kingdom. Some Apocynaceae plants may contain triterpenes such as betulinic acid, which has demonstrated potential anticancer properties and is found in plants such as white birch (*Betula* spp.). Triterpenoids such as betulinic acid, betulin oleanolic acid and ursolic acid were isolated by Siddiqui et al. (1995).

**Sesquiterpenes:** Sesquiterpenes are composed of three isoprene units and are known for their strong and diverse biological activities. While they are more commonly found in plants from other families, certain Apocynaceae plants may contain sesquiterpenes such as caryophyllene and humulene.

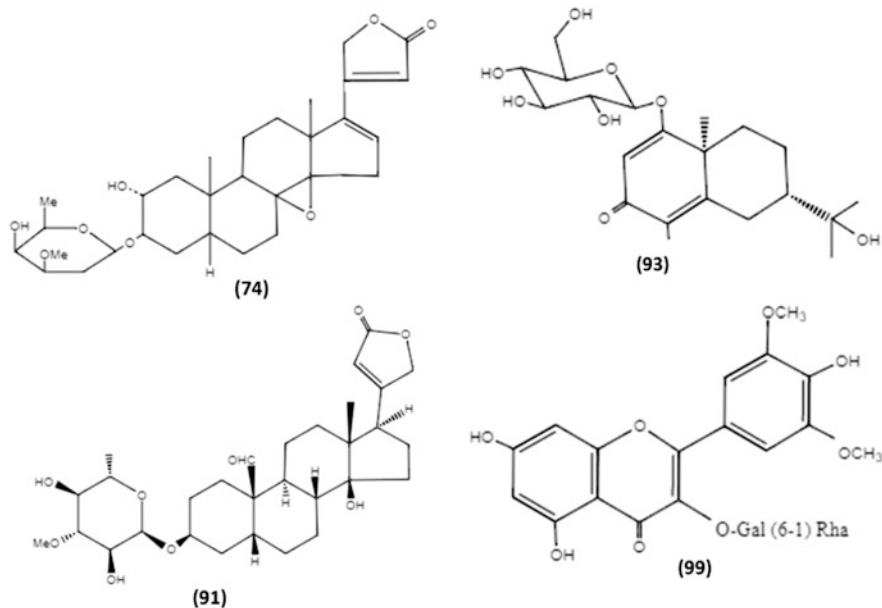
Triterpenoids (**50**, **53**, **55**) were found to be evenly distributed in leaves, roots and fruits (Pakrashi et al., 1968; Naim et al., 1985, 1988; Singh & Uppal, 2015). The sesquiterpene carrisone, triterpene lupeol and oleanolic acid (**71**) were found to exhibit anti-inflammatory activity against proinflammatory mediators such as nitric oxide (NO), TNF- $\alpha$  and IL-1b (Itankar et al., 2011). Recently, a total of 15 triterpenoids, including previously known and newly isolated oleandric acid and oleanerolide (**61**, **64**), from *N. oleander* were evaluated for their anti-inflammatory and anticancer potential (Fu et al., 2005). *W. tinctoria* of this family contains Wrightial (**64**) as a new triterpenoid (Ramchandra et al., 1993). Taraxasterol acetate (**65**) was isolated from the roots and stem of *T. divaricata* (Liang et al., 2006). Alstonic acids A and B as well as 2,3-secofernane (**66**, **71**) triterpenoids were reported from the leaves of *A. scholaris* (Wang et al., 2009) (Fig. 7.3).

## Glycosides

Phenolic glycosides are compounds containing both a phenolic group and a sugar molecule and are present in some Apocynaceae plants. These compounds often



**Fig. 7.3** Chemical structures of common terpenes present in Apocynaceae plants (Bhadane, 2017)



**Fig. 7.4** Chemical structure of glycosides isolated from Apocynaceae plants (Bhadane, 2017)

possess various biological activities and contribute to the medicinal properties of plants. While the prevalence and diversity of phenolic glycosides in Apocynaceae may not be as extensive as in other plant families, here are a few examples of phenolic glycosides that have been reported in certain Apocynaceae plants. The composition of phenolic glycosides may vary among different species within the Apocynaceae family. Cardiac glycosides are a major class of glycosides and are widely used in the treatment of heart failure. The genera *Thevetia* and *Nerium* are reported to possess the highest glycosides (Fig. 7.4). Siddiqui et al. (1986) isolated two cardiac glycosides, kaneroside and neriumoside (74), from the leaves of *N. oleander*. A sesquiterpene glucoside, carandoside (91), was extracted from *C. carandas* stem (Wangteeraprasert & Likhitwitayawuid, 2009). Toki et al. (2008) isolated and characterized anthocyanidin glycosides (93, 99) from orange-red flowers of *C. roseus*.

Oleuropein is a phenolic glycoside present in some Apocynaceae members, such as *Olea europaea* (olive tree). It is known for its antioxidant and anti-inflammatory properties and is associated with numerous health benefits, such as cardiovascular protection and immune system modulation. Another phenolic glycoside, secologanoside, is found in several Apocynaceae plants, particularly in species of *Securidaca*. It has been investigated for its potential analgesic and anti-inflammatory effects. Verbascoside has been isolated and identified in some Apocynaceae plants, including species of *Nerium* and *Vinca*. It possesses antioxidant and anti-inflammatory properties and has been studied for its potential applications in skin health and wound healing. Calotropin is a toxic cardiac glycoside that contains both



a phenolic group and a sugar molecule. It is found in several members of the genera *Calotropis* and *Catharanthus*. Calotropin and related cardiac glycosides have been used traditionally in certain medicinal preparations but should be used with caution due to their potential toxicity.

## Sterols

Sterols are bioactive compounds that are known for their hepatoprotective, anti-inflammatory and antihyperlipidemic activities (Zaki et al., 1981). Sterols play important roles in plant physiology and can have various biological activities. One well-known sterol found in Apocynaceae plants is  $\beta$ -sitosterol.  $\beta$ -Sitosterol is a phytosterol that is widely distributed in the plant kingdom, including some species within the Apocynaceae family. It is chemically similar to cholesterol and has been found to have potential health benefits.  $\beta$ -Sitosterol has been investigated for its cholesterol-lowering effects and its potential anti-inflammatory and immunomodulatory properties. It is commonly used as a dietary supplement for supporting prostate health and managing urinary symptoms. Stigmasterol,  $\beta$ -sitosterol and campesterol (109–110) are present in various parts of these plants (Fig. 7.5) (Ramchandra et al., 1993; Adotey et al., 2012). Stigmasterol is another common sterol found in the Apocynaceae family. It is structurally similar to  $\beta$ -sitosterol and has been studied for its potential cholesterol-lowering and anti-inflammatory effects. Stigmasterol has also been investigated for its anticancer properties and its ability to support cardiovascular health (Jain & Bari, 2010). Akihisa et al. (1988) reported some uncommon sterols (113–114) from unsaponifiable lipids of *W. tinctoria* seeds.

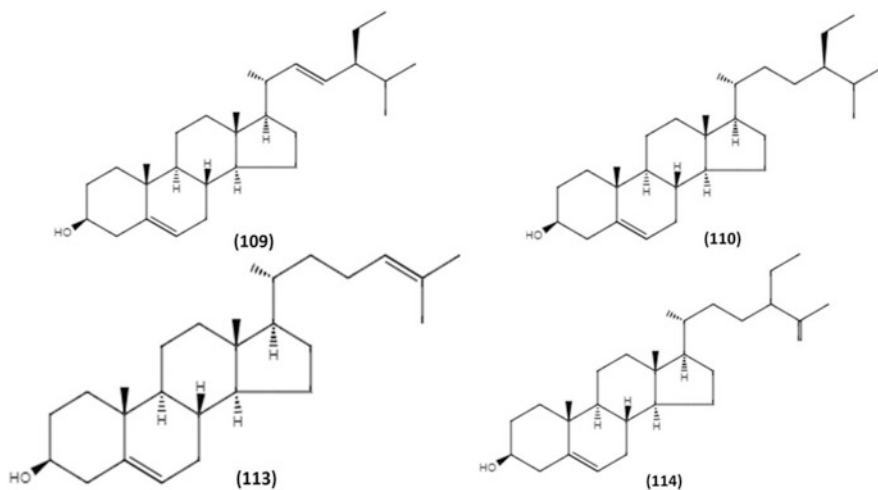


Fig. 7.5 Chemical structure of sterols isolated from Apocynaceae plants (Bhadane, 2017)

Studies on phytochemical aspects of Apocynaceae plants offer potential benefits for human health, as well as their applications in industries such as agriculture, cosmetics, and pharmaceuticals. Phytochemistry helps us identify and isolate the bioactive compounds in Apocynaceae plants that have medicinal properties, which can then be used to develop new drugs or improve existing ones. Understanding the phytochemical aspects of Apocynaceae plants is important in the field of agriculture because it helps us understand the chemical makeup of plants, which can be used to improve crop yields, increase resistance to pests and diseases, and develop new plant varieties. Apocynaceae plants harbor several compounds that have beneficial effects on the skin, such as moisturizing, antiaging, and anti-inflammatory properties. Phytochemistry studies help us identify these compounds and develop new cosmetic products that can improve skin health. Phytochemistry can be used to study the chemical makeup of plants in different ecosystems, which can help us understand the effects of environmental factors such as pollution and climate change on plant growth and health.

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# Chapter 8

## Biological and Pharmacological Properties of Apocynaceae Members



### Introduction

Medicinal plants have been used for centuries by different cultures around the world. The biological activities of these plants are attributed to various phytoconstituents present in them. Apocynaceae plants contain various active bioactive compounds with antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, and anticancer properties. Ethnomedicinal knowledge of many members of this family has led to extensive studies on their biological and pharmacological properties. The pharmacological evaluation of many plants has explored crude extracts as well as purified phytoconstituents. However, very few members of this family have been systematically studied and explored for pharmaceutical/medicinal applications despite their vast ethnomedicinal value. This chapter highlights a detailed outlook of the biological activities exhibited by Apocynaceae members with respect to crude extracts, fractions and single isolated compounds (Table 8.1).

### Antioxidant Activity

The Apocynaceae family includes a number of species with potential antioxidant properties. Antioxidants are compounds that help protect cells from oxidative damage caused by free radicals, which are highly reactive molecules that can contribute to various diseases and aging processes. While not all plants within the Apocynaceae family have been extensively studied for their antioxidant potential, several species have shown promising results.

*Catharanthus roseus* contains various alkaloids, including vincamine and vinblastine, which have demonstrated antioxidant activity. Vincamine has been shown to scavenge free radicals and inhibit lipid peroxidation, protecting cells from oxidative stress. The leaves and flowers of *Allamanda cathartica* have been investigated

**Table 8.1** Biological and pharmacological properties of Apocynaceae members (Reproduced with permission from Bhadane, 2017; Bhadane et al., 2018)

Plant and plant part	Biological activity	Extract/Compound	Effective concentration/Dose	Study model	References
<i>A. scholaris</i> Flowers	Antioxidant (DPPH)	Methanolic and aqueous extract	IC <sub>50</sub> 34.2 and 50 µg/mL	<i>In vitro</i>	James et al. (2011)
	Anticancer	80% methanolic extract	4% crude extract	<i>In vitro</i>	Surendren et al. (2012)
	Anti-HIV	Methanol and butanol extract	10 and 15 µg/mL, respectively	<i>In vitro</i> (using CEM-GFP cell lines infected with HIV-INL4.3)	Sabde et al. (2011)
	Antiarthritic	Ethanolic extract	400 mg/kg	<i>In vivo</i> (Freund's adjuvant induced rat)	
Stem bark	Cardioprotective	Aqueous extract	300 mg/kg	<i>In vivo</i> (Streptozotocin induced rat)	Bandawane et al. (2011)
	Antidiabetic	Aqueous extract	300 mg/kg	<i>In vivo</i> (Streptozotocin induced rat)	Bandawane et al. (2011)
	Anti-inflammatory	Methanolic extract	400 mg/kg	<i>In vivo</i> (carrageenan induced rat paw)	Subraya and Gupta (2012)
<i>C. carandas</i> Leaves	Antioxidant (DPPH and OH <sup>-</sup> radical scavenging)	Methanolic extract	EC <sub>50</sub> 546 and 498 µg/mL	<i>In vitro</i>	Bhadane and Patil (2017)
	Anti-inflammatory	Methanolic extract	1.3 mg/mL	<i>In vitro</i>	Bhadane and Patil (2017)
	Cytotoxic	Carandinol from ethyl acetate fraction of methanolic extract	IC <sub>50</sub> 6.87 µg/mL	<i>In vitro</i> (using HeLa cell line)	Begum et al. (2013)
	Hepatoprotective	Methanol and acetone (3:1) extract	100 mg/kg	<i>In vivo</i> (CCl <sub>4</sub> induced liver injury in albino rat)	Bhati et al. (2014)
Fruit	Antidiabetic				

Roots	Gastroprotective	Carissone and Scopoletin from methanolic extract	IC <sub>50</sub> 20.1 and 24.6 µg/mL, respectively	<i>In vitro</i> (using J774A.1 cell lines)	Galipalli et al. (2015)
	Hepatoprotective	Ethanolic extract	400 mg/kg	<i>In vivo</i> (CCl <sub>4</sub> and paracetamol induced liver injury in albino rat)	Hegde and Joshi (2009)
<i>C. roseus</i>					
Leaves	Hepatoprotective	Ethanolic extract	500 mg/kg	<i>In vivo</i> (simvastatin induced albino rat)	Ahmed and Srinivasa Rao (2013)
	Antimalarial	Ethanolic extract	IC <sub>50</sub> 49.63 µg/mL	<i>In vitro</i> (Antiplasmodial against <i>Plasmodium falciparum</i> )	Ravikumar et al. (2012)
	Anti- HIV	Methanolic extract	2.5 µg/mL	<i>In vitro</i> (using CEM-GFP cell lines infected with HIV-1NL4.3)	Sabde et al. (2011)
	Antibacterial	Ethanolic extract	5 mg/mL	<i>In vitro</i>	Govindasamy and Srinivasan (2012)
Whole plant	Cytotoxic	Leurosine (indole alkaloid) from 95% ethanolic extract	IC <sub>50</sub> 0.73 µM	<i>In vitro</i> (using MDA-MB-231 human breast cancer cell lines)	Wang et al. (2012)
<i>H. antidysenterica</i>					
Leaves	Antioxidant (superoxide and OH <sup>-</sup> radical scavenging)	Methanolic extract	IC <sub>50</sub> 500 and 285 µg/mL, respectively	<i>In vitro</i>	Ganapathy et al. (2011)
Seeds	Antidiabetic	Ethanolic extract	600 mg/kg	<i>In vivo</i> (Streptozotocin induced rat)	Keshri et al. (2012)
	Antiurolithic	70% ethanolic extract	IC <sub>50</sub> 0.27 mg/mL	<i>In vitro</i> (inhibition of CaOx crystal aggregation)	Khan et al. (2012)
			100 mg/kg		
					(continued)



Table 8.1 (continued)

Plant and plant part	Biological activity	Extract/Compound	Effective concentration/Dose	Study model	References
<i>N. oleander</i>	Leaves	Cardioprotective	Oleandrin from methanolic extract	<i>In vivo</i> (CaOx-induced urolithiasis rat)	
				<i>In vivo</i> (using Guinea pigs) <i>In vitro</i> (using ventricular myocytes of mice)	Botelho et al. (2017)
Flower	Antioxidant (DPPH, ABTS, superoxide and OH <sup>-</sup> radical scavenging) Hepatoprotective	Methanolic extract	IC <sub>50</sub> 193, 156, 210 and 211 µg/mL, respectively	<i>In vitro</i>	Singhal and Gupta (2012)
				<i>In vivo</i> (CCl <sub>4</sub> -induced liver injury in rats)	Singhal and Gupta (2012)
				<i>In vivo</i> (Pentylentetrazol induced convulsion in Swiss albino mice)	Singhal and Gupta (2011)
Leaves	Anticandida	Methanolic extract	MIC 1 mg/mL	<i>In vitro</i>	Wankhede et al. (2013)
<i>R. serpentina</i>					
Roots	Anti-HIV	Methanolic extract	10 µg/mL	<i>In vitro</i> (using CEM-GFP cell lines infected with HIV-1NL4.3)	Sabde et al. (2011)
Seeds	Antimalarial	Petroleum ether extract	100 ppm	<i>In vivo</i> (mosquito larvicidal)	Das and Chandra (2012)
	Cytotoxic	Reserpine (indole alkaloid)	IC <sub>50</sub> 14.52 µM	<i>In vitro</i> (drug resistant tumour cell lines)	Abdelfatah and Efferth (2015)
Leaves	Antidiarrheal	Methanolic extract	400 mg/kg	<i>In vivo</i> (Castor oil-induced diarrhea in mice)	Ezeigbo et al. (2012)

<i>T. divaricata</i> Leaves	Antioxidant (metal chelating, OH <sup>-</sup> , DPPH and superoxide radical scavenging)	Ethanollic extract	68% metal chelation by extract (IC <sub>50</sub> 5.3, 5.2 and 5.8 mg/mL for radical scavenging)	<i>In vitro</i>	Anbukkarasi et al. (2016)
	Anti-inflammatory	Hexane of methanollic extract	0.7 µg·cm <sup>-2</sup>	<i>In vivo</i> (croton oil induced edema in albino mice)	Jain et al. (2013)
	Anticandida	Methanollic extract	MIC 1 mg/mL	<i>In vitro</i>	Wankhede et al. (2013)
	Antidiarrheal	Hydroalcoholic and aqueous extract	300 mg/kg	<i>In vivo</i> (Castor oil induced diarrhea in rats)	Raj et al. (2013)
Flower	Antidiabetic	Methanollic extract	300 mg/kg	<i>In vivo</i> (Alloxan-induced mice)	Rahman et al. (2011)
	Gastroprotective	Methanollic extract	500 mg/kg	<i>In vivo</i> (aspirin and ethanol-induced gastric ulcer in rats)	Ali Khan et al. (2013)
	Antifertility and abortifacient activity	Methanollic extract	500 mg/kg	<i>In vivo</i> (using female Wistar rats)	Mukhram et al. (2012)
	Hepatoprotective	Ethanollic extract	400 mg/kg	<i>In vivo</i> (DEN and Fe NTA-induced albino rats)	Poomima et al. (2014)
<i>T. peruviana</i> Leaves	Anticonvulsant	Ethanollic extract	400 mg/kg	<i>In vivo</i> (maximal electroshock, pentylenetetrazol, isoniazid induced convulsion in albino mice)	Basavaraj et al. (2011)
	Wound healing	Hexane extract	200 mg/kg	<i>In vivo</i> (dead space wound rat model)	Rahman et al. (2017)
	Wound healing	Water extract	200 mg/kg	<i>In vivo</i> (dead space wound rat model)	Rahman et al. (2017)
	Anticancer	Methanollic extract	100 mg/kg	<i>In vivo</i> (EAC cell line in albino mice)	Haldar et al. (2015)

(continued)

Table 8.1 (continued)

Plant and plant part	Biological activity	Extract/Compound	Effective concentration/Dose	Study model	References
Seeds	Antimalarial	Ethanolic extract	IC <sub>50</sub> 58.83 µg/mL	<i>In vitro</i> (Antiplasmodial against <i>plasmodium falciparum</i> )	Ravikumar et al. (2012)
Flowers	Gastroprotective	Linalool and 1,8-cineole from volatile oil	33 and 36 mg/kg, respectively	<i>In vivo</i> (ethanol-induced gastric ulcer in mice)	Kumar et al. (2015)
Whole plant	Anticancer	Cardenolides from methanolic extract	200 µg/mL	<i>In vitro</i> (against Hep G <sub>2</sub> , HL-60 and PC 62 cancerous cells)	Barbon et al. (2012)
<i>W. tinctoria</i>					
Leaves	Antimalarial (Larvicidal)	Petroleum ether and aqueous extract	LC <sub>50</sub> 0.37 and 0.11%	<i>In vitro</i> (against filarial larvae <i>Culex quinquefasciatus</i> )	Sakthivadivel et al. (2014)
Fruit	Antimalarial (Larvicidal)	Petroleum ether and aqueous extract	LC <sub>50</sub> 1.31 and 0.09%	<i>In vitro</i> (against filarial larvae <i>Culex quinquefasciatus</i> )	Sakthivadivel et al. (2014)
	Antidiabetic	Methanolic and ethyl acetate extract	300 mg/kg	<i>In vivo</i> (Alloxan-induced mice)	Rani et al. (2012)
Flower	Antioxidant (metal chelating, OH <sup>-</sup> and DPPH radical scavenging)	Ethanolic extract	IC <sub>50</sub> 43.16 µg/mL	<i>In vitro</i>	Ramalakshmi et al. (2012)

for their antioxidant properties. Extracts from *Allamanda cathartica* exhibited significant antioxidant activity in various *in vitro* studies, attributed to the presence of phenolic compounds. *Nerium oleander* (oleander) has been shown to possess antioxidant potential. Its leaf extract has been found to scavenge free radicals and inhibit lipid peroxidation, suggesting a protective effect against oxidative stress. *Plumeria spp.* have been investigated for their antioxidant activity. The flowers and leaves of *Plumeria* contain various phenolic compounds, which contribute to their antioxidant potential. *Tabernaemontana divaricata* is a highly explored member of this family for obtaining natural antioxidants. The antioxidant potential of *Tabernaemontana divaricata* is attributed to the presence of phenolic compounds. Various extracts of *Tabernaemontana divaricata* have demonstrated the ability to scavenge free radicals and inhibit lipid peroxidation in a number of studies (Bhadane, 2017).

The unique property of phenolic compounds is that they act as excellent reducing agents, metal chelators, and free radical quenchers (Rice-Evans et al., 1996). The antioxidant activity of *N. oleander* was evaluated by various *in vitro* methods, such as DPPH and reducing power assays (Singhal & Gupta, 2012). The antioxidative potential of ethanol extracts of *W. tinctoria* and *T. divaricata* was reported using DPPH scavenging activity (Ramalakshmi et al., 2012; Anbukkarasi et al., 2016; Rumzhum et al., 2012). The *in vitro* antioxidant activity of *H. antidysenterica* was attributed to the higher total phenolic content of the plant extract (Ganapathy et al., 2011). James et al. (2011) explored the antioxidative potential of *A. scholaris* fruit extract. The DNA damage inhibition and antioxidative potential of *C. carandas* were studied by Verma et al. (2015).

## Anti-Inflammatory/Analgesic Activity

Inflammation is a natural response of the body's immune system to injury, infection, or tissue damage. It is a complex process involving various immune cells, signaling molecules, and biochemical pathways. Inflammatory diseases can be treated with a variety of medications, including NSAIDs (nonsteroidal anti-inflammatory drugs), but their extended use might have negative side effects. Plants have long been a valuable source of anti-inflammatory compounds, many of which have been used in traditional medicine for centuries. Numerous plant species in the Apocynaceae family have been explored for their possible anti-inflammatory properties. However, the Apocynaceae family has not been fully explored for obtaining anti-inflammatory compounds.

Members of the family, such as *Carissa carandas*, *Plumeria rubra*, *Wrightia tinctoria*, *A. scholaris* and *Vinca major*, are known for their anti-inflammatory activity potential (Arulmozhi et al., 2012). Subraya and Gupta (2012) studied the *in vivo* anti-inflammatory activity of *A. scholaris* using animal models. *T. divaricata* leaf extracts showed significant anti-inflammatory activity in a male albino mouse model Jain et al. (2013). The isolated phytoconstituents from *C. carandas* roots, such as lupeol, stigmasterol, oleanolic acid, carissone and scopoletin, showed

inhibitory potential against pro-inflammatory mediators (Galipalli et al., 2015). Bhadane and Patil (2017) demonstrated the *in vitro* anti-inflammatory activity of a steroidal saponin of *C. carandas* leaves using RBC membrane stabilising potential.

## Anticancer/Cytotoxic Activity

Conventional anticancer compounds have played a significant role in cancer treatment, and they are not without drawbacks. The limitations and drawbacks of conventional drugs are that they exert several side effects on normal cells, and therefore, their applications are restricted. Natural sources, including plants, marine organisms, and microorganisms, have been extensively studied for their potential anticancer compounds. The anticancer and cytotoxic potential of several members of the Apocynaceae family has been explored using animal models. A few compounds from Apocynaceae plants have shown promising anticancer activity.

Alkaloids such as vinblastine and vincristine have been isolated from the Madagascar periwinkle plant (*Catharanthus roseus*) and have been successfully used as chemotherapy drugs for the treatment of various types of cancer, including Hodgkin's lymphoma, leukemia, and some solid tumors. Vinblastine and vincristine interfere with microtubule formation during cell division, leading to cell cycle arrest and cell death. A cardiac glycoside, cerberin, is found in the seeds of *Cerbera odollam*, also known as the "suicide tree." While cerberin is highly toxic, it has demonstrated potent anticancer effects. It has been shown to induce apoptosis (programmed cell death) in cancer cells and inhibit tumor growth in preclinical studies. Oleandrin is isolated from the leaves of *Nerium oleander* and has shown cytotoxic effects against various cancer cell lines, including breast, ovarian, and lung cancer. Oleandrin has been studied for its potential as an anticancer agent, primarily by inducing apoptosis and inhibiting cancer cell proliferation. Cardenolide compounds such as thevetin and thevetoxin were isolated from the seeds and other parts of *Thevetia peruviana*, commonly known as yellow oleander. They have exhibited anticancer activity by inducing apoptosis and inhibiting cell proliferation in various cancer cell lines.

Rumzhum et al. (2012) reported potent cytotoxic activity of *T. divaricata* leaves using an *in vitro* bioassay. Wang et al. (2012) showed cytotoxic activity of different alkaloids isolated from *C. roseus* against breast cancer cells with an  $IC_{50}$  of 0.72  $\mu$ M. Siddiqui et al. (2012) isolated compounds such as odoroside A and B, oleandrin, adynerin and doxorubicin from *N. oleander* with anticancer properties. *A. scholaris* showed antiproliferative effects on cancer/tumor cells (Surendren et al., 2012). Recently, carandinol, an isohopane triterpene from *C. carandas*, showed potent cytotoxic activity (Begum et al., 2013). The crude extract of *T. peruviana* showed considerable antitumour activity in albino mice (Halder et al., 2015). Barbon et al. (2012) demonstrated the growth inhibition of cancer cells using cardenolides isolated from *T. peruviana*. In another study, a reserpine alkaloid from *R. serpentina* was reported for its anticancer potential (Abdelfatah & Efferth, 2015).

## Cardioprotective Activity

The two most significant species in this family that are utilised to treat cardiovascular problems are *N. oleander* and *T. peruviana*. The antiatherogenic activity of *A. scholaris* stem bark extract was assessed by Bandawane et al. (2011), and the extract's ability to reduce LDL cholesterol may have contributed to the prevention of cardiovascular problems. The cardioprotective effect of *N. oleander* (Gayathri et al., 2011) was studied in rats. Roots of *R. serpentina* (Azmi & Qureshi, 2012) exhibited antidiabetic and cardioprotective potential in mice.

## Antidiabetic Activity

Several members of this family have been studied for their antidiabetic activity. For example, *Catharanthus roseus* alkaloids, which are mainly used in cancer treatment, have also been found to possess antidiabetic properties. The leaf extract of *Catharanthus roseus* can reduce blood glucose levels and improve glucose tolerance. The bark extract of *Pterocarpus marsupium* (Indian kino tree) has been traditionally used in Ayurvedic medicine for treating diabetes. Studies have indicated that the extract of this plant can lower blood glucose levels by increasing insulin secretion from pancreatic beta cells and improving insulin sensitivity.

*Gymnema sylvestre* is a well-known plant in traditional medicine for its antidiabetic properties. It is commonly used in Ayurvedic medicine to help control blood sugar levels. Gymnemic acids are known to have antidiabetic effects. These compounds can reduce the absorption of glucose from the intestines and increase insulin production in the pancreas. *Nerium oleander* has also been studied for its antidiabetic potential. The leaf extract of *Nerium oleander* possesses hypoglycemic properties, helping to lower blood glucose levels (Gayathri et al., 2011). The bark of *A. scholaris* showed hypoglycemic activity in rats (Bandawane et al., 2011). *H. antidysenterica* seeds (Keshri et al., 2012); the methanolic extract of flowers of *T. divaricata* (Rahman et al., 2011); and a partially purified fraction of the methanol extract of *C. carandas* fruit (Itankar et al., 2011; Rani et al., 2012) have shown potential antidiabetic activities in various animal models.

## Antimalarial Activity

According to the World Health Organisation (WHO), in 2019, an estimated 229 million cases of malaria were reported worldwide, resulting in approximately 409,000 deaths. *Plasmodium falciparum* and *Plasmodium vivax* are responsible for the majority of malaria cases worldwide. Therefore, the development of antimalarial drugs and the identification of natural compounds with antimalarial properties have

been crucial in the fight against malaria. Plant metabolites have played a significant role in the development of antimalarial drugs and the control of malaria. The use of plant metabolites for the treatment of malaria is well documented in the traditional system of medicine. Plant metabolites such as alkaloids, terpenoids, flavonoids, and phenolic compounds are known for their role in malaria control. For example, artemisinin, derived from the plant *Artemisia annua* (sweet wormwood), and its derivatives (e.g., artesunate, artemether) are potent antimalarial compounds. They exert their action by producing free radicals within the malaria parasite, leading to parasite death. The popular antimalarial drug quinine and its derivatives, such as quinidine, were isolated from the bark of *Cinchona* tree. They were also used to treat certain heart rhythm disorders. Curcumin, an alkaloid found in turmeric, has been demonstrated to have antimalarial activity against both *Plasmodium falciparum* (the most deadly malaria parasite) and *Plasmodium vivax* (Miettinen et al., 2014). Other metabolites, such as berberine and cryptolepine, found in various plants, including *Berberis* species, have been shown to possess antimalarial activity by inhibiting the growth of *Plasmodium* parasites. Saponins derived from plants such as *Quillaja saponaria* or *Sapindus spp.* have shown antimalarial activity. Flavonoids and phenolic compounds from the Apocynaceae family have been investigated for their antiplasmodial activity (Chan et al., 2016). *In vivo* antiplasmodial activity of *A. boonei* (Iyiola et al., 2011); *C. roseus* and *T. peruviana* (Ravikumar et al., 2012); the mosquito larvicidal activity of *R. serpentina* seed extracts (Das & Chandra, 2012) and the anti-malaria and chikungunia larvicidal activity of *W. tinctoria* extract are well documented (Sakthivadivel et al., 2014).

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## Chapter 9

# Agricultural Importance of Apocynaceae Members: Insecticidal Properties



### Introduction

Agricultural insects and pests cause damage to crops, livestock, or stored products. These pests include insects, mites, nematodes, rodents, birds, and plant pathogens such as fungi, bacteria, and viruses. They can cause a significant amount of damage to crops, resulting in lower yields and lower-quality produce. Insects are among the most damaging pests in agriculture. They can cause direct damage to crops by feeding on plant tissues or by transmitting plant diseases. Some of the most common insect pests in agriculture include aphids, caterpillars, beetles, and thrips. In addition to insects, mites can also cause damage to crops by feeding on plant tissues. Controlling pests in agriculture is important for maintaining healthy crops and ensuring high yields. Integrated pest management (IPM) is a sustainable approach for the control of insect pests that involves multiple control methods, including cultural practices and biological and chemical control, that involve the limited use of pesticides while maintaining effective pest control. IPM strategies may include crop rotation, use of resistant plant varieties, introduction of natural predators or parasites, and targeted use of pesticides when necessary. Biopesticides/botanical pesticides are a type of pesticide derived from natural sources such as plants, microbes, and other naturally occurring substances. They are used to control pests and diseases in agriculture, forestry, and public health. There are three main categories of biopesticides: microbial biopesticides (microorganisms such as bacteria, fungi, and viruses), plant-derived biopesticides (these are derived from natural plant extracts or oils and are used to repel, deter, or kill pests), and biochemical biopesticides (naturally occurring substances such as pheromones, enzymes, and plant growth regulators). Biopesticides have several advantages over synthetic pesticides. They are generally less toxic to humans, wildlife, and the environment and have a shorter residual life. They are also more target specific, meaning that they have a lower impact on beneficial organisms such as friendly insects and pollinators (Salunke, 2006).



**Fig. 9.1** *Calotropis procera* (Apocynaceae), a widely used species for insecticidal purposes

Apocynaceae plants have been widely used as a source of insecticidal metabolites. Several plants have been documented to have insecticidal properties (Bhadane et al., 2018). A common medicinal plant found in the tropics of Asia and Africa is *Calotropis procera* (Ait) R. Br., often known as “Arka” in India. It is a spreading shrub or small tree that exudes abundant amounts of milky sap when cut or broken. Leaves are opposite, large, grey-green up to 15 cm long and 10 cm wide, with a pointed tip, two rounded basal lobes, and no leaf stalk; its waxy white flowers are carried in stalked clusters at the ends of the branches; its fruit is grey-green, inflated, and 8–12 cm long, and it has many seeds with tufts (Sharma & Sharma, 1999) (Fig. 9.1). External parts as well as the roots of this plant have been widely utilized in conventional medical practices. According to Mascolo et al. (1988), an ethanol extract of the plant’s flower has antibacterial, anti-inflammatory, antipyretic, analgesic, anticancer (Smit et al., 1995), and antimalarial (Sharma & Sharma, 1999, 2001) properties. In addition, the extracts of *C. procera* in water, ethanol and other solvents show insecticidal, larvicidal, antibacterial, and antiparasitic properties (Moursy, 1997; Markouk et al., 2000). The primary components of *C. procera* latex are flavonoids, cardenolides, triterpenoids, alkaloids, resins, anthocyanins, saponins, tannins, and proteolytic enzymes (Deepak, 1995).

## ***Insects and Pests***

Insects are among the highly diverse forms of life living on earth. They can be found in diverse habitats, such as the ocean, swamps, forests, and deserts, as well as in harsh environments (Imms, 1964). They are highly adaptable to diverse environmental conditions, as their total numbers are far greater than those of other animals. Insects make up over 85% of all animal species known to exist on Earth, yet it is generally believed that just a fifth of all pest species have been recognized and categorized. Since the Palaeozoic era (230–570 million years ago), insects have been prevalent on Earth, and insects that resemble modern species have been discovered in rocks from the Permian era (230–280 million years ago) (FAO, 2006). Most insects have direct benefits for both people and the environment. Despite their enormous diversity, fewer than 0.5% of all known insect species are regarded as pests, and only a small number of these potentially pose a major threat to humans.

## ***Insects and Agricultural Losses***

Insects, weeds, or phytopathogens that affect the crop while it is still in the field cause preharvest losses. At different times during the life of a crop, cutworms, caterpillars, aphids, and fly maggots operate as leaf miners, stem borers, and pod borers (Atwal, 1976). Helicoverpa species, distributed worldwide, have polyphagous feeding habits (Harsulkar et al., 1998; Grover & Pental, 2003). As it is a serious pest of cotton in the USA, it is also called the American bollworm. In India, it causes serious damage to cotton, sorghum and other rabi crops, especially red gram and Bengal gram (Pradhan, 1992; Gujar et al., 2000). Cutworms (*Agrotis* sp.): These are a small group of polyphagous pests active in the rabi season. They are known as cutworms because they cause whole plants or crops such as gram or their twigs to fall on the ground (Pradhan, 1992). The plume moth caterpillar (*Exelastis atoms*) Walsingham (Family: Pterophoridae): This pest causes serious damage to developing seeds of red gram. Leaf miners and other *Agromyzid* flies: Leaf miners of major importance belong to the family of *Agromyzid* flies. Common examples are the pea leaf miner (*Phytomyza atricornis* Meigen), the pea stem borer (*Melanagromyza phaseoli* Coquillett) and the red gram pod fly (*Agromyza obtuse* Mallas). Aphids: A black species of aphid called *Aphis craccivora* K. is a serious pest on leguminous crops, particularly peas. Aphids damage the plant by sucking the cell sap. Other phytopathogenic fungi include *Aspergillus candidus*, *Aspergillus clavatus*, *Aspergillus fumigatus*, *Aspergillus parasiticus*, *Aspergillus restrictus*, *Alternaria alternata*, *Fusarium graminearum*, *Fusarium moniliforme*, *Fusarium roseum*, *Fusarium tricinctum*, *Helminthosporium* spp., *Scopulariopsis* spp., *Penicillium verrucosum*, etc. ICIPE, 2006.

### ***Traditional Pest Control Methods and their Drawbacks***

A number of traditional pest control methods are used in practice to control these insect pests. Traditionally used materials such as inert dust are mixed with the product, which contributes to decreasing pest activity (Arthur, 1996). Fumigation, control by drying, exposure to sunlight, or exposure followed by sieving of the grains, is a well-known technique among farmers in Asia and sub-Saharan Africa. The use of synthetic is also popular among farmers. Pesticides are agrochemicals required to combat the attack of various pests (s) on agricultural and horticultural crops. Synthetic pesticides have rapid action, are target specific and have relatively high residual toxicity. However, their injudicious use has resulted in the disruption of biological control by natural enemies. It also caused the development of resistance in pests (Varma & Dubey, 1999). It resulted in a rise in pest insects that attack stored grains and had unfavorable consequences on nontarget organisms, raising worries for the environment and human health. The public is becoming increasingly concerned about synthetic/chemical pesticides due to resistant strains/insects, release hazardous residues in food and feed, human safety, irregular supply, and high prices. Finding new approaches to combat insect pests at various phases of crop production and storage is therefore essential. A biological approach may offer a practical and sustainable substitute for the management of agricultural pests. It has been successful to employ the bacteria *Bacillus thuringiensis* to combat pests of stored grain and seedlings. *B. thuringiensis* was found to be the best choice for application on grains and pulses. Thus, the importance of microbial, viral, and bacterial insecticides for crop protection is growing. The first microbial pesticides to be registered in the US were bacteria from the genus *Bacillus* (*B. popilliae* and *B. popilliae* *Lentimorbus*) to control the Japanese beetle, *Popillia japonica*. The most popular and effective microbial pesticide ever registered is *B. thuringiensis* (Bt). Industrial bioreactors with huge capacities are used to produce Bt.

Toxin of *B. thuringiensis* (Bt) Berlang. var. Kurstaki, a Gram-positive, aerobic, sporulating bacterium, is effective against lepidopteran pests. It produces a proteinaceous crystalline protoxin. This protoxin, a 130 kDa molecule, is not toxic to insects. After ingestion by the insect, due to the alkaline pH of the insect gut, it is cleaved into smaller subunits (60 and 70 kDa) called  $\delta$ -endotoxins. The activated toxins bind to the receptors present on the larval midgut epithelium, disrupting the membrane and concomitant death of the insect (Khanna, 1998). *Bt israelensis* (*Bti*) was the first *Bt* subspecies discovered by Morgallit in 1976 and has activity only against Diptera (Becker & Margalit, 1993). On the other hand, entomopathogenic fungi are found to produce more than 60 insecticidal compounds that have been isolated and characterized from various fungi possessing a broad spectrum of activity. Avermectins of *Streptomyces avermitilis*, milbemycins of *S. hygroscopicus*, tetranactin from *S. aureus*, piericidins from *S. mobaraensis* and destruxins from *Metarrhizium anisopliae* are some examples of the insecticidal compounds produced by different fungi. The term “integrated pest management” (IPM) has been defined and used in a variety of ways. Previously known as

“integrated control,” is now expanded as IPM that encompasses all biological, cultural, and artificial practices. The most frequent risk that chemical and synthetic pesticides pose is to their natural enemies. The best strategy for decreasing the detrimental effects on natural enemies is to modify pesticide use practices in light of IPM strategies. Alternatively, IPM programs have been run with “pesticide management” goals rather than “pest management” goals (Lewis et al., 1997).

## Botanicals as Pesticides: An Overview

Plants have developed effective countermeasures to withstand herbivorous pests/diseases through the production of highly diverse types of organic compounds and metabolites called secondary metabolites (Salunke et al., 2009). The primary metabolites carbohydrates, lipids, nucleotides and peptides are produced by living cells to control basic life processes. On the other hand, secondary metabolites are obtained from primary metabolites or produced by cells, as the biproducts of their metabolism are involved in protecting plants against various biotic and abiotic stresses, including diseases and pests. A number of plant families produce alkaloids, tannins, terpenes, and phenolics that are widely used for the control of insects and pests. There are several types of secondary plant metabolites with insecticidal/pesticidal properties, viz., repellents, antifeedants, phagostimulants and toxins (Krishnayya & Rao, 1996; Nivsarkar et al., 2001). Many classes of plant proteins and secondary plant substances have been shown to have toxic or antimetabolic effects on insects and have been proposed as possible candidates (Simmonds et al., 1989; Kim et al., 2003; Kotkar et al., 2002; Upasani et al., 2003; Patil et al., 2010). The important character of plant-derived insecticidal metabolites is that they have chronic toxicity on insects, and therefore, their effects are less dramatic than those of synthetic insecticides (Estruch et al., 1997; Park et al., 2003).

Plant-derived pesticides or botanical pesticides, although natural, are not necessarily always safe, and there are a few limitations (Mendki et al., 2001). Most of them are expensive to produce compared to synthetic insecticides and require a large volume of starting material; therefore, an adequate supply of raw material may be a limiting factor in their availability. Moreover, the bioefficacy of botanical pesticides may vary even in identical preparations because of a number of factors. Although natural in origin, they are toxicity issues associated with botanical pesticides in several cases. They are toxic to pests and beneficial insects alike, and repeated use can disrupt biological control. Some commonly used botanical pesticides with their source, mode of action and target insects are shown in Table 9.1.

However, unlike many synthetic pesticides, natural compounds are usually biodegradable and are potentially environmentally safer than many of the synthetic products on the market today. The major benefits of biopesticides are that they are generally safer than conventionally used synthetic pesticides, and they are renewable. Being biodegradable, they can be metabolized, so there is no or less risk of resistance in the target pest. There is a lower risk of developing quick resistance.



**Table 9.1** Some examples of major botanicals and their pesticidal activities (Source: Weinzierl, 1998)

Plant based molecules/ botanicals	Plant source	Probable mechanism of action	Mammalian LD <sub>50</sub>	Target insect(s)
Pyrethrum	Flowers of <i>chrysanthemum cinerariaefolium</i>	Interferes with nerve transmission	> 1000	On fleas, ticks and lice
Rotenone	Roots of <i>derris</i> sp., <i>Lonchocarpus</i> and tropical legumes	Disrupts energy metabolism in mitochondria	Dermal >1000 Oral 25–3000	Beetles
Sabadilla	Seeds of <i>Schoenocaulon officinale</i> and <i>Veratrum album</i>	Interferes with Na and K ion movement in nerve axons	Oral near 4000	Bugs and thrips
Ryania	Stems of <i>Ryania speciosa</i>	Activates calcium ion release channels	Oral near 1000 Dermal near 4000	Caterpillars and thrips
Nicotine	Leaves of <i>Nicotiana</i> sp	Mimics acetylcholine to overstimulate receptor cells	Oral near 3–188	Aphids, thrips and mites
Azadirachtin	Leaves, bark, and seeds of <i>Azadirachta indica</i> and <i>Melia azedarach</i>	Growth hormone analog	Oral >5000	Soft-bodied insect pests
Limonene/ linalool	Citrus oils	Stimulation of sensory nerves	Oral >5000 Dermal >3500	Fleas and ticks

LD<sub>50</sub> = dose required to kill 50% of the test animal population; higher numbers indicate lower toxicity (Source: Weinzierl, 1998)

Most importantly, chances of accumulation of residues in food and fodder, disturbance of soil microflora and hazard to wild life are less compared to synthetic pesticides.

## Plants of the Apocynaceae Family as Bioresources for Pesticidal Molecules

The plant kingdom contains an estimated 500,000–600,000 different species. It is also estimated that 3000 plants belonging to more than 75 plant families are known to exhibit insecticidal activities (Dev & Koul, 1997).

For pesticidal activities, the family Solanaceae is most widely used, followed by Amaranthaceae, Apocynaceae, Fabaceae and Malvaceae. *Calotropis procera* (Ait.) R. Br. and *Calotropis gigantea* R. Br. are two species of family Apocynaceae and subfamily Asclepiadoideae (milkweeds), which are soft-wooded, evergreen,



perennial shrubs. Naturally occurring plant materials or products derived from such plant materials are termed ‘botanicals’ (Jacobson, 1989). They are also referred to as “first generation” insecticides. They include compounds such as Derris, pyrethrum, rotenone, nicotine, sabadilla, physostigmine and quassin, as well as lead arsenate and calcium arsenate. Some of these compounds formed the basis for synthetic “second generation” insecticides. For example, pyrethrum from *Chrysanthemum* flowers gave rise to synthetic pyrethroids, and physostigmine from *Physostigma venenosa* gave rise to structurally similar synthetic carbamates. Botanical insecticides were extensively used (1800–1940) until the discovery of the synthetic insecticide DDT. Table 9.2 provides a review of Apocynaceae members with insecticidal properties.

### **General Procedure Isolation of Insecticidal Metabolites(S) from *Calotropis Procera***

Parts of the identified plants were collected and carried to the laboratory. Different parts, such as leaf stems, flowers, bark, and roots, can be used for the isolation of insecticidal molecules. The plant parts can be used fresh or subjected to sun drying. Plant materials (leaf/stem/root powders of *C. procera*) were extracted in methanol using the Soxhlet apparatus at 65–70 °C for 6–8 h. The extracted material is dried in a rotary vacuum evaporator or drier, and the yield of the product is measured. This extract was dissolved in methanol and used for various experiments as well as for studying the larvicidal, insecticidal and oviposition deterrent activities (Fig. 9.2).

### **Insecticidal Metabolites from Apocynaceae Plants**

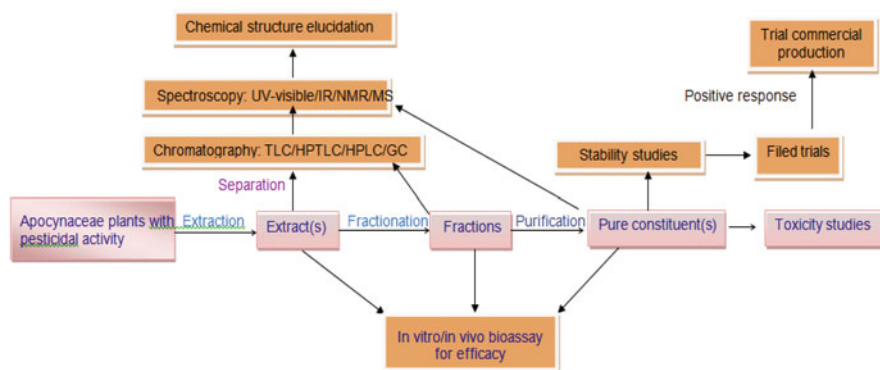
Secondary metabolites play an important role in the response to biotic and abiotic stress-related mechanisms in plants. The adaptive role of secondary metabolites in plant defense against insect pests is gaining importance in our understanding of plant defense. They participate in photosynthesis and cellular energy transfer mechanisms, operate as antioxidants or as enzyme inhibitors, and may be precursors to hazardous compounds or have therapeutic effects (Harborne & Williams 2000; Winkel-Shirley, 2002).

Strong adulticidal activity against the stored grain pest *C. chinensis* was observed with the various secondary metabolites from *A. squamosa*, *R. communis*, *C. procera* and *A. calamus* (Upasani et al., 2003; Salunke et al., 2005) (Table 9.3). Both dose and exposure duration had an impact on the adulticidal action. Other studies have achieved similar outcomes for the toxicity of numerous additional plant bioactive compounds (Kim & Ahn, 2001). According to El-Nahal et al. (1989), exposure

**Table 9.2** Insecticidal properties of Apocynaceae members

Plant and plant part	Pesticidal/insecticidal property	Crude solvent extract/purified compound	Effective concentration/Dose	Study model	References
<i>Catharanthus roseus</i>					
Leaves	Larvicidal	Ethanollic extract	IC <sub>50</sub> 49.63 µg/mL	In vitro (Antiplasmodial against <i>plasmodium falciparum</i> )	Salunke, (2006)
<i>Wrightia tinctoria</i>					
Leaves	Antimalarial (Larvicidal)	Petroleum ether and aqueous extract	LC <sub>50</sub> 0.37 and 0.11%	In vitro (against filarial larvae <i>Culex quinquefasciatus</i> )	Salunke, (2006)
Fruits	Antimalarial (Larvicidal)	Petroleum ether and aqueous extract	LC <sub>50</sub> 1.31 and 0.09%	In vitro (against filarial larvae <i>Culex quinquefasciatus</i> )	Salunke, (2006)
	Antidiabetic	Methanolic and ethyl acetate extract	300 mg/kg	In vivo (Alloxan induced mice)	Prabhu et al. (2018)
<i>Calatropis procera</i>					
Milkweed juice	Larvicidal activity against <i>Musca domestica</i> larvae	Alkaloids, steroids and resinous	80 µg/larvae	In vivo Larvicidal activity against <i>Musca domestica</i> larvae	Mendki et al. (2005), Khatter and Abuldahab (2012)
Leaves	Larvicidal activity against <i>Macrophomina phaseolina</i>	Hydrocarbon, azocompounds, aromatic carboxylic acids	1%, 2.5%, 4%, 5.5% and 7% of	In vivo model of <i>Macrophomina phaseolina</i>	Waheed et al. (2016)
Leaves	Insecticidal activity against <i>Callosobruchus maculatus</i>	Methanol	1.0, 1.5 and 5.0%	In vitro oviposition deterrent activity	Chauhan et al. (2016)
Leaves	Insecticidal activity against <i>C. chinensis</i>	Flavonoids: Rutin and quercetin	5 and 10 mg mL <sup>-1</sup>	In vitro oviposition deterrent activity <i>Callosobruchus chinensis</i>	Salunke et al. (2005)

<i>Calotropis gigantea</i>					
Leaves	Larvicidal activity against <i>Helicoverpa armigera</i>	Aqueous suspensions	2%, 4%, 10%	Leaf disc bioassay method	Prabhu et al. (2018)
Fruits	Larvicidal activity against <i>Helicoverpa armigera</i>	Aqueous suspensions	2%, 4%, 10%	Leaf disc bioassay method	Prabhu et al. (2018)
Flower	Larvicidal activity against <i>Helicoverpa armigera</i>	Aqueous suspensions	2%, 4%, 10%	Leaf disc bioassay method	Prabhu et al. (2018)
Roots	Larvicidal activity against <i>Helicoverpa armigera</i>	Aqueous suspensions	2%, 4%, 10%	Leaf disc bioassay method	Prabhu et al. (2018)
Stem	Larvicidal activity against <i>Helicoverpa armigera</i>	Aqueous suspensions	2%, 4%, 10%	Leaf disc bioassay method	Prabhu et al. (2018)



**Fig. 9.2** Isolation of insecticidal metabolite(s) from *Calotropis procera* (Salunke, 2006)

**Table 9.3** Effect of purified secondary metabolites on different stages of development of *C. chinensis* (Source: Salunke, 2006)

Sr. no.	Secondary metabolite	Emergence after treatment					
		1 <sup>a</sup>	7 <sup>a</sup>	13 <sup>a</sup>	17 <sup>a</sup>	21 <sup>a</sup>	26 <sup>a</sup>
1	<i>A. squamosa</i> Myricetin	12.7 ± 0.8b	21.8 ± 0.8 cd	28.8 ± 0.8b	64.7 ± 0.8c	74.2 ± 0.8c	81 ± 0.6bcd
2	<i>C. procera</i> quercetin	19.2 ± 1.2c	28.2 ± 0.8e	49.3 ± 1d	68.7 ± 0.8d	77.8 ± 1.2d	80.3 ± 0.5b
3	<i>A. squamosa</i> long chain fatty alcohol	8.17 ± 0.8a	12.3 ± 0.8a	25 ± 0.9a	54.8 ± 0.8a	67.8 ± 0.8a	77.8 ± 0.8a
4	<i>A. Calamus</i> Rutin	11.5 ± 1b	20.3 ± 0.8b	28.3 ± 0.5b	60.3 ± 0.5b	71.2 ± 1b	80.2 ± 0.8b
5	Flavone	31 ± 0.9e	44.5 ± 1 g	58.3 ± 0.8f	77 ± 0.9e	79.5 ± 1d	81.7 ± 0.5bcd
6	Rutin	11.5 ± 1b	19.2 ± 0.8b	28.3 ± 0.8b	60.2 ± 0.8b	70.2 ± 1b	80.7 ± 1.2bc
7	Quercetin	20 ± 1.7c	27.5 ± 1e	48.2 ± 0.8d	70.2 ± 1.5d	78.5 ± 0.5d	81.3 ± 0.8bcd
8	Myricetin	12.7 ± 0.8b	22.8 ± 2.6d	30.8 ± 1.2c	63.3 ± 0.8c	71.5 ± 1b	80.3 ± 1b
9	Fisetin	37.8 ± 0.8f	48.2 ± 0.8 h	60 ± 0.9f	78 ± 0.9f	79.5 ± 1d	81 ± 0.9bcd
10	Quercitrin	24.8 ± 0.8d	37.7 ± 1f	54.7 ± 0.8e	75.7 ± 1e	78.3 ± 0.8d	82.2 ± 0.8 cd
11	Solvent control	81.7 ± 1.2 g	81.7 ± 1.2i	81.7 ± 1.2 g	81.7 ± 1.2 g	81.7 ± 1.2e	81.7 ± 1.2 cd
12	Control without treatment	82.3 ± 0.5 g	82.3 ± 0.5i	82.3 ± 0.5 g	82.3 ± 0.5 g	82.3 ± 0.5e	82.3 ± 0.5d

Means within the column followed by the same letter are not significantly different by Tukey's MRT ( $P = 0.05$ ). Means ( $\pm$ SD) of the data are reported.

<sup>a</sup>Days after oviposition; (Dose applied at 10 mg mL<sup>-1</sup>) (Source: PhD Thesis, Salunke et al., 2005)

duration, not dosage, proved to have the greatest impact on the toxicity of the essential oil of *A. calamus* on adults of five stored-product insect species.

Terpenoids are volatile plant-derived substances popularly used as fumigants. According to Ahn et al. (1998), the monoterpene carvacrol functions as an insecticidal fumigant and has a wide range of insecticidal actions against agricultural, stored goods, and medical insect pests. Terpenoids from *A. squamosa* were found to have fumigant action against *C. chinensis*. In closed cups, as opposed to open cups, partially purified terpenoids and long-chain fatty alcohols were significantly more effective against adults of *C. chinensis*. These findings suggest that the chemicals identified from *A. squamosa* may behave as fumigants, which may cause toxic effects to enter the insect body through the respiratory system and produce insecticidal effects. Secondary metabolites are known to reduce oviposition at a 10 mg mL<sup>-1</sup> dose of secondary metabolites of *C. procera*, and no egg laying occurred in most of the combined forms of partially purified secondary metabolite treatments (Salunke et al., 2005). This suggests that flavonoids act as ovipositional deterrents at higher concentrations. The reduced oviposition may be attributed to the triglycerides present in the oils (Hill & Schoonhoven, 1981; Rajapakse & Van Emden, 1997).

The application of plant secondary metabolites at higher doses (10 mg/ml) was able to reduce progeny production, similar to the effect of oil (Rajapakse & Van Emden, 1997). Credland (1992) demonstrated the ovicidal effect of oils obtained from plants that were previously reported to have insecticidal properties. The effect of plant secondary metabolites on eggs may be attributed to their chemical toxicity or physical characteristics, which may reduce the surface tension within the egg similar to those of surfactants/oils (Singh et al., 1978). Flavonoids cause lethal effects not only through direct contact but also by penetrating the cotyledons and inhibiting larval development. Different flavonoids are found to alter molting in insects, causing death (Stamp & Yang, 1996). The majority of plant secondary metabolites either act as antiestrogens or inhibit cytochrome P450 isozyme expression and activity (Mitchell et al., 1993; Tsyrllov et al., 1994). The effect of plant secondary metabolites was reduced when applied 21 and 26 days after oviposition, probably because by that time, most of the larvae became pupae and entered deep inside the grains.

## Botanical Pesticides: A Way Forward

Human, animal and environmental health concerns have prompted governments all over the world to restrict the use of chemical pesticides and other agricultural products. This has resulted in the search for new safe and sustainable alternatives for insect and pest control. Plant secondary metabolites may represent a new class of extremely physiologically active chemicals and an alternative to traditional synthetic materials. The rate of excretion or inactivation of these compounds, changes in the target receptors for these compounds and interactions with endogenous factors that

may override the target responses are just a few examples of how the response to a given compound may be significantly influenced by other factors, both endogenous and exogenous. For this reason, understanding the physiological mechanism by which these active plant secondary metabolites function is crucial for their potential use in agriculture. Additionally, there is a need for the formulation of new strategies for understanding the mode of action of botanical pesticides.

Plant secondary metabolites have multiple modes of action, and the exact mechanism of their activity may need further investigation. There is an important role of plant secondary metabolites in the metamorphosis and reproduction of beetles. There is a relationship between oxidative stress and membrane damage, physiology and nervous system, digestion, ecdysteroid titres and egg laying levels, but further study is needed to ascertain the mechanisms of action in these crucial life cycle events. It can be concluded that the Apocynaceae plant can be considered a reliable, cost-effective source of biopesticides, and it could be an eco-friendly strategy for the protection of crops and has the potential to be included in future insecticide development programs.

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## Chapter 10

# Plant Tissue Culture-Based Approaches for the Production of Pharmaceutically Important Bioactive Compounds from Apocynaceae Members



## Introduction

Plants have been the major and economic source of human and animal medicine for decades. Plant-derived metabolites are widely popular due to the wide knowledge of traditional medicine, their cost effectiveness, wide availability, and low toxicity compared to synthetic compounds. It is estimated that over 80% of the world's population relies on traditional and plant-derived medicine.

Plant products and phytochemicals are widely used in pharmaceuticals, cosmetics, nutraceuticals, etc. However, the overexploitation and injudicious use of medicinal plants have resulted in the extinction of thousands of medicinally valuable species across the globe (De Pasquale, 1984). Due to the presence of several biosynthetic pathways, such as photosynthesis, glycolysis, the Krebs cycle, and shikimate pathways, plant cells are able to produce a variety of complex and biologically active secondary metabolites. These metabolites are grouped into several classes depending on their synthesis pathways, structure and functions. It includes alkaloids, tannins, flavonoids and phenolics, terpenes, saponins, enzymes, steroids, etc. Due to their medicinal value and increasing applications, it is necessary to explore alternate approaches to obtain these pharmaceutically/medicinally important secondary metabolites of plant origin. A number of biotechnological routes can be used for commercial and sustainable production of these medicinally important compounds. **Plant tissue culture** (PTC) is considered an important platform for obtaining medicinally and industrially important secondary metabolites (Table 10.1). Apocynaceae plants have been widely explored for micropropagation, *in vitro* propagation, and hairy root culture using plant biotechnology tools. Other approaches, such as callus and cell suspension culture, have also been widely used to obtain bioactive products similar to those of native plants. Recently, *Agrobacterium rhizogenes*-mediated transformation has been widely explored for the production of enzymes and hormones using hairy root culture.

**Table 10.1** Various types of secondary metabolites obtained through *in vitro* callus and cell suspension culture

Sr. no.	Class of Secondary metabolites	Examples	Method(s) of production	References
1.	Alkaloids	Furoquinolines, lobeline, indoles, isoquinolones, tropane, Piperidine, thebaine, betalaines, catharine, catharine,, apparicine, $\beta$ -carboline, iochrovincine, roscicine, serpentine, leurosinone, cathindine, cavincidine	<i>In vitro</i> callus and cell suspension cultures	Chandran et al. (2020)
2.	Steroids	Digoxin, digitoxin, helleborin, Brassinolide, glycosides, Ouabain, physodine. Catasterone, steroidal	Callus and cell suspension cultures using synthetic media	Chandran et al. (2020)
3.	Terpenoids	Diterpenes, artemisinin, Taxol, paclitaxel, sesquiterpenes, treterpenes, urosilic acid, monoterpenes	<i>In vitro</i> callus and cell suspension cultures	Chandran et al. (2020)
4.	Phenolics and flavonoids	Rutin, quercetin, plumabagin, lupeol, shikimic acid, 2,3-dihydroxybenzoic acid, salicylic acid; salicylic acid glucoside, benzoic acid, gallic acid, glucovanillin, vanillic acid, 2,5-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid glucoside, Rutin and quercetin	<i>In vitro</i> callus and cell suspension cultures	Chandran et al. (2020)
5.	Quinones	Emodin, Rhein, Uglone, shekonin, Isoflavonoids, tannins, Ferulic acid, Caffeic acid	<i>In vitro</i> callus and cell suspension cultures	Chandran et al. (2020)
6.	Phenylpropanoids	Lignans, Isoflavonoids, Phenalinones, anthocyanins	<i>In vitro</i> callus and cell suspension cultures	Chandran et al. (2020)

## Large-Scale Production of Plant Secondary Metabolites Using Callus and Cell Suspension Culture

The *in vitro* culture to obtain plant secondary metabolites consists of two stages. (i) Aggregation of biomass and (ii) synthesis of secondary metabolites (Yue et al., 2016). Plant cells develop a disorganized mass of cells known as a callus when grown *in vitro* with the right inducers and hormones. A high auxin concentration or combination of auxins and cytokinins is typically chosen for callus induction. Callus cultures now have the commercial potential to produce medicinally important secondary metabolites. On the other hand, organized tissues such as shoots, roots,

calli, and cell suspensions are also popular for obtaining pharmaceutically important secondary metabolites.

Carbon and nitrogen sources, vitamins, minerals, and phytohormones play an important role in the induction of callus tissue in an *in vitro* system (Perez-Jimenez et al., 2013). Calluse cells produce secondary metabolites intracellularly through extracellular synthesis. Therefore, secondary metabolites from callus tissue can be extracted using suitable solvents (Karuppusamy, 2009). Screening of callus tissues that yield maximum bioactive metabolites and are genetically stable is preferred for commercial purposes. Thus, inoculum with better characteristics is preferred for cell suspension culture, which ultimately gives a better yield of the desired compound. Cell suspension culture involves the cultivation of callus tissue in submerged cultures where the callus tissue remains in a free floating medium. Cell suspension culture in a submerged culture improves nutrient utilization in plant cells compared to callus culture in solid medium. Moreover, it also yields maximum cell biomass in less time (Raharjo et al., 2010). In cell suspension culture, the yield of the desired metabolites can be increased by adding the appropriate precursors and optimizing the various physico-chemical parameters of the bioprocess. Elicitation may enhance metabolite production. This is achieved by applying chemical or physical stress in submerged cultures. The use of elicitors has become a popular method to obtain an increased production of metabolites that are normally produced at lower levels.

## Media for Callus and Cell Suspension Culture

Murashige and Skoog's (MS) medium (Murashige & Skoog, 1962) is commonly used for plant tissue culture experiments. For the preparation of media, fresh stock solutions of macrosalts, microsals, vitamins, iron, potassium iodide, meso-inositol, glycine, and plant growth regulators (PGRs) are prepared separately. The stock solutions of MS medium are prepared in double distilled water and can be stored at low temperature (4 °C) for a month. The compositions of all stock solutions in MS medium and PGRs commonly used are shown in Tables 10.2 and 10.3, respectively. The pH of the medium was adjusted before use. Semisolid media can be prepared by adding agar powder as a gelling agent.

The plant tissue culture room is sterilized before use by fumigation using a mixture of potassium permanganate and formaldehyde (2:3). Fumigation is carried out at least two times with an interval of 24 h before every inoculation.

**Table 10.2** Chemical composition of MS basal medium

Constituents	Composition (mg/L)
<i>Macrosalts</i>	
NH <sub>4</sub> NO <sub>3</sub>	1650
KNO <sub>3</sub>	1900
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440
KH <sub>2</sub> PO <sub>4</sub>	170
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370
<i>Microsalts</i>	
H <sub>3</sub> BO <sub>3</sub>	6.2
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6
MnSO <sub>4</sub> ·4H <sub>2</sub> O	22.3
<i>Source of potassium iodide</i>	
KI	0.83
<i>Source of iron</i>	
Na <sub>2</sub> EDTA	37.25
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.85
<i>Meso-inositol</i>	100
<i>Source of vitamins</i>	
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
<i>Source of amino acid</i>	
Glycine	2
<i>Gelling agent</i>	
Agar	0.85%

**Table 10.3** List of PGRs used in callus culture

PGRs	Chemical name and formula	Molecular weight
<i>Auxins</i>		
NAA	α- naphthalene acetic acid (C <sub>12</sub> H <sub>10</sub> O <sub>2</sub> )	186.20
2,4D	2,4 Dichlorophenoxy acetic acid (C <sub>8</sub> H <sub>6</sub> Cl <sub>2</sub> O)	221
IAA	Indole 3 acetic acid (C <sub>10</sub> H <sub>9</sub> NO <sub>2</sub> )	203.20
IBA	Indole-3-butric acid (C <sub>12</sub> H <sub>13</sub> N0 <sub>2</sub> )	175
<i>Cytokinins</i>		
BAP	6-benzyl-amino purine (C <sub>12</sub> H <sub>11</sub> N <sub>15</sub> )	225
Kinetin	6-furfurylaminopurine (C <sub>10</sub> H <sub>9</sub> N <sub>5</sub> O <sub>5</sub> )	215.20

## **Practical Approaches for Callus Induction in Apocynaceae Plants (*T. divaricata* and Seeds of *C. carandas*)**

The explant of the selected plant is first subjected to surface sterilization before inoculation in the media. Different sterilants can be used to achieve effective surface sterilization of explants. The juvenile leaves from the healthy plant of *T. divaricata* and seeds of *C. carandas* can be used as explants. The explant surface sterilization procedure was studied and optimized by Bhadane and Patil (2016). Various sterilants have been tested for surface sterilization, such as 0.1%  $\text{HgCl}_2$ , 70% ethanol, 0.1% benzalkonium chloride (available 4% in commercial bleach Lizol), detergent (5% Teepol), Tween 20, antibiotics (0.1% streptomycin sulfate and 0.2% Bavistin, etc.

### ***Sterilization of Explants***

Before surface sterilization, plant material is thoroughly washed with running tap water and air dried. After prewashing, explants are placed into sterile glass bottles, washed with sterile water containing detergent and Tween 20 for 30 min and rinsed with sterile distilled water three times. After washing, the explants were treated with antibiotic solution (0.1% streptomycin sulfate and 0.2% Bavistin) for 15 min. Antibiotic-treated explants are washed three times using sterile double distilled water inside the LAF cabinet.

After surface sterilization using different combinations of sterilants, the explants are finally rinsed with sterile distilled water to wash out the residues from the surface of the explant (Jain et al., 2013) and air dried. Thereafter, the excised leaves of *T. divaricata* can be transferred to MS media fortified with 2.0 mg/L NAA + 0.5 mg/L BAP to check the efficiency of sterilants. Seeds of *C. carandas* are transferred to MS media devoid of PGRs to check the efficiency of sterilants. Data on contamination, explant survival percent and mortality rate, etc., is recorded to evaluate the sterilant efficiency.

### ***Cultural Conditions for Callus Induction***

After standardization of the surface sterilization process, callus induction experiments are carried out. Keng et al. (2008) provided the detailed process of callus induction for Apocynaceae plants. The surface-sterilized leaves of *T. divaricata* and leaves of aseptic seedlings of *C. carandas* were cut to obtain approximately 1 cm<sup>2</sup> pieces. The aseptically prepared explants are cultured on MS medium sucrose and PGRs (Perez-Jimenez et al., 2013; Yang et al., 2008). Plant growth regulators (PGRs), such as auxin and cytokinins, are used for callus induction in Apocynaceae

plants. PGRs are normally insoluble in water; hence, water miscible solvents such as absolute alcohol or 0.1 N HCl can be used to solubilize them prior to use. Stock solutions of PGRs were prepared in double distilled water, filtered to sterilize using a membrane filter and added to MS medium. Some PGRs are sensitive to heat; hence, they are added to the culture medium after autoclaving. MS medium supplemented with various concentrations of auxins (NAA and 2,4 D) either alone or in combination with cytokinins such as BAP and Kinetin can be used for callus induction studies (Rashmi & Trivedi, 2014).

### ***Inoculation and Maintenance of in Vitro Cultures***

The maintenance of sterile conditions during inoculation is important for the success of plant tissue culture work. Before inoculation, hands and arms are washed and sanitized with hand sanitizers such as Dettol® and Savlon. Metalware (forceps, scalpels, spatula, etc.) can be sterilized using 95% ethanol followed by flaming over a burner flame. The laminar air flow (LAF) cabinet was swabbed with Dettol and then with rectified spirit prior to inoculation. The autoclaved media containing glassware, spirit lamps, and necessary metalware are kept in the LAF cabinet. Thereafter, sterilization of the LAF cabinet area is achieved by closing the hood and switching on the UV light for an hour. The UV light was switched off for 10 min, and inoculation work was carried out under LAF (De Pasquale, 1984).

Surface sterilized explants are inoculated in culture vessels containing sterile MS media with PGRs. The culture bottles and test tubes are sterilized over a flame before inoculation of explants. The inoculated cultures are placed in a tray and placed onto the center of culture racks under cool white fluorescent light (Bhojwani & Razdan, 1996). All controlled conditions, such as temperature  $25 \pm 2$  °C, relative humidity  $55 \pm 5\%$  and 16/8 h (dark/light) photoperiod with the intensity of light 2000 lux provided by white fluorescent light during the incubation, were maintained in an incubation room (Patil & Bhalsing, 2015).

### ***Subculture***

After 4 weeks of incubation, a well-developed and proliferated callus from explants can be seen, and it is therefore ready to subculture on fresh medium. Subculturing is carried out at 2-week intervals on fresh MS medium containing the same combinations and concentrations of PGRs. Each subculture passage is carried out up to a selective interval to avoid callus browning (Kotomori & Murashige, 1965; Morel, 1972). The developed callus can be measured by measuring the fresh and dry weights. For determination of the fresh weight of callus, all tissues of callus can be picked up from the media surface using flat-ended tweezers and put into a previously weighed sterile glass vial. Glass vials containing callus tissue were

further weighed on a digital weighing balance. The moisture content of calli can be calculated during fresh and dry weight measurements (Rashmi & Trivedi, 2014). The moisture content can be calculated by using the following formula:

$$\text{Moisture content (\%)} = (B - A) - (C - A) / (B - A) \times 100$$

where A = weight of empty glass vial; B = weight of glass vial with fresh callus tissue, and C = weight of glass vial with dried callus material.

## Extraction of Phytochemicals from Callus Tissue

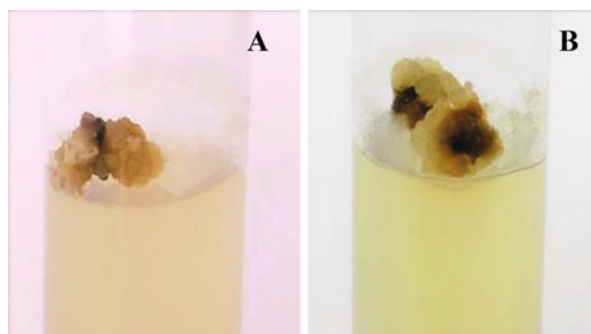
A well-developed callus mass can be obtained after successful contamination-free incubation (Fig. 10.1).

Callus tissues showing better growth and the highest fresh and dry weights can be selected for the extraction of bioactives. The general procedure for the extraction of metabolites from callus tissue is shown in Fig. 10.2. Dried callus was ground to obtain a fine powder. Powdered calli are usually extracted in methanol using the cold maceration method, and phytoconstituents can be extracted (Rajaram et al., 2013). Generally, the dry callus powder is mixed with 1:10 methanol and kept at 4 °C for 24 h. After cold maceration, the extract is centrifuged and filtered using Whatman filter paper.

## Cell Suspension Culture

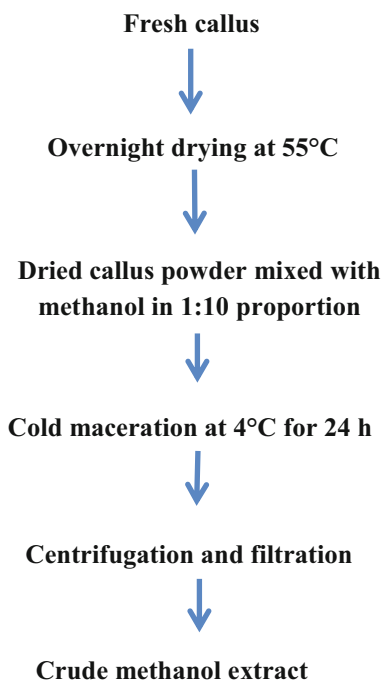
The growth of plant cells in a free moving liquid culture is studied by cell suspension culture. A well-developed callus obtained after standardization of callogenesis and used as inoculum for cell suspension culture. A cell suspension culture can be established in liquid MS medium with continuous stirring. The concentrations of PGRs are crucial for callus induction. Mustafa et al. (2011) optimized the

**Fig. 10.1** A well-developed callus of (a) *T. divaricata* callus (b) *C. carandas*





**Fig. 10.2** General procedure for extraction of callus material



experimental procedure for cell suspension culture. One gram of friable callus of the plant was inoculated separately in an Erlenmeyer flask containing sterile liquid MS medium fortified with sucrose and the respective PGRs.

To initiate the cell suspension culture of *T. divaricata*, approximately 1 g of friable callus was placed in a sterile Petri dish and chopped to obtain pieces. These pieces of callus are then inoculated in liquid MS medium containing NAA and BAP. A friable callus from *C. carandas* can also be prepared in the same way as reported for *T. divaricata* and inoculated in MS liquid medium containing NAA and BAP. After inoculation, the culture flasks were incubated at  $25 \pm 2^\circ\text{C}$  with continuous shaking in a shaking incubator (Fig. 10.3). Various stages in the cell suspension culture of *C. carandas* and *T. divaricata* are shown in Fig. 10.4.

For maintenance of cell suspension culture, cultures are periodically subcultured to fresh liquid medium to achieve maximum cell growth. Subculturing is done at regular intervals of 5 days. Usually, 10 mL of culture is aseptically transferred to fresh medium. Simultaneously, at each subculture interval, the growth and viability of cells in suspension culture was also checked.

Cell growth in a cell suspension culture is measured by several methods based on various parameters, such as determination of packed cell volume, medium conductivity, protein and DNA content and cell viability (Hahlbrock, 1975; Bradford, 1976; Dolezel et al., 2007). Packed cell volume determination and cell viability is used to measure the growth of cells in suspension culture. The packed cell volume (PCV) using a five-day-old cell suspension culture at every subculture can be taken

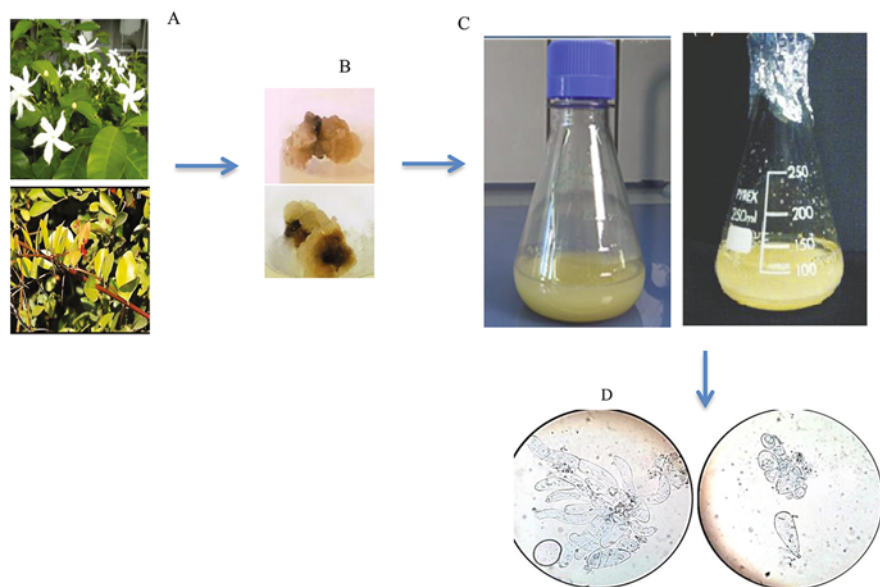
**Fig. 10.3** Shaking cooling incubator used for plant cell suspension culture



aseptically into a Falcon tube and centrifuged at 1000 rpm for 5 min. The PCV of the culture is pelleted at the base of the tube and expressed as percent growth, which can be measured by a calibrated arm on the tube.

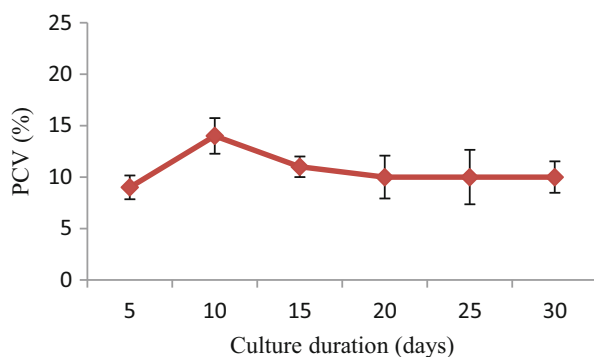
The *T. divaricata* suspension culture showed a maximum PCV of 14% (v/v) at the second passage (Fig. 10.5). On subsequent passages, it remains static. A decrease in the PCV of cells represents a lower yield of biomass or the presence of nonviable cells in culture. A similar trend was observed in the cell suspension culture of *C. carandas*. The maximum biomass yield with a PCV of 21% (v/v) was found at the third passage (Fig. 10.6). No further increase in biomass was reported in subsequent passages. An overall decrease in PCV was reported as the cells were transferred to a new environment (Bhadane, 2018).

During maintenance of the cell line, the cell biomass becomes the reason for the unsuitability of the medium composition for cell growth (Keng et al., 2008). After every five-day interval, a similar size of inoculum is transferred to fresh medium and observed for cell growth and viability. Approximately 20% inoculum gives



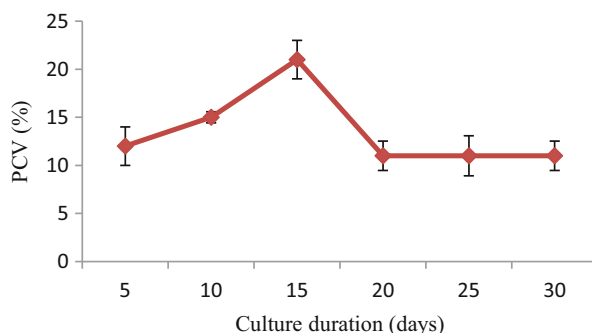
**Fig. 10.4** Various stages of cell suspension culture development of two Apocynaceae plants. (a) Healthy young plant of *C. carandas* and *T. divaricata*. (b) Well developed callus in MS media. (c) 100 mL cell suspension culture. (d) Cell viability test using Evans blue staining from suspension culture showing live and dead cells

**Fig. 10.5** Cell growth (PCV) in *T. divaricata* suspension culture



comparatively more growth than 10 and 15% in cell suspension cultures of *T. divaricata* and *C. carandas*. Determining cell viability is also one of the important aspects when studying cell suspension culture (Bhadane, 2018). It is used as an important parameter to determine growth in cultures. The Evans blue (EB) microscopic assay of Baker and Mock (1994) was used to measure the number of living cells.

**Fig. 10.6** Cell growth (PCV) in *C. carandas* suspension culture



## Biological Activities of the Callus Extract

Plant secondary metabolites are known to have diverse biological and medicinal activities. However, the amount of bioactive secondary metabolites produced by plant cells is limited, and the production of these metabolites is affected by several biotic and abiotic factors, seasonality, etc. Moreover, their extraction and purification from plant material is time consuming and involves technical difficulties. In the last few decades, plant cell culture-based technologies have gained importance for the increased production of secondary metabolites (Eibl et al., 2018; Potenza et al., 2020). The pharmacological activities of the callus extracts of several members of the family Apocynaceae are well documented. The callus from *H. antidysenterica* was reported for the presence of various bioactive metabolites. A number of phytoconstituents, such as terpenes, alkaloids and steroids, were isolated from the callus of this plant (Heble et al., 1976). The *in vitro*-grown callus extract showed excellent antimicrobial activity against human pathogens such as *S. aureus*, *S. typhimurium* and *E. coli* (Mahato et al., 2013). Taha et al. (2011) isolated several glycosides, such as thevetin B, peruvoside, digitoxigenin and neriifolin, from the callus of *T. peruviana* using HPLC. Some reports showed an inferior amount of bioactive metabolites in calli; hence, cell suspension culture was preferred for the large-scale production of phytoconstituents. A large number of reports are available on cell suspension cultures of *C. roseus* and *T. divaricata* and other plants of the Apocynaceae family for the production of alkaloids (Stafford et al., 1985; Van der Heijden et al., 1987; Dagnino et al., 1993; Panda et al., 1992; El-Sayed & Verpoorte, 2002; Zabala et al., 2010; Ibrahim et al., 2007, 2009; Arias et al., 2016).

## Hairy Root Culture

Due to their capacity to create secondary metabolites by expressing tDNA from the Ri plasmid of *Agrobacterium rhizogenes*, hairy root cultures (HRCs) have attracted attention from all around the world (Maldonado-Mendoza et al., 1993). Hairy roots

have the ability to grow due to the development of lateral roots from the first day to the first week, and this ability can be enhanced by exogenously supplying auxin (Baiza et al., 1999). In contrast to plant roots, hairy roots have long-term genetic stability and distinctive secondary metabolite synthesis skills (Parr & Hamill, 1987). In contrast to cell suspension cultures, they can also develop secondary metabolites that coincide with root growth, making it possible to continuously acquire them throughout hairy root culture (Holmes et al., 1997).

For many plants with therapeutic value, HRC has been effectively established. Only *Catharanthus roseus* and *Rauvolfia serpentina* have been identified for *Agrobacterium rhizogenes*-mediated gene transfer using hairy root culture in the Apocynaceae family. There are numerous reports on hairy root cultures of *Rauvolfia serpentina* and *Catharanthus roseus* for the synthesis of alkaloids. Through infection with *Agrobacterium rhizogenes* strain 15,834, Bhadra et al. (1993) discovered that seedlings of *Catharanthus roseus* produced hairy roots that produced indole alkaloids. The hairy roots of *Rauvolfia serpentina* were used to make alkaloids using the same strain and strain A4.

Sim et al. (1994) demonstrated the synthesis of catharanthine and ajmalicine using *A. rhizogenes* (15834)-mediated hairy roots. Catharanthine and ajmalicine were also produced from hairy roots of *Catharanthus roseus*. The production of catharanthine using nitric oxide elicitation (Zhou et al., 2010) and terpenoidal indole alkaloids (ajmalicine and serpentine) was also reported from hairy roots of *Catharanthus roseus* (Batra et al., 2004). New monoterpene alkaloids were obtained from hairy root cultures of *Rauvolfia serpentina* (Sheludko et al., 2002). Recently, Hanafy et al. (2016) reported the synthesis of anticancer compounds such as vinblastine, vincristine and catharanthine in hairy root cultures of *Catharanthus roseus* using MS medium. Table 10.4 provides details of specific metabolites produced using HRC.

In comparison to suspension culture, HRC has a number of benefits, including genotypic and biochemical stability, the ability to differentiate cells, and the ability to grow and multiply cells in hormone-free media. These are significant factors that influence the generation of secondary metabolites in an *in vitro* system. A further benefit is that hairy roots provide an ongoing source for the creation of useful secondary metabolites due to their rapid growth, low doubling time, ease of care, and ability to synthesize a wide variety of chemical compounds (Bourgau et al., 2001). HRC has been used to create a variety of plant secondary metabolites (Giri & Narasu, 2000).

Hairy root culture offers new opportunities for the *in vitro* production of valuable plant secondary metabolites. It has good prospects for the commercial production of pharmaceutically important secondary metabolites from Apocynaceae and other plants. The applications of HRCs are promising due to their stable, high-level secondary metabolite production, fast growth and suitability for growth and adaptation into bioreactor systems. It also has several challenges, such as the unusual rheological properties of hairy root, which makes the purification of metabolites tedious and time consuming.

**Table 10.4** Some examples of high-value compounds produced using hairy root cultures

Sr. no.	Secondary metabolite	Plant	Production method	Reference
1.	Anthraquinone	<i>Rubia tinctoria</i>	HRC using transformation with <i>agrobacterium rhizogenes</i>	Saito et al. (2001)
2.	Atropine and hysocyamine	<i>A. belladonna</i>	Ri T-DNA of <i>agrobacterium rhizogenes</i> and <i>in vitro</i> root growth	Jung and Tepfer (1987)
3.	Hysocyamine and scopolamine	<i>Hyoscyamus niger</i>	Adventitious root culture	Shimomura et al. (1991)
4.	Hysocyamine	<i>Datura innoxia</i>	Adventitious root culture	Shimomura et al. (1991)
5.	Quinine, quinidine	<i>Cinchona ledgeriana</i>	Agrobacterium mediated transformation	Hamill et al. (1989)
6.	Scopolamine	<i>Atropa belladonna</i>	HRC using transformation with <i>agrobacterium rhizogenes</i>	Bonhomme et al. (2000)
7.	Scopolamine	<i>Duboisia leichhardtii</i>	Agrobacterium mediated transformation	Mano et al. (1989)
8.	Scopolamine	<i>Hyoscyamus niger</i>	Agrobacterium mediated transformation	Zhang et al. (2004)
9.	Scopolamine and hysocyamine	<i>D. quercifolia</i>	Agrobacterium mediated transformation	Dupraz et al. (1994)
10.	Scopolamine and hysocyamine	<i>D. candida</i>	Agrobacterium mediated transformation	Christen et al. (1990)
11.	Solasodine	<i>Solanum khasianum</i>	Agrobacterium mediated transformation	Jacob and Malpathak (2004)
12.	Thiophene	<i>Tagetes patula</i>	Agrobacterium mediated transformation	Croes et al. (1989)
13.	Valpotriates	<i>Valeriana officinalis</i>	Agrobacterium mediated transformation	Gränicher et al. (1992)

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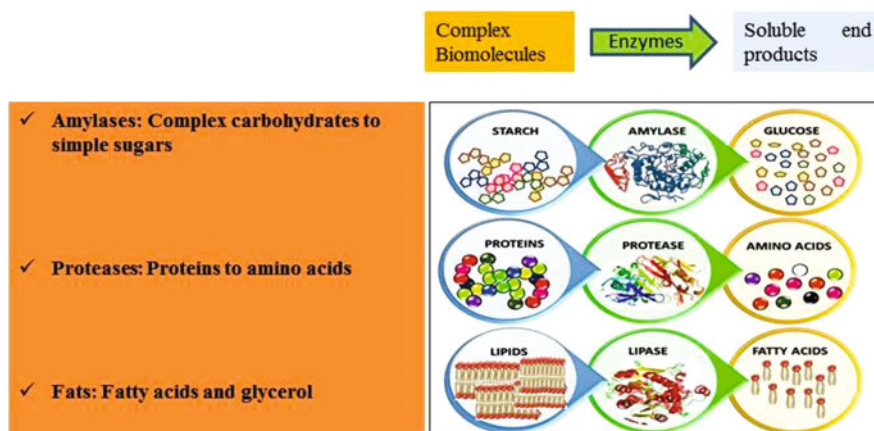
# Chapter 11

## Enzymes and Enzyme Inhibitors from Apocynaceae Plants



### Introduction

Enzymes have been extensively utilised in numerous sectors from last few decades. Recent years have seen a rise in interest in and demand for enzymes with unique features as a result of the development of biotechnology. For the commercial manufacturing of enzymes, microorganisms mainly isolated from soil are used. Over several decades, these industrially important enzymes have mainly been obtained from plant and animal sources. However, extracting enzymes from plant or animal sources is a difficult task due to their limited availability and high cost associated with downstream processing. Therefore, alternate sources such as microorganisms have been extensively used for enzyme production in recent times. Due to their numerous advantages, microbial enzymes have completely replaced enzyme extractions from plant and animal sources (Underkoffler et al., 1957). Microbial enzymes have certain advantages over their plant and animal counterparts. These can be produced in higher quantities within a shorter period, are nonseasonal and are comparatively more stable. The main advantage of using microorganisms instead of plants and animals is that they can be cultured using simple and low-cost media (Wiseman, 1995). Microbial enzymes have wide applications in food, detergent, pharmaceutical, textile, pulp and paper, leather, oleochemical and other industries (Sharma et al., 2001; Hasan et al., 2006). Among industrially important enzymes, hydrolases are widely used. This class of enzymes includes proteases, amylases, and lipases. These enzymes carry out the hydrolysis of their specific substrates, i.e., proteins, starch, and lipids, respectively (Fig. 11.1). Amylases and proteases are used in different industries for various purposes, but it was in the last decade that the use of lipases became more popular. Lipases carry out the hydrolysis of fats and are also involved in esterification and transesterification reactions (Rajendran et al., 2009).



**Fig. 11.1** Biochemical action of digestive enzymes

## ***Proteases***

The alternate name for proteases is proteinases or peptidases, which form the major group of enzymes that hydrolyse peptide bonds. Proteolytic enzymes are categorized into endopeptidases and exopeptidases. Peptide bonds present at the center of proteins are cleaved by endopeptidases and are categorized depending on the presence of catalytic groups on the active site, such as serine, cysteine, carboxyl and metallo endopeptidases (Barrett et al., 2012). Precisely, these exopeptidases breakdown the amino (N) terminus (aminopeptidases) or the carboxy (C) terminus (carboxypeptidases). Proteases have vast applications in various sectors, such as the food and detergent industry, pharmaceutical and agrochemical industries (Gupta et al., 2002; Bougatef et al., 2007).

## ***Amylases***

Amylase is an O-glycosyl hydrolase (EC 3.2.1.) families, which are a ubiquitous set of enzymes that break glycosidic bonds present among two or more sugars or between a sugar and a noncarbohydrate moiety. Amylases are classified into end amylases and exo amylases. Many bacteria produce amylase, which has wide applications in the field of bioengineering and in the food and beverages industry. Traditionally, amylases were obtained from plant, animals, and microbial (bacterial as well as fungal) sources. However, microorganisms are the major source of amylases to fulfil the growing industrial requirements of amylases (Guzman & Paredes, 1995; Van der Maarel et al., 2002; Reddy et al., 2003).

## **Lipases**

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are hydrolases that carry out lipolysis at the lipid-water interface. Lipases are omnipresent in nature and therefore can be isolated from various plants, animals, fungi and bacteria. Lipases catalyze the hydrolysis of triacylglycerol and release glycerol and free fatty acids. Triacylglycerol (TAG) is sparingly soluble in water, and it forms an emulsion, which is the site where lipases perform their action. Triacylglycerol (TAG) has very low solubility in water, and it forms an emulsion, which is the site where lipases perform their action. Lipases are extensively used as biocatalysts in organic chemistry due to their interesting properties, which include easy availability in large quantities, as many of them can be produced in higher amounts from microbes. Microbial lipases are produced intracellularly and secreted extracellularly (Sharma et al., 2012). Commercially, lipases are obtained from *Bacillus*, *Candida*, *Rhizopus*, *Pseudomonas*, etc.

## **Latex Bearing Apocynaceae Members as an Important Source of Biomedical Enzymes**

Plants have been an important source of enzymes such as amylase, proteases, invertase, lipases, papain, bromelain, ficin and lipoxygenase. These enzymes have a number of applications in the food, pharmaceutical, dairy, bakery and alcoholic beverage industries. Several higher plants from various families are used as economical factories for enzyme production.

The Apocynaceae family is also known as the family of latex-bearing plants. Common examples of latex-bearing Apocynaceae plants include *Calotropis procera*, *Calotropis gigantea*, *Synadenium umbellatum*, *Nerium oleander*, *Tarbanamontana divaricate*, *Tinospora cardifolia* and *Wrightia tinctoria*. Latex components include organic compounds, inorganic compounds, minerals and many hydrolytic enzymes (Silva et al., 2003). Latex hydrolytic enzymes include proteases, esterases and lipases, and amylases, which act as anticoagulant enzymes (Yagami et al., 1998). Hydrolytic enzymes play a significant role in plants' resistance to infectious diseases. Latex contains cysteine or serine proteases that possess diverse biological and pharmacological activities. The anticoagulants of plant latex are used in folk medicine, as well as in coronar heart diseases such as strokes and pulmonary embolism (Battjes et al., 2015).

A number of Apocynaceae plants are known to harbor anticoagulating and other proteases in their latex. Latex of *Calotropis gigantea*, *Ficus benghalensis*, *Croton bonplandianum*, *Ficus carica*, *Nerium oleander*, *Synadenium umbellatum*, *Artocarpus heterophyllus*, *Manilkara zapota*, *Ficus religiosa*, *Carica papaya* and *Plumeria acutifolia* have been widely studied for their pharmacological activities (Table 11.1). On the other hand, little work has been done on the latex of Apocynaceae plants for their anticoagulant enzymes.

**Table 11.1** Some examples of Apocynaceae and non-Apocynaceae plants as a source of enzymes

Enzymes	Source plant	Application	Reference
Anticoagulating enzymes	<i>Euphorbia milii</i>	Surgeries and heart diseases	Khuraishi et al. (2019)
	<i>Jatropha malacophylla</i>	blood clot treatment	Khuraishi et al. (2019)
	<i>Thevetia peruviana</i>	Surgeries and blood clot treatment	Khuraishi et al. (2019)
	<i>Euphorbia pulcherimma</i>	Surgeries and blood clot treatment	Khuraishi et al. (2019)
	<i>Artocarpus altilis</i>	Surgeries and blood clot treatment	Khuraishi et al. (2019)
Anticoagulating and proteolytic enzymes	<i>Calotropis gigantea</i>	blood clotting and milk clotting activity	Mishra et al. (2017)
	<i>Nerium oleander</i>	milk clotting activity	Flemmig and Melzig (2013)
	<i>Carica papaya</i>	blood clotting	Flemmig and Melzig (2013)
	<i>Artocarpus heterophyllus</i>	Blood and milk clotting activity	Flemmig and Melzig (2013)
	<i>Plumeria acutifolia</i>	Blood and milk clotting activity	Flemmig and Melzig (2013)
Fibrinolytic enzymes	<i>Wrightia tinctoria</i>	Coagulation and fibrinolysis	Flemmig and Melzig (2013)
Protease	<i>Euphorbia nerifolia</i>	Fibrinolysis	Mahajan et al. (2015)
Proteases and coagulating enzymes	<i>Asclepias curassavica</i>	Fibrinolysis	Karpagam et al. (2013)
coagulating enzymes	<i>Calotropis gigantea</i>	Milk clotting activity	Anusha et al. (2013)
Protease	<i>A. cathartica</i>	Milk clotting activity	Anusha et al. (2013)
Bromelain (mixture of proteases)	<i>Carica papaya</i>	Anti-inflammatory, meat tenderization	Meshram et al. (2019)
Papain (protease)	<i>Carica papaya</i>	Anti-inflammatory agent	Meshram et al. (2019)
Urease	<i>Canavalia ensiformis</i>	Biomedical, blood urea detection	Meshram et al. (2019)
Beta amylase	<i>Hordeum vulgare</i>	Brewing industry	Meshram et al. (2019)
Ficin	<i>Ficus carica</i>	Food industry	Meshram et al. (2019)
Peroxidase	<i>Cochlearia armoracia</i>	Diagnostic applications	Meshram et al. (2019)
Beta glucanase	<i>Hordeum vulgare</i>	Brewing industry	Meshram et al. (2019)

## Enzyme/Protease Inhibitors from Apocynaceae Plants

Several metabolites of Apocynaceae and other plants function as enzyme/protease inhibitors (EIs/Pis), which modulate various functions such as activity of endogenous proteases and protects against biotic and abiotic stresses. PIs are widely studied dmetabolites involved in plant defense which play a significant role insect attack. Small molecular proteins/peptides act as PIs or involved in cell signaling that leads to synthesis of PIs. Pearce (2011) isolated a signal peptide, Systemin, from tomato leaves which was found to be involved in synthesis of multiple defense components. Depending on the kind of PI, PIs can be found in a variety of cell organelles, including the cytoplasmic matrix, cell wall, and nucleus, in reproductive and vegetative tissues. The quantity and kind of PIs present in a plant vary depending on the plant variety, developmental stage, location, harvesting period, and environmental stressors. PIs from plants have generally been categorised according to how specific they are to different kinds of proteases, specifically cysteine, serine, aspartic, and metallo-proteases (Birk, 2003; Barbole et al., 2022) (Fig. 11.2).

## Therapeutic Applications of PIs

Plants have a number of physiological and molecular mechanisms of protection against biotic stress like phytopathogens plant and insects. By deactivating the proteases of the pathogenic organism during infection or infestation, PIs play a significant part in plant defence. For instance, PIs inhibit the proteolysis of ingested dietary proteins by competitively binding to the catalytic region of insect proteases. Due to a decrease in amino acid absorption in the insect stomach, growth and development are delayed, which decreases fertility and survival of the insect (Mishra et al., 2010). PIs from various plants have been thoroughly investigated at the biochemical and molecular level, in vitro insect feeding experiments etc., to investigate their role in insect control. Enzyme or protease inhibitors of plant origin have attracted the attention of researchers because of their application in treating different metabolic and infectious diseases. Medicinal plants are regarded as vital source of bioactive secondary metabolites which are known for their enzyme inhibitory potential. The applications of EIs as novel drugs or lead compounds are enormous and therefore these compounds have been used for the treatment of various metabolic and physiological disorders. Highly specific regulation of proteases is essential for physiological processes scuhas food digestion, blood clotting, apoptosis, and modulationg blood pressure. Due to their unique structure and high specificity, PIs have been explored as drugs to treat a number of physiological and infectious diseases. Several PIs have been successfully explored in cancer treatment, heart diseases, inflammatory and neurological disorders as well as in the managemen of and obesity. For example, Swedberg et al. (2009) isolated a trypsin inhibitor-1 (SFTI-1), a small peptide from sunflower, which can blocked tumor formation and prevented metastasis through blocking human kallikrein-related peptidase 4. In another study, Pin-II-type inhibitors was isolated from potato and successfully used as anti-obesity molecules (Peters et al., 2011). The ensuing paragraphs describe

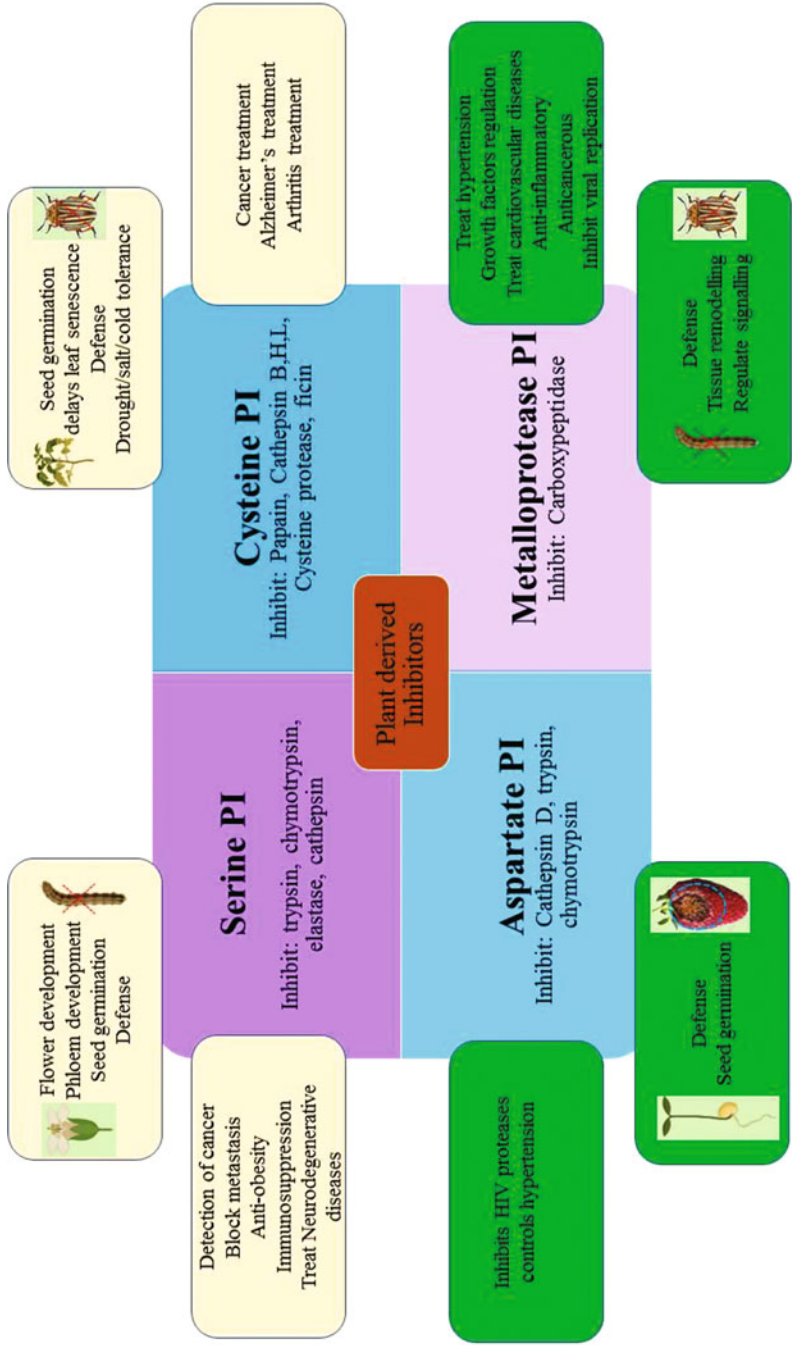


Fig. 11.2 Classification of protease inhibitors (Source: Barbole et al., 2022)

the structure, function and applications of some important class of PIs of plant origin (Fig. 11.2).

### ***$\alpha$ -Glucosidase Inhibitors (AGIs) for Diabetes Control***

Type 2 diabetes affects over 450 million people worldwide, or 6.28% of the world's population. In the entire world, type 2 diabetes is now one of the leading causes of death. It currently reports over 10 lakh deaths annually, with a widespread reporting rate of 6000 cases per 100,000; by the end of 2030, the rate is expected to rise to 7100 patients per 100,000 (Yang et al., 2018). The growing prevalence of diabetes mellitus worldwide, particularly in industrialised nations like America and Europe, calls for rapid attention. A class of oral medications known as alpha-glucosidase inhibitors (AGIs) is used to treat diabetes mellitus type 2. They may be used either alone or in conjunction with other medications. AGIs are popularly used in the patients having low glucose tolerance and delayed-type 2 diabetes. AGIs act by decreasing gastrointestinal assimilation of glucose and thereby prevent sugar absorption.

The presence of new AGIs are being investigated in a variety of plant and fungus species. They lessen postprandial blood glucose and insulin levels, enhancing the ability of the insulin cell to become affected and reducing the stress on the beta-cell (van de Laar, 2008). Nitrogen-containing heterocyclic substances like triazole, quinazoline, imidazole, thiazole, and pyrazole have been reported to possess alpha-glucosidase inhibitors but do not include glycosyl groups. In addition, a number of synthetic and natural flavonoids, including luteolin, anthocyanins, naringenin and baicalein, coumarin, and chromones, as well as their derivatives, have been found as powerful AGIs (Bhatnagar & Mishra, 2022). Triazole and quinazoline compounds have drawn a lot of attention because of their significant pharmacological characteristics. Triazole compounds with a carbazole bond have been found to have inhibitory effect against alpha glucosidase. It has also been demonstrated that substituted quinazolines have antihyperglycemic and anti-glucosidase inhibitory effects. AGIs, in contrast to other oral antidiabetic medications, primarily affect the gut rather than the body's metabolic functions. As a result, a lot of research has been done on the use of AGIs in the management of prediabetic illnesses like impaired glucose tolerance and impaired fasting glucose (Table 11.2).

### ***$\alpha$ -Amylase Inhibitors for Pest Control ( $\alpha$ -AIs)***

Digestive amylases are enzymes from the endo/exo-enzyme family that hydrolyse the glycosidic bonds of large  $\alpha$ -linked polysaccharides like starch and glycogen, to produce smaller oligosaccharides or monosaccharides to meet energy requirements. Thus,  $\alpha$ -amylases play a significant role in energy generation (Pelegrini et al., 2006; Kaur et al., 2014).



**Table 11.2** Natural products from Apocynaceae and other plants known as alpha-glucosidase inhibitors (Source: Bhatnagar & Mishra, 2022)

Sr. no.	alpha-glucosidase inhibitor	Metabolite class	Source	Reference
1.	Acarbose	Pseudotetrasaccharide	<i>Actinoplanes</i>	Li Ting (2018)
2.	Akebonoic acid	Triterpenoid	<i>Akebia trifoliata</i>	Dirir et al. (2021)
3.	Antroquinonol	Meroterpenoid	<i>Fungus</i>	Jhong and Chia (2015)
4.	Berberine	Alkaloid	<i>Berberis sp.</i>	Pan et al. (2003)
5.	Catechin	Flavonoid	<i>Various Plant</i>	Matsui et al. (2007)
6.	Curcumin	beta-diketone	<i>Curcuma longa</i>	Nabavi et al. (2015)
7.	Docosanol	Aliphatic alcohol	<i>Toona sinensis</i>	Jhong and Chia (2015)
8.	Eupafolin	Flavonoid	<i>Artemisia s.</i>	Jhong and Chia (2015)
9.	Fisetin	Flavonoid	<i>Fruits and vegetable</i>	Yue et al. (2018)
10.	Morusin	Flavonoid	<i>Morus alba</i>	Jang et al. (2015)
11.	Myricetin	Flavonoid	<i>Syzygium</i>	Kang et al. (2015)
12.	Psoralidin	Coumestans	<i>Psoralea corylifolia</i>	Wang et al. (2013)
13.	Quercetin	Flavonoid	<i>Various Plant</i>	Oboh and Ademosun (2015)
14.	Rhaponticin	Polyphenol	<i>Rhei Rhizoma</i>	Choi et al. (2006)
15.	Taxumariene F	Diterpenoid	<i>Taxus mairei</i>	Chen et al. (2020)

Inhibition of  $\alpha$ -amylase through the binding of  $\alpha$ -AIs results in prevention of digestion and absorption of dietary starch (Shamki et al., 2012). The  $\alpha$ -AI(s) are the natural products such as proteins or synthetic chemical(s) that control the activity of  $\alpha$ -amylase (McEwan et al., 2010). According to Kadziola et al. (1998), a potent amylase inhibitor must be effective against the target amylase while remaining non-interfering with the actions of endogenous -amylases (such as those expressed during seed germination) and mammalian enzymes.

In addition to being plentiful in cereals and legumes as part of their natural defence mechanisms,  $\alpha$  -amylase inhibitors ( $\alpha$  -AIs) are also equally effective candidates for this usage (Guzman-Partida et al., 2007). Due to their function in the metabolic control of starch, -AIs from cereal plants exhibit inhibitory effect against endogenous amylases (Weselake et al., 1985; Giri & Kachole, 1998). It is well known that plant seeds develop a range of enzyme inhibitors that defend them against infections and insects. The most well researched inhibitors in this class are proteinaceous ones (Christou et al., 2006). Pure inhibitor(s) of insect digestive enzymes generated from plants seem to be a secure and appears to be appealing solution for insect control. A earlier review of the potential of  $\alpha$  -AIs as bioinsecticidal compounds can be found in Carlini and Grossi-de-Sa (2002). For instance, rye, common bean, wheat, and amaranth -AIs work by delaying the rate at which insects

digest the food they consume (Giri & Kachole, 1998; Franco et al., 2002; Dias et al., 2005). According to Bunyatang et al. (2016), the  $\alpha$ -AIs from *Hevea brasiliensis* regulates the absorption of reserve starch and is in charge of sugar accretion for seed growth. Reports on the use of insect amylase and  $\alpha$ -AIs from plant species to regulate postprandial glucose levels predominate over reports on the use of microbial- $\alpha$ -AIs for the same activities (Table 11.3). Large-scale production of  $\alpha$ -AIs from plant sources seems to be difficult because it requires larger space for growing the plants. Moreover, the extraction procedures for  $\alpha$ -AIs from plant sources is complex and time consuming as compared to microbial sources (Jayaraj et al., 2013).

### ***H<sup>+</sup>/K<sup>+</sup>-ATPase Inhibitors***

Increased secretion of acid plays a significant role in the development of gastric ulcers and related disorders. The use of plant-derived substances forms the basis of many modern pharmaceuticals designed for the prevention of diseases such as peptic ulcers. H<sup>+</sup>/K<sup>+</sup> + -ATPase is the proton pump responsible for controlling gastric secretions. Increased secretions of these biomolecules lead to hyperacidity. Plant-derived metabolites have been explored for as inhibitors of H<sup>+</sup>/K<sup>+</sup> + -ATPase. Compounds from Apocynaceae and several other plants have been explored to alleviate gastric acid secretion-related diseases, and thus, these compounds could be potentially used as new lead compounds for the management of peptic ulcer diseases (Hay et al., 2019). However, there is a massive gap in the discovery of effective inhibitors for proton pump inhibitory effects. (Adinortey & N'guessan, 2022). Earlier reports have shown that natural products prevented the gastric ulcers through inhibition of acid secretion in *in vivo* animal studies. Medicinal plants and such as *Solanum nigrum*, *Arctium lappa*, *Garcinia mangostana*, *Azadirachta indica* have been explored for controlling the peptic ulcers. The roots of *Hedranthera barteri* (Onasanwo et al., 2011) and the fruits of *Solanum nigrum* (Jainu & Devi, 2006) showed substantial H<sup>+</sup>/K<sup>+</sup>-ATPase inhibitory potential at 71 and 200  $\mu$ g/ml. Some plants showed minimum H<sup>+</sup>/K<sup>+</sup>-ATPase inhibitory potential (with IC<sub>50</sub> values above 201  $\mu$ g/ml) (Mesía-Vela et al., 2007; Lakshmi et al., 2010). Several other members with substantial H<sup>+</sup>/K<sup>+</sup> ATPase inhibitory potential are shown in Table 11.4 (Adinortey & N'guessan, 2022).

### ***Inhibitors of Other Enzymes of Biomedical Importance***

Some of the crucial proteins involved in disease and pathogenesis are activated by proteases and other enzymes from pathogens like bacteria, fungi, and viruses. The ability of the majority of viral proteases to cleave host proteins to reduce host immunity and promote viral replication is also well-known. Two proteases are implicated in the activation of the SARS-CoV-2 replication machinery and the

**Table 11.3** Important  $\alpha$ -amylase inhibitors of plant origin used for pest control (Kasar et al. 2017, 2021, 2022)

Sr. no.	Source plant	Plant part	Mol. Wt.	Host amylase inhibited	Reference
1.	<i>Colocasias</i>	Seeds	13.9	<i>C. chinensis</i> , <i>T. castaneum</i> , <i>C. cephalonica</i>	Kumari et al. (2012)
2.	<i>Carica papaya</i>	Seeds	4.28	<i>C. maculatus</i>	Farias et al. (2007)
3.	<i>Capsicum annuum</i>	Seeds	9	Mammalian	Diz et al. (2011)
4.	<i>Coffea canephora</i>	Seeds	9	Mammalian	Zottich et al. (2011)
5.	<i>D. esculenta</i>	Tubers	16	Coffee bean and Sweet potato weevil	Sasikiran et al. (2004)
6.	<i>Dipteryx alata</i>	Seeds	14.8	<i>A. grandis</i> , <i>C. maculatus</i> , <i>A. obtectus</i> , Porcine pancreas	Bonavides et al. (2007)
7.	<i>Eleusine coracana</i>	Seeds	14	Porcine pancreatic, <i>C. chinensis</i>	Saxena et al. (2010)
8.	<i>Leucas aspera</i>	Whole plant	28	Mammalian, Maize, <i>B. subtilis</i> , <i>A. oryzae</i>	Meera et al. (2017)
9.	<i>Mucuna pruriens</i>	Seeds	25.6	Human salivary $\alpha$ -amylase	Bharadwaj et al. (2018)
10.	<i>Phaseolus vulgaris</i>	Seeds	30	Mammalian	Ali et al. (2006)
11.	<i>Streptomyces sp. CC5</i>	Cells	8.21	Human pancreatic amylase, Porcine pancreatic amylase	Sun et al. (2015)
12.	<i>Triticale seeds</i>	Seeds	29.2	<i>Eurygaster integriceps</i> Human salivary $\alpha$ -amylase	Mehrabadi et al. (2011)
13.	<i>Triticum aestivum</i>	Seeds	20.0	Human salivary, Porcine pancreatic, Chick pea	Saunders and Lang (1973)
14.	<i>Vigna radiate</i>	Seeds	27	<i>C. maculatus</i>	Wisessing et al. (2010)
15.	<i>Vigna sublobata</i>	Cotyledon	14	Larvae of <i>C. analis</i>	Kokiladevi et al. (2005)
16.	<i>Vigna unguiculata</i>	Seeds	25.1	<i>C. maculatus</i> , <i>A. obtectus</i> , <i>A. grandis</i> , <i>A. oryzae</i>	Alves et al. (2009)

beginning of its pathogenesis: papain-like proteases and chymotrypsin-like cysteine proteases (Vadnere & Patil, 2022). *Staphylococcal* enzymes that degrade host tissue components include nucleases, coagulase, proteases (metalloproteases, serine, and cysteine proteases), hyaluronidase, and lipases. Lipases are known to support the persistence of *S. aureus* in fatty secretions in mammalian skin and therefore directly contribute to their pathogenic potential. Additionally, lipase prevents host granulocytes from phagocytosing pathogenic lipase-producing *S. aureus* cells, demonstrating a direct role for lipase in pathogenesis (Christensson et al., 1985; Rollof et al., 1988).

Another exoenzyme, hyaluronidase (also called hyaluronate lyase HysA), cleaves the hyaluronic acid (HA) polymer at the  $\beta$ -1,4 glycosidic bond, yielding disaccharide

**Table 11.4** Plants with H<sup>+</sup>/K<sup>+</sup> ATPase inhibitory activity (Adinortey & N'guessan, 2022)

Plant name	Family	Part used	Study model	Reference
<i>Acalypha wilkesiana</i>	Euphorbiaceae	Leaf	In vitro	Gupta and Pradeepa (2013)
<i>Annona squamosa</i>	Annonaceae	Twigs Twigs Twigs	In vitro In vitro In vitro	Yadav et al. (2012)
<i>Arctium Lappa L.</i>	Asteraceae	Root	In vitro	Dos Santos et al. (2008)
<i>Carissa carandas</i>	Apocynaceae	Leaf	In vitro	Shukla et al. (2016)
<i>Cecropia glazoui</i>	Cecropiaceae	Aerial	In vitro	Souccar et al. (2008)
<i>Cissus quadrangularis L.</i>	Vitaceae	Tubers	In vitro	Yadav et al. (2012)
<i>Decalepis hamiltonii</i>	Asclepiadaceae	Roots	In vitro	Naik et al. (2007)
<i>Delonix regia</i>	Fabaceae	Stem bark	In vitro	Sachan et al. (2017)
<i>Garcinia kola</i> Heckel.	Guttiferae	Seeds	In vitro	Onasanwo et al. (2011)
<i>Garcinia mangostana</i>	Guttiferae	Fruit peel	In vitro	Nanjarajurs et al. (2014)
<i>Hedranthera barteri</i>	Apocynaceae	Roots	In vitro	Onasanwo et al. (2011)
<i>Pongamia pinnata</i> (L.)	Leguminosae	Seeds	In vitro	Belagihally et al. (2011)
<i>Scoparia dulcis L.</i>	Scrophulariaceae	Aerial parts	In vivo	Mesía-Vela et al. (2007)
<i>Solanum nigrum</i>	Solanaceae	Aerial parts	In vitro	Jainu and Devi (2006)
<i>Tectona grandis</i>	Verbenaceae	Leaf	In vitro	Lakshmi et al. (2010)
<i>Xylocarpus granatum</i>	Meliaceae	Fruit	In vitro	Lakshmi et al. (2010)

units of *N*-acetylglucosamine and d-glucuronic acid. HA is synthesized by plasma membrane of mammalian cells, and serves as a cementing structure of connective tissues (Hynes & Walton, 2000). Tissues with relatively higher HA concentrations are frequently infected with *S. aureus* due to their ability to produce hyaluronidase (Patil et al., 2019). Lipase and hyaluronidase are the major virulence factors during staphylococcal infections. The inhibition of these virulent enzymes can be a simple and effective approach to controlling drug-resistant Staphylococci. A number of medicinal plants have been evaluated for the presence of effective inhibitors targeting several enzymes involved in disease causation and pathogenesis and modulating the host immune response. A number of in vitro and in vivo techniques have been developed for the isolation, screening and evaluation of the inhibitory potential of inhibitors of plant origin. Recently, molecular docking has become an important tool to understand drug-target interactions which is a key step in drug design and discovery (Patil et al., 2022). The docking principles were effectively used to identify the target sites different receptors/targetsof HIV 1 (Schames et al., 2004). Using online tool-AutoDock, this receptor was effectively used to inhibit HIV integrase. The other inhibitors screened using this approach include Type I TGF-beta receptor kinase inhibitor (Singh et al., 2003), Aurora Kinase An inhibitor (Park et al., 2018), Dopamine D3 receptor for anticancer molecules (Varady et al., 2003), etc. Table 11.5 provides the summary of various target proteins/receptors that

**Table 11.5** Protease/enzyme inhibitors from various sources and their biological significance (Source: Patil et al., 2022)

Sr. no.	Target enzyme/protein	Inhibitor/legend	Source plant	Biological significance	Reference
1.	Monoacylglycerol lipase	<i>Rutin</i>	<i>Phaseolus vulgaris</i>	To attenuate the inflammatory response	Saxena et al. (2010)
2.	<b>Cyclophilin D</b>	ZINC161011368	<i>Secale cereale</i>	For the treatment of acute pancreatitis.	Cen et al. (2022)
3.	SARS-CoV-2 3-Chymotrypsin-Like Protease.	<i>Chicoric acid</i>	<i>Wrightia religiosa</i>	preventive nutraceuticals and/or antiviral drugs in COVID-19.	Gyebi et al. (2021)
4.	SARS-CoV-2 3-Chymotrypsin-Like Protease.	<i>Rosmarinic acid</i>	<i>Triticum aestivum</i> ,	preventive nutraceuticals and/or antiviral drugs in COVID-19.	Gyebi et al., 2021
5.	SARS-CoV-2 3-Chymotrypsin-Like Protease.	<i>Myricetin</i>	<i>Wrightia religiosa</i>	preventive nutraceuticals and/or antiviral drugs in COVID-19.	Gyebi et al. (2021)
6.	SARS-CoV-2 3-Chymotrypsin-Like Protease.	<i>Rosmarinic acid</i>	<i>Triticum aestivum</i> ,	preventive nutraceuticals and/or antiviral drugs in COVID-19.	Gyebi et al. (2021)
7.	SARS-CoV-2 3-Chymotrypsin-Like Protease.	<i>Isorhamnetin</i>	<i>Wrightia religiosa</i>	preventive nutraceuticals and/or antiviral drugs in COVID-19.	Gyebi et al. (2021)
8.	SARS-CoV-2 3-Chymotrypsin-Like Protease.	<i>Luteolin</i>	<i>Triticum aestivum</i> ,	preventive nutraceuticals and/or antiviral drugs in COVID-19.	Gyebi et al. (2021)
9.	SARS-CoV-2 3-Chymotrypsin-Like Protease.	<i>Vernomenin</i>	<i>Wrightia religiosa</i>	preventive nutraceuticals and/or antiviral drugs in COVID-19.	Gyebi et al. (2021)
10.	Stearyl coenzyme A desaturase enzyme 1	<i>Stearyl-Coenzyme A</i>	<i>Sorghum bicolor</i>	To alter the metabolism of tumor cells	Huang et al. (2020)
11.	Stearyl coenzyme A desaturase enzyme 1	<i>Aramchol</i>	<i>Zea mays</i>	To alter the metabolism of tumor cells	Huang et al. (2020)
12.	mycobacterial InhA	<i>Enamine 57,340</i>	<i>Oryza sativa</i>	therapeutic applications against TB.	Jayaraman et al. (2021)
13.	mycobacterial InhA	<i>Enamine 57,294</i>	<i>Vigna unguiculata</i>	therapeutic applications against TB.	Jayaraman et al. (2021)
14.	Lactate dehydrogenase	<i>Quercetin</i> ,	<i>Carisa carandas</i>	To arrest tumor invasion and metastasis	Li et al. (2021)
15.	Lactate dehydrogenase	<i>Luteolin</i> ,	<i>Carisa carandas</i>	To arrest tumor invasion and metastasis	Li et al. (2021)

16.	Lactate dehydrogenase	<i>Ursolic acid</i>	<i>Carisa carandad</i>	To arrest tumor invasion and metastasis	Li et al. (2021)
17.	phosphodiesterase 10A	MA8	Secale cereale	alleviating the negative and cognitive symptoms of schizophrenia	Mayasah et al. (2018)
18.	phosphodiesterase 10A	MA98	Eleusine coracana	alleviating the negative and cognitive symptoms of schizophrenia	Mayasah et al. (2018)
19.	Main protease (Mpro) of SARS-CoV-2	Kazinol T		lead compound against the main protease of SARS-CoV-2	Singh et al. (2022)
20.	Aldehyde dehydrogenases (ALDHs)		Eleusine coracana	In the treatment of cyclophosphamide and cisplatin resistance cancer.	Narendra et al. (2021)
21.	acetylcholinesterase inhibitors (AChEIs)	compound D	<i>Dipteryx alata</i>	In the treatment of Alzheimer's disease	Pablo et al. (2015)
22.	butyrylcholinesterase	compound D	<i>Dipteryx alata</i>	In the treatment of Alzheimer's disease	Pablo et al. (2015)
23.	Covid 19 main protease.	<i>Penciclovir</i>	<i>Catharanthus roseus</i>	to develop effective therapeutics to deal with the Covid 19 pandemic	Padhi et al. (2021)
24.	Covid 19 main protease.	<i>Jasmonic acid</i> ,	<i>Catharanthus roseus</i>	to develop effective therapeutics to deal with the Covid 19 pandemic	Padhi et al. (2021)
25.	Covid 19 main protease.	<i>Jasmonic acid methyl ester</i> ,	<i>Catharanthus roseus</i>	to develop effective therapeutics to deal with the Covid 19 pandemic	Padhi et al. (2021)
26.	Covid 19 main protease.	<i>Putaminosin B</i>	<i>Catharanthus roseus</i>	to develop effective therapeutics to deal with the Covid 19 pandemic	Padhi et al. (2021)
27.	Covid 19 main protease.	<i>Putaminosin D</i>	<i>Catharanthus roseus</i>	to develop effective therapeutics to deal with the Covid 19 pandemic	Padhi et al. (2021)
28.	Haemoglobinase Falcipain-2	<i>Nonpeptidic inhibitors</i>	<i>Triticum aestivum</i>	To develop antimalarial drugs to treat resistant strains of <i>Plasmodium</i> .	Rajguru et al. (2022)
29.	Covid 19 main protease.	<i>Bieckol</i>	<i>Phaseolus vulgaris</i>	In the treatment of Covid 19	Rauf et al. (2021)
30.	Covid 19 main protease.	<i>7-Hydroxyeckol hepta-acetate</i>	<i>Amaranthus hypochondracus</i>	In the treatment of Covid 19	Rauf et al. (2021)

(continued)

Table 11.5 (continued)

Sr.	Target enzyme/protein no.	Inhibitor/legend	Source plant	Biological significance	Reference
31.	Covid 19 main protease.	<i>Met165</i>	<i>Triticum aestivum</i>	In the treatment of Covid 19	Rauf et al. (2021)
32.	Covid 19 main protease.	<i>5-Hydroxy-cystofurano-quinol</i>	<i>Secale cereale</i>	In the treatment of Covid 19	Rauf et al. (2021)
33.	Covid 19 main protease.	<i>Sargaquinoic acid</i>	<i>Triticum aestivum</i>	In the treatment of Covid 19	Rauf et al. (2021)
34.	Covid 19 main protease.	<i>Methoxybifurcarenone</i>	<i>Secale cereale</i>	In the treatment of Covid 19	Rauf and Jehan (2017)
35.	Covid 19 main protease.	<i>rutin</i>	<i>Eleusine coracana</i>	In the treatment of Covid 19	Rauf and Jehan (2017)
36.	ERK2 (protein kinase)	Hypol	<i>Calotropis procera</i>	inhibition cell proliferation and oncogenesis	Sofiene et al. (2014)
37.	<i>Glycoprotein Receptor-Binding Domain</i>	<i>solanine, acetoside, and rutin</i>	<i>Solanum melongina</i>	In the treatment of Covid- 19	Teli et al. (2021)
38.	<i>Covid 19 Main protease (Mpro)</i>	<i>Solanine, acetoside, and rutin</i>	<i>Solanum melongina</i>	In the treatment of Covid 19	Teli et al. (2021)
39.	Dipeptidyl-peptidase-4	<i>Butirosin</i>	<i>Amaranthus hypochondracus</i>	In the treatment of Type II Diabetes Mellitus.	Xiong et al. (2021)
40.	Dipeptidyl-peptidase-4	<i>Alogliptin.</i>	<i>Wrightia religiosa</i>	In the treatment of Type II Diabetes Mellitus	Xiong et al. (2021)
41.	Dipeptidyl-peptidase-4	<i>Rutin</i>	<i>Triticum aestivum,</i>	In the treatment of Type II Diabetes Mellitus	Xiong et al. (2021)
42.	Dipeptidyl-peptidase-4	<i>Homatropine</i>	<i>Hordeum vulgare</i>	In the treatment of Type II Diabetes Mellitus.	Xiong et al. (2021)
43.	histone acetyltransferase	<i>Chlorogenic acid (CA)</i>	<i>Phaseolus vulgaris</i>	anti-nonalcoholic fatty liver disease	Xu et al. (2021)
44.	Protein tyrosine phosphatase 1B	<i>H1 to H2O</i>	<i>Triticum aestivum</i>	In the management of diabetes	Yang et al. (2020)

are inhibited using a legand with its PDB ID, binding sites, software used in the study, and the biological significance of the study (Patil et al., 2022).

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## Chapter 12

# Endophytes in Apocynaceae Plants



### Introduction

Endophytes are defined as microbes that inhabit the internal parts of plants regardless of the outcome of the association. They gain entry into the seed, leaf, stem, flower and root of a plant, and they show no harm to the host plant (Yadav, 2018). Endophytes are omnipresent almost in every plant in the internal tissues. They live in intracellular tissues of their plant host plant, and establish a symbiotic relationship with the host. Sometimes, endophytes may revert to some form of pathogenesis in the host (Christel et al., 2000; Tejesvi et al., 2007). Endophytes have been isolated successfully from terrestrial, aquatic and desert plants. Both, bacterial as well as fungal endophytes are reported. However, fungal endophytes are more ubiquitous, versatile, and widespread microorganisms found in the plants growing in diverse environmental conditions. Plants growing in the Arctic, Antarctic (Suryanarayanan et al., 2005), geothermal soils (Fisher et al., 1995), deserts (Rosa et al., 2009), oceans (Wang et al., 2006), mangroves and coastal (Lin et al., 1996) regions harbors highly diverse bacterial and fungal endophytes. Despite their huge diversity, a very endophytic microorganisms have been isolated and studied systematically. Endophytes have been recognized as chief and sustainable source of biologically active natural products and secondary metabolites. Exploring the plant-based drugs using endophytic microorganisms will help overcome geographical and political restrictions involved in the transportation of plants. The metabolites from endophytic microorganisms were explored for their applications in agriculture, medicine, bioremediation, biofuel, etc. (Russell et al., 2011; Shaw et al., 2015). The ability of endophytes to produce a variety of novel secondary metabolites with therapeutic efficacy has recently received much interest in the scientific community (Gupta et al., 2020). Despite this, there is yet to be a substantial breakthrough in the utilization and commercialization of endophytic metabolites containing bioactive compounds on a wide scale (Venugopalan & Srivastava, 2015a, 2015b).



## Plant Endophyte Interactions

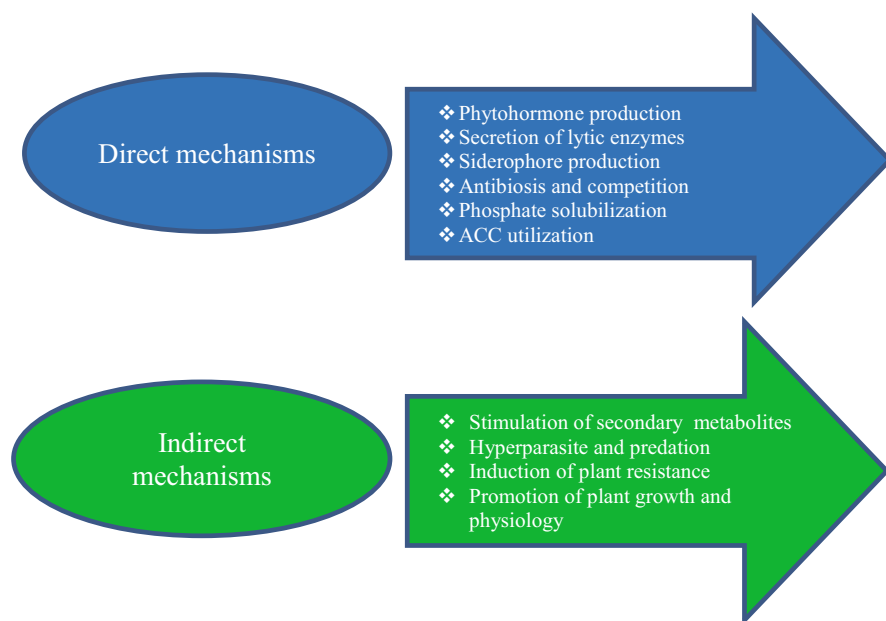
Endophytes are microorganisms with the Greek prefixes “endo” (within) and “phyton” (plant). The interaction between the plant and endophyte are believed to be over 400 million years old, and it is observed that the plants need association of endophytes for their survival in harsh environments (Rodríguez & Redman, 2008). Plants can “select” as endophytes those bacteria or fungi among the highly diverse microbial community that provide them with some benefit. The makeup of a plant's root exudates plays an important role in defining the type of microbe that can be linked with it (Andreozzi et al., 2019). As a result, the ability of endophytic bacteria to utilize the exudates produced by plant roots as a source of energy is critical to their relationship with plants (Kandel et al., 2017). Endophytes can effectively promote plant growth by utilizing various modes of activity and boosting plant tolerance to severe circumstances (Yadav et al., 2018).

According to a study, plants are connected with microbiota that increase stress resistance systems under stress conditions (Marasco et al., 2012; Santoyo et al., 2016). Endophytes are thought to promote plant growth by secreting phytohormones, enhancing nutrient assimilation as well as by improving plant health by protecting them from phytopathogens (Andreozzi et al., 2019). Plant-endophyte interactions cause plants to protect themselves from adverse environmental situations such as heavy metals and drought.

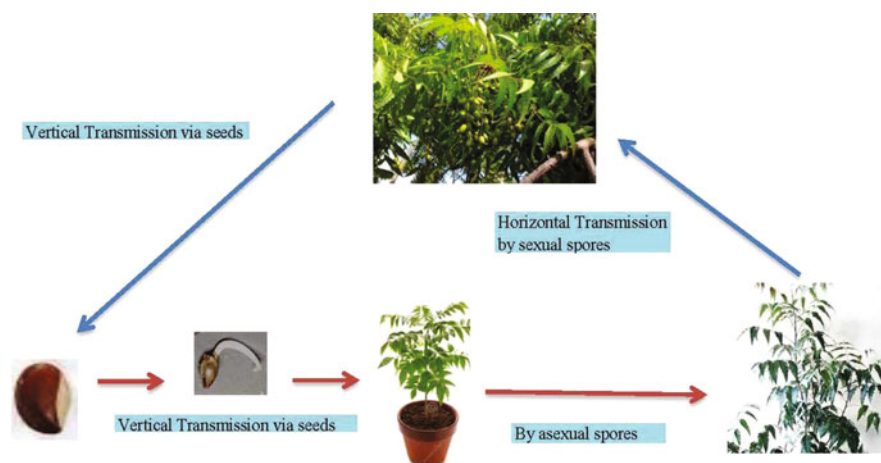
Endophytes possess a very high number of genes that code for plant-beneficial features and have modulatory effects on the growth and development of host plant (Khare et al., 2018). They play an important role in growth promotion by producing the various phytohormones, nutrient acquisition, promoting biodegradation increasing the stress tolerance in the host plant. These endophytic bacteria have been shown to have a variety of biocontrol mechanisms. They use various strategies to combat phytopathogens, including ecological niche competition, antimicrobial agent production, siderophore generation, and induced systemic resistance (ISR). Endophytic microorganisms help boost plant fitness through several direct and indirect mechanisms, such as plant protection from phytopathogens, secretion of lytic enzymes, and phosphate solubilization. The general mechanisms employed by endophytic microbes for plant protection are represented in Fig. 12.1.

The association between the plant and endophytic fungi has begun from the time of evolution as indicated by the fossil record (Rodríguez & Redman, 2008). Endophytes develop a variety of associations with their host plants during their growth inside the plant tissues. These relationships may be symbiotic, mutualistic even parasitic. The levels and complexities of these relationships between the endophytic microorganism and its host vary from host to host and microbe to microbe (Verma et al., 2009). The colonization process is the same for endophytes and fungal pathogens infecting plants, which requires host establishment, including recognition, penetration, and germination. During colonization, fungi have to overcome plant defense mechanisms for stable establishment within their plant host. An endophyte enters inside the plant tissue through various mechanisms. Entry and spread through





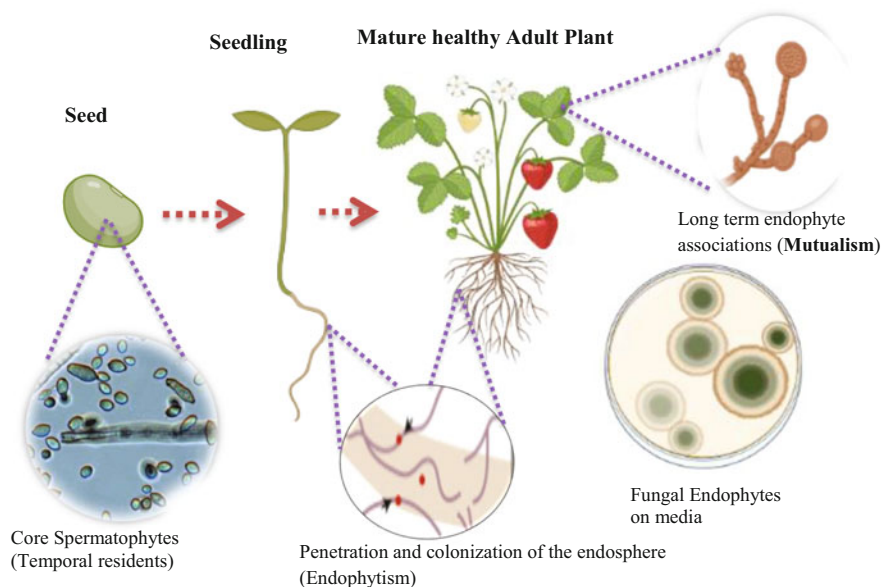
**Fig. 12.1** Mechanisms of plant protection employed by endophytes



**Fig. 12.2** Life cycle of systemic endophytes

the seeds and spreading in different tissues of the plant is well understood (Fig. 12.2).

In the mutualistic relationship, endophytes derive the nutrients from the plant tissues. On the other hand, the endophyte supports the seed germination and produce the phytohormones and other metabolites that favors the growth and the survival



**Fig. 12.3** Mechanism of endophytism

benefits to plant in adverse environmental settings (Verma et al., 2009). The exchange of genes between the plant host and its endophyte that may have occurred evolution favored the symbiotic nature of the interaction between the endophytes and their plant host. As a result of it, the bioactive compounds synthesized by endophytes are normally do not show toxicity in the eukaryotic host system. This character of endophyte derived metabolites makes them suitable for their application as a bioactive pharmaceuticals.

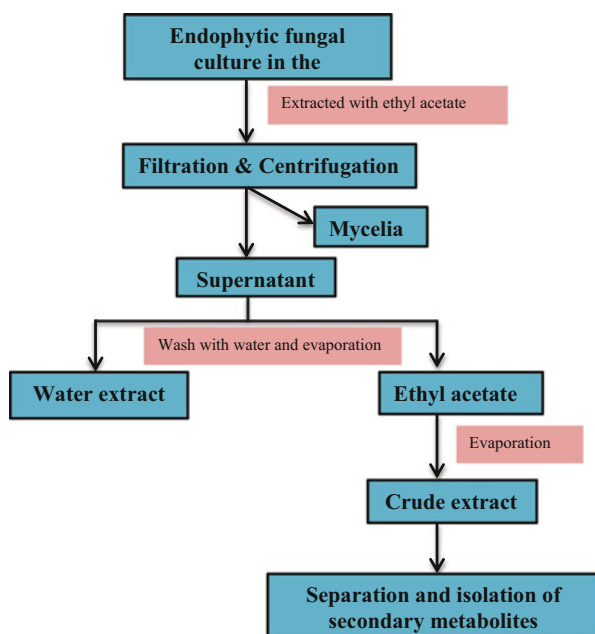
The fungal hyphae grow inside the internal tissues of all the aboveground parts of the plant such as stem, twigs, leaves and fruits and seeds. Presence of endophytes in the seeds helps vertical transfer and spread of endophytes from the mother plant to the offsprings (Fig. 12.3). According to recent research (Clay and Holah, 1999), horizontal transmission is reported in natural grass populations. Fungal spores frequently recognize the host via lectin-like molecules. After germination, the fungi enter plant tissue by breaking the cuticle mechanically or by weakening the cuticle and the epidermal cell wall.

The fungal hyphae then enter in latent state for lifetime or for an extended period. After establishing a stable association with the host, the endophytes offers several survival benefits to their host like increases the stress tolerance, provides protection against the phytopathogens, increases tolerance for drought, salinity, and variations in temperature (Clay, 1990; Rice et al., 1990; Malinowski, 1997; Saikkonen, 1998).

## Strategies for Metabolite Production Using Endophytic Microorganisms

Different researchers have employed a variety of rational methods to acquire endophytic microorganisms with bioactive potential. A crucial first step in the hunt for potential endophytic microorganisms is choosing the plant. The host plant's location, latitude, longitude, and altitude are used to determine its position. The plant(s) under investigation are located, identified, and external tissues like stem, twig, and leaf are taken. These tissues are then transported to the lab processed for endophyte isolation. The surface of the plant material should be treated with 0.5% sodium hypochlorite or 70% ethanol, completely washed in running tap water, and then dried under the sterile air by using the laminar flow hood. The plant material is cut into small pieces (1.5–2 cm<sup>2</sup>), the external tissues are carefully removed and the exposed internal tissues are aseptically placed onto water agar plates. Until the hyphae emerge from the plant tissue explant, the plates are incubated at room temperature or at 28–30 °C for a 12-hour dark/light cycle under illumination. To prevent bacterial contamination, antibiotics are usually added in the isolation medium at the recommended concentrations. During the incubation, the emerging hyphae from the plant explants are transferred to sterile media plates containing Czapek Dox agar or Potato Dextrose agar. The ability of the isolated endophytes to grow in vitro utilizing submerged or solid-state growth methods is examined. The isolated endophytic microorganism (spores in the case of fungal endophytes) is then stored at –70 °C using 15% glycerol as a preservative (Fig. 12.4).

**Fig. 12.4** Extraction scheme for isolation of bioactive compounds



Following isolation, the isolated endophytic microorganism is subjected to identification using morphological and molecular methods. Observations made at the microscopic level are crucial in the tentative identification of endophytic bacteria and fungi. Conidiophore structure and morphology are characterized by macronematous conidiophores taken from the border of conidiogenous pustules during the maturity of conidia for morphological identification of the endophytic fungus (Patil et al., 2015). The most common method for identifying endophytic fungi at the molecular level is ITS sequence analysis. Using 18S rDNA and 5.8S rDNA, several researchers have carried out molecular identification of endophytic fungi (Debbab, 2012).

The isolated endophytic microorganism was cultivated at the shake flask level using synthetic medium at 28 °C for 2–3 weeks on a rotary shaker to provide aeration and agitation. During the incubation period, the medium was monitored for contamination, growth of endophytes, metabolite production and change in pH of the medium. After fermentation (at the stationary phase of the culture), the fermented broth is subjected to recovery of metabolites using organic solvents such as ethyl acetate or chloroform. The fermented broth was filtered using a muslin cloth to remove fungal mycelia. The filtered broth was centrifuged to remove other impurities. Extracted cell debris was discarded, and the filtrate containing the organic phase was separated from the medium (water phase) using a separatory funnel. To remove the salts and polar constituents, the ethyl acetate phase was washed with water twice and concentrated under reduced pressure using a rotary vacuum evaporator (Patil, 2016). An overview of metabolite isolation and characterization is summarized in Fig. 12.4.

## Bioprocess Optimization for the Production of Active Metabolites

The bioprocess for maximum yield of metabolite(s) can be optimized using various physicochemical parameters. This not only helps the rapid growth of the producing microorganism but also favors metabolite production *in vitro* cultivation. Physical parameters: Different effects of temperature, aeration and agitation, and incubation periods were evaluated for optimum production of secondary metabolites. The one variable at a time (OVAT) model is generally used for process optimization studies. The effects of various chemical factors markedly affect the yield of secondary metabolites in bioprocesses. To optimize the production of the metabolites, the effect of different chemical factors by OVAT was studied. The chemical parameters studied included the sole carbon source, sole nitrogen source and pH.

## Screening of Bioactivities of the Crude Extracts and Isolated Compounds

The main focus of many studies on microbial endophytes is to investigate the potential of endophytic bacteria for industrial microbiology (Aly et al., 2010). Endophytes can produce a wide range of secondary metabolites that are identical to those of their host plant because they replicate their biology. Compounds are useful for developing new drugs, according to numerous studies (Joseph & Priya, 2011). Additionally, it has been discovered that some endophytes can produce bioactive secondary metabolites that their plant hosts typically cannot. It has been discovered that the bioactive substances isolated from endophytic microorganisms such as bacteria and fungi possess a variety of therapeutic activities, including antibacterial, antiparasitic, antiviral, anticancer, insecticidal, cytotoxic, neuroprotective, enzymatic, and antioxidant potential.

### *Antimicrobial Compounds from Endophytic Microorganisms*

Antimicrobial compounds are usually substances synthesized by microorganisms as secondary metabolites that inhibit the growth of other microorganisms even at low concentrations. Several metabolites showing antimicrobial properties have been extracted and purified from endophytic microorganisms. The metabolites of endophytic fungi have a broad range of antimicrobial activities. Endophytic microorganisms synthesize chemically diverse structural groups such as terpenoids, alkaloids, phenolics, steroids, aliphatic compounds and flavonoids (Mousa & Raizada, 2013). These compounds show antibacterial, antifungal and antiviral activities not only in humans but also in plants and animals. Phomol, an excellent antibacterial as well as antifungal compound, was produced by an endophytic fungus, *Phomopsis* spp. E02018, isolated the twig issues of *Erythrina crista-galli* (Weber et al., 2004). The endophytic fungus *Xylaria* spp. Isolated from the *Ginkgo biloba* L. produced the novel e compound 7-amino-4-methylcoumarin, which inhibited the growth of several human pathogens and the compound was successfully used as natural food preservative (Liu et al., 2008). Two new compounds called xanalteric acids I and II were obtained from an endophytic fungus, *Alternaria* spp. Obtained from the Chinese mangrove *Sonneratia alba* (Patil, 2016).

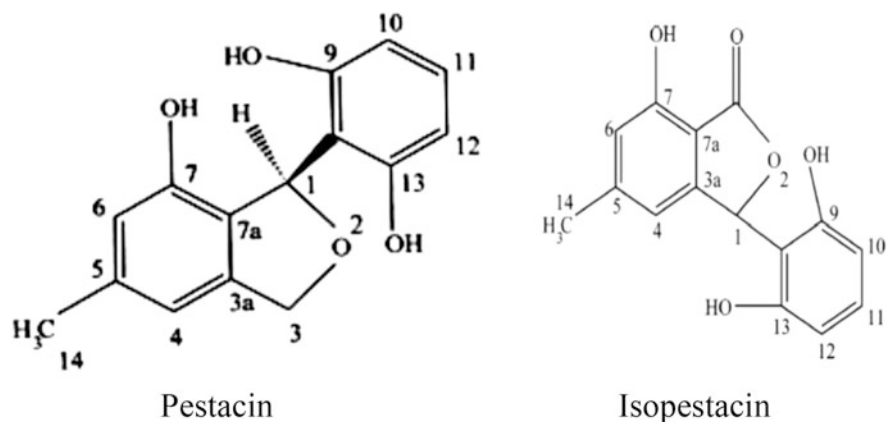


Fig. 12.5 Antioxidant metabolites obtained from *Pestalotiopsis microspora*

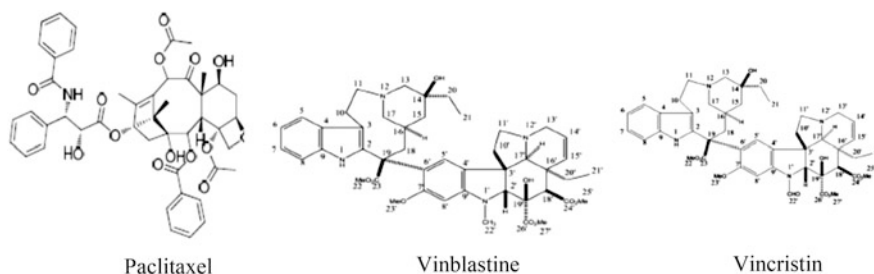
### Endophytic Metabolites with Antioxidant Potential

Reactive oxygen species (ROS) play an important role in the development of degenerative conditions such as aging, cancer, inflammation, and atherosclerosis. (Sandesh et al., 2014). ROS such as hydroxyl radicals ( $\text{OH}^\cdot$ ), superoxide anions ( $\text{O}_2^\cdot$ ), and nitric oxide radicals ( $\text{NO}^\cdot$ ) alters the membrane fluidity, causes protein denaturation and leads to lipid peroxidation through generating oxidative stress that leads to injury, induce apoptosis and death (Anderson, 1996).

A number of antioxidant compounds have been successfully isolated from the broth extracts of endophytic microorganisms. Pestacin and isopestacin have been known for their antioxidant potential. These metabolites were obtained from *Pestalotiopsis microspora*, an endophytic fungus isolated from *Terminalia merobensis* (Fig. 12.5).

### Anticancer Compounds

Adter coronary heart diseases (CHDs), cancer is the second largest cause mortality in the United States and Europe. Breast cancer accounts for 31% of total deaths among adult females, while prostate cancer (31%) is found to be the highest in adult males (American Cancer Society, Cancer Facts and Figures, 2007). Numerous anticancer substances have extracted from from endophytic fungi. A few examples of anticancer compounds produced by endophytes are shown in Fig. 12.6. The world's first multibillion-dollar anticancer medicine, paclitaxel (Taxol), was produced from



**Fig. 12.6** Some examples of anticancer drugs produced by endophytic microorganisms

*Taxomyces andreanae*, an endophytic fungus isolated from the yew plant *Taxus brevifolia* (Suffness, 1995). The discovery of taxol from endophytic fungi proved the bioactive potential of endophytic fungi. Following that, numerous endophytes with the ability to produce the diterpenoid Taxol were found all over the world. According to Lin et al. (1996), Taxol is a well-known powerful chemotherapeutic drug used to treat a range of malignancies, including ovarian and breast tumors. Taxol specifically suppresses the growth of tumor cells by arresting the depolymerization of tubulin during the cell division (Schiff & Horowitz, 1980). Vinblastine and vincristine are potent anticancer drugs isolated from endophytic *Fusarium oxysporum*, isolated from *Catharanthus roseus*. A strain of *Fusarium oxysporum* (AA-CRL-6) produced vinblastine and vincristine in submerged cultures at appreciable amounts (Kumar et al., 2013).

### ***Other Activities of Endophyte-Derived Metabolites***

Hypercholesterolemia is one of the important contributing factor in the development of CHDs such as atherosclerosis and numerous coronary heart disorders. A significant level of blood cholesterol is obtained through endogenous biosynthesis. HMG-CoA reductase (3-hydroxy-3-methylglutaryl-coenzyme A) is a key enzyme in the cholesterol biosynthesis pathway which catalyzes the conversion of HMG-CoA to mevalonate. The secondary metabolites of fungi known as statins competitively block HMG-CoA reductase, decreasing blood cholesterol levels. These antihypercholesterolemic medications are known to have an important source in endophytic fungi. When grown in solid-state fermentation, the endophytic fungus *Aspergillus niger*, which was isolated from *Taxus baccata*, was capable of producing lovastatin (Raghunath et al., 2012). Other compounds with bioactive potential such as anti-inflammatory, antidiabetic, antimalarial, immunosuppressive, and antihypercholesterolemic agents, are also produced by endophytic fungi. According

to numerous reports, endophytic fungi can produce anti-inflammatory substances such as phomol and mevinic acid, which were isolated from the fermentation extrates of the endophytic *Phomopsis* sp. (Weber et al., 2004). Strong in vitro anti-inflammatory action was demonstrated by both substances. The primary secondary metabolite of *Claviceps purpurea* was initially identified as ergoflavin, a substance with anticancer and anti-inflammatory properties. Additionally, it has been isolated from an endophytic fungus called PM0651480 that was discovered in the Indian medicinal plant Bakul, *Mimosops elengi*. Human TNF- and IL-6 were also dramatically reduced by ergoflavin at very low IC50 values (Deshmukh et al., 2009).

## Biotechnological Potential of Endophytes from Apocynaceae Plants

Endophytes are becoming more relevant in terms of biotechnology and industry due to huge biological potential of secreting diverse secondary metabolites. These metabolites have been explored as novel biocontrol agents, antimicrobial agents, antitumour agents, and immunosuppressants. Endophytes have benn also reported to secrete antiviral compounds and produce natural antioxidants, antidiabetic agents, antibiotics, and insecticidal products (Yadav et al., 2018). By utilizing a range of processes and enhancing plant resistance to unfavourable environmental factors, endophytes can successfully promote development (Yadav et al., 2018). Moreover, industrially important products such as enzymes, vitamins, organic acids, and antibiotics have also been reported from endophytic microorganisms. Antibiotic resistance, which is on the rise, especially in human and animal pathogens can be is a major threat to public health if proper precautions are not taken. A detailed account of the biodiversity, bioactive metabolites, and biological activities of endophytic microorganisms from Apocynaceae plants is provided in Table 12.1.



**Table 12.1** Biodiversity and biological activities of endophytes in Apocynaceae plants

Sr. no.	Host plant	Endophytes	Bioactive compounds	Biological activity	Assay method(s)	References
1.	<i>Nerium indicum</i>	<i>Geomyces</i> sp. B	Vincamine alkaloids	The <b>acetyl cholin ester</b> ase (AChE) inhibitory activity	Ellman's in vitro assay	Hemmati et al. (2020)
2.	<i>Catharanthus roseus</i>	<i>Stemphylium amaranthi</i>	Vinblastine	antioxidant activity	DPPH and ABTS in vitro assay	Nascimento (2015)
3.	<i>Catharanthus roseus</i>	<i>Glomastix masseei</i>	Ajmalicine	antioxidant activity	DPPH and ABTS in vitro assay	Nascimento (2015)
4.	<i>Calotropis procera</i>	<i>Phaeoanularia calotropidis</i>	Crude extract	Antimicrobial	Agar well Diffusion method	Parthasarathy et al. (2020)
5.		<i>Guignardia bidwellii</i>	Crude extract	Antimicrobial		Parthasarathy et al. (2020)
6.	<i>Catharanthus roseus</i>	<i>Curvularia verruculosa</i>	Vinblastine	Cytotoxic activity	In vitro cytotoxicity against HeLa cell line	Yin and Sun (2011)
7.	<i>Vinca minor</i>	Unidentified fungus	<b>Vincamine</b>	Not reported	Not reported	Verna et al. (2014)
8.	<i>Vinca rosea</i>	<i>Nigrospora sphaerica</i>	NigrosporaerinA,	Antileukemic, antifungal, antibacterial and antimalarial	In vitro methods	Birat et al. (2022)
9.	<i>Catharanthus roseus</i>	<i>Aspergillus fumigatus</i> EFBL	Epothilone B analogue	antiproliferative activity	In vitro methods <i>In silico</i>	Ayob and Simarani (2016)

(continued)

Table 12.1 (continued)

Sr. no.	Host plant	Endophytes	Bioactive compounds	Biological activity	Assay method(s)	References
10.	<i>Catharanthus roseus</i>	<i>Colletotrichum</i> sp., <i>Macrophomina phaseolina</i> , <i>Nigrospora sphaerica</i> and <i>Fusarium solani</i>	Hydrolytic enzymes; Cellulases, Protease, Amylase	Hydrolytic activity	Not reported	Jasmine and Agastian (2013)
11.	<i>Catharanthus roseus</i>	Actinomycetes	EEA : extract	free radical scavenging activity and marked alpha glucosidase activity	DPPH	Venugopalan and Srivastava (2015a, 2015b)
12.	<i>Catharanthus roseus</i>	<i>Fusarium solani</i>	Camptothecin	Anticancer	Not reported	Ranjan and Jadeja (2017)
13.	<i>Catharanthus roseus</i>	<i>Micrococcus yunnanensis</i> strain rsk5	Antibacterial compound	Antibacterial activity	Against <i>Staphylococcus aureus</i>	El-Sayed et al. (2020)
14.	<i>Cynancum acutum</i>	<i>Sarocladium strictum</i>	Cis-13-octadecenoic acid, sebacic acid, pentamethoxy flavone and n-hexadecanoic acid	Insecticidal activity	Deformation of <i>S. littoralis</i>	Manasa and Nalini (2014)
15.	<i>Tabernaemontana heyneana</i> Wall	<i>Colletotrichum</i> , <i>Curvularia</i> , <i>Fusarium</i> , <i>Phomopsis</i> , <i>Verticillium</i> , and <i>Voluella</i>	L-asparaginase	L-Asparaginase Activity	Nesslerization	Rao et al. (2015)
16.	<i>Cryptolepis buchanani</i>	<i>Phomopsis liquidambaris</i> CBR-15	Antimicrobial metabolites	Antimicrobial activity	Agar well Diffusion method	Gherbawy and Gashgari (2014)
17.	<i>Calotropis procera</i>	<i>Chaetomium globosum</i> <i>Myrothecium verrucaria</i>	Antifungal compound	Antifungal activity.	Agar well Diffusion method	Nagda et al. (2017)
18.	<i>Calotropis procera</i>	<i>Penicillium</i> spp. and <i>Aspergillus</i> spp.,	Phenolics and flavonoids	Antioxidant potential	DPPH	Kudalkar et al. (2012)

19.	<i>Prestonia trifidi</i>	<i>Muscodor sutura</i>	Thujopsene, chamigrene, isocaryophyllene, and butanoic acid, 2-methyl.	antifungal activity	Agar well Diffusion method	Akpotu et al. (2017)
20.	<i>Catharanthus roseus</i>	MR 1B MRB2	Citreoisocoumarin, Paxillin, nigrinicol, fatty acid, cladosporin	Antibacterial and antifungal activity	Agar well Diffusion method	Ramesha and Srinivas (2014)
21.	<i>Plumeria acuminata</i> L.	<i>Colletotrichum sp</i>	Crude extract	Antibacterial and antifungal activity	Agar well Diffusion method	Akshatha et al. (2014)
22.	<i>Plumeria busifolia</i> L.	<i>Fusarium oxysporum</i>	Crude extract	Antibacterial and antifungal activity	Agar well Diffusion method	Akshatha et al. (2014)
23.	<i>Rauwolfia densiflora</i>	<i>Unidentified fungi</i>	Crude extract	Antidiabetic activity	a-amylase inhibitor	Huang et al. (2007)
24.	<i>Tabernaemontana divaricata</i> (L),	<i>Strain AcapF3</i>	Phenolic compound	Antioxidant activity	<i>In vitro</i>	Khiralla et al. (2015)
25.	<i>Rauwolfia verticillata</i> (Lour.)	<i>Strain AcapF3</i>	Phenolic compound	Antioxidant activity	<i>In vitro</i>	Khiralla et al. (2015)
26.	<i>Calotropis procera</i> , <i>Catharanthus roseus</i> ,	<i>Aspergillus sp.</i>	Gallic acid Phenol	Antioxidant activity	<i>In vitro</i>	Palem et al. (2015)
27.	<i>Catharanthus roseus</i>	<i>Talaromyces radicus</i>	Vinblastine and vincristine	Anticancer activity	HeLa cells	Ramesha (2014)
28.	<i>Nerium oleander</i>	<i>Cochliobolus Kusanoi</i>	Oosporein	Antimicrobial and Anticancer activity	Cytotoxicity	Li et al. (2007)
29.	<i>Trachelospermum jasminoides</i>	<i>Verticillium sp. Tj33</i>	Verticase	fibrinolytic activity	<i>In vitro</i>	Zhang et al. (2008)
30.	<i>Trachelospermum jasminoides</i>	<i>Cephalosporium acremonium</i> IFB-E007	Cephalosol	Antimicrobial	<i>In vitro</i>	Ayob et al. (2017)
31.	<i>Catharanthus roseus</i>	<i>Nigrospora sphaerica</i>	Vinblastine	Cytotoxic activity	breast cell line cancer (MDA-MB 231)	Corrado and Rodrigues (2004)

(continued)

Table 12.1 (continued)

Sr. no.	Host plant	Endophytes	Bioactive compounds	Biological activity	Assay method(s)	References
32.	<i>Aspidosperma tomentosum</i>	<i>Phomopsis</i> sp.	Crude extract	Antibacterial and antifungal activities.	bioautographic TLC agar-overlay technique	Bhagat et al. (2016)
33.	<i>Vinca rosea</i>	<i>Alternaria alternata</i>	cholinesterase inhibitor, altenuene	Antioxidant and insecticidal activity	Dot blot assay	Akshatha et al. (2016)
34.	<i>Rauwolfia densiflora</i>	<i>Streptomyces globosus</i>	Crude extract	Antioxidant activity	DPPH activity	Song et al. (2005)
35.	<i>Trachelospermum jasminoides</i>	<i>Cephalosporium</i> sp. IFB-E001	Graphis lactone A	Antioxidant activity	DPPH activity	Gao et al. (2018)
36.	<i>Cynanchum auriculatum</i>	<i>Stemphylium lycopersici</i> BG01	Physcione	Antioxidant activity	DPPH and ABTS scavenging activity	Gao et al. (2018)

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# Chapter 13

## Micropropagation-Based Conservation Strategies for Apocynaceae Plants



### Introduction

Being the largest family in the plant kingdom, Apocynaceae consists of over 400 genera with more than 4600 species distributed in five subfamilies: Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae, and Asclepiadoideae. A number of plants from this family are **cultivated** as ornamentals for their attractive flowers and foliage. The members are most abundant in the tropics and subtropics. Plants of the family Apocynaceae are native in Indian Subcontinent. The countries such as Pakistan, China, Bangladesh and Sri Lanka are known for dominant presence of Apocynaceae plants (Mahmood et al., 2011). In the Indian subcontinent, the Himalayan ranges of the southernmost mountains at altitudes of 600–1200 m above sea level are richly colonized by Apocynaceae plants. The western states of India are geographically and culturally rich and diverse and are widely exploring plant diversity for various ethnomedicinal purposes (Vineeta et al., 2022).

Applications of medicinal plants and their products for the primary health needs is an integral part of day to day life of tribal people. Indian tribal people rich knowledge of medicinal plants and therefore medicinal plants are widely used for health benefits. Historically, tribal tribes in the area have investigated the therapeutic potential of many of the local plants that are a part of their ecology. Alkaloids abundant in this family of plants provide them with great therapeutic value. Additionally, many species are planted for decorative purposes. People in rural regions utilize some Apocynaceae species as food and some of them as poisons (Aiyambo, 2010). Apocynaceae plants have been used in different traditional systems of medicine, such as Indian, Chinese, and Thai (Van Beek et al., 1984; Bhat et al., 2012). The Indian subcontinent has a dominant presence of Apocynaceae plants. Approximately 84 species or 30 genera are present in India. Some important members of this family, viz. *C. roseus*, *A. scholaris*, *R. serpentina*, *H. antidysentriaca*, *N. indicum* and *T. peruviana* have been documented in

Ayurveda, Siddha, Unani and Homeopathy for the treatment of various diseases and disorders.

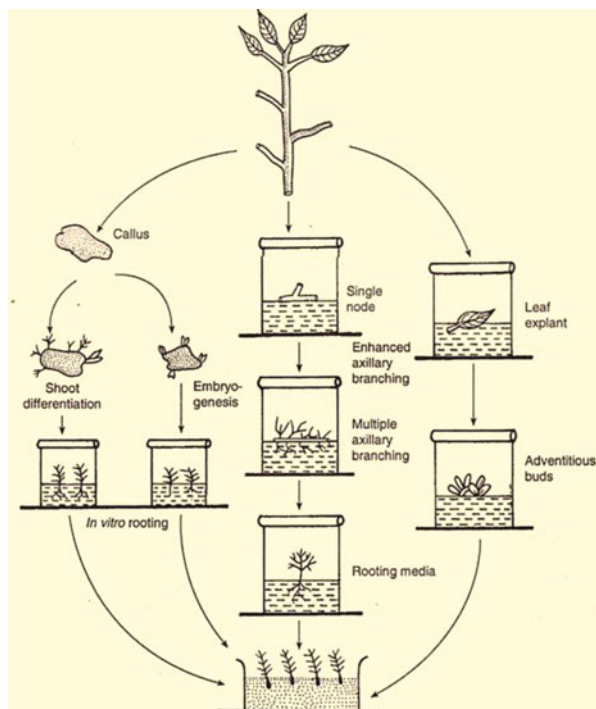
In African countries such as Ghana, Uganda, Nigeria and Senegal, Apocynaceae members such as *T. crassa*, *V. africana* and *Rauvolfia vomitoria* are commonly used for similar ethnomedicinal uses. The leaf, stem, root and flower extracts of these plants have been widely used for anti-arthritic activity in the treatment of [rheumatism](#) and parasitic skin infections, including [leprosy](#), used as [diuretic](#), insecticidal as well as in the treatment of fever, skin diseases as well as in wound healing (Bhat et al. [2012](#)).

The Western Ghats of India are a biodiversity hotspot and rich in medicinal plants that harbor approximately 3000 [plants of medicinal](#) importance (Vineeta et al., [2022](#)). The tribals of Western Ghats are using these plants for their primary health care. The high biodiversity of medicinal plants in the Western part of India is attributed to the varying ecosystems in this region. *Caralluma europaea* is another plant of this family native to Morocco, Algeria, Tunisia, Libya, Egypt, Jordan, Spain, and Italy. Various plants are used traditionally to treat diabetes mellitus, flu, caught, [kidney stones](#), cysts, [respiratory infections](#), cancer, [digestive disorders](#), [urogenital infections](#), [metabolic disorders](#), and cardiovascular problems (Ouassou et al., [2021](#)).

## Micropropagation Based Conservation of Apocynaceae Plants

Injudicious use and overharvesting of medicinal plants from the Apocynaceae family, many species of the family are facing the danger of extinction. Therefore, there is a need of immediate efforts to design the conservation strategies for this family. Plant cells have the totipotent capacity to regenerate the complete plant from explant tissue using the tools and techniques of plant tissue culture technology. Currently, secondary metabolites with applications in medicine, food, and cosmetics are mostly produced on a big scale from medicinal and aromatic plants (Karuppusamy, [2009](#)). Many species of medicinal and aromatic plants have already gone extinct as a result of excessive commercial use, and the ones that survive fall into the category of endangered. The preservation of superior genotypes of plants is thought to be best accomplished through micropropagation. Plant genotypes can be preserved through effective shoot tip/meristem culture, lateral bud culture, and somatic embryogenesis. The development of buds from tobacco explants resulted in the identification of kinetin and other useful cytokinins (Miller & Skoog, [1953](#)). It has been documented that coconut milk can induce somatic embryogenesis in carrot explants (Steward, [1958](#)). Due to the totipotent nature of the cell, single-cell somatic embryogenesis has the capacity to regenerate entire plants. Forster et al. ([2007](#)) investigated embryogenesis and organogenesis from pollen culture of datura anthers.

**Fig. 13.1** Various approaches to micropropagation (Verma, 2017)



Micropropagation is widely used for raising genetically identical plants, pathogen-free plants, large-scale plant production, gene conservation, etc. The plant can be multiplied asexually (by vegetative propagation) or sexually (via seed). Gametes are fused during sexual reproduction, which produces seeds with a high degree of variability. Cloning takes place during micropropagation. They are spread either by vegetative propagation or asexual modes of reproduction. In both cases, a single individual is multiplied *in vitro* to create genetically identical plants. Several methods are practiced for the micropropagation of medicinal and other economically important plants. These methods are summarized in Fig. 13.1, Table 13.1.

## Advantages of Micropropagation

1. A large number of clones can be created in a given time using the plant tissue in limited quantities.
2. Micropropagation is used as an effective alternative for the large-scale production of plant species that are difficult to grow using traditional practices.
3. A large number of plantlets can be kept up in small spaces.
4. Clonal expansion is possible throughout the year.

**Table 13.1** Summary of micropropagation methods and their advantages and drawbacks

Sr. no.	Micropropagation method	Brief description	Advantages	Drawbacks
1.	Callus culture	The explant is placed in an artificial media and cultured for callus development. The well-developed callus is transferred into a fresh medium containing <b>desired hormones</b> for roots, shoots induction. After a few weeks, the plantlet is subjected to hardening	Can be used to successfully accelerate the growth of many different herbaceous plants	High biochemical variability and slow growth rate
2.	Meristem culture	The leaf primordial and meristem tissue are placed in a nutrient medium and allowed to grow. A rooted plantlet is created after a few weeks. When it reaches the desired height or growth, it is transplanted into the soil	A disease-free plant can be produced using this method	
3.	Suspension culture	Individual cells or callus cells are suspended and allowed to grow in liquid media and it is continuously aerated on a rotary shaker at a specific temperature	Suitable for secondary metabolites production	More susceptible to contamination. Less yield of product. The formation of clumps and cells does not separate after division
4.	Protoplast culture	To repair the cell wall and the callus, the isolated explant/plant cell is grown in the appropriate medium. In the right circumstances, the cell forms a cell wall, which is followed by a stimulation of cell division that results in differentiation into a new plant		It is difficult to strip the cell wall of root tips and embryos and therefore generally not used as an explant for the protoplast culture

(continued)

**Table 13.1** (continued)

Sr. no.	Micropropagation method	Brief description	Advantages	Drawbacks
5.	Embryo culture	In this method, the embryos are extracted and placed into a medium with desired plant growth regulators with proper nutrients in aseptic conditions	Has wide application in crop improvement	

5. The global trade and exchange of medicinal and horticultural plant material without the danger is possible.
6. Micropropagation is an important method for seed creation.
7. Because micropropagation has distinct favorable conditions than conventional engendering techniques, this method has a better chance of producing major plant-based phytopharmaceuticals.

## Applications and Merits of Micropropagation Over Conventional Plant Breeding

1. Small amounts of explant tissue are sufficient for micropropagation to produce thousands of clones in a single year. The same number of plants could not be produced using conventional methods in the same amount of time.
2. Plant species that are either slow-growing or difficult to cultivate using conventional bulk propagation can be effectively propagated using micropropagation.
3. Vegetative propagation of valuable plants for large-scale production is possible through micropropagation. Large-scale production of plant types that are genetically pure and free of diseases is possible, and the creation of new kinds takes much less time.
4. A small amount of physical space is needed for a lot of plants. This aids in the storage of genetic material and the preservation of endangered species.
5. Using meristem tip culture, disease-free varieties are obtained through micropropagation. Expansion of *in vitro* stocks can be performed throughout the year.
6. Increased yields and vigor of plants in floriculture and horticulture species can be achieved.
7. International transfer of plants can be made easier without any risk of spreading of phytopathogens and plant diseases.
8. Because the requirement for genetic conservation is crucial for seed production, micropropagation is especially helpful for the production of seeds in some crops.

Micropropagation, due to its several advantages over conventional propagation methods, has become popular and a prospect for the production of important plant-based bioactive natural products. Irrespective of the availability of plants, micropropagation is considered an alternative approach to conventional methods for the controlled production of medicinally important biochemicals.

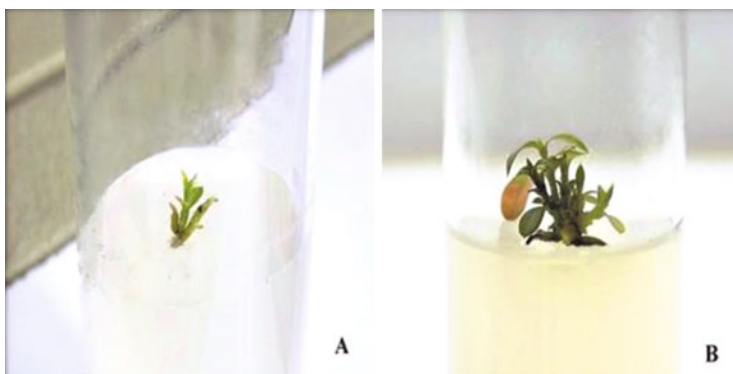
## ***In Vitro* Propagation of Apocynaceae Members**

The shoot apex of the native disease-free plant or aseptically germinated seedling is used for *in vitro* propagation of *C. carandas*. Surface sterilization of explants is not needed when aseptically germinated seedlings are used for multiple shoot induction, which ultimately reduces the chances of contamination. Aseptic seedlings were obtained after seed germination on MS medium. Before inoculation, seeds are surface sterilized and inoculated on MS medium, and the shoot apex of aseptic seedlings can be used for multiple shoot induction in *C. carandas*. The role of cytokinins is critical for shoot multiplication on the *C. carandas* shoot apex. In addition, cytokinins and many other metabolites may have a synergistic or antagonistic effect on shoot induction. For example, when quercetin was used in combination with cytokinins, it favored shoot induction, and the results obtained were comparatively better than those of other treatments (Bhadane et al., 2018).

## **Multiple Shoot Induction**

The shoot apex of *in vitro* raised aseptic seedlings or the apex tissue from disease-free plants was excised and cultured on MS medium supplemented with different combinations and concentrations of 6-benzylaminopurine (BAP) alone or in combination with kinetin. The effect of several organic and inorganic molecules can also be evaluated. Bhadane et al. (2018) evaluated the effect of quercetin and AgNO<sub>3</sub> on multiple shoot induction. Quercetin acts as an auxin transporter inhibitor (Brown et al., 2001), while AgNO<sub>3</sub> acts as an inhibitor of ethylene action (Beyer, 1976). In these experiments, shoot apices from aseptic seedlings were cultured on MS medium fortified with the abovementioned combinations of PGRs combined with quercetin at 0.75 and 1.5 mg/L. Similarly, in another set, MS medium augmented combinations of BAP and kinetin combined with AgNO<sub>3</sub> at 0.5 and 1.0 mg/L. Explants cultured on MS medium devoid of PGRs served as controls.

Cytokinins possess the ability to break buds and are involved in cell division, shoot initiation, and multiplication from explants (George et al., 2008; Mazri, 2015). BAP and kinetin are commonly employed for multiple shoot induction in the *C. carandas* shoot apex. Several studies reported lower efficiency of cytokinins when they were used alone for shoot multiplication. The combinations of two or more cytokinins were found to be effective for shoot induction in *Curcuma longa*



**Fig. 13.2** (a) Shoot apex of *C. carandas* explant on MS medium. (b) Multiple shoot induction on MS medium after 4 weeks

(Thingbaijam et al., 2012), *Pyrus pyrifolia* (Kadota & Niimi, 2003), and *Phoenix dactylifera* (Al-Khateeb, 2006). Studies on shoot multiplication showed the effectiveness of BAP over other cytokinins in *Cucumis anguria* (Jeyakumar et al., 2014). A higher BAP to lower kinetin ratio for shoot enhancement in *C. carandas* resulted in effective shoot regeneration (Bhadane et al., 2018). This combination of cytokinins showed a positive effect on shoot induction. The newly developed shoots were initiated from the cut edges of the shoot apex after 13 days of incubation. The use of BAP alone at 1.0 mg/L was unable to show shoot initiation from the explant. However, increasing concentrations of BAP had a poor impact on multiple shoot induction (Fig. 13.2a, b). The maximum length ( $1.73 \pm 0.16$  cm) of shoots was obtained in MS media supplemented with 4.0 mg/L BAP and 0.5 mg/L kinetin (Bhadane, 2017). The mean number of shoots per explant and the average length of shoots per explant in all treatments were found to be comparable with each other (Table 13.2).

This indicates that different concentrations of BAP used in combination with kinetin at 0.5 mg/L increase the rate of shoot induction in *C. carandas* (Bhadane et al., 2018). Development hormones, specifically auxins and cytokinins, are the most important components of tissue culture media because they have a higher impact on cell growth and differentiation than other components. It is known that cytokinins may distinguish between shoot buds, shoot tips, and nodal explants. For the initiation of shoot buds from nodal explants, BAP is the most often employed cytokinin (Ram & Shekhawat, 2011).

The shoot regeneration frequency is positively modified by the synergistic impact of cytokinin and auxin. Other growth additives, such as adenine sulfate, polyamines (low molecular weight compounds), diamines, triamine, and casein hydrolysate and tetramine spermine, positively affect cell division and differentiation (Gopal et al., 2020).

Secondary metabolites such as phenolics and flavonoids are known for their role as the inhibitors of auxin transport, *in vitro* as well as *in vivo* (Brown et al., 2001).

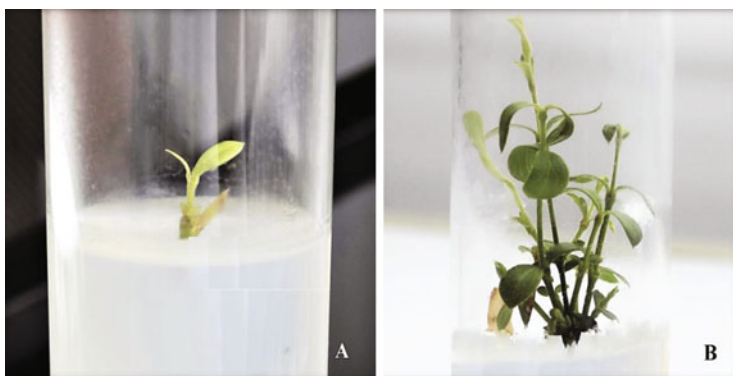
**Table 13.2** Effect of cytokinins with quercetin on shoot multiplication (Bhadane, 2017)

BAP (mg/L)	Kinetin (mg/L)	Quercetin (mg/L)	Mean number of shoot per explant	Mean length (cm) of shootlet per explant
1.0	—	—	0 ± 0.00 <sup>o</sup>	0.00 ± 0.00 <sup>m</sup>
2.0	—	—	2 ± 1.51 <sup>n</sup>	1.20 ± 1.05 <sup>k,l</sup>
3.0	—	—	3 ± 1.35 <sup>m,n</sup>	1.39 ± 0.53 <sup>i,k,l</sup>
4.0	—	—	3 ± 0.67 <sup>l,m</sup>	1.64 ± 0.21 <sup>i,j</sup>
1.0	0.5	—	2 ± 1.54 <sup>m,n</sup>	1.08 ± 0.74 <sup>l</sup>
2.0	0.5	—	4 ± 1.14 <sup>k,l</sup>	1.45 ± 0.24 <sup>i,j,k</sup>
3.0	0.5	—	5 ± 1.30 <sup>i,k</sup>	1.58 ± 0.19 <sup>i,j</sup>
4.0	0.5	—	5 ± 1.47 <sup>i,k</sup>	1.73 ± 0.16 <sup>h,i,j</sup>
1.0	—	0.75	6 ± 1.08 <sup>i,j</sup>	1.81 ± 0.30 <sup>h,i</sup>
2.0	—	0.75	7 ± 1.87 <sup>h,i</sup>	2.18 ± 0.21 <sup>f,g</sup>
3.0	—	0.75	7 ± 1.14 <sup>h,i</sup>	2.27 ± 0.21 <sup>f,g</sup>
4.0	—	0.75	8 ± 1.04 <sup>g,h</sup>	2.40 ± 0.28 <sup>e,f</sup>
1.0	—	1.5	9 ± 1.03 <sup>f,g</sup>	2.03 ± 0.17 <sup>g,h</sup>
2.0	—	1.5	10 ± 1.70 <sup>c,d,e</sup>	2.45 ± 0.23 <sup>d,e,f</sup>
3.0	—	1.5	10 ± 1.37 <sup>d,e,f</sup>	2.18 ± 0.27 <sup>f,g</sup>
4.0	—	1.5	9 ± 1.40 <sup>e,f,g</sup>	2.54 ± 0.28 <sup>c,d,e,f</sup>
1.0	0.5	0.75	11 ± 2.31 <sup>c,d</sup>	2.81 ± 0.22 <sup>b,c,d</sup>
2.0	0.5	0.75	15 ± 1.62 <sup>a</sup>	3.24 ± 0.28 <sup>a</sup>
3.0	0.5	0.75	11 ± 2.37 <sup>c,d</sup>	2.76 ± 0.30 <sup>b,c,d</sup>
4.0	0.5	0.75	12 ± 1.96 <sup>c</sup>	2.90 ± 0.28 <sup>b,c</sup>
1.0	0.5	1.5	11 ± 1.79 <sup>c,d</sup>	2.74 ± 0.25 <sup>b,c,d,e</sup>
2.0	0.5	1.5	13 ± 1.56 <sup>b</sup>	2.92 ± 0.21 <sup>a,b</sup>
3.0	0.5	1.5	11 ± 1.79 <sup>c,d</sup>	2.77 ± 0.13 <sup>b,c,d</sup>
4.0	0.5	1.5	11 ± 1.75 <sup>c,d</sup>	2.82 ± 0.30 <sup>b,c,d</sup>

The values correspond to means ± standard deviation of 10 replicates. Means in each column followed by the same letter (a to o) were not statistically different according to DMRT (Duncan's Multiple Range Test) at  $p < 0.05$

Quercetin may have a synergistic effect with cytokinins on shoot multiplication. During plant growth, auxins are synthesized in meristematic tissues and are responsible for apical dominance with reduced growth of axillary buds. Cytokines induce axillary bud formation and stimulate multiple shoot initiation. Therefore, if the medium is supplemented with auxin transport inhibitors such as flavonoids, it can improve the shoot multiplication ability of *in vitro* cultured plants. Bhadane et al. (2018) demonstrated the effect of different concentrations and combinations of PGRs and quercetin on shoot multiplication in a few members of the Apocynaceae family. To induce multiple shoot induction, the synergistic effect of quercetin and kinetin was studied. It was observed that the use of quercetin with kinetin and BAP favored multiple shoot induction. The maximum numbers of shoots were obtained in MS media supplemented with combinations of BAP:kinetin:quercetin at 2.0:0.5:0.75 mg/L after 4 weeks (Fig. 13.3a, b) This combination of PGRs and quercetin not





**Fig. 13.3** (a) Shoot apex explant of *C. carandas* inoculated on MS medium with BAP, kinetin and quercetin combination on day one. (b) Multiple shoot induction on MS medium with BAP, kinetin and quercetin after four (Bhadane, 2017)

only increased multiple shoot induction but also showed a significant increase in mean shoot length.

The combination of Quercetin, kinetin and benzyl adenine was used for shoot multiplication studies on *Oldenlandia umbellata* (Krishnan & Siril, 2015). They reported maximum shoot induction in MS medium supplemented with 5  $\mu\text{M}$  BA and 5  $\mu\text{M}$  quercetin. According to them, quercetin has endogenous auxin transport inhibitory potential, which ultimately enhances cytokinin action with an increase in shoot number. On the other hand, synthetic auxin transport inhibitors such as 2,3,5-tri iodobenzoic acid (TIBA) and 1-naphthylphthalamic acid (NPA) showed a progressive effect on shoot multiplication in *Rosa hybrida* (Singh & Symal, 2000) and *Alnus glutinosa* (Lall et al., 2005). Jacobs and Rubery (1988) demonstrated that quercetin acts as a competitive inhibitor of NPA.

Recently, there has been renewed interest and progress in the use of  $\text{AgNO}_3$  in multiple-shoot induction. It is used as the source of nitrate, which favours the shoot induction process. In addition, it has been reported to inhibit ethylene action in plants. Plants generally evolve or produce ethylene during injury by various mediators. In plant tissue culture, ethylene is produced in aseptically cut explants placed on medium. This ultimately inhibits the *de novo* regeneration of shoots and retards growth. Due to its potency, easy availability, stability, and solubility in water, it has been widely used in plant tissue culture for promoting growth and shoot induction (Kumar et al., 2009).

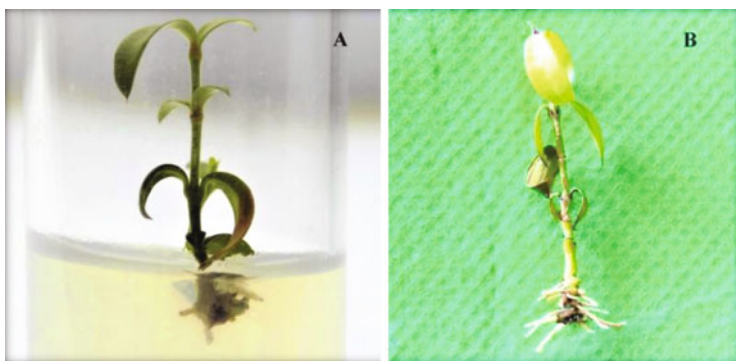
Sridevi and Giridhar (2014) reported an increase in shoot growth in *Coffea dewevrei* using  $\text{AgNO}_3$  at 40  $\mu\text{M}$  + BA at 8.88  $\mu\text{M}$  + IAA at 2.85  $\mu\text{M}$ . They also reported that a reduced concentration of  $\text{AgNO}_3$  in the medium promotes somatic embryogenesis of *Coffea dewevrei* calli. According to studies by Bayer (1976), silver ions are effective in blocking exogenously applied ethylene response towards senescence and growth retardation. These features of  $\text{AgNO}_3$  were studied for the

regeneration of monocot and dicot plants using *in vitro* tissue culture techniques (Bais et al., 2000; Giridhar et al., 2003).

### ***In Vitro* Rooting of Shoots**

Once the microshoots are obtained from the multiplication of shoot induction with a length of  $>2$  cm, they can be used in *in vitro* rooting to regenerate plantlets. Before cutting the shoots from the clump, they are subjected for washing using sterile deionized water. The dried explants are cultured MS medium fortified with various concentrations of IAA and IBA alone. After 30 days of culture, developed roots can be observed in root lengths, thickness, etc. Studies on *C. carandas* and *T. divaricata* showed that the quarter strength MS medium favors rooting at lower levels of IAA and IBA. Tiny roots can be seen during the second and third weeks of incubation (Fig. 13.4a, b).

For successful rooting, *in vitro*-raised plantlets are transferred to auxin-containing medium. Previous studies on other members of the Apocynaceae family, Goel et al. (2009) and Nagesh and Shanthamma (2011), showed that IAA, IBA, and NAA were effective for *in vitro* rooting. In addition, the concentration of auxin and major stocks in the basal medium also affects *in vitro* rooting. According to Purohit and Kukda (2004), increased concentrations of medium constituents as well as auxins are the reasons for calluses to shootlets instead of root formation. In their study on *Wrightia tinctoria*, they used reduced concentrations of major salts of MS medium to  $\frac{1}{4}$  strength for *in vitro* rooting. This modified medium favored maximum rooting without callusing. These facts were advantageous for designing the rooting experiment in our study; therefore, we used two different auxins (IAA and IBA) alone at 0.25 mg/L – 2.0 mg/L in  $\frac{1}{2}$  strength and  $\frac{1}{4}$  strength MS medium.



**Fig. 13.4** *In vitro* rooting (a) Rooting to the shoots cultured on MS medium with IAA. (b) *In vitro* rooted plant with multiple roots after 4 weeks (Bhadane, 2017)

## Stepwise Acclimatization (hardening) of *In Vitro* Raised Plantlets

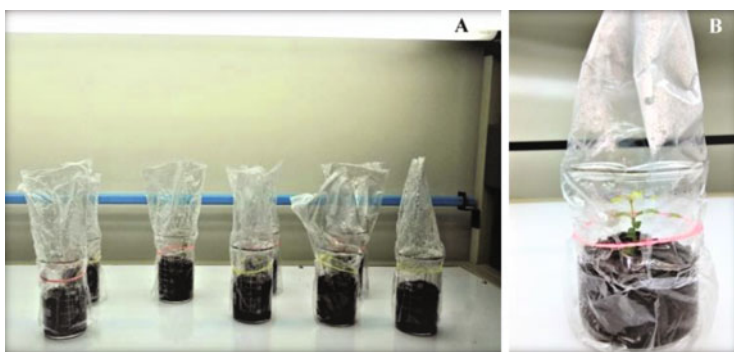
*In vitro* developed plants well developed roots are removed from the culture vessel and the roots of each plant are washed with sterile distilled water. These plantlets are further acclimatized in glass/plastic pots containing sterile garden soil and vermicompost, and irrigated with diluted MS salt solution. The pots can be covered with polybags to maintain proper moisture inside the container. Acclimatization of these plantlets is done in the culture room under controlled conditions at  $25 \pm 2^\circ\text{C}$  and  $55 \pm 5\%$  relative humidity with 16/8 h photoperiod for 4–6 weeks. After acclimatization, these plantlets are transported in a plastic pot containing natural soil. For proper hardening, all pots were placed in a case covered with a polythene sheet and maintained in a shed net house for 4 weeks before subsequent transfer to the field (Bhadane, 2017).

During hardening in a shed net house, plantlets show varying levels of survival. Upon completion of acclimatization and hardening, plantlets can transfer to the field. The entire process takes 8 weeks for the complete regeneration of *C. carandas* (Fig. 13.5).

Upon completion of acclimatization and hardening, plantlets are transferred to the field. The entire process takes 8 weeks (Fig. 13.6).

It can be concluded that *in vitro* propagation techniques developed to conserve *C. carandas* and the other members of this family can be useful for other plants under the endangered category.

Plants such as *Holarrhena antidysenterica* and *Carissa carandas* are pharmacologically very significant, as they possess several bioactivities. Additionally, these plants grow slowly, and their seeds have poor viability and germination. The delicate *Carissa carandas* seeds must be sown right away after being removed from the fruit because they cannot be kept for a longer period of time. Any attempt at preservation causes them to lose their ability to germinate. Therefore, to address these challenges,



**Fig. 13.5** (a) Acclimatization of *in vitro* rooted plantlets in containers covered with polythene bags; (b) magnified view (Bhadane, 2017)

**Fig. 13.6** Acclimatized and established plantlets (after 8 weeks) in plastic pots containing natural soil (Bhadane, 2017)



*in vitro*-raised medicinally important, recalcitrant, and fruiting plants of this family can also be effectively conserved by the *ex situ* method. There are reports on phenotypic and genetic variability among wild plants grown in different regions. Hence, there is the possibility of variability in *in vitro* raised and *ex situ* conserved plants. It is possible to determine the genetic variability/stability of *in vitro* raised and *ex situ* conserved plants by molecular markers. Further transcriptome analysis tools can be used to obtain gene sequences to develop molecular markers for the plants under study.

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# Chapter 14

## Practical Applications of Apocynaceae Plants in Nanotechnology



### Introduction

Nanotechnology is a branch of applied science and technology whose basis includes the control and synthesis of matter on a molecular level in the range of 1–100 nm. The concepts and ideas of nanoscience and nanotechnology were first used by the physicist Richard Feynman in 1959 (Feynman, 1959). The term “nanotechnology” was defined by Tokyo Science University Professor Norio Taniguchi in 1974. Nanotechnology is the multidisciplinary integration of biotechnology, nanotechnology, chemical processing, material science, and system engineering in biochips, molecular motors, nanocrystals and nanobiomaterials (Huang et al., 2007). Nanoparticles (NPs) are particles less than 100 nm in diameter that have a very high surface area to volume ratio, which leads to both physical and chemical differences in their properties compared to bulk material (Rai et al., 2008; Baker et al., 2013; Iravani, 2011).

A nanometer (nm) is one-billionth of a meter, which is a microscopic particle with at least one dimension less than 100 nm. A DNA molecule is 2.5 nm wide, a protein approximately 50 nm, and a flu virus approximately 100 nm. Human hair is approximately 10,000 nm thick. Nanoparticles are of great scientific interest because they link the gap between bulk materials and atomic or molecular structures. A bulk material has constant physical properties irrespective of its size, but at the nanoscale, it is different. Several well-characterized bulk materials have been found to possess the most interesting properties when studied at the nanoscale (Thakkar et al., 2010).

Noble metal nanoparticle research has great scientific interest due to its potential applications in biomedical, optical, magnetic, electronic fields and information storage. Metal nanoparticles have superior properties to their bulk counterparts, i.e., They have powerful mechanical strength, thermal stability, catalytic activity, electrical conductivity, magnetic properties (Murray et al., 2001), optical properties, etc. Nanoparticles have size-dependent properties on the nanometer scale, while bulk materials have more constant physical properties than their size (Fendler & Tian,

1998). Due to their smaller size, nanoparticles have optical properties that produce quantum effects (Dai & Bruening, 2002; Klabunde & Richards, 2009).

Research has reported the applications of metal nanoparticles (MNPs) in several fields. MNPs have attractive spectral, optical and electronic properties. MNPs also have wide applications in catalysis and biosensing applications. Biosensing platforms are selective and specific bioanalyte detectors (Doria et al., 2012). The fields of electronics, magnetism and optoelectronics, and information storage are some of the applications of MNPs. Silver nanoparticles (AgNPs) are used in photonics, microelectronics, and medicine, and they are commonly used for their antimicrobial activity against a broad variety of microorganisms (Nangare & Patil, 2020). Copper nanoparticles (CuNPs) are reported as a disinfectant for wastewater, catalysts, conducting applications, antibacterial activity, etc. The CuNPs may cause fluorescence quenching, dye degradation, dye aggregation, dye deaggregation, and fluorescence enhancement for biosensing and biolabelling applications (Din & Rehan, 2017). Ferrous nanoparticles (FeNPs) are widely used in remediation, in the treatment of organic or inorganic pollutants in water, in dye removal and as antibacterial agents (Fahmy et al., 2018).

## Methods of Nanoparticle Synthesis

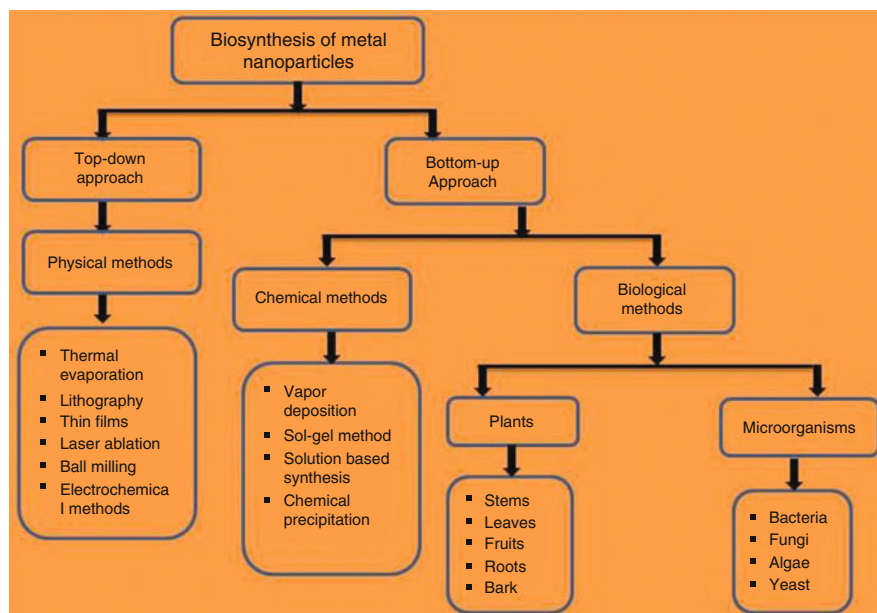
Nanoparticles are synthesized via physical, chemical and biological methods. NP synthesis methods are broadly classified as top-down and bottom-up approaches (Fig. 14.1). The top-down approach refers to the carving or successive cutting of suitable bulk material into particles at the nanoscale. Bottom-up or self-assembly refers to the buildup of material at the nanoscale from the bottom: atom-by-atom, molecule-by-molecule, or cluster-by-cluster. Lithographic techniques and grinding and milling are typical examples of top-down methods, whereas colloidal dispersion is an example of the bottom-up method (Ranjit & Baquee, 2013).

### *Physical Methods*

#### **Mechanical Attrition/Ball Milling**

Milling is a characteristic “top-down” approach that belongs to the mechanical alloying process. The purpose of milling is to reduce the particle size and blend particles into new phases, which can be carried out at room temperature. It can be performed on high-energy mills, centrifugal-type mills, vibratory-type mills, and low-energy tumbling mills. Various types of high-energy milling techniques are available to prepare nanoparticles, such as the Attrition Ball Mill, Planetary Ball Mill, Vibrating Ball Mill, Low Energy Tumbling Mill and High Energy Ball Mill (Rajput, 2015; Ghorbani, 2014).





**Fig. 14.1** An overview of different methods of nanoparticle synthesis

Milling is performed by the stirring action of an agitator. It has a vertical rotator central shaft with horizontal arms, i.e., impellers. The rotation speed and the milling temperature must be in greater control. The common drawbacks of this technique were the low surface area, highly polydispersed size distribution and partially amorphous state of the powder. The powders were highly reactive with oxygen, hydrogen and nitrogen (Rajput, 2015; Ghorbani, 2014).

### Pulsed Laser Ablation (PLA)

Pulsed laser ablation in liquid media (PLAL) is a flexible technique used for the synthesis of several kinds of NPs and metal-oxide NPs, such as noble metals, alloys, oxides and semiconductors. The controlled synthesis of nanomaterials was carried out through rapid reactive quenching of ablated species at the plasma and liquid interface. Using a single-step method, well-crystallized NPs can easily be achieved due to the high energetic state of ablated species. Using different combinations of ablated inorganic species and surfactant molecules in liquids, organic and inorganic nanocomposites can be synthesized (Sasaki et al., 2006; He et al., 2008).

The PLA technique possesses the advantages of simplicity of the procedure, as it requires a small amount of chemical substances and inexpensive equipment for controlling the ablation atmosphere, which cannot be achieved by vapour-based techniques or chemical solution route processes. During this process, the surface of

the target material is heated by using a laser beam. As a result, the metal atoms from the target are vaporized or sublimated, and the vaporized material condenses into clusters or particles with or without chemical reaction. Usually, the nanoparticles prepared by PLA in deionized water have a rather large size distribution because of the postablation coalescence of nanoclusters (Sasaki et al., 2006; He et al., 2008).

### **Chemical Vapour Deposition**

The formation of a nonvolatile solid film on a heated substrate by the reaction of vapour-phase or gas-phase chemicals in an activated environment (heat, light, plasma). The substrate is exposed to gaseous molecules that react and decompose on the surface of the substrate and produce the expected product. The method is important for synthesizing thin films and coatings. This process is relatively simple, uses inexpensive equipment, and particle formation can be controlled by a variety of process parameters, such as reactor temperature, precursor concentration, or residence time in the reactor (Moravec et al., 2011).

### **Electrochemical Method**

Electrochemical deposition is a technique that has been widely used for the synthesis of metal nanoparticles. Electrochemical deposition occurs at the interface of an electrolyte solution containing the metal to be deposited and an electrically conductive metal substrate.

Mostly in the electrochemical method of nanoparticle synthesis, a two-electrode system is used. The method is inexpensive and yields high-purity nanoparticles compared to other bottom-up approaches. By monitoring the electrolytic concentration and the applied current density, the size of the particles can be manipulated. AgNPs are usually synthesized by this method, which does not require any expensive chemicals and materials or special reagents such as surfactants and reducing agents. AgNPs are synthesized electrochemically with 0.01 M AgNO<sub>3</sub> as an electrolyte. Glassy carbon electrodes and silver metal were used as working electrodes and counter electrodes, respectively (Singaravelan & Alwar, 2015).

Ultrasonication, photo irradiation, radiolysis, spray pyrolysis, vaporization, solvothermal, chemical reduction and reverse micelles have been successfully used for the synthesis of NPs (Anamika Mubayi et al., 2012).

## ***Chemical Methods***

### **Sol–Gel Method**

The sol–gel method is a wet-chemical technique widely used for nanoparticle synthesis. A two-step sol–gel method to prepare a nanostructured Pt/CeO<sub>2</sub>–ZrO<sub>2</sub>–Al<sub>2</sub>O<sub>3</sub> catalyst with high thermal stability was reported. The first step includes the preparation of a dispersion colloid of a noble metal particle suspended with aluminum isopropoxide Ce(NO<sub>3</sub>)<sub>4</sub> and Zr(NO<sub>3</sub>)<sub>4</sub> and condensed into a gel, whereas the second step involves the hydrolysis of metal oxide precursors (Long et al., 2007). It is possible to modify the surface of synthesized zinc oxide nanoparticles by the sol–gel method with selected organic compounds. The method is inexpensive, requires a low processing temperature and reliable, repeatable, controllable reaction conditions, and narrow nanoparticles can be produced (Kołodziejczak-Radzimska & Jesionowski, 2014).

### **Chemical Precipitation**

The chemical precipitation method is attractive due to its low cost, high purity, short preparation time, high homogeneity, well-crystallized product, and relatively low reaction temperature (Lassoued et al., 2017). A wide-ranging literature review concludes that the chemical precipitation method is a notable method of ZnO nanoparticle synthesis (Jay Chithra et al., 2015). Chemical precipitation is used widely in microelectronics and plastic electronics because it is an inexpensive, low-temperature process with controllable process parameters for particle size (Jay Chithra et al., 2015). The method yields the smallest ZnO nanoparticles with the best optical and photocatalytic activity compared with physical and chemical methods (Liu et al., 2012). The synthesis involved a reaction between constituent materials in a suitable solvent. The precipitate was separated by centrifugation. The Cd NPs synthesized by chemical precipitation were heated and dried at 50 °C using double-distilled water as the solvent and thioglycerol as the capping agent (Singh & Chauhan, 2009; Rajput, 2015).

In top-down methods such as size reduction from a suitable starting material, imperfections were observed in the surface structure of the products (Rai et al., 2008). The surface chemistry and other physical properties of nanoparticles are highly dependent on the surface structure, which limits the use of these methods. Other physical methods, such as UV irradiation, aerosol and lithography, are also not considered environmentally friendly. In chemical methods (traditional wet methods), hazardous substances such as sodium borohydride, tetrakis(hydroxymethyl) phosphonium chloride (THPLC), poly-n-vinyl pyrrolidone (PVC), and hydroxylamine have been used for the synthesis of nanoparticles. The factors limiting the use of synthesized nanoparticles by these methods for biomedical applications include contamination from precursor chemicals, use of toxic solvents,

hazardous byproducts generated during synthesis, etc. (Nath & Banerjee, 2013; Karazhanov & Ravindran, 2010; Narayanan & Sakthivel, 2010; Sharma et al., 2019).

Physical and chemical methods were preferred for narrow particle sizes and uniform distributions. However, specific chemicals, such as reducing and capping agents, which may be environmentally destructive and costly, involve tedious and severe experimental conditions with high energy intake (Ahmed et al., 2016).

Additionally, the use of conventional reducing agents such as sodium borohydride, hydrazine hydrate and polyvinyl pyrrolidone (PVP), sodium polyacrylate and cetyltrimethylammonium bromide (CTAB), etc., as capping agents may adsorb on the surface of the nanoparticle and may be liable for various biological risks due to their general toxicity. Thus, it is important to develop reproducible “green” processes for the biogenic synthesis of nanomaterials (Luque & Varma, 2012; Ahmed et al., 2016).

In the last few years, chemical methods have been used for the production of large quantities of nanoparticles that are complicated, outdated and expensive and produce hazardous toxic wastes that are harmful to the environment as well as human health (Din & Rehan, 2017). Biological entities offer a nontoxic, clean and eco-friendly approach to synthesize a varied range of nanoparticles, physicochemical properties, shapes and composites (Husen & Siddiqi, 2014). Plants have been used for the synthesis of metallic nanoparticles due to their availability, cost-effectiveness, environmentally friendly nature and nonhazardous byproducts (Din & Rehan, 2017).

## Biological Methods

In bottom-up synthesis, the nanostructured building blocks of the nanoparticles are formed first and then assembled to produce the final particle (Thakkar et al., 2010; Mukherjee et al., 2001). Biosynthesis of a nanoparticle is a type of bottom-up approach that employs a biological system or its components for the formation of nanoparticles (Baker et al., 2013). The bioreduction of raw metallic salt into metal nanoparticles is the basis of biogenic nanoparticle synthesis.

The ‘biogenic approach’ involves nanoparticle synthesis via the biological approach without adding any reducing agent or stabilizer, which are replaced by molecules produced by living organisms, i.e., bacteria, fungi, yeast, algae, higher plants, etc. Biological methods involve the use of plants, animals and microorganisms for the synthesis of nanoparticles.

### *Microorganism-Mediated Nanoparticle Synthesis*

Microorganisms (such as algae, fungi, yeast, bacteria, and actinomycetes) have been widely used for the synthesis of various nanoparticles. Studies on the biosynthesis of

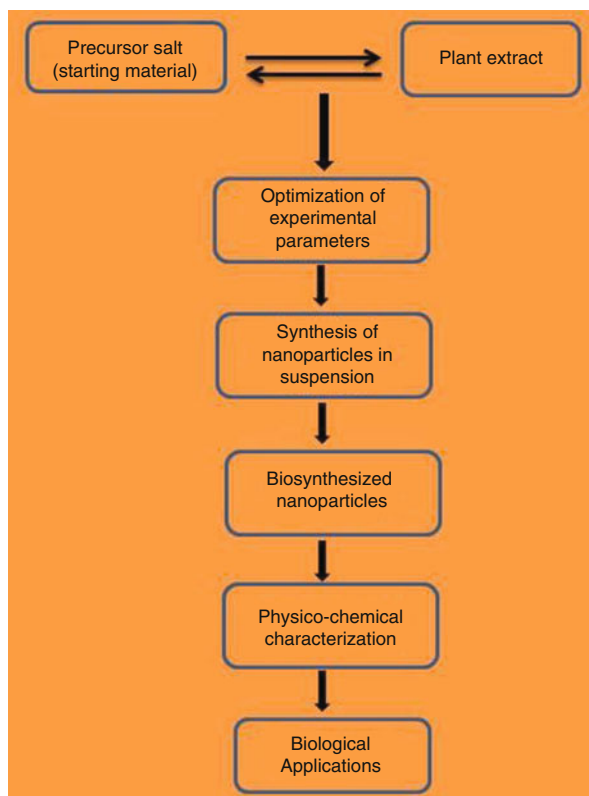
silver (Ag), gold (Au), cadmium sulfide (CdS), zinc sulfide (ZnS), platinum (Pt), and palladium (Pd) nanoparticles using microorganisms are well documented (Sastry et al., 2003; Gahlawat & Choudhury, 2019; Talebi et al., 2010). Biosynthesis of nanoparticles using microorganisms is widely used but has limitations such as maintenance of aseptic conditions, culture maintenance, and time-consuming processes (rate of synthesis is slow). Moreover, it is expensive compared to plant-mediated biosynthesis of nanoparticles (Rai et al., 2008). Other disadvantages related to microbes include spontaneous mutations that lead to loss of their biosynthesis potential due to long-term preservation and maintenance of microbial cultures (Baker et al., 2013). Furthermore, nanoparticles with limited size and shapes are synthesized by microbial sources compared to plant-derived materials (Kharissova et al., 2013).

### ***Plant-Mediated Synthesis Of Nanoparticles***

Phytomediated nanoparticle synthesis is a simple, eco-friendly and cost-effective method for the synthesis of nanoparticles (Rai et al., 2008). It can be easily scaled up, and plant metabolites are generally free from toxic contaminants (Mittal et al., 2013). Medicinal and aromatic plant usage for various diseases was suggested by the traditional Indian system. This opens up new avenues for the green chemical synthesis of biocompatible nanomaterials using plant parts (Alam et al., 2013). The use of plant extracts for making nanoparticles is inexpensive, easily scaled up and environmentally benign (Mittal et al., 2013). Plant tuber extracts such as *curcuma longa*, *ginger officinale*, and *Dioscorea bulbifera*. were enriched with different reducing and capping agents, which help in the rapid and stable formation of different metal nanoparticles (Ghosh et al., 2012; Singh et al., 2011; Shameli et al., 2012; Sathishkumar et al., 2009).

The synthesis mediated by plant extracts is based on the reduction/oxidation reaction. In this reaction, the phytochemicals and enzymes present in biological materials favor the conversion of metal compounds into specific nanoparticles (Bhuyan et al., 2015). The plant parts used are leaves, roots, latex, seeds, stems and tubers (Thakkar et al., 2010; Kharissova et al., 2013). The metal accumulation potential of plants is a fundamental basis explored for the biosynthesis of nanoparticles (Baker et al., 2013; Irvani, 2011). Plant-mediated nanoparticle synthesis is an easy one-step protocol. In phytomediated nanoparticle synthesis, the rate of reduction of the metal ion is comparatively faster compared to the microbial source. Plant-derived nanoparticles are relatively stable, and the process is reproducible compared to the microbial process (Kharissova et al., 2013). Moreover, plants are a more suitable option than microbes at a large scale due to their easy availability, lack of requirement for aseptic conditions, rapid synthesis, and easy handling (Sathishkumar et al., 2018). The biosynthesis processes predominantly use water extracts that require normal temperature and air pressure, making the process energy efficient (Liu et al., 2012).

**Fig. 14.2** Flow chart for the biosynthesis of metal nanoparticles



Plant extracts contain various biomolecules that act as a reducing agent (reduction of metallic salt to zero-valent metal nanoparticle) and capping agent/stabilizing agents (provides nanoparticle surface capping) (Mittal et al., 2013; Baker et al., 2013). This is because plant extracts contain different concentrations and combinations of organic reducing agents such as amino acids, vitamins, polysaccharides, polyphenols, organic acids, citric acid, dehydrogenase, flavonoids, functional groups (alcohol, aldehyde, amines, carboxylic acids, ketones and sulfahydryl), heterocyclic compounds hydrogenase, intracellular CO<sub>2</sub>, membrane proteins, NADP reductase, peptide, phenolics, polyphenol, polyols, proteins, quercetin, reducing sugars, saponins, secondary metabolites, sesquiterpenes, tannic acid, tartaric acid and terpenoids (Akhtar et al., 2013; Baker et al., 2013). The general protocol for the biosynthesis of metal nanoparticles using plant extracts is shown in Fig. 14.2.

In the “Reduction” cum “Stabilization” approach, the plant extract containing biomolecules was simply mixed with the corresponding aqueous metal salt solution at room temperature. As a result, bioreduction occurs within a few minutes. The mono- or divalent states of metal ions were converted to a zero-valent state, which was visually observed by the color change of the solution (Malik et al., 2014; Mittal et al., 2013). In the “chelation” cum “stabilization” approach, polyphenols and

flavonoids can act as chelating agents. Due to chelation, the hydroxyl functional group of polyphenols coordinates with the metal ions forming metal-phenolate complexes. Additionally, the complexes undergo thermal decomposition, leading to the formation of metal oxide nanoparticles. In both approaches, the biosynthesized nanoparticles might be capped by the plant extract containing biomolecules, which may further prevent aggregation of nanoparticles. The secondary metabolites of plants that act as capping agents will stabilize the growth of biosynthesized nanoparticles. Amino acids or proteins containing hydroxyl and carbonyl functional groups usually act as capping agents and strongly adhere to the surface of nanoparticles to avoid aggregation with nearby NPs (Imran Din & Rani, 2016; Makarov et al., 2014; Mallikarjuna et al., 2011).

The reduction process of metal ions to metal nanoparticles is affected by different factors, such as the concentration of metallic salt, the concentration of plant extracts, pH, biosynthesis temperature, and reaction time (Baker et al., 2013). Phytosynthesis of nanoparticles mainly depends on the choice of solvents, reducing agent, capping agent, etc. (Alam et al., 2013). The concentration of extract affects the shape of nanoparticles such as triangular, spherical, hexagonal, etc. (Mittal et al., 2013).

The mechanism of nanoparticle synthesis by plant extract was explained on the basis of the positive functional groups present in the plant extract, such as positive amino groups, sulfahydril groups and carboxylic groups, which allowed the metal ions to become more closed to binding sites and approved the reduction of metal ions to their zero-state metal ions, i.e., metal nanoparticle formation at selective pH (Iravani, 2011). The oxidation of the polysaccharide hydroxyl group in the carbonyl group plays an important role in the reduction of metal salt into nanoparticles (Akhtar et al., 2013).

## **Applications of Nanoparticles**

There is a wide range of medical applications that lead to the development of diagnostic, therapeutic and pharmaceutical applications. Due to the similar size of nanoparticles and biological molecules, nanoparticles can be useful for both in vivo and in vitro biomedical research and applications.

### ***Medical Applications***

Drug delivery is the most promising application of colloidal nanoparticles, which are used as carriers of a drug for site-specific delivery. For these applications, the size, charge and surface chemistry of magnetic nanoparticles are important and strongly affect both blood circulation time and bioavailability of the particles within the body.

A glucose-sensing platform using gold and silver nanoparticles was also reported (Aslan et al., 2004).

### ***Antioxidant Activity***

Nanomaterials represent one of the most promising frontiers in research for improved antioxidants. Inorganic metal nanoparticles (NPs) possess intrinsic antioxidant properties due to the surface properties of the material itself (Valgimigli et al., 2018). Nanoparticles have a higher ability to scavenge free radicals due to their characteristic features of small size and high surface area to volume ratio. The antioxidant activity of many metal nanoparticles, such as silver (Sharma et al., 2012; Bunghez et al., 2012) silver, gold (Veeraapandian et al., 2012), silver, copper, iron and zinc (El-Refai et al., 2018), copper (Das et al., 2013), iron and nickel (Saikia et al., 2010), and zinc (Muthuraman et al., 2014), has been reported.

### ***Anticancer Activity***

New tools for cancer imaging and treatment are available to researchers with recent developments in nanotechnology. Nanotechnology has enabled the development of nanoscale devices that can be conjugated with numerous functional molecules simultaneously, comprising anticancer drugs, antibodies, tumor-specific ligands, and imaging probes (Wang et al., 2008). Therapeutic nanoparticles can enhance efficacy and reduce side effects compared with conventional cancer therapeutic drugs (Wang et al., 2009). The high cytotoxic effect against human liver cancer cells (HepG2) was shown by AgNPs biosynthesized using the medicinal plant dandelion *Taraxacum officinale* (Saratale et al., 2018). The reduced cell viability and increased cytotoxicity in HCT-116 colon cancer cells have been shown by biosynthesized gold and silver nanoparticles (Kuppusamy et al., 2016).

The in vitro anticancer efficacy of *guava* and *clove* extracts synthesized from silver and gold NPs against four dissimilar cancer cell lines has been reported (Raghunandan et al., 2011).

A mini-review “On the anticancer activities of silver nanoparticles” summarizing the utilization of AgNPs in cancer treatment is also available in the literature (Abdel-Fattah & Ali, 2018).

### ***Antimicrobial Activity***

The antimicrobial activity of nanoparticles has mostly been considered against human pathogenic bacteria and fungi. The bactericidal activity of nanoparticles in



part depends on their size, shape and stability. The antimicrobial and medicinal properties of silver are well recognized (Prabhu & Poulouse, 2012), whereas silver and its salts have short-term and restricted antimicrobial activity. This problem can be resolved with nanoforms of silver, which can act as suitable antimicrobial agents with inertness and stability (Huang et al., 2011; Ghosh et al., 2012).

Several mechanisms for the microbicidal activity of silver nanoparticles (AgNPs) have been proposed (Dakal et al., 2016): (a) cell membrane structural changes due to deposition of AgNPs on cells (Sondi & Salopek-Sondi, 2004); (b) AgNPs form free radicals (Kim et al., 2007). Ag ions from AgNPs (Feng et al., 2000) deactivating several active enzymes (the thiol group of enzyme interaction with Ag leads to inactivation of enzyme) (Matsumura et al., 2003). Soft acids (Ag) react with soft bases (cells) (Morones et al., 2005). Bacterial signal transduction was altered by AgNPs by destroying cell DNA. Here, soft acids (Ag) and DNA contain sulfur and phosphorus as soft bases (Hatchett & White, 1996).

AgNPs are a real bactericide as AgNP deposition on the bacterial cell wall leads to structural changes that ultimately cause cell death (Sondi & Salopek-Sondi, 2004). Gram-negative bacteria contain less peptidoglycan than Gram-positive bacteria cell walls (Ankanna et al., 2010; Maliszewska & Sadowski, 2009). AgNPs inhibit DNA replication, as fungal DNA forms a complex with AgNPs after penetration (Kolya et al., 2015; Nasrollahi et al., 2011).

Silver and gold nanoparticles have potential applications in antibacterial and antifungal activities against even multidrug registrant pathogenic microbes (Vijayakumar et al., 2017). Furthermore, AgNPs in combination with antibiotics have improved bactericidal activity (Fayaz et al., 2010; Ghosh et al., 2012).

## *Applications in Biosensing*

The current demand for more specific and highly sensitive biomolecular diagnostics can be fulfilled with the application of functionalized MNPs. The type of conjugation between biomolecules and MNPs for biofunctionalization may be electrostatic interactions, chemisorption, and affinity-based (Doria et al., 2012). Nanomaterial optical sensors are a type of chemical sensor classified as luminescence-based and absorbance-based depending on the origin of the optical signal. Luminescence-based sensors are based on the principle of detection by photoluminescence and chemiluminescence, whereas absorbance sensors are based on spectral absorbance changes (Shi et al., 2004). Optical sensors have resistivity to electromagnetic noise, fire resistance and remote control capability and information transfer through an optical fibre network compared to conventional electrochemical sensors (Shi et al., 2004). The optical properties of MNPs vary depending on the spacing between polymeric aggregates, which is explored for colorimetric detection of various kinds of analytes, such as metal ions, organics or biomolecules, within a simple and low-cost way (Shi et al., 2004). The MNP optical properties were also explored to improve the sensitivity of conventional SPR systems. Gold nanoparticle aggregates appear red

when interparticle distances are noticeably greater than the average particle diameter, whereas when interparticle distances are less than average, they appear blue (Kreibig & Genzel, 1985; Yang et al., 1995). A highly selective heterogeneous colorimetric polynucleotide detection method based on mercaptoalkyloligonucleotide-modified gold nanoparticle probes was reported in which the color change is due to a redshift in the surface plasmon resonance of the Au nanoparticles (Elghanian et al., 1997; Reynolds et al., 2000; Storhoff et al., 2000). A label-free optical biosensor (Colorimetric Gold Nanoparticle Sensor) to quantify biomolecular interactions in real time on a surface in a commercially available UV–visible spectrophotometer and of a colorimetric end-point assay using an optical scanner was reported. The spectrophotometric sensor shows concentration-dependent binding and a detection limit of 16 nM for streptavidin (Nath & Chilkoti, 2002). An aggregation-based immunoassay for anti-Protein A using gold nanoparticles was reported. The aggregation of gold nanoparticles coated with protein antigens in the presence of their corresponding antibodies results in an absorption change at 620 nm, which was the basis of the assay monitored with an absorption plate reader. A two orders of magnitude dynamic range and a 1 µg/ml limit of detection for anti-protein A were reported. The new technique could be used for fast, high-throughput screening of antibodies in clinical diagnostic applications (Thanh & Rosenzweig, 2002).

### ***Applications in Catalysis***

Green catalysis involves the use of environmentally benign catalysts for easy removal from the reaction media and is recyclable with high efficiency. The reactivity of nanoparticles depends on their size, shape, composition and surface atomic arrangement. One of the major reasons is the high surface-to-volume ratio of metal nanoparticles, which makes them promising materials for catalysis. Nanoparticle catalysis has been investigated for both homogeneous and heterogeneous systems. A shape-dependent catalytic activity of platinum nanoparticles in colloidal solution was reported (Narayanan & El-Sayed, 2004). The textile, paper, paint, plastic and pharma industries have extensive usage of organic synthetic dyes, which are pollutants (environmentally unsafe) (Bhakya et al., 2015). Organic dyes can be efficiently degraded using biosynthesized nanoparticles (Indana et al., 2016; Ganapuram et al., 2015; Seralathan et al., 2014).

### **Characterization of Nanoparticles**

The reduction of a particular metal nanoparticle can be monitored using the UV–vis visible spectrum, which is a simple tool for the detection of the surface plasma resonance (SPR) property of the metal nanoparticle. XRD analysis confirms the metallic state of a particular metal nanoparticle. The diffraction pattern provides

information on translational symmetry—size and shape of the unit cell from peak positions and information on electron density inside the unit cell. It also provides information on deviations from a perfect particle if the size is less than 100 or 200 nm, extended defects and microstrain from peak shapes and widths. The surface morphology and size of bio-reduced metal nanoparticles were characterized by transmission electron microscopy (TEM) (Ghosh et al., 2011). The FT-IR analysis of metal nanoparticles along with plant extract was performed before and after bio-reduction of nanoparticles to identify the probable biomolecules responsible for the reduction and capping of nanoparticles (Shameli et al., 2012).

## ***Silver Nanoparticles***

Silver nanoparticles (Ag NPs) are one of the most commercialized nanomaterials and have unique optical, electrical and thermal properties. They are widely applied as biocides for their strong antimicrobial activity. Due to its strong antimicrobial activity, silver is used in both medical treatment and industrial processes for its inhibitory effects. Therefore, silver nanoparticles have wide applications, such as diagnostic applications, antibacterial applications, fabrics, manufacturing materials, electronic devices, and solving environmental problems.

The application of nanoparticles depends upon their size, shape, surface morphology, composition and fine structure (Alam et al., 2013). Silver nanoparticles have numerous applications in the fields of dentistry, clothing, catalysis, mirrors, optics, photography, electronics and the food industry (Rai et al., 2009).

The antimicrobial and medicinal properties of silver are recognized (Panáček et al., 2006; Prabhu & Poulose, 2012). The Ag ion and its salt antimicrobial activity is short duration and restricted, which is resolved by using inert and stable nanoforms of silver (Huang et al., 2011; Ghosh et al., 2012). Additionally, AgNPs in combination with antibiotics have improved bactericidal activity (Fayaz et al., 2010; Ghosh et al., 2012). The green synthesis of silver nanoparticles with antibacterial effects has been reported. (Zhang et al., 2020; Gomathi & Suhana, 2021; Nakhjiri et al., 2017; Pethakamsetty et al., 2017; Rautela & Rani, 2019; Premkumar et al., 2018).

AgNP biosynthesis have reported been using plant part extracts as reducing agents. An overview of the synthesis of silver nanoparticles by physical, chemical, and biological synthesis is reviewed by Iravani et al. (2014). AgNPs synthesis, effects of various reaction parameters, characterization techniques, properties and their applications were summarized by Pethakamsetty et al. (2017). Plant extract-mediated AgNP synthesis for antimicrobial applications was reported by Ahemed et al. (2016). A green synthesis of silver nanoparticles using the different plant products of different taxonomic groups with a single step protocol was reported (Gupta & Chauhan, 2017). A summary of the green phytochemical methods used for AgNP biosynthesis is presented in Table 14.1, highlighting different precursors,

**Table 14.1** Different precursors, plants, plant parts, and the ratio of extract and metal salts used for the biosynthesis of AgNPs (Pawar, 2021)

Sr. no.	Precursor salt	Precursor concentration (mM)	Plant source	Plant part used	The ratio of extract: metal salt (ml)	Nanoparticle size (nm)	Applications	Reference
1	Silver nitrate	1	<i>Carica papaya</i>	Green unripe fruits	10:90	10–50	Antibacterial activity	Jain et al. (2009)
2	Silver nitrate	1	<i>Ocimum sanctum</i>	Leaves	0.5:10	3–20	Not reported	Malikarjuna et al. (2011)
3	Silver nitrate	1	<i>Ipomoea batatas</i> L. (Sweet potato)	Tubers	10:90	1–10	Antibacterial activity	Sivakumar et al. (2015)
4	Silver nitrate	1	<i>Dioscorea oppositifolia</i>	Tuber	05:95	17–25	Catalytic activity towards reduction of 4-nitrophenol	Ghosh et al. (2016)
5	Silver nitrate	1	<i>Curcuma longa</i>	Rhizomes	02:08	18 ± 0.5	Antibacterial activity	Alsammaraie et al. (2018)
6	Silver nitrate	1	<i>Momordica cymbalaria</i>	Fruit and tuber	10:90	10–50	Antibacterial and cytotoxic potential	Hemalatha et al. (2019)
7	Silver nitrate	1	<i>Nervilia zeylanica</i> (Vatha Kodi)	Leaf	10:90	10–50	Catalytic activity by reduction of an azo dye methyl orange	Vijayan et al. (2019)
8	Silver nitrate	25	<i>Ocimum sanctum</i>	Stem and root	40:10	10 ± 2	Not reported	Ahmad et al. (2010)
9	Silver nitrate	5	<i>Jatropha curcas</i>	Latex	20:20	10–20	Not reported	Bar et al. (2009)
10	Silver nitrate	1	<i>Chaetomorpha linum</i>	Seaweed	100:20	3–44	Not reported	Kannan et al. (2013)
11	Silver nitrate	1	<i>Solanum xanthocarpum</i> L.	Berries	20:10	10–18	Antimicrobial and Urease Inhibitory Activity	Amin et al. (2012)

12	Silver nitrate	3	<i>Theobroma cacao</i> :	Husk, pulp, seed	20:40	6–18	Antibacterial and photocatalytic activity	Thaikayala et al. (2019)
13	Silver nitrate	1.75	Banana ( <i>Musa paradisiaca</i> )	Peel	1:50	23.7	Antimicrobial activity	Ibrahim (2015)
14	Silver nitrate	1.75	<i>Premna herbacea</i>	Leaves	0.5:10	10–30	Antibacterial activity	Kumar et al. (2013)
15	Silver nitrate	1	<i>Tinospora cordifolia</i>	Stem	15:200	9–12	Antibacterial activity	Singh et al. (2014)

plant parts used for extracts, their concentrations, and applications of biosynthesized AgNPs.

A green AgNP biosynthesis method including AgNO<sub>3</sub> reduction through *papaya* fruit extract was reported. AgNPs are highly toxic against different multidrug-resistant human pathogens (Jain et al., 2009). An eco-friendly simple route for the reduction of silver salt using the leaf broth of *Ocimum sanctum* was reported (Mallikarjuna et al., 2011). Tuber extract of *sweet potato* was used for the biosynthesis of AgNPs under different reaction times. The biosynthesized AgNPs demonstrated strong antioxidant and effective antibacterial activity against human pathogenic bacteria (Sivakumar et al., 2015).

Biosynthesis of gold (AuNPs) and silver (AgNPs) nanoparticles using *Dioscorea oppositifolia* tuber extract is also reported. The biosynthesized AuNPs and AgNPs demonstrated effective catalytic action in 4-nitrophenol reduction by NaBH<sub>4</sub> to 4-aminophenol by pseudofirst-order rate kinetics (Ghosh et al., 2016). Green synthesis of AgNPs was reported using the aqueous extract of turmeric powder, where plant biomaterials were used as reducing agents as well as capping agents. Green synthesized AgNPs show high and efficient antimicrobial activities against two food-borne pathogens (*Escherichia coli* O157:H7 and *Listeria monocytogenes*) (Alsammarraie et al., 2018). Green synthesis of AgNPs by using aqueous fruit and tuber extracts of *Momordica cymbalaria* was reported along with their antibacterial and cytotoxic potential (Hemalatha et al., 2019). A simple, one-pot, inexpensive, and eco-friendly method for the biosynthesis of AgNPs using *Nervalia zeylanica* leaf extract as a reducing and stabilizing agent by a microwave-assisted strategy was reported. The AgNPs showed better scavenging activities than the leaf extract. AgNPs show excellent catalytic activities and antimicrobial activities (Vijayan et al., 2019).

The room-dried stem and root of *Ocimum sanctum* were explored in the biosynthesis of AgNPs at room temperature (Ahmad et al., 2010). *Jatropha curcas* latex was demonstrated as a reducing as well as a capping agent in the biosynthesis of AgNPs (Bar et al., 2009). Seaweed (*Chaetomorpha linum*) extract was explored for the biosynthesis of AgNPs (Kannan et al., 2013). The methanol extract from *Solanum xanthocarpum* berry as a green route for the synthesis of AgNPs was reported. In vitro, the anti-*Helicobacter pylori* activity of synthesized AgNPs demonstrated a stronger anti-*H. pylori* activity compared to AgNO<sub>3</sub> or metronidazole and equally potent to tetracycline (Amin et al., 2012).

Extracts of different parts of *Theobroma cacao*, such as the husk, pulp, and seed, were reported for the biosynthesis of AgNPs. The biosynthesized AgNPs demonstrated nearly 30% methylene blue degradation within 60 min and antibacterial activity against *Bacillus subtilis* and *Escherichia coli* (Thatikayala et al., 2019). The banana peel extract as a reducing and capping agent was demonstrated in the biosynthesis of AgNPs. The biosynthesized AgNPs had effective antibacterial activity against representative pathogens of bacteria and yeast (Ibrahim, 2015). The aqueous leaf extracts of *Premna herbacea*, a local herb of Bodoland, Assam, India, were reported as reducing as well as capping agents in the biosynthesis of

AgNPs. Significant antibacterial activity was found against *Shigella dysenteriae* and *E. coli* (Kumar et al., 2013).

The biosynthesis of AgNPs from the stem of *Tinospora cordifolia* was performed. The biosynthesized AgNPs were evaluated for their antibacterial activity against MDR strains of *P. aeruginosa* (Singh et al., 2014). Marathe et al. (2021) studied the AgNPs biosynthesis using haloalkaliphilic *Streptomyces* spp. The AgNPs were found significantly active against phytopathogenic fungi, *Fusarium verticillioides* and *Ustilago maydis* (Marathe et al., 2021).

## **Copper Nanoparticles (CuNPs)**

Copper nanoparticles have wide applications as heat transfer systems, antimicrobial materials, superstrong materials, sensors and catalysts. Copper nanoparticles are very reactive because of their high surface-to-volume ratio and can easily interact with other particles (Subhankari & Nayak, 2013; Yallappa et al., 2013). Researchers have reported green biosynthesis of CuNPs with pharmacological activities such as antibacterial, antioxidant and catalytic activities (Thiruvengadam et al., 2019; Gondwal & Joshi nee Pant, 2018; Aher et al., 2017; Tahvilian et al., 2019; Harikumar & Aravind, 2016). One-step biofabrication of copper nanoparticles from *Aegle marmelos* aqueous leaf extract and evaluation of its anti-inflammatory and mosquito larvicidal efficacy was reported (Angajala et al., 2014). Antidiabetic and antioxidant properties of copper nanoparticles synthesized by the medicinal plant *Dioscorea bulbifera* were reported (Ghosh et al., 2015). The green synthesis of copper oxide nanoparticles using *Caesalpinia bonducella* seed extract and evaluation of electrocatalytic properties for electrochemical detection of riboflavin (Vitamin B2) was reported (Sukumar et al., 2020).

## **Biosynthesis of CuNPs Using Plant Extracts**

A summary of the green phytochemical methods used for CuNP biosynthesis is using extracts of several Apocynaceae and other plants is presented in Table 14.2, showing different precursors, plant parts, combinations of extracts, their concentrations, and applications of CuNPs.

A study indicated the use of an aqueous extract of *Abutilon indicum* for the synthesis of CuO NPs by a superficial method (solution combustion method), which had considerable antioxidant activity and antibacterial activity against *Klebsiella* and *Bacillus subtilis* (Ijaz et al., 2017). CuNP biosynthesis was carried out after the addition of *Alchornea laxiflora* aqueous leaf extract to the copper sulfate solution. The superior catalytic activity of the biosynthesized CuNPs was observed compared to conventional acetic acid in the oxidation of dibenzothiophene (DBT). Biosynthesized CuNPs may be an economical substitute material for oxidative desulfurization in the oil industry (Olajire et al., 2018). *Asparagus adscendens* leaf

**Table 14.2** Different precursors, plants, plant parts, and the ratio of extract and metal salts used for the biosynthesis of CuNPs (Pawar, 2021)

Sr. no	Precursor concentration (mM)	Precursor	Plant source	Plant part used	Extract conc (ml)	Metal salt (ml)	Nanoparticle size (nm)	Application	Reference
1	–	Cu (NO <sub>3</sub> ) <sub>2</sub> ·3H <sub>2</sub> O	<i>Abutilon Indicum</i>	Leaf extract	0.3	–	16.78	Antimicrobial, antioxidant and photocatalytic dye degradation activities	Ijaz et al. (2017)
2	1	CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>Alchornea laxiflora</i>	Leaf extract	20	80	3.29	Catalytic activity	Olajire et al. (2018)
3	1	CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>Asparagus adscendens Roxb.</i>	Root and Leaf extract	10	100	50–60	Antimicrobial activities	Thakur et al. (2018)
4	10	Cu (CH <sub>3</sub> COO) <sub>2</sub> ·H <sub>2</sub> O/methanol	<i>Borreria hispida</i>	Linn. extract	50	100	84–158	Antioxidant activity	Venugopalan et al. (2020)
5	100	CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>Citrus medica</i>	Linn. (Idilimbu) juice	–	–	10–60	Antimicrobial activity	Shende et al. (2015)
6	10	Copper sulfate	<i>Cissus amotiana</i>	Plant extract	10	90	60–90	Antibacterial and antioxidant activity	Rajeshkumar et al. (2019)
7	1	Copper (II) sulfate pentahydrate	<i>Citrus sinensis</i>	Aqueous fruit extract	100	–	10.2	Antibacterial potentials	Jahan et al. (2021)
8	20	Cu(NO <sub>3</sub> ) <sub>2</sub>	<i>Cocoa pod</i>	Cocoa pod extract	1.5 gm	–	20–50	Catalytic activity	Nguyen et al. (2020)
9	5	CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>Ripened Duranta erecta</i>	Aqueous fruit extract	15	85	70	Catalytic reduction of carcinogenic azo dyes Methyl orange and Congo red	Ismail et al. (2019)



10	40	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	<i>Cordia myxa</i>	L. Leaves	100	–	20–35	Reducing, stabilizing and capping agent	Thamer et al. (2018)
11	100	Copper acetate dihydrate solution	<i>Curcuma longa</i>	Curcuma longa extract	50	100	5–20	Antimicrobial activity	Jayarambabu et al. (2020)
12	5	Copper nitrate	<i>Eryngium campestre</i>	Leaf extract	10	90	50–60	Not reported	Khodaie and Ghasemi (2018)
13	40	Copper (II) ion	<i>Moringa oleifera</i>	Leaves extract	80	20	35.8–49.2	Antioxidant and Antimicrobial Activities	Das et al. (2020)
14	–	Copper sulfate pentahydrate	<i>Seedless dates</i>	Seedless dates extract	100	10	78	Not reported	Mohamed (2020)
15	25	$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , Cu $(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$ and Cu $(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$	<i>Vaccinium species</i>	Bilberry fruits	9	1	2–10	Antimicrobial activity	Benassai et al. (2021)
16	10	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	<i>Eucalyptus globulus L.</i>	Leaves extract	10	100	10–130	Against fruit rot pathogen of chilli crop	Iliger et al. (2021)
17	10	$(\text{Cu}(\text{NO}_3)_3 \cdot 3\text{H}_2\text{O})$	<i>Eryngium caucasicum Trautv</i>	Leaves extract	5	50	40	Antioxidant and antimicrobial properties	Hasheminya and Dehghannya (2020)
18	100	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	<i>Ginger Lily</i>	Leaves extract	100	–	40	Antibacterial activities	Nagore et al. (2020)
19	–	$\text{CuSO}_4$	<i>Hawthorn berries</i>	Hawthorn berries extract	10	30	200	The antimicrobial activities	Dlugosz et al. (2020)
20	40	Copper chloride	<i>Cochlospermum gossypium</i>	Bark extract	–	–	14	Antimicrobial property, biomedical application	Seku et al. (2018)

(continued)

Table 14.2 (continued)

Sr. no	Precursor concentration (mM)	Precursor	Plant source	Plant part used	Extract conc (ml)	Metal salt (ml)	Nanoparticle size (nm)	Application	Reference
21	3	Copper chloride	<i>Jatropha curcas</i>	Leaves extract	20	80	10	CT-DNA binding and photocatalytic activity	Ghosh et al. (2020)
22	3	Cu (CH <sub>3</sub> COO) <sub>2</sub> ·H <sub>2</sub> O	<i>Magnolia Champaca</i>	Floral Extract	10	–	20–40	Antioxidant & Toxicity Assay using Danio Rerio	Santhosh Kumar and Shanmugam (2020)
23	–	Copper sulfate	<i>Mitragyna parvifolia</i>	Plant Bark extract	10	90	12–23	Antimicrobial study	Kotval et al. (2018)
24	3	CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>Thymus vulgaris</i> L.	Leaf extract	50	50	23–94	Catalytic activity	Issaabadi et al. (2017)
25	1	CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>Phoenix Dactylifera</i> L.	Leaves extract	1	100	22–28	Medical application	Berra et al. (2018)
26	1	CuSO <sub>4</sub>	<i>Plectranthus Amboinicus</i>	Leaves Extract	2	10	21.74	–	Parthasarathy et al. (2020)
27	–	CuCl <sub>2</sub>	<i>Rheum palmatum</i> L.	Root extract	–	30	10–20	Catalytic activity	Bordbar et al. (2017)
28	–	Copper sulfate pentahydrate	<i>Citrus limon</i>	Fruit extract	100	–	5–28	Antibacterial activity	Amer and Awwad (2020)
29	5	CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>Syzygium alternifolium</i>	Stem bark	10	100	5–13	Antimicrobial and anticancer activity	Yugandhar et al. (2017)
30	1	Cu (CH <sub>3</sub> COO) <sub>2</sub> ·H <sub>2</sub> O	<i>Syzygium aromaticum</i>	Bud extract	10	50	20	Physical, optical, antimicrobial properties, Fungicidal activity	Rajesh et al. (2018)
31	25	Copper chloride	<i>Tinospora cardifolia</i>	Leaves extract	2	10	63.3	Antimicrobial evaluation	Sharma et al. (2019)

32	7.5 × 10 <sup>-3</sup> M	CuCl <sub>2</sub> ·2H <sub>2</sub> O	<i>Azadirachta indica</i>	Leaves extract	–	–	48	–	Nagar and Devra (2018)
33	3	Cu (CH <sub>3</sub> COO) <sub>2</sub> ·H <sub>2</sub> O	<i>Eclipta prostrata</i>	Leaves extract	20	80	23–57	Antioxidant and cytotoxic activities	Chung et al. (2017)
34	1	Copper sulfate	<i>Nerium oleander</i>	Leaves extract	100	100	21	–	Sebeia et al. (2019)
35	5	CuCl <sub>2</sub> ·2H <sub>2</sub> O	<i>Plantago asiatica</i>	Leaf extract	100	20	7–35	Cyanation of aldehydes	Nasrollahzadeh et al. (2017)
36	20	Copper sulfate	<i>Ziziphus spinachristi</i> (L.) Willd	Fruit extract	–	–	5–20	Adsorption of triphenylmethane dye and antibacterial assay	Khani et al. (2018)

and root extracts were used for the biosynthesis of CuNPs. The antimicrobial efficacy of CuNPs was established against Gram-negative and Gram-positive pathogenic microbes (Thakur et al., 2018).

The crude methanol extract of the whole dried plant *Borreria hispida* (Linn) was used for the synthesis of CuNPs. The antioxidant efficacy of the CuNPs was found to be higher than that of the plant crude extract (Venugopalan et al., 2020). An innovative biogenic synthesis of CuNPs was studied using citron juice. Its efficacy against a wide variety of microorganisms, such as *Escherichia coli*, followed by *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Propionibacterium acnes*, and *Salmonella typhi*, was studied (Shende et al., 2015). *Cissus arnotiana* extract was reported for the biosynthesis of CuNPs. The biosynthesized CuNPs demonstrated better antibacterial activity against Gram-negative bacteria such as *E. coli* and antioxidant properties than the standard ascorbic acid (Rajeshkumar et al., 2019). The use of orange *Citrus sinensis* fruit extract for the biosynthesis of CuNPs through microwaves was reported. These biosynthesized CuNPs demonstrated their effectiveness in the management of human pathogens, i.e., *Escherichia coli* and *Staphylococcus aureus* (Jahan et al., 2021). Biosynthesis of CuNPs using *cocoa pod* extract as a reducing agent, an efficient and green technique, was reported. CuNPs loaded on different inert supports were tested for catalytic deep oxidation of aromatic hydrocarbons (AHs) (Nguyen et al., 2020).

The fruit extract of *Duranta erecta* was reported for the green synthesis of CuNPs. In the presence of  $\text{NaBH}_4$  as a reducing agent, CuNPs exhibit outstanding catalytic reduction for methyl orange and Congo red. They can be used to purify natural water from organic effluents (Ismail et al., 2019). An aqueous extract of *Cordia myxa* L leaves was used as a reduction, stabilization and capping agent, and a simple and low-cost approach to CuNP preparation was reported (Thamer et al., 2018). A novel green route for the synthesis of CuNPs was reported using an aqueous extract from a *Curcuma longa* solution. CuNPs capped with *C. longa* extract have shown promising antibacterial activity against Gram-positive and Gram-negative bacteria. Compared to Gram-negative bacteria, the inhibition zone of CuNPs for Gram-positive bacteria is greater (Jayarambabu et al., 2020). *Eryngium campestre* leaf extract was used to investigate the synthesis of CuNPs. The synthesis of CuNPs was completed with pH 6, temperature 35°C and time 80 min (Khodaie & Ghasemi, 2018). A hydroalcoholic extract of *Moringa oleifera* leaves was reported to biosynthesize CuNPs. The synthesized CuNPs have antioxidant, antibacterial, and antifungal properties (Das et al., 2020). *Seedless date* extract contains a high content of phenolics, and flavonoids were used to biosynthesize Cu/Cu<sub>2</sub>O NPs (Mohamed, 2020). Green synthesis of CuNPs using aqueous extracts of *bilberry* waste residues from the production of fruit juices and nonedible false bilberry fruits was tested. CuNPs can be considered a cost-effective sanitization method with a broad range of action from green synthesis (Benassai et al., 2021).

A simple, beneficial and green method for CuNP biosynthesis using *Eucalyptus* and *Mint* plant extracts was reported. The CuNPs were tested and proven to be highly successful for their efficacy against the fungus *C. capsici* (Iliger et al., 2021). An aqueous extract of *E. caucasicum Trautv* leaves was used as a reducing agent for

green and environmentally friendly CuNP biosynthesis. Antibacterial characterization of CuNPs revealed remarkable antibacterial properties against *E. coli*, *S. typhimurium*, *B. cereus*, and *S. aureus* (Hasheminya & Dehghannya, 2020). Using ginger lily leaf extract as a natural reducing and stabilizing agent, a simple, renewable, eco-caring technique was reported to phylogenically synthesize luminescent CuO NPs (Nagore et al., 2020). A green method for AgNP and CuNP preparation was reported using water and ethanol extracts of dried hawthorn fruit extract as a stabilizer and reductant. They have antibacterial activity against selected pathogenic microorganisms (*A. niger*, *E. coli* and *S. cerevisiae*) (Długosz et al., 2020). An eco-friendly approach for CuNP synthesis was carried out by hydrothermal processes using carboxymethyl gum konagogu as a capping agent from the bark of *Cochlospermum gossypium*. CuNPs had excellent antibacterial and antifungal activity against pathogenic strains (Seku et al., 2018).

A simple green chemistry method for CuNP biosynthesis using *J. curcas* leaf extract was reported. CuNPs have potential photocatalytic activity against methylene blue (MB) (Ghosh et al., 2020). Biosynthesis of CuO NPs using *Magnolia champaca* floral extract as a reducing agent and stabilizing agent was reported (Santhosh Kumar & Shanmugam, 2020). *Mitragyna parvifolia* plant aqueous bark extract was used as a reducing agent for the green synthesis of CuNPs. The antibacterial activity of CuNPs was determined (Kotval et al., 2018). A cost-effective and environmentally friendly technique for the synthesis of bentonite-supported copper nanoparticles (bentonite/CuNPs) using *Thymus vulgaris* leaf extract as a mild, sustainable and nontoxic reducing agent and an efficient stabilizer without adding any surfactants was reported. They had excellent catalytic activity in the presence of NaBH<sub>4</sub> in water at room temperature for Congo Red and Methylene Blue degradation. (Issaabadi et al., 2017). Copper oxide nanoparticles were synthesized using *Phoenix dactylifera* L leaves from the El Oued area (Southeast of Algeria) (Berra et al., 2018). The use of *Plectranthus amboinicus* extracts as reducing agents for CuNP biosynthesis was explored (Parthasarathy et al., 2020). A green method was used to prepare CuO NPs using *Rheum palmatum* L. root extract as a reducing and stabilizing agent. Very mild reaction conditions, cleanliness, no need for organic solvents, poisonous and unsafe reagents, and simplicity are some of the many benefits of this process. The CuO NPs demonstrated strong activity as a heterogeneous catalyst in the reduction of 4-nitrophenol, methylene blue, and rhodamine B at ambient temperature. (Bordbar et al., 2017).

Green synthesis of CuNPs without any harmful materials using *Citrus limon* fruit extract was reported. CuNPs demonstrated excellent antibacterial activity against pathogenic *E. coli* and *S. aureus* bacteria (Amer & Awwad, 2020). A cost-effective, eco-friendly, green approach for CuO NP synthesis from *S. alternifolium* stem bark extract was reported, which had antimicrobial and anticancer action (Yugandhar et al., 2017). Using *Syzygium aromaticum* bud extract as a reducing agent, CuNPs were synthesized at room temperature through a simple, fast and eco-friendly pathway. The CuNPs showed excellent bactericidal action against *Bacillus* spp. and marked fungicidal activity against *Penicillium* spp. of microorganisms (Rajesh et al., 2018).

The biosynthesized CuNPs capped with *Tinospora cardifolia* were formulated and integrated into the fabric. The antimicrobial behavior of formulated CuNPs along with fabric was evaluated, which showed that the percent efficacy of copper nanoparticle-coated fabric for Gram-positive and Gram-negative bacteria was 101% and 74% at the maximum concentration, respectively (Sharma et al., 2019). The CuNPs were biosynthesized using *Azadirachta indica* leaf broth as a reducing agent in which synthesized CuNPs were strongly crystalline and cubical (Nagar & Devra, 2018). The *E. prostrata* leaf extract was used as a reducing agent for biosynthesizing CuNPs. The biosynthesized CuNPs showed significant antioxidant potential, whereas cytotoxicity against tested HepG2 cells has been demonstrated in in vitro anticancer studies (Chung et al., 2017).

The biological extract of *Nerium oleander* leaves was used as a reducing agent for the biosynthesis of CuNPs. They were used as nanoadsorbents for methylene blue and crystal violet (Sebeia et al., 2019). Green synthesis of CuNPs by in situ CuCl<sub>2</sub> reduction using *Plantago asiatica* leaf extract as a reductant and stabilizer was explored. The catalytic activity of the CuNPs was evaluated (Nasrollahzadeh et al., 2017). A green chemistry approach for Cu-NP biosynthesis using *Ziziphus spinachristi* fruit extract was explored. They act as a green and effective nanoadsorbent for crystal violet and also had antibacterial activity (Khani et al., 2018).

## ***Ferrous Nanoparticles***

Metallic iron oxide nanoparticles have a variety of scientific and technological applications, such as biosensors, antimicrobial activity, food preservation, magnetic storage media, ferrofluids, magnetic refrigeration, magnetic resonance imaging, hyperthermic cancer treatments, cell sorting and targeted drug delivery (Behera et al., 2012; Kumar, Singh, & Pandey, 2015; Kumar, Suresh, Nagabhushana, et al., 2015; Wei et al., 2016; Nagajyothi et al., 2017; Asghar et al., 2018; Devi et al., 2019). A summary of the green phytochemical methods used for FeNP biosynthesis is presented in Table 14.3, which summarizes the different precursors, plant parts used for biosynthesis, extracts, and their concentrations as well as the application of FeNPs.

Iron nanoparticles synthesized by the green method were found to be toxic to bacteria such as *Escherichia coli* and *Staphylococcus aureus* (Pandey et al., 2012). Green synthesis of iron oxide nanoparticles using *Carica papaya* leaf extract was reported for photocatalytic degradation of remazol yellow RR dye and antibacterial activity (Bhuiyan et al., 2020). Iron nanoparticles were applied for the remediation of heavy metals from soil and disinfection of water (Mahdavi et al., 2013). Membrane-supported nanoparticles were used for the degradation of the common and highly important pollutant trichloroethylene (TCE) (Kharissova et al., 2013).

Using *Agrewia optiva* and *Prunus persica* leaf extracts, biogenic synthesis of FeNPs was carried out using a simple, nontoxic, sustainable, low-cost, quick and environmentally friendly process (Mirza et al., 2018). The FeNPs were

**Table 14.3** Different precursors, plants, plant parts, and ratios of extract and metal salts used for the biosynthesis of FeNPs (Pawar, 2021)

Sr. no	Precursor	Precursor concentration (mM)	Plant used	Plant part used	Ratio of extract to metal salt (ml)	Nanoparticle size (nm)	Applications	Reference
1	FeCl <sub>2</sub> ·4H <sub>2</sub> O	100	<i>Agrewia optiva</i> and <i>Prunus persica</i>	Leaves	30	15–60 13–70	Antioxidant activity, antibacterial activity	Mirza et al. (2018)
2	FeCl <sub>3</sub>	10	<i>Cinnamomum Verum</i>	Bark	1:1	20–50	Antioxidant, anti-inflammatory and anti-diabetic	Sivakami et al. (2020)
3	Fe <sub>3</sub> O <sub>4</sub>	---	<i>Padina pavonica</i> <i>Sargassum acinarium</i>	Seaweeds	1:1	10–19.5 21.6–27.4	Biomedical uses	El-Kassas et al. (2016)
4	FeSO <sub>4</sub> ·7H <sub>2</sub> O	100	<i>Mangifera Indica</i> <i>Murraya Koenigii</i> <i>Azadiracta indica</i> <i>Magnolia champaca</i>	Leaves	1:2	100–150	For domestic wastewater treatment	Devatha et al. (2016)
5	FeSO <sub>4</sub>	20	<i>Mimosa pudica</i>	Root	3	60–80	Antibacterial activity targeted drug delivery	Nirimathee et al. (2016)
6	FeSO <sub>4</sub>	1	<i>Ruellia tuberosa</i>	Leaf	50	20–80	Antibacterial activity	Vasantharaj et al. (2019)
7	FeCl <sub>3</sub> ·6H <sub>2</sub> O	100	<i>Falcaria vulgaris</i>	Leaf	9	25	Antioxidant, noncytotoxic, skin wound healing, antifungal, and antibacterial	Zangeneh et al. (2019)
8	FeCl <sub>3</sub>	10	<i>Eucalyptus globulus</i>	Leaf	20	100		Balanurugan et al. (2014)
9	FeCl <sub>2</sub>	1	<i>Punica granatum</i>	Seed	1:2	25–55	Photocatalytic activity, antioxidant and antimicrobial activities	Bibi et al. (2019)

(continued)

Table 14.3 (continued)

Sr. no	Precursor	Precursor concentration (mM)	Plant used	Plant part used	Ratio of extract to metal salt (ml)	Nanoparticle size (nm)	Applications	Reference
10	FeSO <sub>4</sub>	10	<i>Lawsonia inermis</i> and <i>Gardenia jasminoides</i>	Leaves	50	21 and 32	Antibacterial activity	Naseem and Farrukh (2015)
11	FeCl <sub>2</sub>	500	<i>Terminalia bellirica</i> (TB); <i>Moringa oleifera</i> fruit (MOF) and <i>Moringa oleifera</i>	Fruit and Leaves		21.32 and 45	Antimicrobial and anti-oxidant activity	Jegadeesan et al. (2019)
12	FeCl <sub>2</sub> ·4H <sub>2</sub> O	100	<i>Nettle</i> and <i>Thyme</i>	Leaves	Ratio of 2:3	20–80	Antibiotic activity	Leili et al. (2018)
13	FeCl <sub>3</sub> ·6H <sub>2</sub> O	100	<i>Couroupita guianensis</i> Aubl.	Fruit	1:1	17 ± 10	Antimicrobial activity	Sathishkumar et al. (2018)
14	Ag- Fe <sub>2</sub> O <sub>3</sub>	100	<i>Psidium guajava</i>	Leaf	1:1	50–90	Not reported	Biswal et al. (2020)
15	FeCl <sub>3</sub>	100	<i>Moringa oleifera</i>	Leaf and Seed	–		Antibacterial activity	Katata-Seru et al. (2018)
16	FeCl <sub>2</sub> ·4H <sub>2</sub> O	100	<i>Rosa damascene</i> 33 (RD), <i>Thymus vulgaris</i> (TV), and <i>Urtica dioica</i> (UD)	Leaves	–	100	Antioxidant activity	Fazlzadeh et al. (2017)
17	—	–	<i>Chromolaena odorata</i>	Root	–	5.6–16.8	Magnetic resonance imaging, cancel cell amelioration, biosensors	Nnadozie and Ajibade (2020)
18	FeCl <sub>3</sub> ·6H <sub>2</sub> O	200	<i>U. dioica</i>	Leaves	9	21–71	Anti-inflammatory, anti-hyperglycaemic, antioxidant, diuretic, natriuretic, antitumor, and analgesic	Ebrahiminezhad et al. (2017)



19	FeCl <sub>3</sub>	10	<i>Trigonella foenum-graecum</i>	Seed	5	7–14	Antibacterial activity	Radini et al. (2018)
20	FeCl <sub>3</sub> ·6H <sub>2</sub> O	100	<i>Laurus nobilis</i> L.	Leaves	30	8.03 ± 8.99	Antimicrobial activity	Jamzad and Kamari (2020)
21	FeCl <sub>3</sub>	400	<i>Phoenix dactylifera</i>	Leaf	50	2–30	Antioxidant activity	Abdullah et al. (2020)
22	FeCl <sub>3</sub> ·6H <sub>2</sub> O	100	<i>Daphne mezereum</i>	Leaf	9	6.5–14.9	Not reported	Beheshtkhoo et al. (2018)
23	FeCl <sub>3</sub>	1	<i>Murrayakoenigii</i>	Leaves	10	12	Not reported	Prasad et al. (2017)
24	FeCl <sub>3</sub>	10	<i>Green tea</i>	Leaves	1:1	116	Antioxidant	Gottimukkala et al. (2017)
25	Fe (NO <sub>3</sub> ) <sub>3</sub> ·9H <sub>2</sub> O	–	<i>Platanus orientalis</i>	Leaf	10	38	Antifungal activity, antimicrobial	Devi et al. (2019)
26	FeSO <sub>4</sub>	–	<i>green tea</i>	green tea extract	1:2	40–50	Not reported	Huang et al. (2014)
27	FeCl <sub>3</sub>	–	<i>Eichhornia crassipes</i>	Leaf	1:1	20–80	Antioxidant activity.	Wei et al. (2017)
28	FeSO <sub>4</sub> ·7H <sub>2</sub> O	–	<i>Eriobotrya japonica</i>	Leaves	1:1	171.2	Not reported	Önal et al. (2017)
29	FeCl <sub>3</sub>	100	<i>Nephrolepis auriculata</i>	Plant	2:1	40–70	Not reported	Yi et al. (2019)
30	FeCl <sub>3</sub>	100	<i>Cassia fistula</i> Linn	Leaf	50	20–50	Anti-inflammatory and antibacterial activity	Ranjith et al. (2018)
31	FeSO <sub>4</sub>	100	<i>eucalyptus</i>	Leaf	2:1	20–80	Antioxidant activity	Wang et al. (2014)
32	FeCl <sub>3</sub> ·6H <sub>2</sub> O	10	<i>Lagenaria siceraria</i>	Leaves	1:1	30–100	Antimicrobial Activity	Kanagasubbulakshmi and Kadirvelu (2017)
33	FeCl <sub>3</sub>	500	<i>Amaranthus dubius</i>	Leaves	40	43–220	Antioxidant activity	Harshiny et al. (2015)
34	FeCl <sub>3</sub>	1	<i>Azadirachta indica</i>	Leaf	1:1	50–100	Antifungal, antidiabetic, antibacterial,	Pattanayak and Nayak (2013)
35	FeCl <sub>3</sub> ·6H <sub>2</sub> O	–	<i>Aerva lantia</i>	Flower	50	38	Anti-inflammatory	Clarina et al. (2018)

biosynthesized using *Cinnamomum verum* bark extract as a reducing agent reported for biomedical applications. The antioxidant, anti-inflammatory and antidiabetic assays demonstrated the productive actions of low concentration Fe NPs (Sivakami et al., 2020). The  $\text{Fe}_3\text{O}_4$ -NPs were synthesized using a completely green biosynthetic method by reduction of ferric chloride solution using *brown seaweed* water extracts. The biosynthesized  $\text{Fe}_3\text{O}_4$ -NPs showed bioremoval capacity for Pb (91%) (El-Kassas et al., 2016). A quick, fast and eco-friendly method was used to synthesize FeNPs using different leaf extracts, such as *Mangifera indica*, *Murraya Koenigii*, *Azadiracta indica* and *Magnolia champaca*, which were used for domestic wastewater treatment. *Azadiracta indica* showed 98.08% phosphate, 84.32% ammonia nitrogen and 82.35% chemical oxygen demand removal among the various plant-mediated synthesized iron nanoparticles (Devatha et al., 2016). Superparamagnetic FeO NPs have been synthesized successfully from the root extract of *M. Pudica* (Niraimathee et al., 2016).

FeONPs were synthesized using *Ruellia tuberosa* leaf aqueous extract as a reducing and stabilizing agent. Higher antibacterial activity against *Escherichia coli* and *Klebsiella pneumoniae* and less antibacterial activity against *Staphylococcus aureus* were shown by FeONPs. The synthesized FeONP photocatalytic potential was demonstrated by up to 80% of the degrading crystal violet dye under solar irradiation (Vasantharaj et al., 2019). The potential of *F. vulgaris* aqueous extract for the biosynthesis of FeNPs was explored. It has useful antioxidant, noncytotoxic, skin wound healing, antifungal, and antibacterial impacts (Zangeneh et al., 2019). The  $\beta$ -iron oxide NPs were synthesized by adding the leaf extract of *Eucalyptus globulus* to an aqueous solution of ferric chloride. This method was capable of producing crystalline  $\beta\text{-Fe}_2\text{O}_3$  (Balamurugan et al., 2014).  $\text{Fe}_2\text{O}_3$  NPs were developed using pomegranate (*Punica granatum*) seed extracts via the green path. The synthesized  $\text{Fe}_2\text{O}_3$  showed excellent photocatalytic activity with a maximum degradation of 95.08% (Bibi et al., 2019). Green biosynthesis of FeNPs using *Lawsonia inermis* and *Gardenia jasminoides* leaf extracts showed excellent antibacterial activity against the human pathogens *Escherichia coli* and *Staphylococcus aureus* (Naseem & Farrukh, 2015). A simple biogenic synthesis of FeNPs from three plant sources using aqueous extracts, *Terminalia bellirica* (TB), *Moringa oleifera* fruit (MOF), and *Moringa oleifera* leaves (MOL), was carried out. Biosynthesized FeNPs have excellent thermal, antimicrobial and antioxidant properties (Jegadeesan et al., 2019). For the synthesis of novel nano zero-valent FeNPs for the removal of cephalixin adsorption, *nettle* and *thyme* leaf extracts have been used. These materials could be considered promising adsorbents for the removal of antibiotics and probably other organic contaminants from aqueous solutions (Leili et al., 2018).

To produce magnetic  $\text{Fe}_3\text{O}_4$  nanoparticles using edible *C. guianensis* (CGFE) aqueous fruit extract, a sustainable green chemistry approach was been developed. The synthesized CGF $\text{Fe}_3\text{O}_4$  NPs induce a substantially enhanced cytotoxic effect against HepG<sub>2</sub> cells at a minimum dose of 44.5  $\mu\text{g}/\text{ml}$  (Sathishkumar et al., 2018).

For the preparation of  $\alpha\text{-Fe}_2\text{O}_3$ -Ag nanocomposite, guava (*Psidium guajava*) leaf extract was used, which can be used as an adsorbent for the decontamination of medium-sized aqueous chromium (VI) ions (Biswal et al., 2020). The green

synthesis of FeNPs using *Moringa oleifera* leaf and seed extracts was reported by mixing different ratios of plant extracts with iron chloride solution. Furthermore, the use of biosynthesized FeNPs for the removal of nitrate ions ( $\text{NO}_3$ ) from surface and groundwater was explored (Katata-Seru et al., 2018). Nano zero-valent iron (NZVI) particles were synthesized from plant extracts such as *Rosa damascene*, *Thymus vulgaris*, and *Urtica dioica*, which have the potential to effectively adsorb hexavalent chromium even at high concentration levels (Fazlzadeh et al., 2017). FeNPs were prepared using the root extract of *Chromolaena odorata* (Nnadozie & Ajibade, 2020). By using *U. Dioica* leaf extract as a capping and shielding agent, zero-valent FeNPs have been synthesized. (Ebrahimezhad et al., 2017). *Trigonella foenum-graecum* was found in the seed extract and was used for biosynthesis of FeNPs, which was very efficient for methyl orange dye degradation. The minimum inhibitory concentrations (MICs) of Fe NPs for *E. coli* and *S. aureus* were 32  $\mu\text{g/ml}$  and 64  $\mu\text{g/ml}$ , respectively (Radini et al., 2018). The FeONPs were synthesized in the phase of hematite ( $\alpha\text{-Fe}_2\text{O}_3$ ) by the aqueous extract of *Laurus nobilis L.* leaves in a simple and eco-friendly method. The nanoparticles were moderately selective on *Listeria monocytogenes*, a Gram-positive bacterium, and *Aspergillus flavus* and *Penicillium spinulosum* fungi (Jamzad & Bidkorpeh, 2020). The biosynthesis of stable FeNPs was carried out using *Phoenix dactylifera L.* as a reducing agent. The antioxidant and antiradical activities of the bio-reduced iron nanoparticles were measured (Abdullah et al., 2020). FeNPs were synthesized using *Daphne mezereum* aqueous leaf extract as a reducing and stabilizing agent. The synthesized FeNPs were more effective in degrading methyl orange dye catalysts (Beheshtkhoo et al., 2018). *Murraya konenigii* leaf extract was used to form an RGO/ $\text{Fe}_3\text{O}_4$  nanocomposite that was capable of removing Pb(II) from aqueous solution (Prasad et al., 2017). *Camellia sinensis* leaf extract was used to synthesize FeNPs in an environmentally friendly and long-term manner (Gottimukkala et al., 2017). An aqueous phase green synthesis of FeNPs using *Platanus orientalis* leaf extract as a reduction and capping agent was performed. The NPs were stable, crystalline, and impurity-free and had effective antimicrobial activity. The antifungal action of the FeNPs against *M. Piriformis* was 1.6-fold higher than that against *A. niger* (Devi et al., 2019).

The green synthesis of FeNPs using tea extracts was performed, and the dye degradation capacity was evaluated. The high degradation of malachite green (81.56%) was obtained, which may be due to the high polyphenol/caffeine content in green tea extracts (Huang et al., 2014). The biosynthesis of FeNPs using aqueous extracts of *Eichhornia crassipes* was reported, which showed 89.9% Cr (VI) extraction (Wei et al., 2017). The green synthesis of FeNP aqueous extract from *Eriobotrya japonica* leaves was used to biosynthesize FeNPs could be an excellent catalyst for the decolorization and removal of toxic dyes from aqueous solutions (Önal et al., 2017). Using *Nephrolepis auriculata* extracts, the efficient synthesis of FeNPs was reported, which showed 90.93% Cr(VI) removal (Yi et al., 2019). FeNPs were successfully synthesized using an aqueous solution of *Cassia fistula* Linn leaf extract by reducing the ferric chloride solution. The procedure was nonlethal, straightforward, environmentally friendly, and relatively inexpensive. The synthesized FeNPs had antibacterial activity against pathogenic bacteria such as

*Staphylococcus* sp, *Pseudomonas* sp, *Bacillus* sp and *Klebsiella* sp. (Ranjith et al., 2018). The spheroidal FeNPs were biosynthesized via a one-step process using *eucalyptus* leaf extracts at room temperature. FeNPs showed that 71.7% of total N and 84.5% of COD were extracted and used for in situ eutrophic wastewater remediation (Wang et al., 2014).

The *Lagenaria siceraria* leaf extract was capable of green synthesis of  $\text{Fe}_3\text{O}_4$ -NPs. The antimicrobial properties of synthesized  $\text{Fe}_3\text{O}_4$ -NPs were assessed against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* (Kanagasubbulakshmi & Kadirvelu, 2017). The biosynthesis of FeNPs was reported using aqueous extracts of *Amaranthus dubius* leaves reduced from ferric chloride. Synthesis of *A. dubius* leaf extract was a safer alternative to toxic reduction, including  $\text{NaBH}_4$  (Harshiny et al., 2015). Nanoscaled zero-valent FeNPs were synthesized under atmospheric conditions from *Neem* plant leaf extract (Pattanayak & Nayak, 2013). The eco-friendly chemical-free biosynthesis of FeNPs using *polpala* flower extract as a reducing agent was simple and green and used as a recyclable magnetic nanocatalyst for organic reactions (Clarina et al., 2018).

### **Gold Nanoparticles (AuNPs)**

Gold nanoparticles (AuNPs) are widely studied for their biological and diagnostic properties. Gold nanoparticles are considered promising new metal NPs because of their efficient use in catalysis, gene expression profiling, optoelectronics, optics, and disease diagnostics and treatment. A number of medicinal plants have been used for the biosynthesis of AuNPs. AuNPs are made using either phytochemicals or other extract constituents that are not stable for a long time. Table 14.4 provides a detailed outlook of AuNP biosynthesis using various Indian medicinal plants.

### **Zinc Nanoparticles (ZnNPs)**

Zinc (Zn) is an inorganic metal oxide that forms diverse types of nanomaterials, such as nanotubes, nanowires, nanoclusters and zinc nanoparticles (ZnNPs). ZnNPs have attracted considerable attention because of their large surface area to volume ratio and low cost. The biological activities of ZnNPs include UV-filtering, antifungal, antioxidant, antibacterial and catalytic properties. Plant-based synthesis of ZnNPs has been widely studied (Lingaraju et al., 2016; Choudhary et al., 2017).

Phytoconstituents present in plant extracts act as reducing agents (reduction of metallic salt to zero-valent metal nanoparticles) and capping agents/stabilizing agents (provides nanoparticle surface capping) (Mittal et al., 2013; Baker et al., 2013). Various organic reducing agents, such as amino acids, vitamins, polysaccharides, polyphenols, organic acids, citric acid, dehydrogenase, flavonoids, functional groups (alcohol, aldehyde, amines, carboxylic acids, ketones and sulfahydryl), heterocyclic compounds hydronase, intracellular  $\text{CO}_2$ , membrane proteins,

**Table 14.4** Different precursors, plants, plant parts, and the ratio of extract and metal salts used for the biosynthesis of AuNPs

Sr. no.	Precursor metal	Precursor concentration (mM)	Plant used	Plant part used	Size (nm)	Application	Reference
1.	AuCl <sub>3</sub>	500	<i>Aegle marmelos</i>	Leaves	4–10	Antioxidant activity	Noruzi et al. (2011)
2.	AuCl <sub>3</sub>	100	<i>Aloe vera</i>	Plant extract	50/350	Antifungal, antidiabetic, antibacterial	Chandran et al. (2006)
3.	AuCl <sub>2</sub>	1	<i>Canna indica</i> , <i>Quisqualis indica</i>	Leaves and flower	30–130	Antifungal, antidiabetic, antibacterial	Shukla and Vankar (2012)
4.	AuCl <sub>3</sub>	100	Cinnamon	Bark	35	Antioxidant activity	El Mitwalli et al. (2020)
5.	AuCl <sub>2</sub>	20	<i>Croton Caudatus Geisel</i>	Leaves	20	Antioxidant activity	Giljohann et al. (2010)
6.	AuCl <sub>2</sub>	400	<i>Cuminum cyminum</i>	Seeds	1–10	Antifungal activity, antimicrobial	Sneha et al. (2011)
7.	AuCl <sub>2</sub>	10	<i>Curcuma pseudomontana</i>	<a href="#">Rhizome</a>	20	Antioxidant activity	Kumari and Arti (2014)
8.	AuCl <sub>3</sub>		<i>Lawsonia inermis</i>	Leaves	20	Antifungal activity, antimicrobial	El Mitwalli et al. (2020)
9.	AuCl <sub>3</sub>	100	<i>Mangifera indica</i>	Leaves	17–20	Antibacterial activity	Philip (2010)
10.	AuCl <sub>3</sub>	10	<i>Mentha</i> , <i>Ocimum</i> , <a href="#">Eucalyptus</a>	Leaves	3–16	Anti-inflammatory	Jha and Prasad (2012)
11.	AuCl <sub>2</sub>	100	<i>Monordica charantia</i>	Fruit	30–40	Antioxidant activity	Pandey et al. (2012)
12.	AuCl <sub>2</sub>	10	<i>Murraya koenigii</i>	Leaves	20	Antibacterial activity	Philip et al. (2011)
13.	AuCl <sub>2</sub>	200	<i>Nyctanthes arbortristis</i>	Flower extract	19.8	Antifungal activity, antimicrobial	Pandey et al. (2012)
14.	AuCl <sub>2</sub>	100	<i>Parkia biglobosa</i>	Leaves	1–35	Antibacterial activity	Giljohann et al. (2010)
15.	AuCl <sub>3</sub>	100	<i>Phyllanthus amarus</i>	Leaves	65–99	Antifungal activity, antimicrobial	Annamalai et al. (2011)

(continued)

Table 14.4 (continued)

Sr. no.	Precursor metal	Precursor concentration (mM)	Plant used	Plant part used	Size (nm)	Application	Reference
16.	AuCl <sub>3</sub>	100	<i>Rosa hybrid</i>	Rose petals	10	Antifungal activity, antimicrobial	Kumar et al. (2012)
17.	AuCl <sub>2</sub>	100	<i>Sorbus aucuparia</i>	Leaf extract	16–18	Antibacterial activity	Dubey et al. (2010)
18.	AuCl <sub>2</sub>	--	<i>Stevia rebaudiana</i>	Leaves	8–20	Antioxidant activity	Mishra et al. (2010)
19.	AuCl <sub>2</sub>	1	<i>Tamarind</i>	Leaves	20–40	Antifungal activity, antimicrobial	Giljohann et al. (2010)
20.	AuCl <sub>3</sub>	100	<i>Tanacetum vulgare</i>	Fruit	11	Antioxidant activity	Giljohann et al. (2010)
21.	AuCl <sub>2</sub>	100	<i>Terminalia chebula</i>	Plant extract	6–60	Antioxidant activity	Kumar et al. (2012)
22.	AuCl <sub>3</sub>	10	<i>Trigonella foneum-graecum</i>	Leaves	15–25	Antioxidant activity	Pandey et al. (2012)]

NADP reductase, peptide, phenolics, polyphenol, polyols, proteins, quercetin, reducing sugars, saponins, secondary metabolites, sesquiterpenes, tannic acid, tartaric acid and terpenoids, are reducing and stabilizing agents in the reaction system (Akhtar et al., 2013; Baker et al., 2013). These metabolites are synthesized by various plant tissues, such as roots, stems, leaves, fruits and seeds. These phytochemicals lower the metal's valence to zero and then calcinate it to add oxide. Zinc ions interact with the polyphenols, terpenoids, and flavones in the plant extract to form a complex. After that, zinc hydroxide ( $\text{Zn}(\text{OH})_2$ ) is formed via hydrolysis, and then ZnO nanoparticles are synthesized (Choudhary et al., 2017). Several members of the Fabaceae, Rutaceae, Apocynaceae, Solanaceae and Lamiaceae families are most commonly employed for the production of ZnNPs (Table 14.5).

A number of plants from the family Apocynaceae, Lamiaceae, have been used to produce ZnO nanoparticles of different sizes and shapes and biological activities. All experiments displayed nanoparticles in the same size range with spherical and hexagonal disc shapes, which were characterized by XRD and TEM analysis. One of the important members from the Apocynaceae family, *Calotropis procera*, has been potentially used to synthesize ZnNPs with spherical shapes in the range of 5–40 nm in size (Singh et al., 2011). Some reports on the plant-assisted synthesis of zinc nanoparticles are listed below in Table 14.5.

### ***Titanium Nanoparticles (TiNPs)***

Titanium and dioxide nanoparticles (TiNPs and  $\text{TiO}_2$ NPs) are becoming popular due to their electrical band structure, quantum efficacy, high surface area and stability, and chemical inertness. Therefore, TiNPs are considered the most versatile and prominent oxides, with an annual production of over 20,000 tons in 2020. TiNPs have wide insecticidal and pharmaceutical applications. For example, TiNPs are used to lower the toxicity of reactive dyes (Pawar et al., 2011), as antimicrobial and pharmaceutical dosage forms (Ajmal et al., 2019), in wastewater treatment and cosmetics (Ziental et al., 2020). TiNP synthesis is largely carried out using biological methods and has attracted scientific attention recently because of its low cost, environmental friendliness and reproducibility. Microbes such as bacteria, fungi, algae and plants are widely used for the biosynthesis of TiNPs. A number of non-Apocynaceae plants have also been reported for the biosynthesis of TiNPs. The extract of *Eclipta prostrata* is used to produce TiNPs with spherical shapes and sizes ranging from 36 nm to 68 nm (Nakayama et al., 2020). Subhashini and Nachiyar (2017) studied TiNPs using the leaf extract of *Albizia saman* as well as by using the extract of *Curcuma longa*. TiNPs with various shapes and sizes have been synthesized by a number of green synthesis methods. TiNPs have been characterized using a number of spectroscopic and microscopic techniques, including XRD, FTIR, SEM and EDX, which reveal the structure and particle size of the TiNPs. Table 14.6 provides a detailed outlook of the plant-assisted synthesis of TiNPs.

**Table 14.5** Different precursors, plants, plant parts, and the ratio of extract and metal salts used for the biosynthesis of ZnO nanoparticles

Sr. no.	Precursor metal	Precursor concentration (mM)	Plant used	Plant part used	Size (nm)	Application	Reference
1.	ZnO	--	<i>Acalypha Indica</i>	Leaves	100–200	Antibacterial and antifungal	Jamdagni et al. (2018)
2.	ZnO	100	<i>Aloe vera</i>	Leaves	22.18	Environmental applications	Varghese and George (2015)
3.	ZnO	100	<i>Artemisia pallens</i>	Leaves along with stem	50–100	Studying glucosemetabolism	Gomathi and Suhana (2021)
4.	ZnO	10	<i>Artocarpus gomeziianus</i>	Fruit	50	Studying metabolism of intracellular substances	Oudhia et al. (2015)
5.	ZnO	100	<i>Azadirachta indica</i>	Leaves	50	Antioxidant activity.	Noorjahan et al. (2015)
6.	ZnO	20	<i>Azadirachta indica</i>	Leaves	25	antifungal, antidiabetic, antibacterial,	Oudhia et al. (2015)
7.	ZnO	20	<i>Beta vulgaris</i>	Root	52–76	Studying glucose metabolism	Kumar, Singh, and Pandey (2015), Kumar, Suresh, Nagabhushana, et al. (2015)
8.	ZnO	100	<i>Brassica oleraceae</i>	Leaves	1–100	Studying glucose metabolism	Oudhia et al. (2015)
9.	ZnO	100	<i>Calotropis gigantea</i>	Leaves	1.5–8.5	Studying glucosemetabolism	Choudhary et al. (2017)
10.	ZnO	10	<i>Catharanthus roseus</i>	Leaves	23–57	Studying glucosemetabolism	Bhumi and Savithramma (2014)
11.	ZnO	20	<i>Cayratia pedata</i>	Leaves	52.24	Studying glucose metabolism	Jayachandran et al. (2021)
12.	ZnO	10	<i>Coptidis rhizome</i>	Rhizome	2.9–25.2	Antibacterial and antifungal	Oudhia et al. (2015)



13.	ZnO	1	<i>Curcuma longa</i>	Root	20–80	Antioxidant activity	Raut et al. (2015)
14.	ZnO	100	<i>Eucalyptus globules</i>	Leaves	52–70	Antifungal, antidiabetic antibacterial	Ahmad et al. (2020)
15.	ZnO	1	<i>Euphorbia hirta</i>	Leaves	20–50	Antioxidant activity	Ahmad Kalra (2020)
16.	ZnO	100	<i>Ginger</i>	Rhizome	23–26	Anthelmintics	Awwad et al. (2014)
17.	ZnO	100	<i>Hibiscus rosa-sinensis</i>	Leaves	30–35	Studying metabolism of intracellular substances	Devi and Gayathri (2014)
18.	ZnO	100	<i>Nephelium lappaceum</i>	Peel	20	Antifungal, antidiabetic, antibacterial	Azizi et al. (2014)
19.	ZnO	1	<i>Nyctanthes arbor-tristis</i>	Flowers	12–32	Studying metabolism of intracellular substances	Jamdagni et al. (2018)
20.	ZnO	100	<i>Ocimum tenuiflorum</i>	Leaves	11–25	Environmental applications and global warming	Raut et al. (2015)
21.	ZnO	100	<i>Olea europea</i>	Leaves	18–30	Antioxidant activity	Awwad et al. (2014)
22.	ZnO	200	<i>Plectranthus amboinicus</i>	Leaves	20–50	Anthelmintics	Devi and Gayathri (2014)
23.	ZnO	100	<i>Ruta graveolens</i>	Stem	28	Studying microbial denitrification and global warming	Lingaraju et al. (2016)
24.	ZnO	–	<i>Sargassum muticum</i>	Leaves	30–57	To create strategies for reducing the effects of ZnO NPs on microbial denitrification	Azizi et al. (2014)
25.	ZnO	1	<i>Senna auriculata</i>	Leaves	2	Studying metabolism of intracellular substances	Jamdagni et al. (2018)
26.	ZnO	1	<i>Solanum nigrum</i>	Leaves	20–30	Studying glucose metabolism	Ramesh et al. (2015)

(continued)

Table 14.5 (continued)

Sr. no.	Precursor metal	Precursor concentration (mM)	Plant used	Plant part used	Size (nm)	Application	Reference
27.	ZnO	10	<i>Tecoma castanifolia</i>	Leaves	70–75	Antifungal, antidiabetic, antibacterial	Sharmila et al. (2019)
28.	ZnO	1	<i>Zingiber officinale</i>	Root	30–50	Antioxidant activity	Raj and Jayalakshmy (2015)

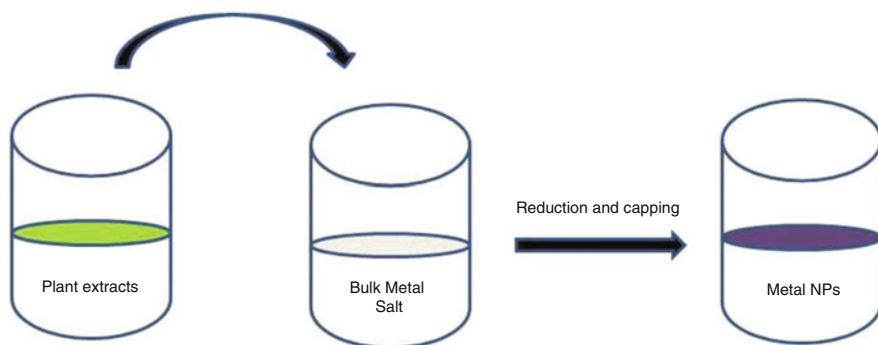
**Table 14.6** Different precursors, plants, plant parts, and the ratio of extract and metal salts used for the biosynthesis TiO<sub>2</sub> nanoparticles

Sr. no.	Precursor metal	Precursor concentration (mM)	Plant used	Plant part used	Size (nm)	Application	Reference
1.	TiO <sub>2</sub>	1.5 mM	<i>Annona squamosa</i>	Leaves	43–45	To kill breast cancer cells	Prasad et al. (2017)
2.	TiO <sub>2</sub>	1.5 mM	<i>Azadirachta indica</i>	Leaves	15–50	Plastics, anticancer	Wei et al. (2017)
3.	TiO <sub>2</sub>	0.80 mM	<i>Azadirachta indica</i>	Leaves	124	Antimicrobial	Harshiny et al. (2015)
4.	TiO <sub>2</sub>	1 mM	<i>Calotropis gigantea</i>	Flower	10–52	Antifungal	Ranjith et al. (2018)
5.	TiO <sub>2</sub>	0.5 mM	<i>Cassia auriculata</i>	Leaves	38	Antibacterial	Yi et al. (2019)
6.	TiO <sub>2</sub>	0.5 mM	<i>Citrus sinensis</i>	Fruit peel	19	Antifungal	Wang et al. (2014)
7.	TiO <sub>2</sub>	1.5 mM	<i>Euphorbia prostrata</i>	Leaves	81–84	Antimicrobial	Ranjith et al. (2018)
8.	TiO <sub>2</sub>	2.5 mM	<i>Jatropha curcas</i>	Latex	25–50	Textile	Yi et al. (2019)
9.	TiO <sub>2</sub>	2.5 mM	<i>Jatropha curcas, citrus aurantium</i>	Leaves	25–50	Plastics, anticancer	Gottmukkala et al. (2017)
10.	TiO <sub>2</sub>	1 mM	<i>Ledebouria revoluta</i>	Bulb	47	Toothpaste, coatings,	Prasad et al. (2017)
11.	TiO <sub>2</sub>	0.80 mM	<i>Mentha arvensis</i>	Leaves	20–70	Cosmetics	Huang et al. (2014)
12.	TiO <sub>2</sub>	2.5 mM	<i>Nyctanthes arbor-tristis</i>	Leaves	100–150, 100–200	Antimicrobial	Yi et al. (2019)
13.	TiO <sub>2</sub>	1.8 mM	<i>Ocimum basilicum</i>	Leaves	50	Antimicrobial	Ranjith et al. (2018)
14.	TiO <sub>2</sub>	0.5 mM	<i>Pouteria campechiana</i>	Leaves	73–140	Pharmaceuticals papers inks	Gottmukkala et al. (2017)
15.	TiO <sub>2</sub>	2.0 mM	<i>Psidium guajava</i>	Leaves	32.58	Textile	Önal et al. (2017)
16.	TiO <sub>2</sub>	0.5 mM	<i>Salvia officinalis</i>	Leaves	15–20	Antibacterial	Wang et al. (2014)
17.	TiO <sub>2</sub>	0.55 mM	<i>Solanum trilobatum</i>	Leaves	70	Antimicrobial	Kanagasubbulakshmi and Kadirvelu (2017)
18.	TiO <sub>2</sub>	0.55 mM	<i>Syzygium cumini</i>	Leaves	22	Food products	Devi et al. (2019)

## Practical Approaches for the Biosynthesis of Metal Nanoparticles Using Apocynaceae Plants

Generally, the aqueous extract of part of the selected plant is used for the biosynthesis of metal nanoparticles. The plant parts include leaves, roots, stems, rhizomes, tubers, etc., of the selected plant were chopped into thin slices and dried at room temperature in the dark. The dried material was ground to a fine powder using a grinder and sieved to obtain uniformly sized particles. A specific amount of powdered material was boiled in water, and the extract was filtered. The rich plant metabolites can be used for further experimentation as well biosynthesis studies. The extract obtained was also subjected to phytochemical analysis. The extracts were analysed for the presence of various phytochemicals, such as carbohydrates, amino acids, steroids, flavonoids, and alkaloids (Pawar, 2021).

The process of phytosynthesis of metal nanoparticles is summarized in Fig. 14.3. The plant extract is used as a reducing agent for the biosynthesis of metal nanoparticles. In brief, the aqueous extract of selected plants (usually 2–10% w/v) is added to the solution of selected metal salt (for example, 1 mM  $\text{AgNO}_3$  can be diluted to 0.1 mM, 1 mM, 2 mM, 5 mM, 10 mM and 20 mM) in an Erlenmeyer flask (Pawar & Patil, 2020). The reaction mixture was kept at room temperature for 5 h under static conditions. The bioreduction of silver nitrate can be preliminarily evaluated through visual observation. The color of the reaction mixture changes from colorless to brown. The reaction can also be monitored by scanning UV absorbance (200–800 nm) at 1 h intervals using a UV–visible spectrophotometer. After incubation for 5–6 h, the reaction mixture is centrifuged to settle the biosynthesized nanoparticles at the bottom of the centrifugation tube. The pellet is redispersed in double-distilled water, and recentrifugation is carried out for better separation of nanoparticles. The collected pellets of biosynthesized AgNPs were dried and powdered samples of AgNPs were used for further experimentation (Pawar & Patil, 2020).



**Fig. 14.3** General procedure of biosynthesis of metal nanoparticles using plant extracts

Plants or plant parts can be effectively used as reducing and capping agents in the biosynthesis of metal nanoparticles. The phytochemicals present in the tuber extract played a significant role in the bioreduction and stabilization of the synthesized MNPs. A number of plants of Apocynaceae have been explored for the biosynthesis of metal and metal oxide nanoparticles. Some important members of this family, viz. *C. roseus*, *C. procera*, *C. gigantea*, *A. scholaris*, *R. serpentina*, *H. antidysentriaca*, *N. indicum*, *C. carandas*, *Cassia auriculata*, *Wrightia tinctoria*, *Cynanchum sarcomedium* and *T. peruviana* have been widely used for the biosynthesis of metal and metal oxide nanoparticles. Table 14.7 highlights Apocynaceae members used for the biosynthesis of various metal and metal oxide nanoparticles.

## Characterization of Biosynthesized MNPs

The characterization is important to ensure that the prepared particles are at the nanoscale. In material science, “characterization” includes the structure and properties of the material to be explored through general and broad processes. The characterization of biosynthesized NPs can be carried out using various spectroscopic and microscopic techniques. The spectroscopic method involved UV–Visible and FT-IR spectroscopy, microscopy-based nanomaterial characterization using SEM, and EDX. X-ray-related characterization techniques such as X-ray diffraction analysis (XRD), dynamic light scattering (DLS), zeta potential, etc., were also carried out to determine the shape and size of the NPs (Titus & Samuel, 2019). The details of characterization are mentioned in the following paragraphs. The characterization techniques used for biosynthesized metal nanoparticles are highlighted in Fig. 14.4.

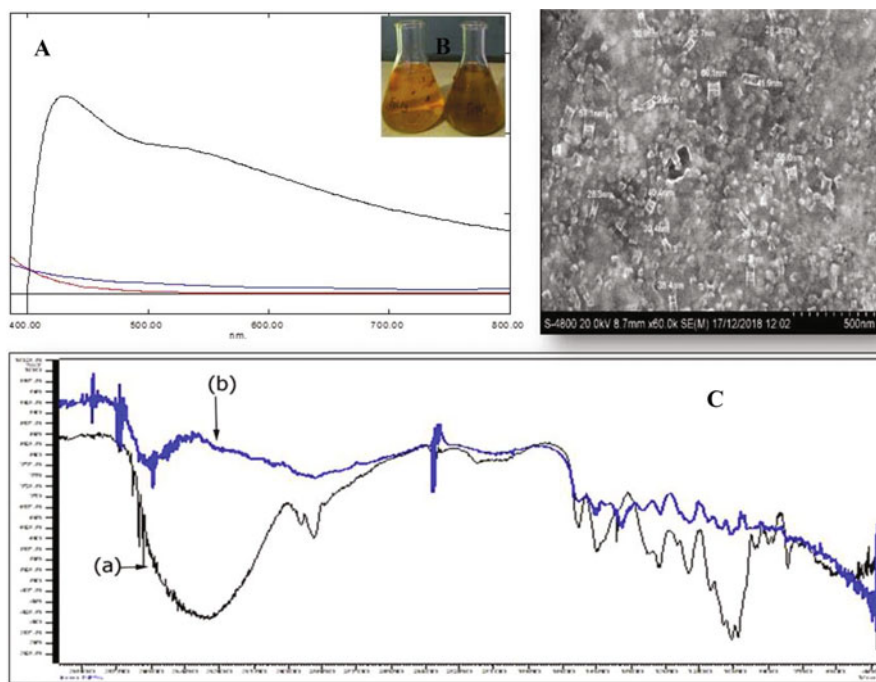
### UV–Visible Spectrophotometer

A UV–vis visible spectrophotometer with a wavelength range of 200–1100 nm consisting of a deuterium and tungsten lamp as a light source with 1 nm resolution is used for measuring the absorption. The matching pair of quartz cuvettes with a path length of 1 cm can be used for analysis, and the data are processed using the software. The reaction mixture formed in the biosynthesis of metal nanoparticles is the combination of metal salts, and plant extracts can be directly scanned in the UV–vis visible range of 200–800 nm. The UV–vis visible absorbance spectra of various biosynthesized MNPs (AgNPs, CuNPs and FeNPs) using plant extracts were recorded and compared with control samples of the respective metal salts, and the test extracts were scanned in the same range. The reaction mixtures are monitored periodically.

**Table 14.7** Apocynaceae members used for biosynthesis of nanoparticles

Sr. no.	Precursor metal	Precursor concentration (mM)	Plant used	Plant part used	Size (nm)	Application	Reference
1.	AgNO <sub>3</sub>	1.5 mM	Calotropis gigantea.	Leaves	43–45	Antibacterial activity against Gram-positive bacteria.	Mathew et al. (2020)
2.	Chitosan	1.5 mM	Catharanthus roseus	Leaves	15–50	Protection against salinity stress.	Saxena et al. (2021)
3.	TiO <sub>2</sub>	1 mM	Calotropis gigantea	Flower	10–52	Antifungal	Ranjith et al. (2018)
4.	TiO <sub>2</sub>	0.5 mM	Cassia auriculata	Leaves	38	Antibacterial	Yi et al. (2019)
5.	Pd	0.5 mM	Allium fistulosum	Leaves	19	Antioxidant, antifungal and antibacterial activity.	Vinodhini et al. (2022)
6.	Pd	1.5 mM	Basella alba	Leaves	81–84	Antioxidant, antifungal and antibacterial activity.	Vinodhini et al. (2022)
7.	Pd	2.5 mM	Tabernaemontana divaricate	Leaves	25–50	Antioxidant, antifungal and antibacterial activity.	Vinodhini et al. (2022)
8.	ZrO <sub>2</sub>	2.5 mM	Wrightia tinctoria	Leaves	25–50	Gram-positive and Gram-negative bacteria.	Naeimi et al. (2021)
9.	Ni	1 mM	Calotropis procera	wood	47	Role in catalytic performance, oxidation	Naeimi et al. (2021)
10.	NiO	0.80 mM	Calotropis procera	wood	20–70	Role in catalytic performance, oxidation	Naeimi et al. (2021)
11.	ZnO	2.5 mM	Carissa spinarum	Leaves	100–150, 100–200	Antimicrobial against Bacillus and Staphylococcus.	Saka et al. (2022)
12.	AgNO <sub>3</sub>	1 mM	Cynanchum viminale.	Leaves	50	Biomedical applications	Bhagyanathan and Thoppil (2018)
13.	AgNO <sub>3</sub>	1 mM	Cynanchum sarcomedium	Leaves	73–140	Biomedical applications	Bhagyanathan and Thoppil (2018)

14.	TiO <sub>2</sub>	2.0 mM	Catharanthus roseus	Leaves	32.58	Biomedical applications	Bhagyanathan and Thoppil (2018)
15.	TiO <sub>2</sub>	0.5 mM	Salvia officinalis	Leaves	15–20	Antibacterial	Wang et al. (2014)
16.	TiO <sub>2</sub>	0.55 mM	Catharanthus roseus	Leaves	70	Antimicrobial	Kanagasubbulakshmi and Kadirvelu (2017)
17.	TiO <sub>2</sub>	0.55 mM	Syzygium cumini	Leaves	22	Food products	Devi et al. (2019)



**Fig. 14.4** Characterization techniques used for biosynthesized metal nanoparticles

### ***Fourier Transform Infrared Spectroscopy (FTIR)***

FT-IR spectroscopy was performed to determine the presence or absence of functional groups using an FT-IR spectrometer. The diffuse reflectance scan (DRS) sampling method can be used in the  $4000\text{--}400\text{ cm}^{-1}$  range. To analyse the sample by the DRS method, a solid dispersion of the sample can be prepared with KBr (IR spectroscopy grade) (Pawar & Patil, 2020).

The biosynthesized MNPs obtained can be centrifuged at 10,000 rpm for 20 min at room temperature, and the pellet of each MNP is collected. The process of centrifugation and redispersion of the collected pellet in water is repeated three times to ensure better separation of nanoparticles from other phytochemical adsorbed material. The pellet of each MNP was dried, and a powder sample of each MNP was used for further experimentation (Ghosh et al., 2012).

The dried powder sample of each MNP was mixed with KBr (IR spectroscopy grade) at a proportion of 1:100 using a small mortar and pestle to obtain a small particle size distribution. The solid dispersion of each MNP in KBr was used as the sample for FT-IR spectroscopy. The mixture is then placed in a sample cup 3–4 mm deep and approximately 10–15 mm in diameter to record the FT-IR spectra of each sample. For FT-IR spectroscopy of the plant extract, 1 ml of tuber extract was dried



to obtain a powder. The plant tuber extract powder was mixed with KBr at a proportion of 1:100. The sample was ground and mixed uniformly using a small mortar and pestle to obtain a small particle size distribution (Pawar, 2021).

### ***Scanning Electron Microscopy (SEM)***

A scanning electron microscope with a resolution of 3.5 nm and an accelerating voltage in the range from 0.5 kV to 30 kV is commonly used to study the surface morphology and size of biosynthesized MNPs. The JSM-5600 is a conventional SEM with a tungsten filament electron source (Pawar & Patil, 2020).

Conductive tape (double-sided carbon tape) is affixed on a polished aluminum surface (Pawar & Patil, 2020). Samples of biosynthesized MNPs are placed at the center of the carbon tape individually while performing the analysis. The pressure is applied, and excess free particles are removed by blowing. The samples were kept in a vacuum chamber for one h and then loaded on the SEM platform (Pawar & Patil, 2020). It is equipped with a secondary electron detector for topographic contrast imaging and an Oxford Inca EDX system for compositional analysis (Pawar, 2021).

### ***Energy Dispersive X-ray Analysis (EDX)***

EDX is used in conjunction with SEM. This technique gives an overall mapping of the sample by analysing near-surface elements and estimates the elemental proportion at different positions (Titus & Samuel, 2019).

An electron beam with an energy of 10–20 keV strikes the conducting sample's surface, causing X-rays to emit from the material, and the energy of the emitted X-rays depends on the material under examination (Titus & Samuel, 2019).

### ***X-ray Diffraction (XRD)***

The degree of crystallinity, element identification and composition, and structural state can be derived from XRD data analysis (Titus & Samuel, 2019).

The MNP sample was prepared by spreading the sample on a microscopic glass slide and exposed to diffraction at an operating voltage of 40 kV, a current of 30 mA and a scan rate of 10°/min with Cu-K $\alpha$  wavelength radiation in a 2 $\theta$  configuration from 20–80°. The software modules for easy X-ray powder diffraction data acquisition and evaluation can be used for interpretation of the data.

## ***Dynamic Light Scattering (DLS)***

Light scattered from a laser, which travels through the colloidal solution, is measured by DLS. Modulation of the intensity of the scattered light is analysed as a function of time, and the particle size can be derived based on this information (Titus & Samuel, 2019).

## ***Zeta Potential***

Zeta potential is a measure of the effective electric charge on the nanoparticle surface and quantifies the charge stability of colloidal particles (Titus & Samuel, 2019). Biosynthesized MNPs using plant extracts are dispersed in double-distilled water. The instrument software then automatically determines the appropriate electric field strength, adjusts the reference beam intensity to ensure the optimal signal-to-noise ratio, and collects and analyses the data.

## **Conclusion**

Plants of the Apocynaceae family can be effectively used as reducing and capping agents in the biosynthesis of metal nanoparticles. The exploration of plant capability to reduce metal salt to zero-valent metal depends on many experimental parameters. The biosynthesis of metal nanoparticles using these plants is simple, cost-effective and eco-friendly. Biosynthesis of AgNPs, CuNPs, ZnONPs, AuNPs and FeNPs using a number of plants of this family is well studied. The phytochemicals present in the plant extract played a significant role in the bioreduction and stabilization of the synthesized MNPs. The synthesized MNPs were characterized by UV–VIS, FT-IR, SEM, EDX and X-ray diffraction spectroscopy. Biosynthesized metal nanoparticles have a wide range of biological activities; therefore, Apocynaceae plants can be considered valuable resources for future studies in nanobiotechnology.

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