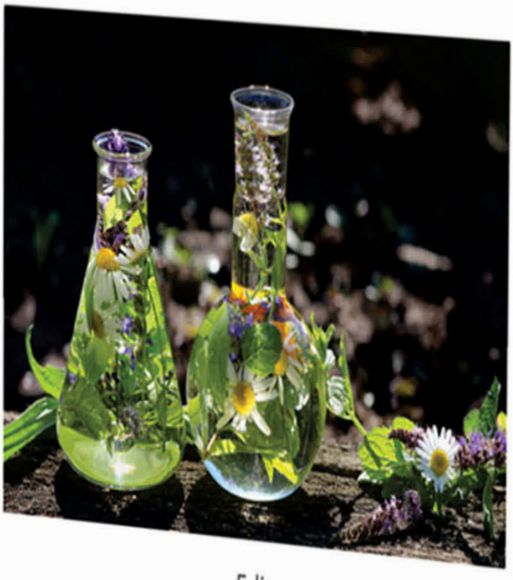
Phytochemistry

Volume 1

Fundamentals, Modern Techniques, and Applications



Editors

Chukwuebuka Egbuna • Jonathan Chinenye Ifemeje
Stanley Chidi Udedi • Shashank Kumar

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PHYTOCHEMISTRY

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Edited by

Chukwuebuka Egbuna

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ABBREVIATIONS

2D	Two-dimensional
3-BP	3-bromopyruvate
3D	Three-dimensional
67LR	67-kDa laminin receptor
AA	Arachidonic acid
AAS	Atomic absorption spectroscopy
AES	Atomic emission spectroscopy
ALA	α-linolenic acid
ANOVA	Analysis of variance
AO/EB	Acridine orange/ethidium bromide
AP-1	Activator protein 1
APE1	Apurinic/apyrimidinic endonuclease-1
ASE	Accelerated solvent extraction
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
BB	Biobreeding
BER	Base excision repair
BSS	Beta-sitosterol
CAGR	Compound annual growth rate
CaOx	Calcium oxalate
CCC	Countercurrent chromatography
CC	Column chromatography
CHI	Chalcone isomerase
CHS	Chalcone synthase
CNS	Central nervous system
CoQ10	Coenzyme Q10
COX-2	Cyclooxygenase-2
CPC	Centrifugal partition chromatography
CPDB	Carcinogenic potency database
CPU	Central processing unit
CSC	Cancer stem cell
DHA	Docosahexaenoic acid
DHTQ	Dihydrothymoquinone
DMAPP	Dimethylallyl diphosphate

DMRT	Duncan's Multiple Range Test
DNA	Deoxyribonucleic acid
DNAPK	DNA-dependent protein kinase
DNP	Dinitrophenylhydrazine
DTH	Delayed-type hypersensitivity
EGCG	Epigallocatechin-3-O-gallate
ent-CDP	Ent-copalyl-diphosphate
EPA	Eicosapentaenoic acid
ER	Estrogen receptor
ERK	Extracellular-signal-regulated kinase
FC	Flash chromatography
FDA	Food and Drug Administration
FTIR	Fourier-transform infrared spectroscopy
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
GC-MS	Gas chromatography–mass spectrometry
GLA	γ-linolenic acid
GLN	Glyceollins
GLUT	Glucose transporter
GPU	Graphics processing unit
GSK-3β	Glycogen synthase kinase-3 β
HIF-1	Hypoxia-inducible factor-1
HIT	Herb ingredients target
HPLC	High-performance liquid chromatography
HR	Homologous recombination
HTS	High-throughput screening
IC_{50}	Half maximal inhibitory concentration
ICH	International Conference on Harmonization
ICP-MS	Inductively coupled plasma mass spectroscopy
ICP-OES	Inductively coupled plasma optical emission spectroscopy
IFN	Interferon
IL	Interleukins
IMP	Inosine monophosphate
iNOS	Inducible nitric oxide synthase
IPP	Isopentenyl pyrophosphate
IQ	Installation qualification
IR	Infrared
JA	Jasmonic acid
LC-MS	Liquid-chromatography mass spectrometry
LD50	50% lethal dose

LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LGA	Local government area
LOQ	Limit of quantitation
LRP5/6	Lipoprotein receptor-related proteins 5/6
LS	Light scattering
LT	Leukotriene
MAE	Microwave-assisted extraction
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate transporter
MD	Molecular dynamics
MEP	Methylerythritol phosphate
MGMT	Mismatch and errors repair
MMR	Mismatch repair
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NER	Nucleotide excision repair
NF-κB	Nuclear factor-κB
NHEJ	Non-homologous end joining
NIST	National Institute of Standards and Technology
NK	Natural killer
NMR	Nuclear magnetic resonance
NOD	Nonobese diabetic
NO	Nitric oxide
NP	Nanoparticle
Nrf2	Nuclear factor E2-related factor 2
OECD	Organization for Economic Co-operation and Development
OPLC	Optimum performance laminar chromatography
OQ	Operating qualification
PAL	Phenylalanine ammonia lyase
PA	Pyrrolizidine alkaloid
PCA	Principal components analysis
PDB	Protein Data Bank
PEP	Phosphoenolpyruvate
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases
PGE2	Prostaglandin E2
PhA	Phytoalexins
PhT	Phytoantipicins
PKA	Protein kinase A

PK	Pyruvate kinase
PM	Plasma membrane
PP	Phenylpropanoid
PQ	Performance qualification
PR	Pathogenesis-related
PRP	Proline-rich protein
PSA	Phytochemical Society of Asia
PSE	Phytochemical Society of Europe
PSNA	Phytochemical Society of North America
PUFA	Polyunsaturated fatty acid
QSAR	Puantitative structure–activity relationship
R_f	Retention factor
RNA	Ribonucleic acid
RNF43	Ring finger 43
ROS	Reactive oxygen species
RP	Reverse phase
SAM	S-adenosyl-L-methionine
SA	Salicylic acid
SD	Standard deviation
SFE	Supercritical fluid extraction
SPE	Solid-phase extraction
SPF	Specific pathogen free
SPSS	Statistical Package for the Social Sciences
SRBC	Anti-sheep red blood cells
STZ	Streptozotocin
syn-CDP	Syn-copalyldiphosphate
TCA	Tricarboxylic acid cycle
TCMID	Traditional Chinese Medicine Integrative Database
TLC	Thin-layer chromatography
TNF	Tumor necrosis factor
UAE	Ultrasound-assisted extraction
UV	Ultraviolet
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
VLC	Vacuum liquid chromatography
WHO	World Health Organization
XRF	X-ray fluorescence
ZNRF3	Zinc and ring finger 3

FOREWORD

I feel honored for being invited to write the Foreword to the book *Phytochemistry, Volume 1: Fundamentals, Modern Techniques, and Applications*. I am very happy to write this foreword to a book of this kind that has great scope and scholarly contents. A panoramic review of this book indicates that it is well-articulated and written by professionals from diverse academic backgrounds. I congratulate the editors and contributors for their excellent work. The design is exceptional and gives firm background information about modern phytochemistry. The style of grouping the chapters into four parts of fundamentals, methods, computational, and research applications is one that makes it different from other books of phytochemistry. The present book provides a framework starting from the introduction, biosynthesis of phytochemicals to their effects in living systems and to the state-of-the-art modern techniques with insights on the discovery of medicinally active compounds through *in silico* and *in vitro* studies.

As a molecular biologist and a computational chemist with technical and scientific expertise in drug discovery, specialized in network pharmacology, ligand, and structure-based drug design, and with many years of experience in pharmaceutical R&D, biotech, and scientific software development, I will say that the field of phytochemistry is growing at a very astonishing rate because of the renewed interests in the search for safe natural drugs with minor side effects. The plant kingdom, which harbors over 400,000 species of plants, offers the opportunity for the discovery of novel compounds that will be useful for the treatment of diseases. Until today, only about 15% of these plants has been studied closely for their medicinal potentials despite the increasing need for new drugs.

Modern phytochemistry involves the application of high-throughput techniques and modern omics tools. It encompasses the study of plant natural products through metabolic profiling of the various biosynthesis pathways, optimization of the production of plant natural products, and importantly, the utilization of computational docking and simulation tools to identify molecular targets for the discovery of novel therapeutic

compounds. Personally, I feel obliged to present this book to the scientific community. I recommend it to teachers, students, researchers, and everyone with interests in phytochemistry. It is with immense pleasure that I sincerely thank the Editor-in-chief, Chukwuebuka Egbuna, for inviting me to write this Foreword.

—**Timea Polgar PhD**
VP, R&D, Founder
Envision Biotechnology

PREFACE

Phytochemistry is a branch of science that deals with the study of the chemicals produced by plants, particularly the secondary metabolites. It takes into account the synthesis of secondary metabolites, its metabolisms in plants, and their effects in other living organisms. It also encompasses the medicinal, industrial, and commercial applications of plant natural products. Phytochemistry is multifaceted, and this made it complicated and problematic for authors to compose a good book of phytochemistry. Till now, there has been no comprehensive book on phytochemistry other than compendiums of research articles from aftermath of conferences or seminars. They have served a useful purpose, however, and could be excused in times when phytochemistry was without a very clear conceptual framework.

This book *Phytochemistry: Fundamentals, Modern Techniques, and Applications* is a comprehensive book on phytochemistry written by professionals from key institutions around the world. The authors are experts in their academic and research niche. The chapters are drawn carefully and integrated sequentially to aid flow, consistency, and continuity. This book will be very useful to researchers, teachers, students, phytochemists, plant biochemists, food and medicinal chemists, nutritionists and toxicologists, chemical ecologists, taxonomists, analytical chemists, industrialists, and many others.

The chapters are grouped into four parts. Part I covers the fundamentals of phytochemistry. Part II details the state-of-the-art modern methods and techniques in phytochemical research. Part III is an overview of computational phytochemistry and its applications. Part IV presents novel research findings in the discovery of drugs that will be effective in the treatment of diseases. Each chapter has a short abstract that briefly explains the scientific basis of the chapter and what readers should expect. The in-text references ensure that all information is authentic and verifiable.

Chapter 1 by Egbuna et al. is an introductory chapter that discusses the general scope of phytochemistry, its modern history, and the relationship it has with other sciences. It also discusses the prospects for phytochemists and the future projections of phytochemical research. Chapter 2 is a comprehensive description of the biosynthesis of phytochemicals. Tijjani et al. structurally presents the various biosynthetic pathways and the mechanisms

involved. Hanan and Musarat present the mechanisms of plants defenses through the induction of phytoalexins (PhA) in Chapter 3. They also discuss the biosynthesis of PhA and its regulation with emphasis on the variations that exist within different plant families. Chapter 4, written by Onyekere et al., is an overview of the biological roles of phytochemicals in animals. Aslanipour presents the immunomodulatory roles of phytochemicals in Chapter 5, while author Anywar discusses phytochemicals as nutraceuticals and pharmafoods in Chapter 6. Nwafor and Orabueze in Chapter 7 details the role of phytochemistry in plant classification. The authors also details the various phytochemical markers of taxonomic importance. Chapter 8 by Datir and Jha is an overview of metabolomics in phytochemistry while highlighting the various high-throughput techniques in modern phytochemistry.

Banjo et al. in Chapter 9 describes the various extraction methods and techniques. Orabueze and Nwafor in Chapter 10 provides an overview of the techniques utilized in phytochemotaxonomy. Chapter 11 by Javad and Naz presents the different chromatographical techniques used for the isolation and characterization of phytochemicals. Kujore of Cecil Instruments in Chapter 12 describes the use of ultraviolet/visible spectroscopy and high-performance liquid chromatography (HPLC) with emphasis on principles, calibrations, and qualifications. Verma and Gavankar in Chapter 13 details the use of HPLC and high-performance thin-layer chromatography as sophisticated tools in phytochemical research. Mtewa and Amanjot discusses the various techniques in the elemental profiling of plant materials in Chapter 14. Egbuna et al. in Chapter 15 presents the various phytochemical tests methods used in the qualitative and quantitative analysis of phytochemicals. The chapter also details reagent preparation and the various calculations commonly encountered in phytochemistry. Adedeji et al. in Chapter 16 details the various animal models in phytopharmacology with emphasis on *Drosophila melanogaster* as a model. Neagu and Constantin in Chapter 17 describes the various protocols involved in the toxicological testing of plant natural products. Chapter 18 by Haider et al. is an overview of the roles of biostatistics in phytochemical research with emphasis on essential oil studies.

Vallinayagam et al. in Chapter 19 presents the various databases and tools utilized in drug discovery under computational phytochemistry. Kushwaha et al. in Chapter 20 presents research findings of the stemness modulation by phytochemicals to target stem cells. In a separate development in chapter 21, Dash et al. presents findings on targeting cancer cell carbohydrate metabolism by phytochemicals through in silico studies. Nagy in Chapter 22 emphasizes herbal discovery through an automated platform

capable of establishing a handshake of the ethnobotanical uses of medicinal plants with scientific evidence by the Envision Biotechnology approach.

Priya et al. in Chapter 23 presents cancer research findings on the effects of *Tectona grandis* bark extract on human breast cancer cell line (MCF-7). Authors present a photomicrographs of the anticancer activities of *T. grandis*. Chapter 24 by Shaista et al. is a phytochemical analysis of *Nigella sativa* L. seed aqueous extract by gas chromatography–mass spectrometry and Fourier-transform infrared. Faleyimu et al. in Chapter 25 presents phytochemical studies of five Nigerian indigenous vegetables.

In summary, this book is great in scope and one that is invaluable. The volume took many months to come to completion. I sincerely appreciate the unflinching supports of the chapter contributors, volunteer reviewers, and my co-editors. I also extend my sincere appreciation to my family for their support and patience during the editorial process of this book. My gratitude also goes to Apple Academic Press for their guidance from the onset of this book project and to the management of ResearchGate social platform where the project originated.

I recommend this book to everyone with interests in phytochemistry or related areas. I will welcome reviews, suggestions, and areas that will need improvements with an open heart. Thank you.

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PART I
Fundamentals of Phytochemistry

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CHAPTER 1

INTRODUCTION TO PHYTOCHEMISTRY

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ABSTRACT

Phytochemistry is the study of the chemicals produced by plants, particularly the secondary metabolites, synthesized as a measure for self-defense

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against insects, pests, pathogens, herbivores, ultraviolet exposure and environmental hazards. Phytochemistry takes into account the structural compositions of these metabolites, the biosynthetic pathways, functions, mechanisms of actions in the living systems as well as it's medicinal, industrial, and commercial applications. The proper understanding of phytochemical is essential for drug discovery and for the development of novel therapeutic agents against major diseases. This chapter introduces phytochemistry, discusses the history of modern phytochemistry, the relationship of phytochemistry with other sciences and the importance of phytochemistry. It also provides information on the sources and classification of phytochemicals, prospects for phytochemists, the usefulness of computational phytochemistry, biostatistics and the advances in phytochemical research.

1.1 INTRODUCTION

It will be recalled that in the food chain, plants are referred to as the producers because they had the ability to trap energy from sunlight, harness and assemble some basic units which they transform through some chemical process into complex high energy-yielding compounds that are readily available to organisms. Their generosity became overwhelmingly and practically complex to comprehend at a glance. A field has to emerge – "phytochemistry." Phytochemistry is the study of chemicals produced by plants, particularly the secondary metabolites. It takes into account their structural compositions, the biosynthetic pathways, functions, and mechanisms of actions in the living system. The study of phytochemicals has been instrumental in the discovery of new plant natural products which are of commercial values in various industries such as the traditional and complementary medicine systems, pharmaceutical industries, nutraceuticals, and dietary supplement industries. Not left out is the cosmeceuticals industries, clothing and textiles industries, food, wine, and beverage industries, the military among others. Owing to the consistent threat of microorganisms, environmental hazards to public health, the significance of phytochemistry in the medical and pharmaceutical industries for the quest for the discovery of new drugs has overshadowed their essence in other industries.

Phytochemicals have been in existence since time immemorial and are known to be responsible for the organoleptic properties (color, taste, flavor, aroma, and odor) of plants, such as the smell of garlic, ginger, and the deep purple color of blueberries. The ability of plants to exhibit

curative potentials and the characteristic difference that exists within them may also have awakened early interests for the knowledge about their chemical compositions. In the plant kingdom, these variations are quite glaring. One example is the Four O'Clocks plant (*Mirabilis jalapa*), called the marvel-of-Peru, or beauty-of-the-night because of its ability to open in mid-afternoon through the night and closes in the early morning. *Mirabilis*, a Latin word meaning wonder, also radiates some pleasant fragrances and exhibits flowers of different colors such as a white, red, pink, yellow, and some two-toned blooms simultaneously on the same plant. This phenomenal features in their biodiversity can be understood through the study of some chemical networks and interactions within the plants and its external environment. Plants are diverse and widely distributed from lands, rocky hills, mountains to marine environments. There are over 400,000 species of plants in the world (Pitman and Jørgensen, 2002), out of which only a small fraction of about 35,000–70,000 species of plants have been screened for their medicinal use (Veeresham, 2012). The medicinal potentials of phytochemicals are exhibited from the least primitive to higher plants. According to Fabricant and Farnsworth (2001), about 80% of 122 plant-derived drugs are related to their original traditional uses. Reportedly, as at the dawn of 21st century, 11% of the 252 drugs considered as basic and essential by the World Health Organization (WHO) were exclusive of flowering plant origin (Veeresham, 2012).

In the evolutionary study of phytochemicals, it was believed that there was little free oxygen in the atmosphere when plants first evolved. The direct consequence of this is that as plants metabolize, the oxygen concentration in the world increased. This polluted the environment and to deal with it, plants began to synthesize antioxidants molecules to protect it from highly reactive species that are cytotoxic to the plant cells. Moreover, the damaging effects of microbes on the cell structures of plants especially the important biomolecules has left plants with no options than to synthesize more bioactive compounds to protect it (see Chapter 3 for more details). This evolutional theory is supported by recent evidence in the compositional patterns of phytochemicals in plants. For instance, plant parts such as the leaves, flowers, stems, barks, roots, and seeds that are prone to insects, pests, microbial attacks, and the harsh environment have more amounts of phytochemicals than other parts of the plants. Another supportive evidence is the variation that exists in the same species of plants grown in the harsh environment and those in areas with less environmental stress (see Volume 3, Chapter 12 and 13 for more information).

Prior to the in-depth understanding of phytochemicals, the first tool employed by man is the "error and trial tools" which helped man to distinguish between edible and non-edible plants. Many casualties were recorded at that time. Grazing animals are not left out, they graze, identify, and avoid toxic plants through their sense of smell. The study continued and was widely utilized by the oldest medical system, the Chinese and the Indian Ayurvedic medicine, for the treatment of various diseases such as cancer, cardiovascular diseases, and stroke. The knowledge became prominent in the 19th and 20th century due to the extensive research using sophisticated hybrid chromatography and spectroscopy for the extraction, isolation, characterization, and purification of phytochemicals (see Chapter 8–14 for detailed information).

This time around, research is ongoing and individual molecules are constantly been discovered. The search for the discovery of new drugs and repurposing of existing ones have driven the study of phytochemistry to a new era employing in silico study techniques, applying simulation, and molecular docking procedures of bioinformatics and cheminformatics.

1.2 BRIEF HISTORY OF MODERN PHYTOCHEMISTRY

The ethnobotanical studies of medicinal plants for the treatment of diseases have existed since antiquity. For instance, the discovery of quinine marked the first successful use of chemical compounds to treat infectious disease (David and Jacoby, 2005). This was considered as the most important medical discovery of the 17th century (Achan et al., 2011). But in practical terms, the use of the quinine source, that is, the bark of the Cinchona (quinaquina) tree dated back as at the 16th century. However, the beginning of the isolation of plant chemical compounds marked the early stages of modern phytochemistry. One such example is the isolation of alkaloids by the brilliant pharmacist named Friedrich Wilhelm Adam Serturner (1783–1841) in the latter part of 18th century (Krishnamurti and Rao, 2016). This isolation not only led to the synthesis of new drugs but also to the purification of plant extracts used as medicines. It is important to note that apart from being the first to isolate an alkaloid, morphine, Friedrich Wilhelm was the first person to isolate an active ingredient associated with a medicinal plant or herb. Not long enough, his discoveries transformed pharmaceutical chemistry from a state of alchemy to an acknowledged branch of science (Krishnamurti and Rao, 2016).

Under similar circumstance, the scientists Pierre Joseph Pelletier and Joseph Caventou in 1820 isolated quinine from the herb *Cinchona officinalis*, a unique drug with the indication to be used against malaria (Dobson, 2001). Since then (within the last 300 years), many other compounds have been successfully isolated and characterized such as digitalis (1785), picrotoxin (1812–1884), curare (1856–1958), and salicin (1860–1877) (Dikshit, 2017). With the progress in biotechnology during the 1970's, a trend in the synthesis of various derivatives of plant metabolites by mimicking the biosynthetic pathways has led to the production of more stable, consequently, more effective albeit less poisonous compounds which are of commercial value. The in vitro synthesis of phytochemical is detailed in Volume 3 of this book.

Because terpenes represent a diverse nonetheless a problematic class for extraction, the scientists Croteau and Cane in the 1980's became the first to determine terpene-synthesizing enzyme, called terpene synthase which has led to the discovery of alternative pathways for the synthesis of terpenoids, monoterpenes, diterpene, and so forth (Hartmann, 2007). The year 1990 became a significant period for modern phytochemistry because of the development of sophisticated techniques.

1.2.1 STRUCTURE–ACTIVITY RELATIONSHIP

A feature of numerous modern-day drug is that they resemble the natural products from the structural point of view, hence, without the existence of these compounds in nature, scientist would never be able to treat the countless number of diseases. A typical illustration is morphine, a model substance of several anesthetics and salicylic acid, a model for the creation of acetylsalicylic acid (Lydon and Duke, 1989). Another supporting evidence of the stereochemistry of drugs (structural resemblance) which resulted in problems is thalidomide (an analog of glutethimide, a sedative), a sleeping substance administered to pregnant women with little-known aftermath effects. During that period, numerous children born from pregnant mothers who were administered it suffered phocomelia. This led the company to suffer legal issues and has to withdraw the product from the market. In reality, thalidomide contained a racemic mixture of both isomers, ((−) (S) and (+) (R) thalidomide), whereas the isomer (−) (S) thalidomide had teratogenic effects, the other (+) (R) thalidomide doesn't. Eventually, this drug has found usage in cancer therapy (Fabro and Smith, 1967).

1.2.2 TRENDS

During the first decade of the 21st century, there was a decrease in the interest of advancing the knowledge of plant-based chemistry by scientists and pharmaceutical companies for greater interest in synthetic drugs because they were easily mass-produced compared to the natural ones (Schmidt and Ribnicky, 2008). However, due to reported side effects in patients, numerous products were withdrawn from the market. A study has shown that the effect of natural remedies persist higher for patients receiving treatment for their long-lasting diseases. In view of this, there appears to be an increased usage of plant products since 2010, which provides slower effects yet with fewer side effects than synthetic medications. At present, new versions of the pharmacopoeia are also adding recent knowledge about phytochemicals to their volumes as well as gaining extended sophisticated products related to the modification of plant enzymes to easily obtain therapeutic substances. Worthy of note is that some remarkable phytochemicals have been discovered from the marine environment. Marine-derived compounds have recently gained a considerable interest because of the wide variety of pharmacological applications. A detailed overview of marine phytochemistry can be found in Volume 3 of this book.

1.3 RELATIONSHIP WITH OTHER SCIENCES

Phytochemistry is an important part of a number of disciplines. There have been controversies and speculations as to the place of phytochemistry in science. Some scientists consider it as a subfield of botany and chemistry while others believed it should be part of the food and medicinal chemistry because of its wide application in drug discovery. Categorically, phytochemistry is a fulcrum and an aspect of many biosciences and would be difficult to single out as a stand-alone science (Fig. 1.1). For instance, phytochemistry is an important part of Systematic Botany, Taxonomy, Ethnobotany, Conservation biology, Plant Genetic and metabolomics, Evolutionary Sciences and Plant Pathology. The field of Pharmacy and Pharmacognosy, Complementary and Alternative medicine, Ethnomedicine, Biochemistry, Microbiology, Bioinformatics and Computational Chemistry employs the knowledge of phytochemistry in the discovery of bioactive compounds. The field of biotechnology and process engineering, nutrition and food sciences, organic chemistry, employs the knowledge of phytochemistry in the production of natural products with increase phytochemical yields. In the control of

environmental pollution, the knowledge of phytochemistry is essential in applying bioremediation techniques such as phytoremediation to mop up harmful substances (detailed in Volume 3).

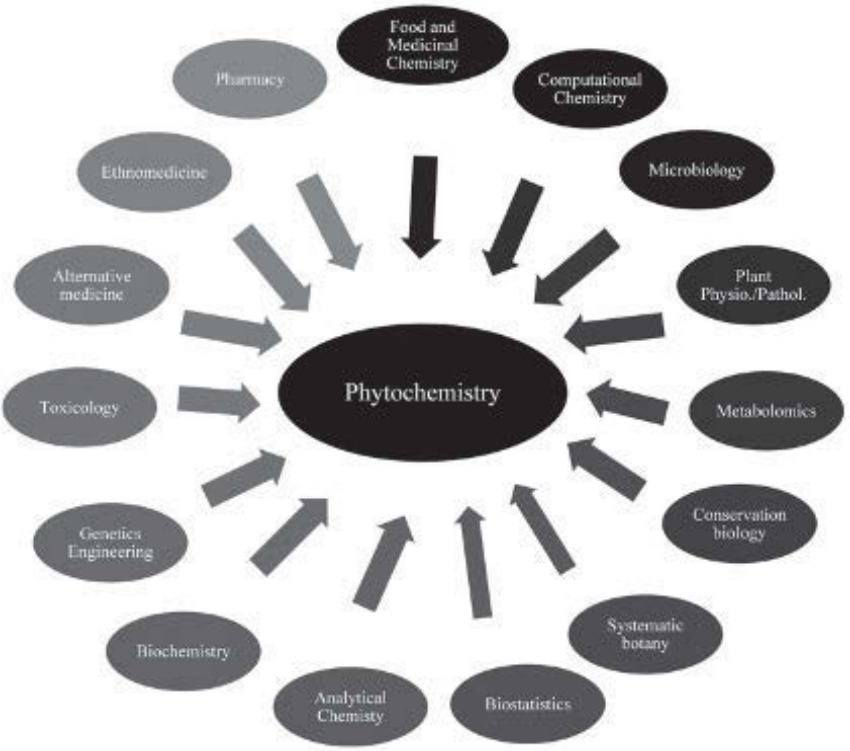

FIGURE 1.1 Some contributing fields to phytochemistry.

1.4 IMPORTANCE OF PHYTOCHEMISTRY

The knowledge of phytochemistry is essential in the:

1. Search for the discovery of new drugs and repurposing of existing ones (see Chapter 20–25 for more information).
2. Characterization and standardization of traditional herbal drugs in the crude form (see Chapter 9–15, 22 for a comprehensive information on the various techniques involved).
3. Assessment of the toxicity levels of plants (see Chapter 16 and 17 for details).

4. Understanding of plant physiology, biosynthetic pathways, and metabolomics (detailed in Chapter 2, 3 and 8).
5. Identification and classification of plants (see Chapter 7 for more information).
6. Study of inter and intraspecific chemical variability within plants (see Chapter 3 for details).
7. Biotechnology and genetic engineering for the optimization and synthesis of classic compounds (detailed in Volume 3).
8. Plant pathology (discussed in Chapter 3).
9. Development of environmentally friendly, biofungicides, insecticides, pesticides, and herbicides (detailed in Volume 3).
10. Food preservations (see Volume 3 for more information).
11. Phytoremediation of toxic substances such as poisons and heavy metals (presented in Volume 3).

1.5 PHYTOCHEMICALS, CLASSIFICATION, SOURCES, AND FUNCTIONS

Phytochemicals are simply plant-derived chemicals. The word "phyto" comes from the Greek word plant. It is used to refer to the secondary metabolites produced by plants. As noted earlier, these metabolites are usually synthesized as a measure for self-defense against insects, pests, pathogens, herbivores, ultraviolet exposure, and environmental hazards. Phytochemicals differ from the essential nutrients (primary metabolites) such as the carbohydrates, proteins, fats, minerals, and vitamins that are needed for the day to day maintenance of the plants. Sometimes, phytochemicals are used to refer to functional foods with antioxidant properties, nutraceuticals, phytonutrients, anti-nutrients, phytotoxins, and so forth.

1.5.1 CLASSIFICATION OF PHYTOCHEMICALS

There are tens of thousands of phytochemicals. So far, there could not have been a consistent classification system because of their numerous number and the pace by which new phytochemicals are discovered. A simple classification system divided phytochemicals into three chemically distinct groups. They are the phenolics, terpenes, N, and S containing compounds (Table 1.1).

TABLE 1.1 Classification of Common Phytochemicals.

Major classes	Subclasses	Representatives
Phenolics	Polyphenols	Flavonoids, isoflavonoids, chalconoids, lignans, stilbenoids (e.g., resveratrol), curcuminoids, tannins (e.g., protocatechuic and chlorogenic acids)
	Aromatic acid	Phenolic acids (e.g., gallic acid, tannic acid, vanillin, ellagic acid), hydroxycinnamic acids (e.g., coumarin)
Terpenes	Monoterpenes (C_{10})	Geraniol, limonene, pyrethroids, myrcene
	Sesquiterpenes (C_{15})	Costunolides
	Diterpenes (C_{20})	Abietic acid, cafestol, gibberellins
	Triterpenes (C_{30})	Azadirachtin, phytoecdysones
	Polyterpenes $(C_5)_n$	Tetraterpenes, for example, carotenoids, rubber
Terpenoids	Carotenoids (tetraterpenoids)	β-carotene, lycopene, phytoene
	Xanthophylls	Lutein, zeaxanthin
	Triterpenoid	Saponins, ursolic acid
	Steroids	Tocopherols (vitamin E), phytosterols (β-sitosterol, campesterol)
N (organonitrides)	Alkaloids	Nicotine, morphine, caffeine, theobromine, theophylline
	Cyanogenic glucosides	
	Nonprotein amino acids	Canavanine, azetidine-2-carboxylic acid
S (organosulfides)	Allicin, alliin, piperine Glutathione, phytoalexins	
Others	Phytic acid, oxalic acid, tartaric acid, malic acid, quinic acid	

1.5.2 SOURCES OF PHYTOCHEMICALS

Phytochemicals are found in fruits, vegetables, whole grains, spices, legumes, herbs, shrubs, and trees (Table 1.2). They get accumulated in plant parts at different concentrations such as in the leaves, fruit, bark, stem, roots, seeds, and flowers. Some phytochemicals are also synthesized by other living organisms such as fungi, although the mechanism by which they synthesize it might differ. However, many foods containing phytochemicals

are already part of our daily diet except for some refined foods such as sugar or alcohol. The easiest way to get more phytochemicals is to eat varieties of at least five to nine servings of fruits or vegetable per day representing colors of rainbows. Chapter 6 of this book gave a holistic view of phytochemicals acting as nutraceuticals.

1.5.3 FUNCTIONS OF PHYTOCHEMICALS IN THE LIVING ORGANISMS

Phytochemicals perform quite a number of roles in the living organisms (Table 1.2), and the mechanism by which they accomplish it has not been fully understood. However, phytochemical functions as:

1. Antioxidants by preventing oxidative damage of important biomolecules such as nucleic acids, proteins, and fats (elaborated in Volume 2).
2. Antimicrobial agents: antibacterial, antifungal, antiviral, anti-trypanocidal agents (discussed in Volume 2).
3. Stimulation of immune system (see Chapter 5 for comprehensive details).
4. Modulation of detoxifying enzymes.
5. Anti-inflammatory functions.
6. Reduction of platelet aggregations.
7. Physiological activities such as interfering with the binding of pathogens to cell receptors.

Others include antimalarial activity, antidiarrheal, antihelminthic, hepatoprotective, anti-atherosclerosis, anti-allergy, antidiabetic, antimutagenic, wound healing, pain relief, and antihypertension. Phytochemicals are also used in the treatment of a sore throat, cough, toothache, ulcers, menstrual bleeding, improvement of sperm count, dysentery treatment, stomach upset, vertigo, and appetite enhancing. Many other functions of phytochemicals exist depending on the plant. About 80% of the world most useful drugs are from plants. Chapters 4 and 5 of this book gave an overview of the biological functions and immunomodulatory properties of phytochemicals. Volume 2 of this book contains many chapters that discusses the various applications of plant natural products for the treatment of diseases.

TABLE 1.2 Types of Phytochemicals, Sources, and its Biological Effects.

Phytochemicals	Sources	Biological effects	References
Carotenes			
α-carotene	Carrots, sweet potatoes and winter squash, pumpkins, maize, tangerine	Antimetastatic agent, provitamin A, immuno-enhancement, cataracts, and macular degeneration	Liu et al. (2015); Rodriguez-Amaya (2015)
β-carotene	Carrots, sweet potatoes and winter squash, dark, leafy greens, red, orange and yellow fruits, and vegetables	Coloring agent and as provitamin A, antimetastatic agent, immuno-enhancement, cataracts, and macular degeneration, anti-autism agent	Avraham et al. (2017); Mehrad et al. (2018); Ravanfar et al. (2018)
Terpenes and Terpenoids			
Triterpenoid	Soybeans, beans, other legumes, maize, alfalfa	Anticancer agents, antidiabetic effect, anti-inflammatory agents, anti-oxidants, and so forth.	Grishko and Galaiko and (2016); Salvador et al. (2017); Xu et al. (2018)
Diterpenes	Mustard, bugleweeds, common skullcap, germanders, and so forth	Antioxidant, antifeedant, antimicrobial, anti-inflammatory	Faiella et al. (2014); Grishko and Galaiko (2016); De Oliveira et al. (2017)
Monoterpenes	Oils of citrus, cherries, spearmint, dill, garlic, celery, maize, rosemary, ginger, basil, citrus oils, caraway, mints	Applications in drugs, flavors, and fragrances, advanced biofuels, enzyme inhibition, effects on bio channels	Zebec et al. (2016); Mewalal et al. (2017); Zhang et al. (2017)
Steroids	Almonds, cashews, peanuts, sesame seeds, sunflower seeds, whole wheat, maize, soybeans, many vegetable oils, avocado, rice bran, wheat germ, corn oils, fennel, peanuts, soybeans, hawthorn, basil, buckwheat	Neuroactive, neuroprotective, and immunomodulatory increase muscle and bone synthesis, regulate many aspects of metabolism and immune function, influence sex differences, and support reproduction	Chrbolka et al. (2017)
Astaxanthin	Salmon, green algae, Krill, arctic shrimp, red snapper	Nutraceuticals, cosmetics, and the food and feed industries, pigmentation, aquaculture, antioxidant, and so forth.	Rodriguez-Amaya (2015)

TABLE 1.2 *(Continued)*

Phytochemicals	Sources	Biological effects	References
β-Cryptoxanthin	Kamut, corn flour, quinoa, noodles, egg	Provitamin A activity, antioxidant, reduces inflammatory disorders, effective against rheumatoid arthritis, antimetastatic potential.	Rodriguez-Amaya (2015)
Lutein	Colorful fruits and vegetables	Reduced risk of age-related macular degeneration and cataracts, found in macular pigment of human retina	Olaf Sommerburg (1998); Rodriguez-Amaya (2015)
Phenolic compounds			
Natur al monophenols	Parsley, celery leaf, rosemary, sage, oregano, thyme, pepperwort, wild bergamot	Antimicrobial, antioxidant	Gutiérrez-Larraínzar et al. (2012)
Polyphenols and others			
Flavonoids	Red, blue, purple pigments, tea, strawberries, gooseberries, cranberries, grapefruit, apples, peas, brassicas (broccoli, kale, brussels sprouts, cabbage), chives, spinach, endive, leek, tomatoes	Antioxidant, antimicrobial, enzyme inhibition	Ouédraogo et al. (2018); Xu et al. (2018)
Isoflavonoids	Soy, alfalfa sprouts, red clover, chickpeas, peanuts, kudzu, other legumes	Antimycobacterial agents, estrogenic agents, wound healing agents, antimicrobial potential	Araya-Cloutier et al. (2017); Coronado-Aceves et al. (2017); Ergene Oz et al. (2018);
Aurones	lemons, oranges, and grapefruit	Active against hepatitis C, neuro-generatic agents, antifungal agents, antimicrobial, anticancer, antiviral, antimalarial, antioxidant, anti-inflammatory, anticarcinogenic	Liew et al. (2015); Boucherle et al. (2017); Liew et al. (2017)

TABLE 1.2 *(Continued)*

Phytochemicals	Sources	Biological effects	References
Chalconoids	Licorice root, citrus peel, apple peel	Anticancer, antimalarial, antimicrobial, anti-inflammatory, anti-protozoal, anti-oxidant, antiproliferative agents, enzyme inhibition.	Singh (2014); Mostofi et al. (2015); Mirzaei et al. (2017)
Flavonolignans	Artichokes, milk thistle	Antioxidant agents, antiviral agents, hepato-protective agents, inhibiting blood coagulation	Althagafy et al. (2013); Pyszkova et al. (2016); Bijak et al. (2017);
Lignans	Apricots, strawberries, Broccoli, kale, oats, wheat	Cytotoxic against human tumor cell lines, antioxidant, neuroprotective, cognition enhancement, anti-inflammatory, enzyme inhibition	Jiang et al. (2017); Sowndhararajan et al. (2017); Xu et al. (2017);
Stilbenoids	Grape (skins and seeds, grape wine), nuts, peanuts, Japanese knotweed root	Cytotoxic against human tumor cell lines, antioxidant, phytoalexins activity, anti-inflammatory activity, enzyme inhibition	Basheer et al. (2017); Cao et al. (2016); Hu et al. (2018)
Curcuminoids	Turmeric, mustard.	Anti-turmeric potential, anti-diabetic potential, drug interactive ability, painkiller, anti-microbial agents.	Bahramsoltani et al., (2017); De Meloa et al. (2017); Verma et al. (2017)
Tannins	Tea, berries, horse chestnut (*Aesculus hippocastanum*), cranberry juice, peanut skin	Enzyme inhibition and speed up agents, albumin interactive agents, wound healing promoters, anti-microbial agents,	Adamczyk et al. (2017); Barrett et al. (2018); Sekowski et al. (2018);
Aromatic acid	Peppermint, licorice, peanut, wheat, vanilla beans, cloves, soy	Anti-oxidant agents, anticancerous effect, antibacterial agents	Cvijetic et al. (2018); Zhang et al. (2018)
Glucosinolates	Broccoli, cabbage, kale, cauliflower, turnip, mustard greens	Antioxidant, antitumeric effects, high doses cause toxicity, lower doses stimulate appetite, heart-protective agent	Bieganska-Marecik et al. (2017); Radošević et al. (2017)
Betalains	Beets, chard, Amaranthus tricolor	Antioxidant, anti-tumeric, anti-lipidemic, and antimicrobial activity	Gengatharan et al. (2015); Kumar et al. (2015)

TABLE 1.2 *(Continued)*

Phytochemicals	Sources	Biological effects	References
Chlorophylls	Colorful fruits and vegetables.	Antioxidant, photosensitizers, clastogenic activity, wound healing, anti-inflammatory, photodynamic therapy	Dos Reis et al. (2015); Roca et al. (2016)
Amines	Beetroot	Cytotoxic nature, International Agency for Research on Cancer declared heterocyclic aromatic amines as carcinogenic	Canales et al. (2017); Gu et al. (2018); Papageorgiou et al. (2018)
Carbohydrates	Wheat, barley, rye, oat	Source of energy, maintains cells, tissue, organ structures, some have role in maintaining stomach acidity, additives, role in insulin resistance, brain functions regulator	Arens (2018); Barazzoni et al. (2017); Gerschenson et al. (2017).

1.5.4 ROLE OF PHYTOCHEMICALS IN PLANT DISEASE MANAGEMENT

Plants synthesize a large number of secondary metabolites numbering above 200,000 that do not play a direct role in their growth but help them to survive in the environment especially by providing defense against diseases and pests. The wide variety of secondary compounds is synthesized mainly by the isoprenoid, phenylpropanoid, alkaloid or fatty acid, or polyketide pathways. The biosynthesis of phytochemicals is detailed in the next chapter.

Plant disease management involves the reduction in the economic loss of plants due to diseases caused by pathogens. Plants have evolved several mechanisms which have led to the production of tens of thousands of phytochemicals. Earlier, plant-based chemicals constitute a very small portion which was overlooked. Since the introduction of Food Quality Protection Act of 1996, there has been a vast market opportunity for agro-allied-based chemicals used in plant disease management in the United States and most of North America (Isman, 2000).

A lot of essential oils in plants have shown a high potential for getting rid of insects. A range of essential oils such as cinnamaldehyde, α-pinene, extracts from clove (*Syzygium aromaticum,* major oil being eugenol) and star anise (*Illicium verum*) has been shown to have fumigant and antifeedant effect on red flour beetle (*Tribolium castaneum*), and the maize weevil (*Sitophilus zeamais*) (Ho et al., 1995, 1997; Huang and Ho, 1998; Huang et al., 1998). Eugenol and oils from the holy basil (*Ocimum suave*) have also shown to be effective against *Sitophilus granarius* and *Prostephanus truncatus* (Obeng-Ofori and Reichmuth, 1997). Essential oils of cumin, star anise, oregano, and eucalyptus have been shown to be active against greenhouse pests such as cotton aphid (*Aphis gossypii*) and carmine spider mite (*Tetranychus cinnabarinus*) (Tuni and Sahinkaya, 1998). Volume 3 of this book contains comprehensive chapters on the role of essential oil in pest and disease management.

Plant-derived aldehydes and ketones play key roles against pathogenic fungi. Among aliphatic aldehydes and ketones, cinnamaldehyde has been shown to have the most potent activity against fungi especially two species of *Penicillium* that causes disease in humans (*P. cyclopium* and *P. frequentans*). The effects of perillaldehyde and citral were slightly weaker but potent enough. *Penicillium ulaiense*, an important pathogen causing molds in citrus, and other *Penicillium* spp. causing molds in apple and pear can be targeted using these aliphatic aldehydes that have one or more double bonds

conjugated to their carbonyl group. Among aromatic aldehydes, cuminaldehyde had been shown to have fairly potent antifungal activity (Kurita et al., 1981). The essential oils of *Thymbra spicata* and *Satureja thymbra* plants used as spices in Mediterranean cuisine have been shown to inhibit phytopathogenic fungi such as *Fusarium moniliforme*, *Rhizoctonia solani*, and *Phytophthora capsici* at a concentration of 400–800 μg/mL. Thymol and carvacrol have been identified as the major constituents in the essential oils involved in the fungicidal property, followed by monoterpenes γ-terpenin and p-cymene (Muller et al., 1995).

Phenolic compounds play a significant role in plant defense against bacteria and fungi. One important phenolic compound is coumarin. Halogenated coumarin, often brominated, chlorinated, or iodinated, is more stable than coumarin. It has been shown to be particularly effective against plant pathogenic fungi such as *Macrophomina phaseolina* (charcoal rot), *Phytophthora* spp. (damping off and seedling rot), *Rhizoctonia* spp. (damping off and root rot), and *Pythium* spp. (seedling blight). These four fungi are from different families, showing the broad spectrum activity of halogenated coumarins. In addition, halogenated coumarins have polymer seed coating abilities and less phytotoxicity, making them good candidates for natural pesticide development. In another study, 7-hydroxylated coumarin has been shown to be effective against parasitism of *Orobanche cernua* in sunflower (Serghini et al., 2001).

Tannins are another class of phenolic compounds that provide defensive properties. Though tannins are mostly known to provide defense against herbivores due to their astringent properties, they also play some fungicidal roles. They are active against *Colletotrichum circinans*, a fungus that causes smudge in onions. Tannins are also known to be inhibitory for fungal spore germination. Tannins are also known to be inhibitory for fungal spore germination (Mazid et al., 2011).

Throughout evolution, plants have come up with multiple defense mechanisms against different pathogens and predators. With the development of high throughput technologies, the understanding of the mechanisms of plant–pathogen interactions has widen. Subsequent chapter address this in details.

1.6 PHYTOCHEMIST, SKILLS, AND FUTURE PROSPECTS

A chemist or chemical scientist is one that is involved in research activities related to chemical analysis, confirmation of elements, elucidation

of the structure of chemical compounds for industrial purposes. But a phytochemist is a specialist who is interested in the study of chemical interactions in plants based on the knowledge of chemical science which is employed for a successful isolation of its components and the determination of its molecular structure through the study of its properties. The phytochemists have a good command over medicinal plants through the study of plant physiology, morphology, internal structure elucidation, and metabolic activities. A phytochemist is one who is knowledgeable about the identification, characterization of different natural products by using biochemical analysis to understudy the chemical composition of different plant products. The utilization of plants for medicinal purposes is not a new approach. However, periodically, plants are explored for extraction of chemical compounds which are beneficial to humans in several aspects. Once a plant-derived product is confirmed to exhibit curative potentials, the product will be recommended for drug designing, clinical approach, and finally to pharma industries.

1.6.1 SKILLS AND EXPERTISE REQUIRED OF A PHYTOCHEMIST

A phytochemist is required to be knowledgeable about the basics of plant science, isolation, and identification of molecules from plants. The knowledge and expertise on different analytical techniques for extraction, characterization, and quality assessment is a prerequisite. In addition, a phytochemist should be familiar with natural products induction, metabolomics profiling (nuclear magnetic resonance [NMR], mass spectrometry [MS]), micro-fractionation, natural products database, e-bioprospecting. Expertise in the state-of-art techniques including the various extraction methods, for example, solvent extraction methods, superficial fluid extraction, microwave-assisted extraction, chromatographic fingerprinting, and marker compound analysis is required. Advances in chromatographic techniques (liquid chromatography–MS; liquid chromatography–NMR), gas chromatography–mass spectroscopy, anti-microbial and antioxidant studies will help in a comprehensive analysis of natural product extracts. In the recent years, studies are being conducted in relation to the stress induction of natural products under metabolomics view (plant metabolomics). The use of metabolomics in conjunction with direct NMR profiling approaches is important for the understanding of metabolic reactions in plants. Chapter 8 of this book provides an overview of plant metabolomics and its applications.

1.6.2 FUTURE PROSPECTS FOR PHYTOCHEMISTS

Today, a huge sector of the population is relying on medicinal plants for their preventive and curative properties. WHO stated that those traditional medicine/ethnomedicine are still being used to treat different ailments. Nearly 70% of the populations rely on these medicinal practices. People from remote areas and semi-urban regions of the globe still depend on either crude or purified product from the plant's origin. Standardization and quality control is an important factor in ethnomedicinal formulations. At present, the phytochemists aim to apply modern techniques to preserve and maintain the standard and quality of these plant products. Findings by phytochemists through research investigations are supporting the ethnomedicinal formulations used by the tribal doctors. Several chemical compounds derived from plants are undergoing clinical trials and some of them are in preclinical treatment. Similarly, research is to be focused on the possibility of developing new products in combination with natural compounds from ethnomedicine. They identified the diversity of chemical compounds observed with phytochemicals which have led to the formulations of novel drugs against multidrug-resistant pathogens.

Phytochemists are seriously embarking on research activities involving the extraction of natural compounds present in plants. Some of these phytochemicals have the ability to suppress the activity of cancer cells by encouraging cell cycle inhibition and apoptosis. There is a lot of demand for natural products of plant origin and these by-products are to replace the synthetic products in view of their side effects on human health. Therefore, a lot of attention is needed toward natural products in which a phytochemist plays a key role in this context. Owing to the increasing demand for novel drugs, so many important and vital compounds regularly being manufactured by the industries generate employment opportunities for experts in this field. In addition, the increasing acceptance of the chemical diversity of natural products is well suited to provide the core scaffolds for future drugs. There will be further developments in the use of novel natural products and chemical libraries based on natural products in drug discovery campaigns.

1.7 COMPUTER-AIDED PHYTOCHEMICAL STUDIES

Advances in the knowledge of genome sequencing have led to increase in knowledge of therapeutic targets for pharmaceutics research (de Ruyck et al., 2016). Just as the knowledge of high-throughput crystallography

and NMR methods have developed over the time and contributed to the acquisition of atomic structures of proteins and protein–ligand interactions to an increasing level of detail (Gore and Desai, 2014), the knowledge of computational phytochemistry has also grown to give insight into protein interactions with their ligands. Computational phytochemistry method has been utilized by research-based pharmaceutical industries to study structure–activity relationships (Hughes et al., 2011).

This aspect of phytochemistry among other computer-aided drug discovery program have gain extensive use in studies of drug candidates, to increase their efficiency and development pipeline, based on their purpose and required interest (Zhang, 2011). Among such programs are docking techniques. Docking program is a software technique that allows the user to fit a molecule (ligand) into target (protein)-binding sites. It can also be used to predict the structure of the molecular interactions between these pairs (ligand/protein). The ligands are often relatively the smaller molecules which conformations (ligand–receptor complexes), binding energies or affinities, and nature of interactions are assessed in the binding site of their receptors, which are relatively larger macromolecules. The various software, databases, and tools for molecular docking and dynamics simulations are detailed in Chapter 19 of this book. Its applications are discussed further in Volume 2.

Molecular docking studies have diverse applications. It is a powerful and important modeling tool utilized in modern drug discovery. They are cheap, convenient, and not time-consuming, as several samples could be asses in lesser time. Prior to in vivo studies, molecular docking studies are used to access lead compounds for further studies. Its applications to phytochemicals studies are of immense importance, some of which are summarized below;

1. **Identification of natural phytochemical:** Molecular docking tools are used in identifying natural phytochemical as well as in the repurposing of existing commercial drugs that are effective in treating disease. In applying this knowledge to phytochemical studies, the constituents in a particular plant are tested individually for their activities against key enzymes in selected diseases.
2. **Development of phytochemical database:** There exist the importance of means to obtain phytochemicals in the format required for in silico studies. Data-based design for this purpose have been identified (Barlow et al., 2012). The availability of phytochemical dataset with their various two-dimensional/three-dimensional structure, possible target proteins, simplified molecular input line entry

specification, MOL2 files, and chemical class may enable their use in docking studies. This will ease the process of identification of lead compounds from natural products and their development into phytomedicines (Pathania et al., 2015).

3. **Sorting out lead compounds:** Molecular docking studies are applied to a large database, in order to identify hit compounds. The defined program and the various scores obtained from each compound can be compared to identify such hit compounds.
4. **Optimization of lead phytochemicals:** Molecular docking can be used to predict and subsequently develop a more potent, and effective drug candidates from selected phytochemicals. The develop candidate end up with optimized ligand–protein interactions.
5. **Identification of the mechanism of action:** When in search of the mechanism of action of certain active phytochemicals, molecular docking studies may be applied in order to identify the nature of the interaction of the compound with the protein. Their binding affinities and nature of interactions can similarly be analyzed.

1.8 BIOSTATISTICS AS A TOOL FOR PHYTOCHEMISTS

Biostatistics is the application of statistics to topics relating to biology. Biology, is a natural science concerned with life and living organisms, branches out to a handful of other related fields such as ecology, zoology, anatomy, microbiology, biochemistry, and so on. Statistics as the best breed of mathematical analysis provides objective and rational methods that enable research questions to be answered and rational questions to be asked for research after research.

The scope of biostatistics ranges from formulating the research question until the presentation or publication of the results. Biostatisticians and practitioners spend a considerable amount of time in developing research designs, evaluating methodologies, and analyzing statistical results. However, just like how it is used in any other fields, statistics in biology can often be misused. And as the application moves into life applications such as medical and pharmacological studies, statistics must be practiced efficiently and truthfully.

A misconception exists that the role of statistics come into play after data collection – when the analysis is done to determine rationale findings regarding the data. However, statistics should be well accounted all throughout the research process. Good research problems start with simple

and measurable objectives. Even for qualitative studies, there must be a clear set of indicators to be used in the research. A sound literature review needs good statistics for the research to gain some traction regarding its significance. Methodologies and data analysis must be systematic and abiding with the fundamentals of the different fields not only biology but most commonly with chemistry and mathematics. Lastly, publication and result dissemination require a grasp of the understanding of how statistics can be used to relay findings in scientific yet technical manner. Essentially, the role of biostatistics is to keep every aspect of a research, from the objectives down to result publication, as efficient and valid as possible.

1.8.1 BIOSTATISTICS IN PHYTOCHEMICAL RESEARCH

Phytochemical research has been able to coexist with biostatistics smoothly. The study of how chemicals from plants may potentially affect humans and other organisms has been objectified and quantified through statistics. Specifically, it is in pharmacology and drug development that biostatistics has been considered not only vital but necessary. The application of biostatistics in pharmacology answers three key notions – validity, significance, and consistency.

The use of biostatistics for validity refers to a sound formulation of objectives, research idea, and importance. One researcher may pick a random plant, derive a concoction, and then test its antimicrobial properties. However, a good phytochemical research involves an assessment of the community needs and formulating measurable objectives. This can only be done by looking into the statistics (statistics in its plural form) related to the plant. This could be about its folkloric use, history, preparation as well as how many studies were done regarding this plant. The validity also comes from the statistics found from these accompanying literature whether future studies can bare fruitful results. Measurable objectives also contribute to validity. Using statistical concepts such as the types of variables and varying levels of the measure will ensure sound objectives and will later on be the basis for statistical analysis.

Testing for significance has been the highlight between the relationship of pharmacology and statistics. To test for significance, treatments (usually in dosages) are compared with a positive treatment (a commercially manufacture drug) and a negative control. This phase of the research is fairly straightforward; nonetheless, the analysis is highly dependent on a sound methodology and appropriate statistical test/analysis. Most often researches

do not amount to anything due to bad or mismatched statistical testing. This is where the role of a biostatistician is crucial as they bridge the gap of knowledge between masters of two different fields. A research could only be valid if the statistical methodology on it fits the actual objectives.

With that, consistency allows the public to assess at a glance the research and development that has come through for a specific pharmacological study. For example, ASCOF® Lagundi herbal drug that came from *Vitex negundo* went through almost 42 years of research and development. Summative studies were done to determine the consistency of the findings regarding its anti-inflammatory and anti-histaminic properties before it was commercially made available to the public. Simply, using statistics to evaluate results ensures public safety and protects the best interests of every stakeholder.

1.8.2 ROLE OF A BIOSTATISTICIAN

A lot of statistical tests are utilized for pharma research. A biostatistician must be equipped with three core skills to ensure a productive study. First, he must be equipped with a portfolio of parametric/nonparametric tests and other statistical methods. Sampling or sampling calculations is often an overlooked aspect of pharmacological studies. A practitioner must be able to understand the purpose of the study, the research design, and methodology only then can he start with the sampling technique. A practitioner should at least be equipped with a standard set of parametric and its nonparametric counterpart, with their corresponding post hoc analyses. Classic tests such as analysis of variance, t-tests, and logistic regression analysis have always been effective in pharmacological studies.

Second is that a practitioner must be able, and willing, to understand both sides of the coin – statistics and biology. Compromise has been the key in clinical trials to fit goals with appropriate statistical tests. A practitioner must be able to discuss results on a life science perspective without losing the technicality statistics brings onto the study. Last, practitioners must be able to adapt to technology and developments in the growing field of biostatistics.

1.8.3 TOOLS FOR BIOSTATISTICS

Research and development in biostatistics have always associated with the release of multiple automated software that made the mitigation between

biology and statistics easier. Microsoft's Excel spreadsheet has been able to adapt to the increasing demand by publishing downloadable macros that can be used for biostatistics, econometrics, and applied statistics. SPSS and R GUI has been the go-to professional package and academic statistical packages, respectively. SPSS offers user-friendly interface that enables non-practitioners to run data, meanwhile, R GUI has been the most dynamic statistical software tool as it takes advantage of the user's in-depth understanding of statistics to program through codes the different tests. With the increasing demand, specific programs have been made available for commercial usage. OpenEpi and Stata have been widely used in clinical trials. Meanwhile, EpiInfo has been the go-to software for medical professionals, researchers in clinical trials, and even by industrial manufacturers. Chapter 18 of this book detailed the various statistical tools used in phytochemical research with web links to download them while using essential oil studies to make references to the features of few.

1.9 MAJOR PHYTOCHEMICAL SOCIETIES AND FUNDING AGENCIES

There are several phytochemical groups, institutes, research centers all over the world. The activities of Phytochemical Society of North America (PSNA), Phytochemical Society of Europe (PSE), and Phytochemical Society of Asia (PSA) deserves mention.

1.9.1 PHYTOCHEMICAL SOCIETY OF NORTH AMERICA

The PSNA is a nonprofit organization with membership subscription fee. Its membership is open to anyone with an interest in phytochemistry. It aims to promote research on the chemistry and biochemistry of plant constituents, the physiology and pathology effects upon plant and animals, and the industrial applications of phytoconstituents. PSNA transcended from Plant Phenolics Group of North America to this day PSNA with Mabry, T. J. as it is first President as a Society in 1966. PNSA provides professional development opportunities such as research presentations (oral or poster), annual conferences, travel awards, the Frank and Mary Loewus Student Travel Award for undergraduate students, graduate students, and postdoctoral researchers. More information can be accessed from its website: http://www.psna-online.org/index.html.

1.9.2 PHYTOCHEMICAL SOCIETY OF EUROPE

PSE is a membership organization for everyone with the interest in phytochemistry. It promotes the advancement of the chemistry and biochemistry of plants constituents and its applications in industry and agriculture. PSE organizes two to three conferences yearly in the United Kingdom and Continental Europe and gives awards to deserving phytochemists. The society metamorphosed from Plant Phenolics Group to Phytochemical Group and to a Society in 1967 and finally adopted its present name in 1977. More information can be accessed from its website: http://phytochemicalsociety.org/.

1.9.3 PHYTOCHEMICAL SOCIETY OF ASIA

The PSA was founded in 2007 at Kuala Lumpur, Malaysia with Prof. Yoshinori Asakawa (Japan) elected as the founding President and Prof. Iqbal Choudhary M. (Pakistan) as the Vice President. The aim of the society is to promote collaborative research between scientists and in the growth and advancement of research in the field of natural products from of the region and outside the region. It has different types of membership such as regular membership, student membership, honorary members, life members, institutional membership with different subscription rates. More information can be accessed from its website: http://phytochemsoc-asia.com/.

1.9.4 FUNDING AGENCIES FOR RESEARCHERS IN PHYTOCHEMISTRY

Government funding programs are available in many countries for phytochemical and related investigations. Besides, international organizations such as Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services, International Union for Conservation of Nature and WHO are offering funds towards the progress of these investigations.

1.10 ADVANCES IN PHYTOCHEMICAL RESEARCH

So far, a substantial progress has been made for the discovery of new phytochemicals which could serve as a lead compounds for the development of new drugs. Drug discovery is capital intensive which usually starts with

the screening and identification of active ingredient in traditional plants. According to Business Wire, the global drug discovery informatics market is expected to reach the US $2.84 Billion in 2022 from the US $1.67 Billion in 2017 at a CAGR (Compound Annual Growth Rate is the mean annual growth rate of an investment over a specified period of time longer than 1 year) of 11.2%. The discovery of new drugs usually is carried out by pharmaceutical and biotechnology companies with the assistance from the universities. The search for the discovery of new drugs must be continued to stem out the ravaging effects of diseases in plants and animals. Many more phytochemicals abound yet undiscovered in plants because only a small fraction of the plants has been studied. The development of new analytical techniques and validation of the existing ones will greatly help in phytochemical research.

Computer-aided drug discovery is part of the modern drug discovery tool which helps to drive a fast and efficient discovery process through the identification of screen hits by molecular docking, modeling, and dynamics simulations. Target compounds are assessed for their binding affinity to target receptor cells, its selectivity, efficacy or potency, metabolic stability, and oral bioavailability. Once a compound is identified to fulfill the requirements, it will undergo phase I, II, and III clinical trials.

One drawback in the development of new drugs, is that many plant-derived compounds still cannot be synthetized and so are not commercially available. This is why the access to some drugs is quite expensive. More needs to be made in the area of phytochemistry including government and non-governmental agencies for fundings, grants, consultation, and collaboration in research projects and so on. The other way to improve the quality of research output is to establish the field of phytochemistry as a single stand-alone science. Although few institutions have done so there is a need for more tertiary institutions to do so.

Phytochemicals are naturally present in many foods but it is expected that through bioengineering, new plants will be developed, which will contain higher levels. This would make it easier to incorporate enough phytochemicals into the food.

1.10.1 PROGRESS IN PHYTOCHEMICAL RESEARCH

1.10.1.1 AS NANOPARTICLE (NP) SYNTHETIC PRECURSORS

Phytochemicals can act as cheap and raw materials for different classes of nanoparticle (NP) synthesis, which has been discussed in details in Volume 2.

Phytochemicals can be selectively utilized to synthesize the NP of interest, for example, having red emission, having good ability to carry drugs, and so forth.

1.10.1.2 AS A FABRICATING SOURCE OF NPS

Literature goes in favor of the phytochemicals as NPs surface decorating agents for enhancing the phytochemical activity of the related NPs. It is also very interesting that this field of research is nascent, and requires a lot of research which would definitely boost both the areas of phytomedicine and nanotechnology. A lot of reports are available for the applications of phytochemicals as fabricating source (Ahmad et al., 2017).

1.10.1.3 ANTITUMOR EFFECTS

Cancer is a leading cause of deaths worldwide. A lot of research has been done to overcome this notorious disease, while still much is needed to completely haul this disease. Chapters 20, 21, and 23 are research discoveries of potential anticancer drugs. Many chapters in Volume 2 and 3 of this book contain novel research in this direction.

Phytochemicals could contribute to the cancer treatment in the following ways:

a) Drugs source: certain important chemicals, such as taxol can directly be isolated from plants, and could be potentially used as an anticancer agent (Bo et al., 2016).
b) Along with a source of drugs isolation, phytochemicals could also be used to obtain NPs having anticancerous effects, as well as NPs using as agents for anticancerous drugs delivery, as well as photoluminescent agents in their treatment (Angelova et al., 2017, Kapinova et al., 2017, Kaur et al., 2017).

1.10.1.4 CUTANEOUS CARCINOMA

The prevention of this heinous disease by phytochemicals is of main concern these days as compared to their chemoprevention. It has been considered that phytochemicals are safely relieving multiple pathological processes,

including oxidative damage, epigenetic alteration, chronic inflammation, angiogenesis, and so forth (Wang et al., 2017).

1.10.1.5 OTHER DISEASES

Research outcomes from the study of the pharmacological effects of phytochemicals in the treatment of various diseases are largely being dependent on as interest is growing due to the safety of natural herbal products compared to the synthetic ones with side effects. Many medications for some ravaging diseases remain a mirage despite the enormous work in drug discovery. Diseases such as diabetes, a well-known disease of rich and poor, as well as found in developed and developing nations, have a lot of space to be treated phytochemically. The study of plant-derived chemicals is continuous with many sophisticated techniques being developed to assist in the discovery of compounds which will be of medical significance and to other industries for the betterment of life. An ample discoveries have been made but more is yet to be done.

KEYWORDS

- **phytochemistry**
- **phytochemicals**
- **terpenes**
- **flavonoids**
- **alkaloids**

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CHAPTER 2

BIOSYNTHESIS OF PHYTOCHEMICALS

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ABSTRACT

Phytochemicals are plant secondary metabolites derived from distinct biosynthetic pathways in plants. Their reactions are in some cases complex and require several enzyme catalyses. They are of diverse activities due to their numerous structural biological activities, which are relayed based on their importance in various pharmacological effects. The pentose phosphate, shikimate, malonyl-CoA, nucleotide metabolic, and mevalonate pathways are among the contributing pathways for phytochemical biosynthesis. These biosynthetic pathways, enzymes, and precursors of alkaloids, anthocyanidins, anthraquinones, flavonoids, glycosides, lignans, phenolics, saponins, steroids, and terpenoids are discussed further in this chapter. These pathways have been studied, in some cases to provide alternative means of obtaining phytochemicals in larger quantities with the aim to apply them in bioengineering, pharmaceutics, and nutraceuticals as well as in other fields.

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2.1 INTRODUCTION

Phytochemicals are products of plant metabolism devoid of growth and developmental functions in plants. They are required for other functions, which may include their defense and environmental survival. Their roles in photosynthesis, respiration, transport of solute, translocation, nutrient assimilation, and differentiation are not specified (Hartmann, 1998). Thus, they are termed secondary metabolites due to their noninvolvement in major biochemical activities and differentiating them from the primary metabolites, which are necessary for plants growth and survival. Moreover, they are considered nonessential and side products of the primary metabolites (Fig. 2.1). Phytochemicals are of diverse activities due to their numerous structural biological activities (Pandey and Kumar, 2013; Compean and Ynalvez, 2014), making them the important class of compound for drug development. Their biosynthetic pathways are focused on in this chapter, with some pathway discussed in details.

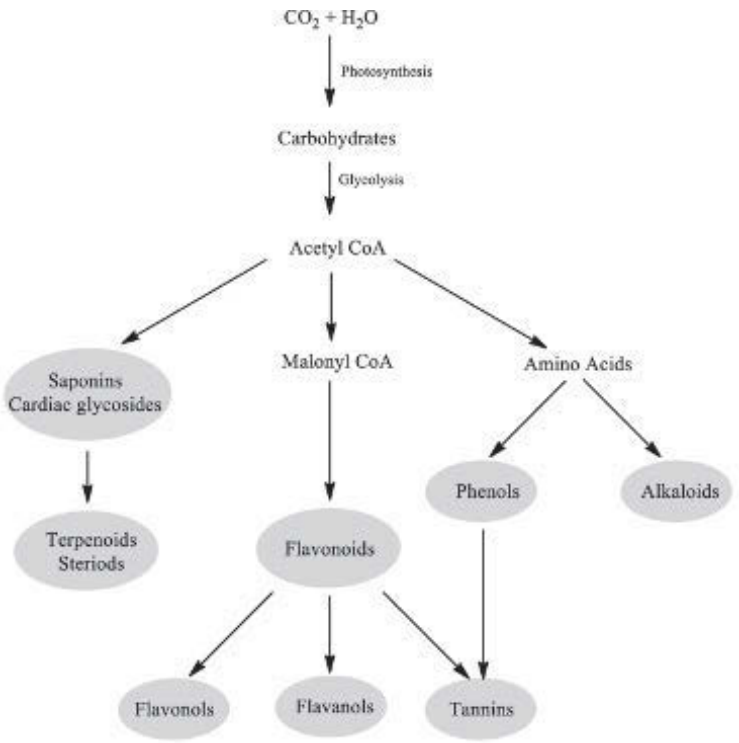

FIGURE 2.1 Interplay between the primary metabolites and the secondary metabolites.

2.1.1 CLASSIFICATION OF PHYTOCHEMICALS

Classifications of phytochemicals are done with no fixed criteria. Several phytochemicals have been identified and classified base on their physical and chemical characteristics (ACS, 2000; Arts and Hollman, 2005; Heneman and Zidenberg-cherr, 2008), while several others are yet to be identified (Zhang et al., 2015). Figure 2.2 attempts the classification of phytochemicals based on their chemical structures. The phenolics, flavonoids, and lignans are the major class from which other phytochemicals are drowned. Flavonoids are further divided into flavonols, flavanols, flavanones, and others as illustrated below. Based on the biosynthetic origins of phytochemicals, they can be classified into three major groups (Mazid et al., 2011): (1) the nitrogen-containing alkaloids or the sulfur-containing compounds such as camalexin; (2) the nonnitrogen-containing saponins, cardiac glycosides, terpenoids, and terpenoid-derived compounds, which include several steroids; and (3) the flavonoids, phenolics, and polyphenolics class of compounds.

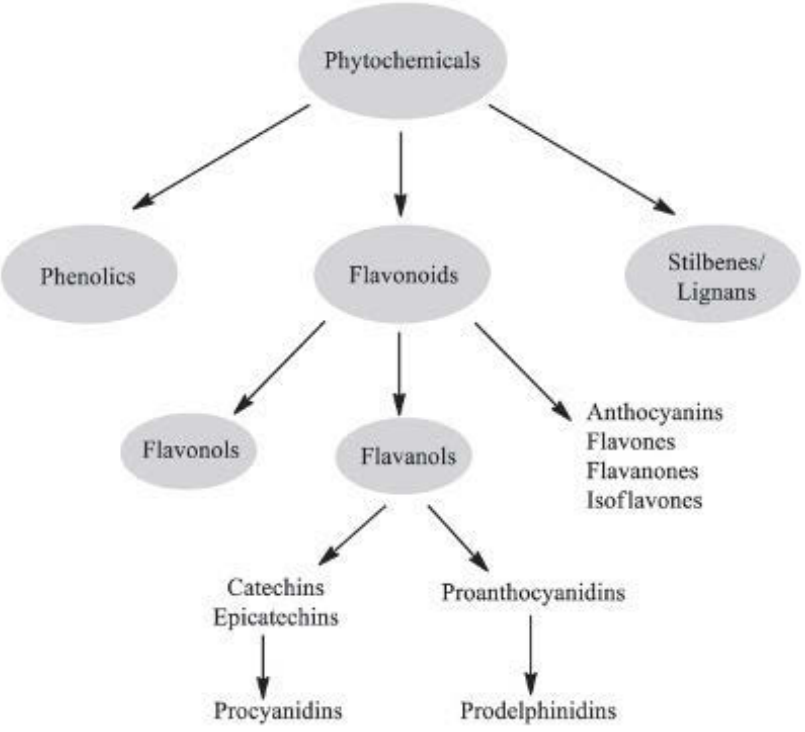

FIGURE 2.2 Simple classification of phytochemicals based on their chemical structures.

2.1.2 IMPORTANCE AND BENEFITS OF PHYTOCHEMICALS

Phytochemicals have diverse importance and relevance in different fields. They are important in pharmaceutical research, providing treatment and management options for different diseases. Phytochemicals are also important in the field of nutraceuticals and the science of food additive (Smetanska, 2008).

1. **Pharmaceutics:** The development of resistance to certain disease has posed many challenges to existing drugs, which have been known for their potent activities. The search for new and potent phytochemicals has provided therapeutic options for several diseases. Several researches on phytochemicals have been done extensively and the outcome is presently used as therapeutic options that are widely accepted and in use (Tijjani et al., 2017). Phytochemicals have beneficial roles in management and treatment of coronary heart disease; they prevent oxidations of low-density lipoprotein (LDL) and can inhibit the synthesis of cholesterol or its absorption (Mathai, 2000).
2. **Nutraceutics:** Phytochemicals have wide applications, both directly and indirectly in nutraceutics. The attractive colors of fruits and some edible plants are a result of the various plant secondary metabolites in them. The red colors of pumpkin, yellow color of corn, and the orange color of tomatoes are properties of their carotenoid contents. Fragrance in many aromatic plants is properties of different terpenes (Laxminarain, 2013). Similarly, the purple color in grapefruits is properties of their anthocyanins contents. Phenolics present in various teas, as tannins are responsible for their astringent taste when taken.
3. **Food additive:** Beside phytochemical benefits in nutraceutics, disease management, and treatment, they are also a good source of several food additives. Their additions improve food palatability and acceptability; they are better accepted by consumers when compared to artificial food additives (Smetanska, 2008). Anthocyanins are applied in soft drinks to give it a red color.

2.2 BIOSYNTHESIS OF PHYTOCHEMICALS

The synthesis of phytochemical is of critical importance to plants. Environmental necessities for survival are believed to trigger this synthesis;

such environmental threats include changes in climate, soil requirements, the presence of herbivores, pollinators, and microorganism (Stepp and Moerman, 2001; Mazid et al., 2011). The various reactions involved in plant biochemical synthesis are said to be generally reversible under the influence of specific enzymatic reactions. In general, plant secondary metabolites are either synthesis in vivo or hydrolyzed.

2.2.1 CONTRIBUTING PATHWAYS FOR THE BIOSYNTHESIS OF PHYTOCHEMICAL

A number of pathways contribute to the biosynthesis of phytochemicals (Fig. 2.3). One or two pathways can also be linked to the synthesis of a particular phytochemical. The contributing pathways include pentose phosphate pathway, a source of various sugar moieties found in the phytochemicals. The shikimic acid pathway generates the amino acids, which are further modified during the biosynthesis of alkaloids, phenolics, flavonoids, and lignins. The malonyl-CoA pathway synthesizes anthraquinone, anthocyanidin, and anthocyanin. Nucleotide metabolic pathway contributes to the synthesis of purine alkaloids, while terpenoids and steroids are biosynthesized majorly through mevalonate and non-mevalonate pathway.

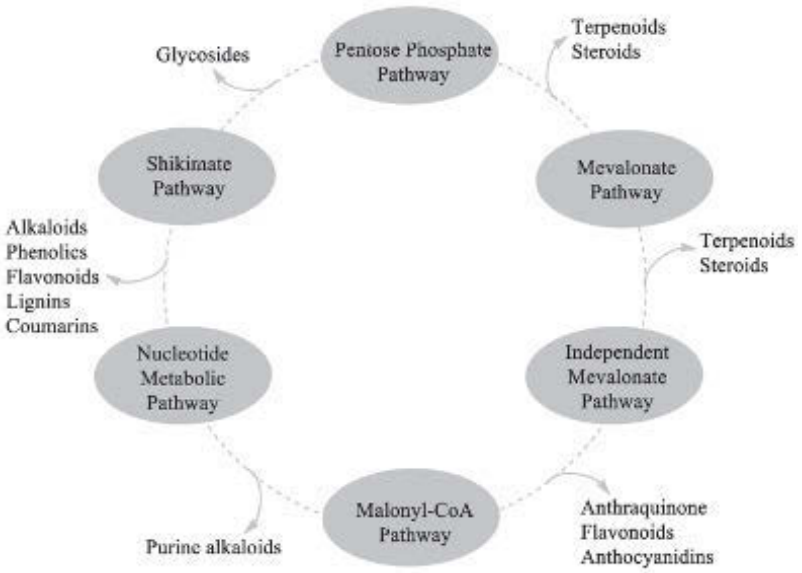

FIGURE 2.3 Contributing pathways to phytochemical biosynthesis.

2.2.1.1 PENTOSE PHOSPHATE PATHWAY

The pentose phosphate pathway provides various sugars, which are conjugated in glycosides. Some of the carbohydrates include arabinose, D-glucose, galactose, glucorhamnose, or L-rhamnose (Pretorius, 2003). The pathway occurs in both plants and animals in two phases (oxidative and nonoxidative) where a hexose phosphate sugar is oxidized to a pentose phosphate sugar with the release of CO_2 (Heldt et al., 2011). The pathway also provides NADPH for biosynthetic reactions. First, the enzyme glucose 6-phosphate dehydrogenase oxidizes glucose 6phosphate to 6-phosphogluconolactone (Fig. 2.4). The reaction is highly exothermic, irreversible, and utilizes $NADP^+$ to generate NADPH. 6-phosphogluconolactone is hydrolyzed by lactonase

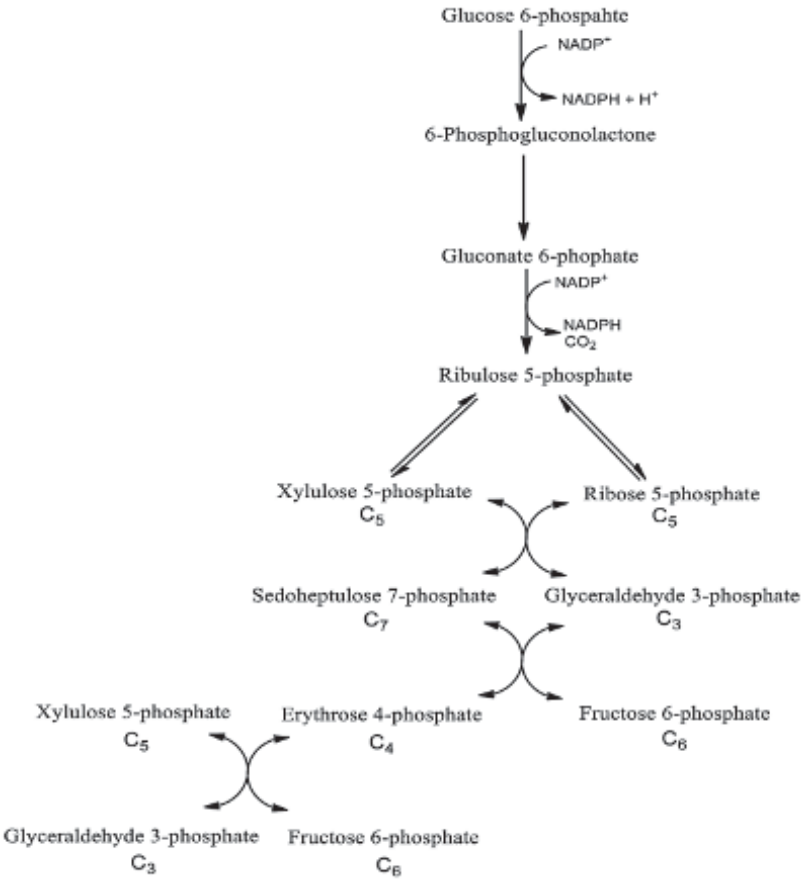

FIGURE 2.4 Pentose phosphate pathway.

to gluconate 6-phosphate (Heldt et al., 2011). Ribulose-5phosphate is formed by the oxidation reaction catalyzed by gluconate 6-phosphate dehydrogenase with the release of NADPH and CO_2. In the oxidative phase, xylulose 5phosphate and ribose 5phosphate are isomerized from ribulose 5phosphate by the enzyme ribulose phosphate epimerase and ribose phosphate isomerase, respectively. While in the nonoxidative phase, xylulose 5phosphate and ribose 5phosphate are converted to sedoheptulose 7phosphate and glyceraldehyde 3-phosphate by a transketolase. The C_{10} compounds, sedoheptulose 7-phosphate, and glyceraldehyde 3-phosphate are converted by a transaldolase to erythrose 4-phosphate and fructose 6-phosphate. Similarly, the C_9 compounds, xylulose 5-phosphate, and erythrose 4-phosphate are converted by a transketolase to glyceraldehyde 3-phosphate and fructose 6-phosphate (Heldt et al., 2011).

2.2.1.2 SHIKIMATE PATHWAY

The shikimic acid pathway located exclusively in plant plastids is for the synthesis of alkaloids, flavonoids, coumarins, lignans, and some cyanogenic glycosides. Others are cinnamates, quinones, and phenolics. The precursors for this pathway are erythrose 4-phosphate and phosphoenolpyruvate, the intermediates from the pentose phosphate pathway and glycolysis, respectively. First, a condensation reaction between erythrose 4-phosphate and phosphoenolpyruvate occurs in two steps catalyzed by dehydroquinate synthase to give rise to a cyclic dehydroquinate compound with the release of two phosphate groups (Fig. 2.5). From the product, a water molecule is removed, which results in the reduction of the carbonyl group to form 3-dehydroshikimate catalyzed by 3-dehydroquinate dehydratase (Heldt et al., 2011). Shikimic acid from which the name of the pathway is derived is then synthesized in the reaction catalyzed by the enzyme shikimate dehydrogenase. The 3′ hydroxyl group is then protected by a form of phosphorylation using ATP by shikimate kinase and liberation of ADP to form shikimate 3-phosphate (Heldt et al., 2011). The free hydroxyl group at the 5′ carbon reacts with one molecule of phosphoenolpyruvate to form 5′-enolpyruvyl shikimate-3-phosphate. Chorismate is formed thereafter by the hydrolysis of pyrophosphate by chorismate synthase (Heldt et al., 2011).

In another series of enzyme-catalyzed reactions, tryptophan is formed from which alkaloids are synthesized. Also, prephenate and arogenate are formed from which phenylalanine and tyrosine are synthesized. Prephenate is produced by the rearrangement of the side chain from 5′ carbon to 1′ carbon

by chorismate mutase, while arogenate is formed by the transamination of the keto group at the 1′ carbon by an amine transferase (Heldt et al., 2011). The removal of water and the decarboxylation of arogenate by prephenate dehydratase give rise to phenylalanine from which phytochemicals such as flavonoids, coumarins, and lignans groups can be synthesized. The oxidation of arogenate by NAD^+, followed by its decarboxylation results in the formation of tyrosine from which cyanogenic glycosides and other alkaloids are synthesized.

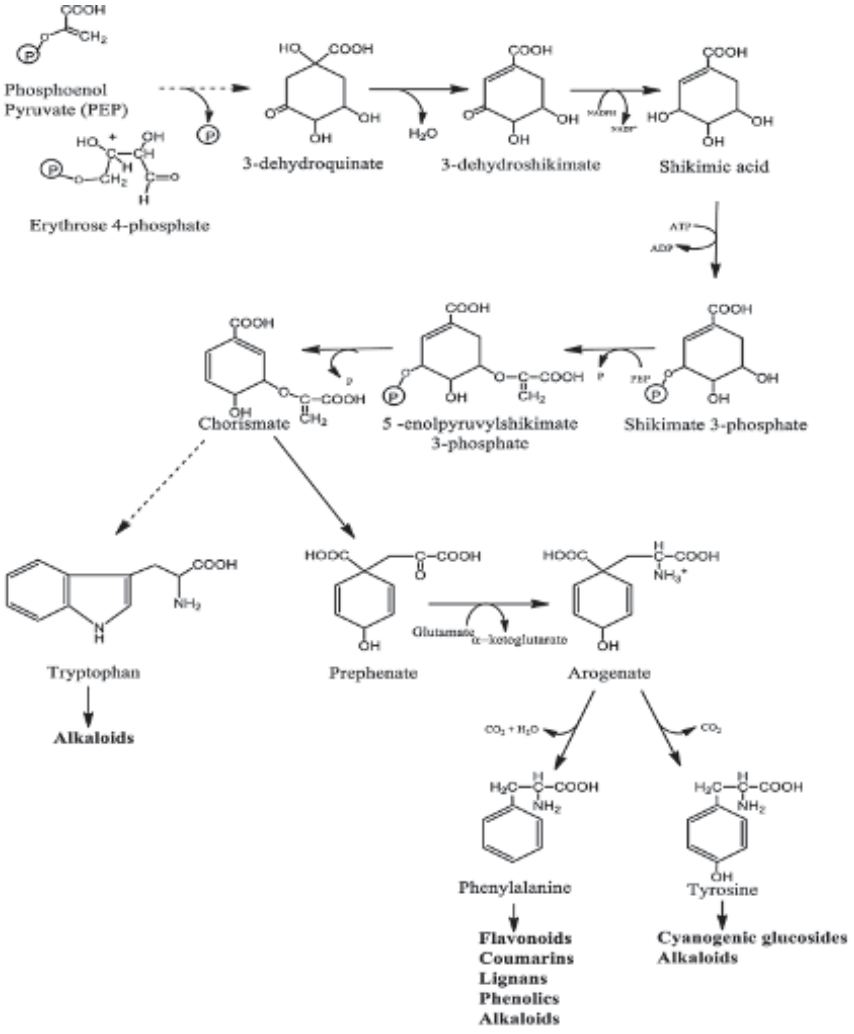

FIGURE 2.5 Shikimate pathway for the biosynthesis of phytochemicals.

2.2.1.3 MALONYL-COA PATHWAY

Malonyl-CoA is synthesized from acetyl-CoA by the action of acetyl-CoA carboxylase which requires biotin as a cofactor (Fig. 2.6). It is an irreversible reaction and the rate-limiting step in fatty acid biosynthesis. Malonyl-CoA is a key metabolite in the synthesis of anthraquinones, anthocyanins, and flavonoids.

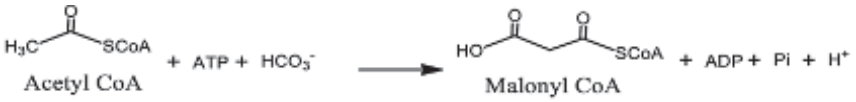

FIGURE 2.6 Malonyl-CoA biosynthesis from acetyl-CoA.

2.2.1.4 MEVALONATE PATHWAY

Mevalonate pathway for the synthesis of terpenes, terpenoids, and steroids is localized in the cytosol. The first reaction in the pathway involves the synthesis of acetoacetyl-CoA catalyzed by the enzyme acetoacetyl-CoA thiolase (Fig. 2.7). HMG-CoA is synthesized from acetoacetyl-CoA catalyzed by the enzyme HMG-CoA synthase. The next reaction is catalyzed by HMG-CoA reductase to produce mevalonic acid and then mevalonic acid phosphate by mevalonate kinase. Mevalonate monophosphate is phosphorylated again by

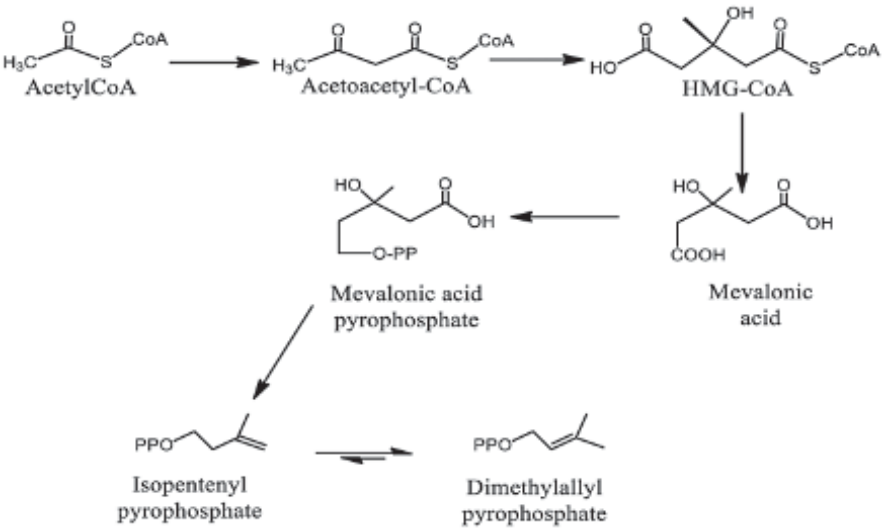

FIGURE 2.7 Mevalonate pathway.

phosphomevalonate kinase where a phosphate group is added. Mevalonate 5-diphosphate decarboxylase catalyzes the formation of isopentenyl pyrophosphate (IPP). A 1,3-allylic rearrangement converts IPP to dimethylallyl diphosphate (DMAPP) in a reaction catalyzed by IPP isomerase. The detailed mechanism of these enzymes has been reported elsewhere by Dewick (Dewick, 2002).

2.2.1.5 NON-MEVALONATE PATHWAY

The non-mevalonate pathway is also known as the triose phosphate or pyruvate pathway. Other names are 1-deoxy-D-xylulose-5-phosphate pathway or the 2C-methyl-D-erythrito-4-phosphate pathway. The pathway has been ascertained in bacteria, algae, and higher plants (Rohmer, 1999). In the reaction steps (Fig. 2.8), glyceraldehyde 3-phosphate condenses with pyruvate to form 1-deoxy-D-xylulose-5-phosphate (Rohmer, 1999; Hunter, 2007). In

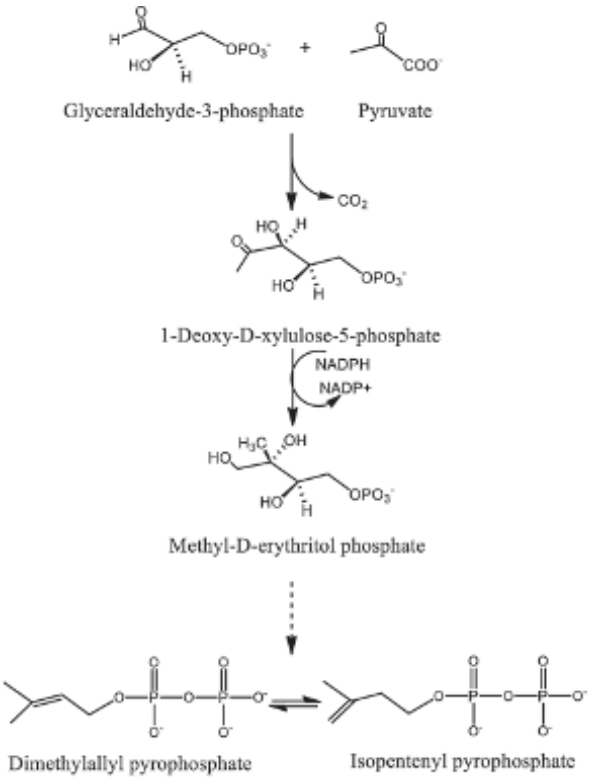

FIGURE 2.8 Non-mevalonate pathway.

the next reaction, which is NADPH dependent, forms the key intermediate 2C-methyl-D-erythritol-4-phosphate, from which IPP and DMAPP are formed (Rohmer, 1999; Hunter, 2007). Enzymes of the non-mevalonate pathway are recently being researched for the development of drugs against infections in pathogenic prokaryotes because the pathway occurs in them rather than in humans (Sassetti et al., 2001; Akerley et al., 2002; Kobayashi et al., 2003). Drugs from such research are targeted at diseases such as malaria and tuberculosis.

2.2.1.6 NUCLEOTIDE METABOLIC PATHWAY

The purine nucleotide metabolic pathway contributes to the biosynthesis of some phytochemicals. These types of phytochemicals, which belong to the alkaloid family, are referred to as the purine alkaloids (Zulak et al., 2006). The reaction pathway has been reviewed extensively by Ashihara et al. (2008). Xanthosine plays a central role and provides the xanthine skeleton frame of caffeine. It is contributed by four different pathways, which include the de novo synthesis of purine metabolism, degradation of adenine nucleotide to form AMP, degradation of guanine nucleotide to form GMP, and the S-adenosyl-L-methionine (SAM) cycle of adenosine formation to adenine.

Ribose 5-phosphate through a series of enzyme-catalyzed reactions generates inosine monophosphate (IMP) (Fig. 2.9). IMP dehydrogenase catalyzes the conversion of IMP to xanthosine monophosphate, followed by its conversion to xanthosine. The subsequent steps are reactions leading to the synthesis of caffeine. First, xanthosine is methylated by N-methyltransferase, an SAM depending enzyme to form 7-methylxanthosine catalyzed by 7-methylxanthosine synthase. The product is then hydrolyzed by N-methyl nucleosidase to 7-methylxanthine. Theobromine synthase and caffeine synthase catalyze the last two steps forming theobromine and caffeine, respectively (Ashihara et al., 1996). Several enzymes of this pathway have been isolated from plant sources as well as some of the genes encoding them (Suzuki et al., 1992; Kato et al., 1999; Kato et al., 2000; Mizuno et al., 2003; Uefuji et al., 2003; Ashihara and Suzuki, 2004).

Theobromine (3,7-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) are examples of purine alkaloid synthesis through this pathway. They are found in tea, coffee, and other nonalcoholic beverages (Ashihara et al., 2008).

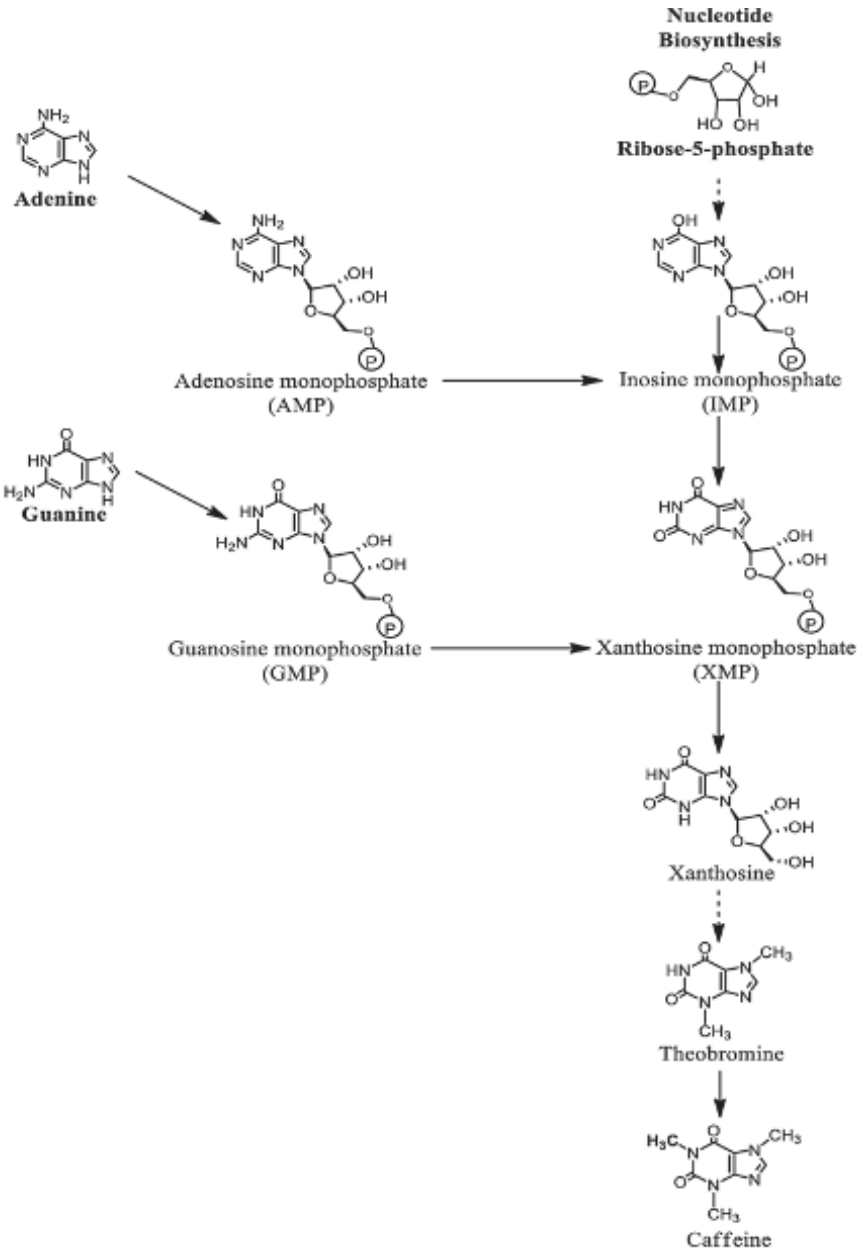

FIGURE 2.9 Nucleotide metabolic pathway for biosynthesis of purine alkaloids.

2.2.2 BIOSYNTHESIS OF FLAVONOIDS

Flavonoids are ubiquitous phytochemicals, existing in a wide range of fruits and plants, including the lower and higher plants (De Groot and Rauen, 1998). They are the most studied plant phenols (Dai and Mumper, 2010). Vegetables, tea, and coffee are other sources rich in flavonoids (Pridham, 1960). Flavonoids are subgrouped into flavonols (kaempferol), flavones (luteolin), flavanone (naringenin), chalcones (butein), and anthocyanidins (pelargonidin) (Harborne et al., 1975).

A common pathway relates biosynthesis of flavonoids. Their precursors are derived from the shikimate and the acetate-malonate pathway (Hahlbrock and Grisebach, 1975; Wong, 1976) with chalcone being the first intermediate from the flavonoid reaction pathway (Fig. 2.10). Several flavonoids are thus generated from reactions involving chalcone (Hahlbrock, 1981). Flavanone, flavone, isoflavone, dihydroflavonol, flavonol, catechin, epicatechin, and anthocyanidin are among the compounds obtained through this pathway.

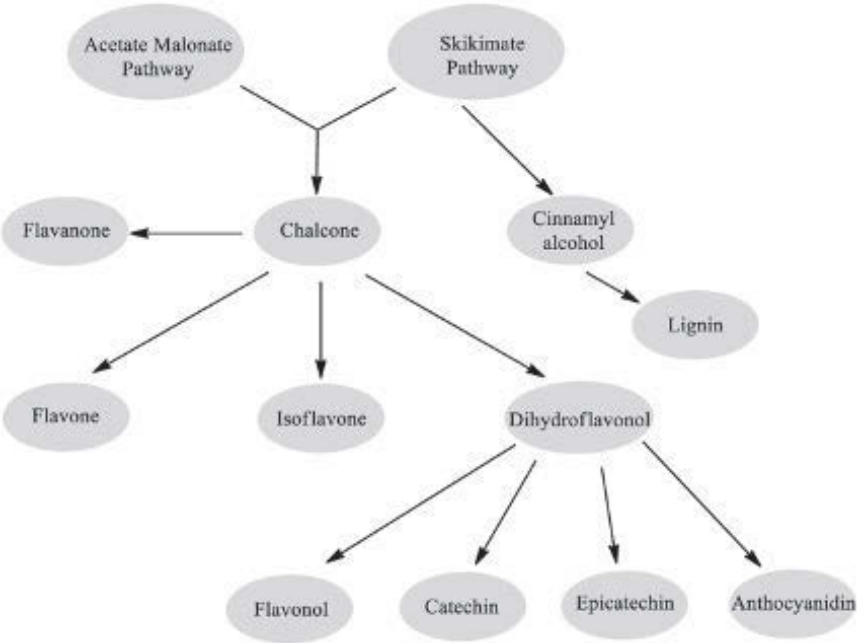

FIGURE 2.10 Biosynthetic pathway of flavonoids.

Flavonoids are a six-member structure linked to a benzene ring to form a pyrone flavone or flavonol. It could also form the dihydro derivatives of

pyrone, which are flavanones or flavanols. When the substituted benzene ring is at position 2, a flavanone is formed or when it is at position 3, an isoflavone is formed. Flavonoids may exist in nature as conjugated at position 3 or 7 with several glycosidic linkages. The carbohydrate moieties are derived from the pentose phosphate pathway, and can be L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose (Pretorius, 2003). Catechin biosynthesis is illustrated in Figure 2.11. The condensation reaction between three molecules of malonyl-CoA and one molecule of cinnamoyl-CoA (Mora-pale et al., 2013) catalyzed by chalcone synthase is the committed step for their biosynthesis. Chalcone formed is then isomerized to a flavanone by chalcone isomerase. The final steps of the reaction is catalyzed by an NADPH-dependent enzyme dihydroflavonol 4-reductase to form leucoanthocyanidins (Martens et al., 2002), which are converted by leucoanthocyanidin reductase to catechin (Tanner et al., 2003).

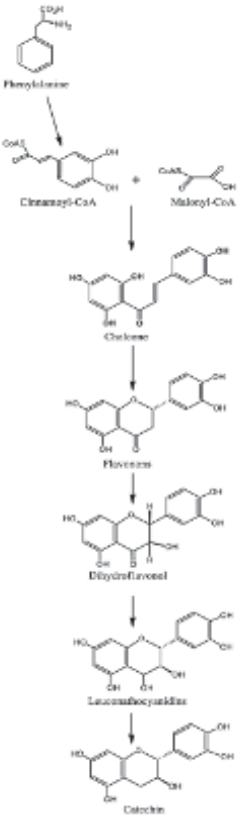

FIGURE 2.11 Biosynthetic pathway of catechin.

2.2.2.1 BIOACTIVITY OF FLAVONOIDS

Biological activities of flavonoids are diverse and well documented. Some of their activities include anti-inflammatory, antimicrobial, anti-allergic, antioxidant, cytotoxic, and antitumor (Tapas et al., 2008). In diabetic treatment, flavonoids have been used to inhibit digestive enzymes with reduced side effects (Bedekar et al., 2010). Specifically, catechin has been reported to trigger the secretion of insulin from pancreatic β-cells (Chemler et al., 2007).

2.2.3 BIOSYNTHESIS OF LIGNAN

Lignans are plant phytochemicals with highly branched polymers. The name (lignan) was first introduced by Haworth (Haworth, 1936), where he describes it as plant compounds with dimeric phenylpropanoids (Fig. 2.12). Dimeric phenylpropanoids are linked through C6–C3 attached by a central carbon at carbon 8 (C8) to form the basic structure of lignans. Another form of linkage that exists through a C5–C5′ is named neolignans (Umezawa, 2003).

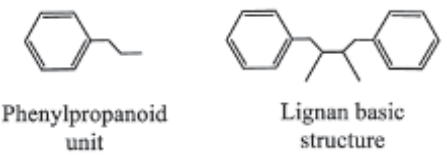

FIGURE 2.12 Basic structure of lignins.

Biosynthesis of lignans is from two aromatic amino acids, phenylalanine or tyrosine by the dimerization of either three of substituted hydroxycinnamoyl alcohols (Fig. 2.13), which are the main precursors of all lignins and lignans. The alcohols are oxidized into free radicals by ubiquitous peroxidase enzymes (Mazid et al., 2011). Through this reaction, the peroxidase induces an electron oxidation of the phenol group, thus causing a delocalization of the unpaired electron through the different resonance forms. Few other steps proceed after the oxidation – formation of a pinoresinol and it is reduced by the enzyme pinoresinol reductase to form lariciresinol. Several lignans are generated from this point onward through this pathway (Fig. 2.14).

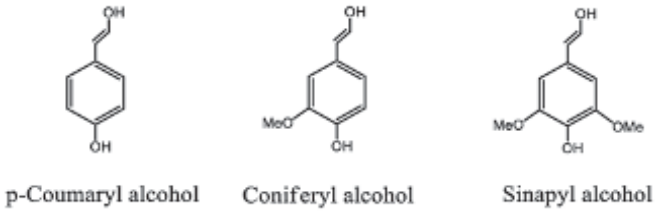

FIGURE 2.13 Chemical structures of lignan precursors.

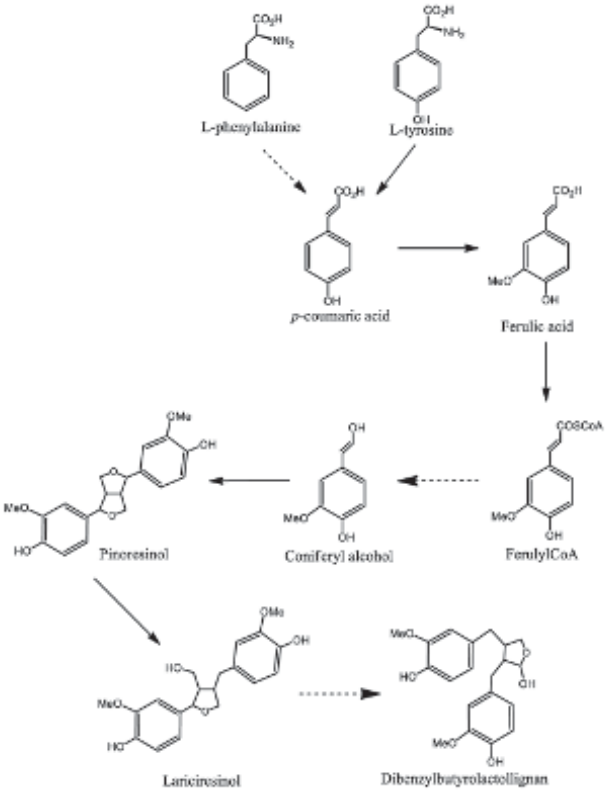

FIGURE 2.14 Biosynthetic pathway of lignan.

2.2.3.1 BIOACTIVITY OF LIGNANS

Lignans have a wide range of applications. They are applied in cancer therapy for their ability to reduce cell proliferation in colon cancer cells, inhibit metastatic secondary tumors, and decrease the levels of markers in

a rat model of colon cancer (Delmas et al., 2006). Lignans are also reported to be anti-inflammatory (Saleem et al., 2005), antimicrobial (Saleem et al., 2005), antioxidant (Fauré et al., 1990; Saleem et al., 2005; Pan et al., 2009), immunosuppressive (Saleem et al., 2005), and hepatoprotective (Negi et al., 2008). Silymarin are flavonolignans formed by the combination of flavonoid and lignan structures by oxidative coupling (Dewick, 2002); they have been used for centuries in the treatment of liver, spleen, and gallbladder disorders (Shaker et al., 2010).

2.2.4 BIOSYNTHESIS OF ANTHOCYANINS

Anthocyanins belong to the water-soluble type of phytochemicals, which provide bright color to many flowers and fruits due to their ability to change color at low pH or pH above neutral scale. Their synthesis is majorly through the phenylpropanoid pathway through the formation of malonyl-CoA, chalcone, and catechin (Fig. 2.15). Finally, following the formation of dihydroflavonols through leucoanthocyanidins, anthocyanidins

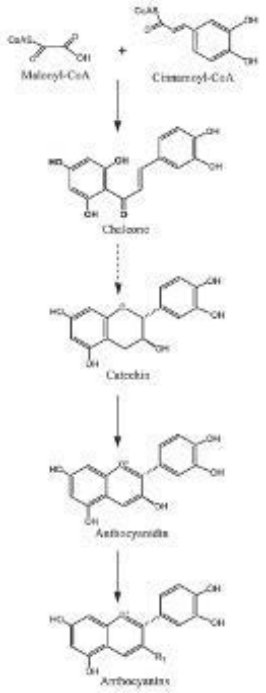

FIGURE 2.15 Biosynthetic pathway of anthocyanins.

are biosynthesized (Mora-pale et al., 2013). Anthocyanin biosynthesis has been recognized as one of the first branches in the general phenylpropanoid pathway, in which their biosynthetic enzymes have been identified (Allen et al., 2008). Enzymes catalyzing the reactions, including chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, and anthocyanidin synthase (leucoanthocyanidin dioxygenase) have been studied extensively (Shirley et al., 1995; Winkel-Shirley, 2001; Lepiniec et al., 2006).

2.2.4.1 BIOACTIVITY OF ANTHOCYANINS

Anthocyanins are beneficial as antioxidants, anti-inflammatory, anti-carcinogenic, and anti-microbial compounds. They are also reported to be beneficial in preventing cardiovascular and diabetic complications (Ghosh and Konishi, 2007; Toufektsian et al., 2008; Jing et al., 2008; He and Giusti, 2010; Pascual-Teresa et al., 2010).

2.2.5 BIOSYNTHESIS OF PHENOLICS

The phenolic class of phytochemicals possesses a hydroxyl group as part of their chemical structure, which is directly bonded to their aromatic hydrocarbon. They have a complex and large chemical constituent (Walton et al., 2003). The simplest member of this class is the phenol, with the chemical formula (C_6H_5OH). Tannins are a heterogeneous polyphenolic compound with high molecular weight that are capable of forming reversible and irreversible complexes with other compounds such as alkaloids, minerals, nucleic acids, polysaccharides, and proteins (Mueller-Harvey and Mcallan, 1992; Vansoest, 1994; Schofield et al., 2001). Tannins are compounds with the characteristic features similar to tan leather. They are known for their acidic reactions due to the presence of phenolic or carboxylic groups (Kar, 2007). They are categorized as condensed and hydrolyzable tannins. Plant phenolics are biosynthesized from the shikimic acid pathway through phenylalanine. Phenylalanine is deaminated by the enzyme phenylalanine ammonia-lyase (PAL) to cinnamic acid (Fig. 2.16). Cinnamic acid is the first precursor from which several phenolics can be synthesized. Hydroxycinnamic acids are produced from a simple ester formation with a glucose or hydroxycarboxylic acids. Simple phenolics are also synthesized from a ρ-coumaric acid (hydroxycinnamic acid) from the same pathway.

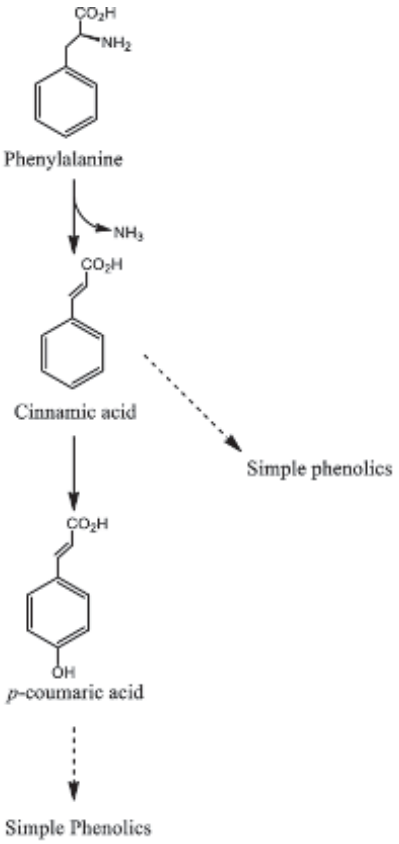

FIGURE 2.16 Biosynthetic pathway of phenolics.

2.2.5.1 BIOACTIVITY OF PHENOLICS

Phenolics are an important class of phytochemicals due to their several beneficial roles in humans. Examples of phenolics with important bioactivities include gallic acid, caffeic acid, cinnamic acid, and vanillic acid (Fig. 2.17). They possess antioxidant defenses against free radical-related diseases. Phenolics reduce toxicity arising from streptozotocin by neutralizing the free radicals generated in the pancreas (Uttara et al., 2009). They are also reported to possess anti-inflammatory, antidepressant, antispasmodic, antitumor, antiulcer, and cytotoxic activities (Silva et al., 2007; Ghasemzadeh et al., 2010). The tannins found its usefulness in textile dyes and antioxidants in industries during the production of beer, wine, and fruit juice (Gyamfi and Aniya, 2002).

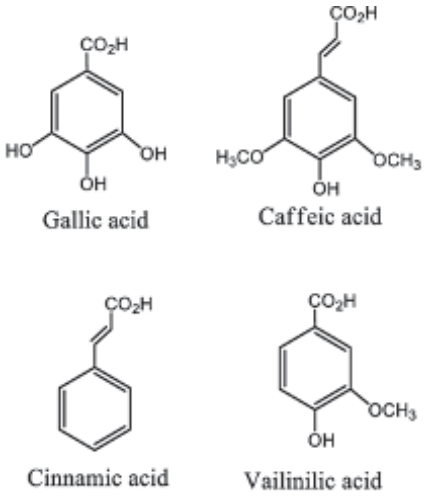

FIGURE 2.17 Structure of some important phenolic compounds.

2.2.6 BIOSYNTHESIS OF ALKALOIDS

Alkaloids derive their name linguistically from the Arabic word *al-qali* (Kutchan, 1995) signifying their "alkaline" nature and describing the presence of a nitrogenous base (Mueller-Harvey and McAllan, 1992). They turn red litmus paper to blue, confirming their alkaline properties. Morphine was the first alkaloid identified from the opium poppy (*Papaver somniferum*) in 1806. More than 10,000 alkaloids have been identified since then with their various structures elucidated (Southon and Buckingham, 1989).

Alkaloids are synthesized from a number of amino acids, which include aspartic acid, lysine, tryptophan, and tyrosine (Pearce et al., 1991). Most of which are aromatic amino acids. Their synthesis was assumed to be from the abnormalities of amino acid synthesis, most likely from a mutation in the control mechanisms, which result to the conversion of excess amino acids to alkaloids as a means of disposal (Waller and Nowacki, 1978).

In the biosynthesis of terpenoid indole alkaloids (Fig. 2.18), tryptophan is first decarboxylated to tryptamine by the enzyme tryptophan decarboxylase (Leete, 1961). Protoalkaloid (tryptamine) serves as the substrate for the first committed step of the reaction catalyzed by strictosidine synthase (Stöckigt and Zenk, 1977). The carbohydrate source, deoxyxylulose is converted to secologanin. Secologanin is then condensed with tryptamine by strictosidine synthase (Stöckigt and Zenk, 1977). Strictosidine synthase is a

stereospecific enzyme, which is of biotechnological interest and its product 3α(S)-strictosidine is the first monoterpenoid indole alkaloid in this pathway (O'Connor and Maresh, 2006).

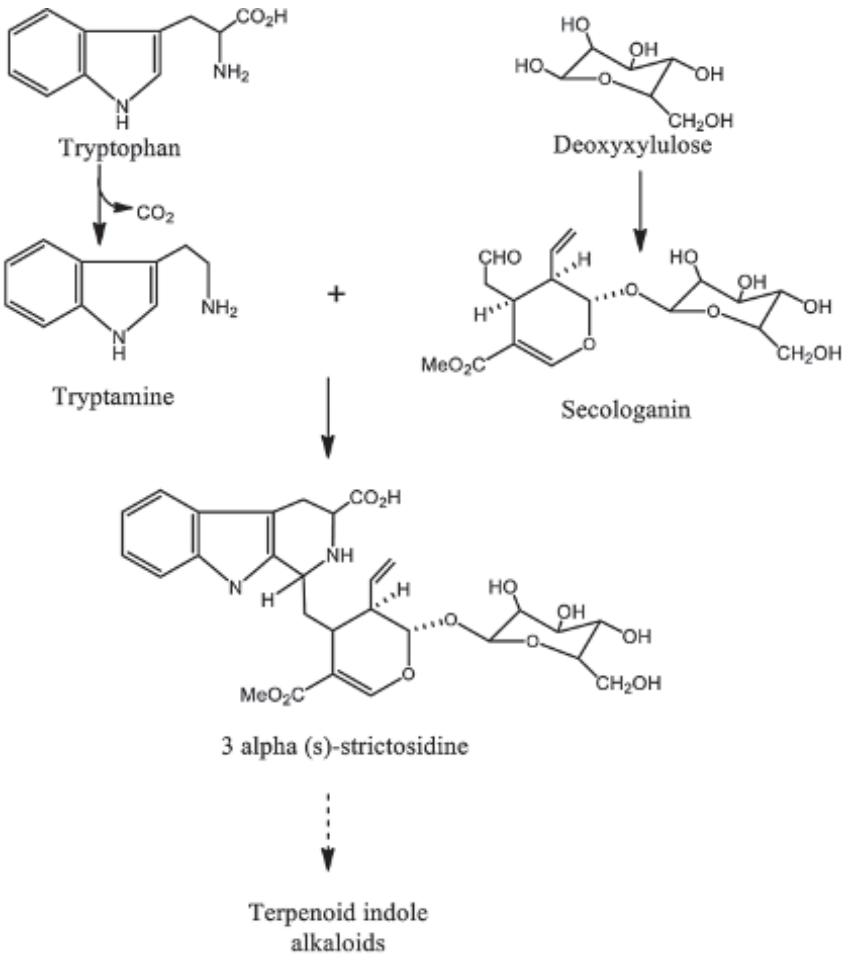

FIGURE 2.18 Biosynthetic pathway of terpenoid indole alkaloids.

2.2.6.1 BIOACTIVITY OF ALKALOIDS

Caffeine, morphine, and nicotine are examples of alkaloids with stimulating properties and are examples of good analgesic (Rao et al., 1978). The alkaloid quinine (Fig. 2.19) is a known antimalarial compound (Rao et al., 1978)

obtained from the cinchona plant and it is used in the treatment of symptomatic erythrocyte stages of malaria infection. Yohimbine, an example of terpene indole alkaloids, is an adrenergic receptor blocker, which has been isolated from the plant *Rauvolfia serpentina*. Ajmalicine and camptothecin are other examples of terpene indole alkaloids isolated from *Catharanthus roseus* and *R. serpentina, Ophiorrhiza pumila,* and *Camptotheca acuminata*, respectively. They are antihypertension and anti-tumor compounds, respectively.

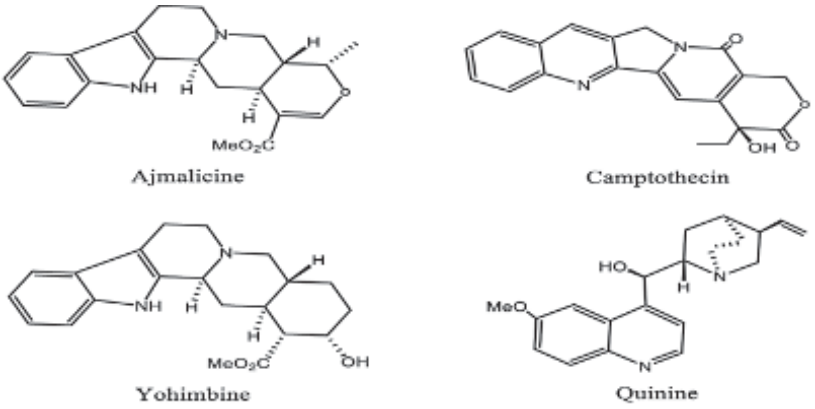

FIGURE 2.19 Chemical structure of some important physiologically active terpenoid indole alkaloids.

2.2.7 BIOSYNTHESIS OF TERPENOIDS

Terpenoids are polycyclic compounds with a 5-carbon basic isoprene (C_5H_8) structure. They differ by the number of isoprene units in their basic structures as well as the functional groups they possess. The number of isoprene units serves as a means of classification (Langenheim, 1994). Terpenes are phytonutrients widely distributed in green foods and grains.

Terpenoids are synthesized from two pathways, which leads to the formation of its two biosynthetic precursors, namely IPP and DMAPP (Chang et al., 2007; Meng et al., 2011). They are the fundamental building blocks in the biosynthesis of biological compounds with isoprenoid units. This includes steroids, carotenoids, saponins, and limonoids. The first pathway for isoprenoid synthesis is through the mevalonic acid pathway (Fig. 2.20). This is specific for some bacteria, plants, and higher eukaryotes (Zurbriggen et al., 2012; Zhou et al., 2012), while the mevalonic independent pathway, also known as the 1-deoxy-d-xylulose-5-phosphate pathway

(Fig. 2.21), is specifically for plants and most bacterial strains (Hoeffler et al., 2002). IPP and DMAPP are restructured by subsequent additions of IPP to form various units of terpenoids. First, hemiterpenes (C_5) and monoterpenes (C_{10}) are produced and then sesquiterpenes (C_{15}) by the addition of an IPP unit. Triterpenes (C_{30}) are synthesized from two units of triterpenes. Steroids are also generated from triterpene units (Fig. 2.20). Similarly, from the non-mevalonic pathway, terpenoids are synthesized from IPP through geranyl diphosphate and secologanin.

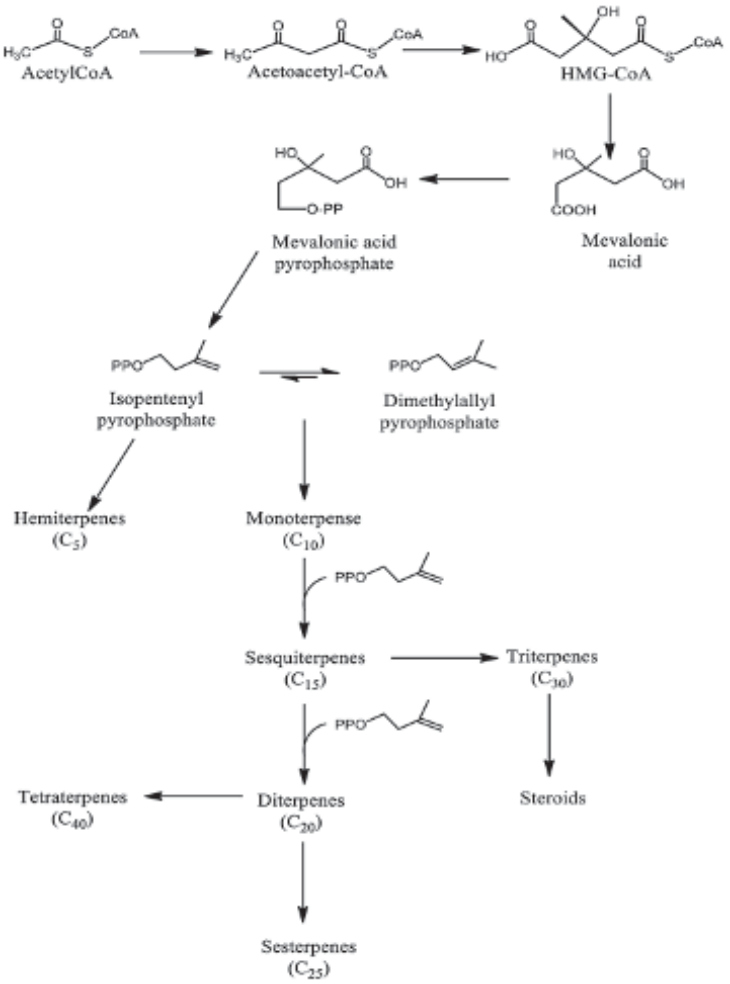

FIGURE 2.20 Biosynthetic pathway of terpenes through the mevalonic pathway.

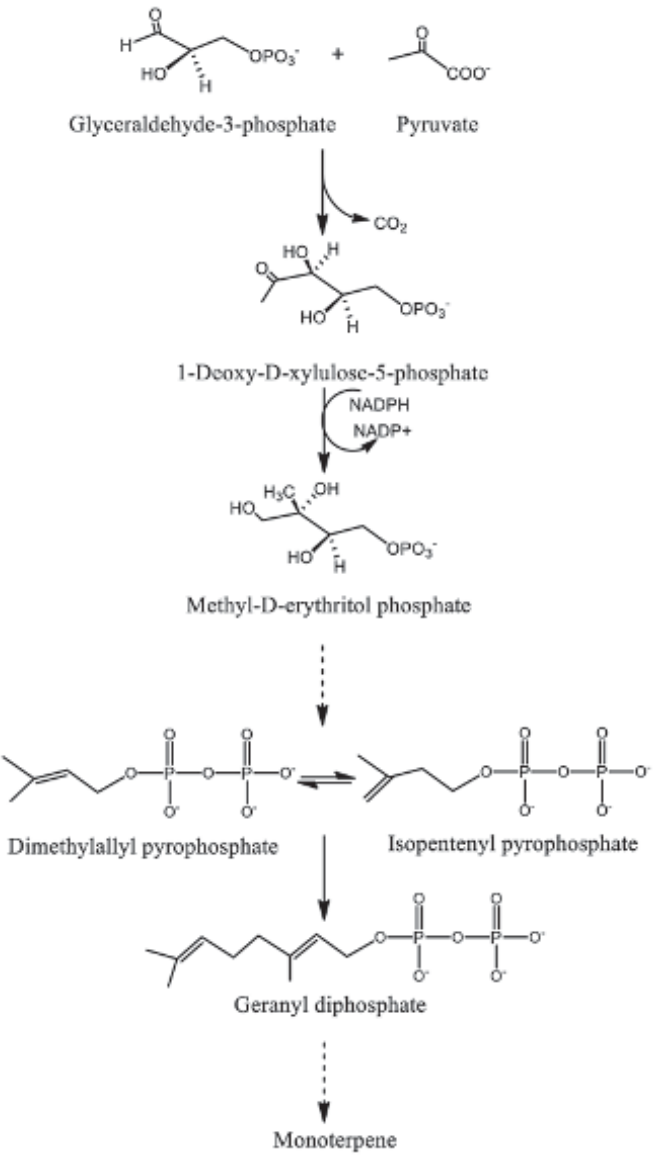

FIGURE 2.21 Biosynthetic pathway of terpenes through the mevalonic independent pathway.

Studies have indicated that monoterpenoids, diterpenoids, and tetraterpenoids are preferably biosynthesized in plastids through the mevalonic independent pathway, while triterpenoids and sesquiterpenes are mostly biosynthesized in the cytosol through mevalonic acid pathway (Sawai and

Saito, 2011). Studies have also indicated that secologanin is derived from the mevalonic independent pathway by feeding ^{13}C-glucose in *C. roseus* cell culture (Contin et al., 1998) and that same mevalonic independent pathway leads to the synthesis of secologanin in *O. pumila* (Yamazaki et al., 2004).

2.2.7.1 BIOACTIVITY OF TERPENOIDS

Terpenoids are of several biological relevances. Their protective role is derived from their bitter taste; hence, they protect some plants from being eaten by animals (Degenhardt et al., 2003). Pyrethroids are a monoterpene ester which exists in the leaves and flowers of the plant *Chrysanthemum*. They are strong insecticidal compound by which *Chrysanthemum* plant protect itself from insects. Lycopene and β-carotene (Fig. 2.22) are two structurally related compounds, transported primarily in LDLs. Their conjugated double bonds enhance their powerful antioxidant nature, thereby protecting LDLs from oxidations during transportation (Goulinet and Chapman, 1997).

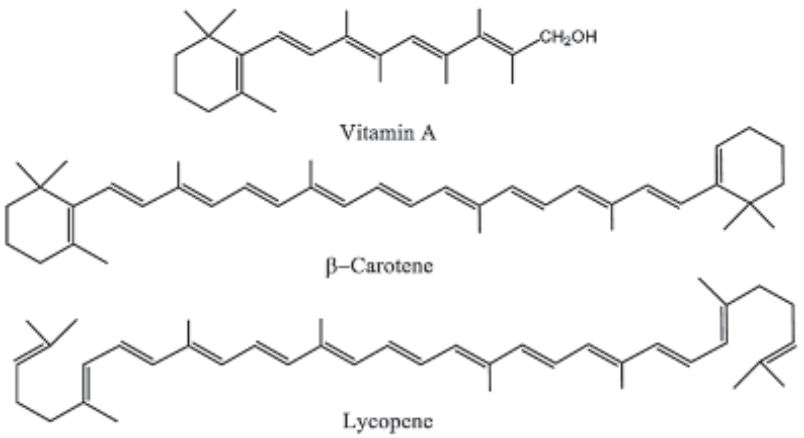

FIGURE 2.22 Chemical structures of some important terpenes.

2.2.8 BIOSYNTHESIS OF SAPONINS

Saponins are among the groups of plants nonnitrogenous secondary metabolites, which are widely distributed in plants. Their name is derived from their ability to form stable foam in aqueous solutions when shaken. On hydrolysis, an aglycone moiety called sapogenin is released. Steroidal and

triterpenoidal sapogenin are the two known types of sapogenins with a sugar moiety attached to the hydroxyl group on carbon 3. Triterpenoid saponins are examples of saponins synthesized from isoprenoid units (Yendo et al., 2014). Three isopentyl pyrophosphate are combined in an head to tail linkage to form farnesyl pyrophosphate (Fig. 2.23). In the next reaction, catalyzed by squalene synthase, two farnesyl pyrophosphate units are combined with a tail-to-tail manner to form squalene (Vincken et al., 2007). Squalene epoxidase oxidizes the product to 2,3-oxidosqualene. The committed step of the reaction is the cyclization of 2,3-oxidosqualene, a reaction catalyzed by oxidosqualene cyclase followed by oxidation, substitution, and glycosylation to form various triterpenoid saponins.

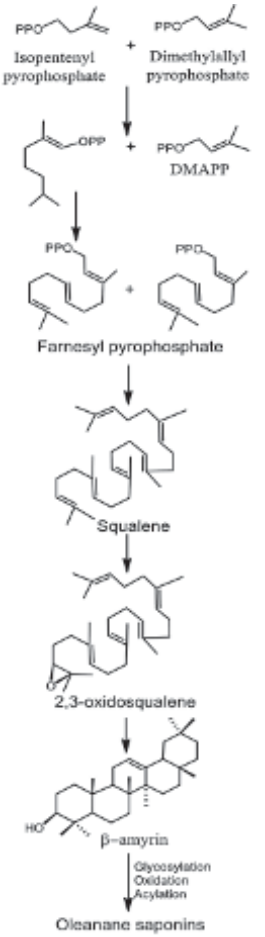

FIGURE 2.23 Biosynthesis of triterpenoid saponins.

2.2.8.1 BIOACTIVITY OF SAPONINS

Saponins have a diverse range of biological activities. Members of this phytochemical group are reported to have anti-inflammatory, antileishmanial, antimutagenic, antiviral, hemolytic, and hepatoprotective properties (Rahimi et al., 2009). Saponins from *Medicago truncatula* (Fig. 2.24) have been studied for their various functions such as antimicrobials, fungicides, insecticides, and other functions (Tava et al., 2011).

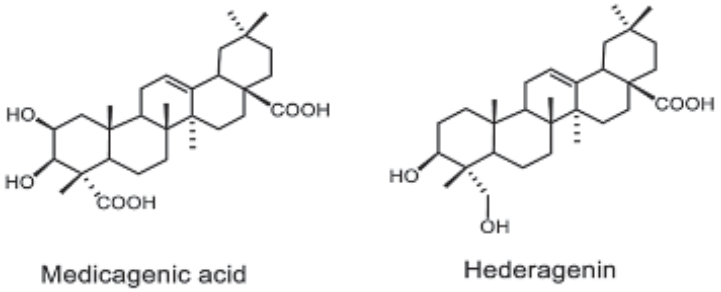

FIGURE 2.24 Examples of sapogenins from *Medicago truncatula.*

2.2.9 BIOSYNTHESIS OF GLYCOSIDES

Glycosides are products of the condensation of various sugars with different types of organic hydroxyl compounds or few cases in thiol compounds. They are colorless, crystalline carbon, hydrogen or oxygen compounds or nitrogen and sulfur compounds. Due to their nature, glycosides are water soluble. Chemically, they contain a glucose (carbohydrate moiety) and an aglycone (noncarbohydrate moiety) (Kar, 2007; Firn, 2010). The sugar moiety is derived from the pentose phosphate pathway and may be a monosaccharide or disaccharide including either of the following sugars: D glucose, D-xylose, L-rhamnose, L-arabinose, and L-fructose, while the aglycon may be a coumarin, a flavonoid, a lignan, a phenolic, or a terpene compound. Following the various combinations above, aldehyde glycosides, anthraquinone glycosides, cardiac glycosides, cyanogenic glycosides, phenolic glycosides, or saponin glycosides may be formed (Fig. 2.25).

The aglycones are synthesized from the shikimate pathway through phenylalanine or tryptophan. PAL deaminates phenylalanine to cinnamic acid with the release of ammonia from phenylalanine. In the biosynthesis of Arbutin, glucose is conjugate with hydroquinone (Fig. 2.26). Aldoximes

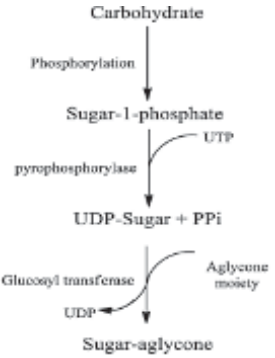

FIGURE 2.25 A typical pathway for biosynthesis of glycosides.

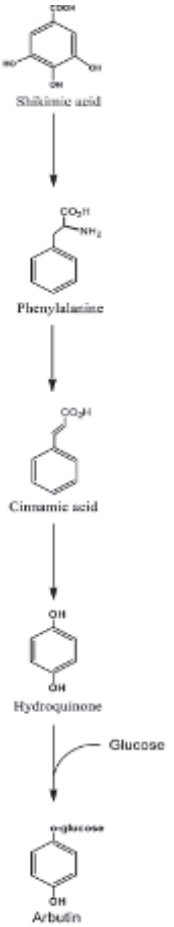

FIGURE 2.26 Biosynthetic pathway of Arbutin.

are important intermediates in the biosynthesis of cyanogenic glycosides (Fig. 2.27) and their reaction is catalyzed by cytochrome-P_{450} enzymes. Cyanogenic glycosides are conjugates of various sugar moieties with an N-protective group from the aldoximes. They release their poisonous compounds like cyanide (HCN) and sulfide (H_2S) when the plant containing them is digested. Therefore, they serve to protect such plants from feeding by insects or animals (Taiz and Zeiger, 1995).

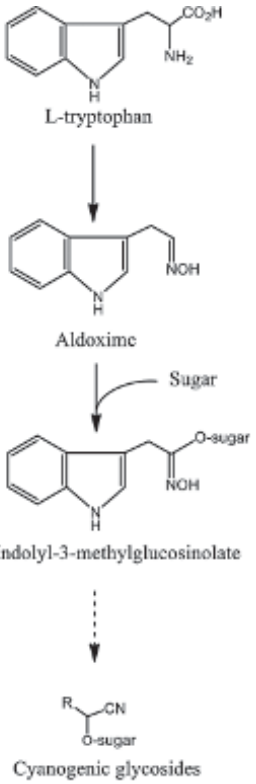

FIGURE 2.27 Biosynthesis of the cyanogenic glycoside.

2.2.9.1 BIOACTIVITY OF GLYCOSIDE

Cyanogenic glycosides are themselves not toxic except by the release of their HCN and H_2S contents which are volatile and poisonous. Their primary roles in plants involve the chemical defense and mediating their interactions with insects (Zagrobelny et al., 2004).

2.2.10 BIOSYNTHESIS OF ANTHRAQUINONES

The basic structure of anthraquinones consist of three rings (Fig. 2.28). Ring A is believed to be obtained from the shikimic acid pathway through the synthesis of shikimic acid, chorismic acid, and thiamine diphosphate (Han, et al., 2001). Ring B is derived from tricarboxylic acid pathway through the formation of α-ketoglutarate. The ring closure is achieved by the formation of a succinylbenzoic acid intermediate (Han et al., 2001). The ring C is derived from isopentenyl diphosphate (IPP) through the terpenoid biosynthetic pathway (Han et al., 2001).

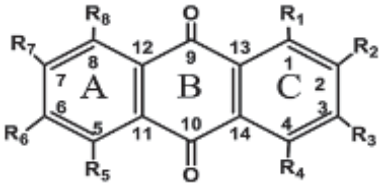

FIGURE 2.28 Basic structure of anthraquinones.

The two distinctive pathways for the biosynthesis of anthraquinones in higher plants are the polyketide and shikimic acid pathway. The polyketide pathway occurs in families of higher plants such as Leguminosae, Rhamnaceae, and Polygonaceae and in fungi (Simpson, 1987; Inouye and Leistner, 1988). The reaction involves a seven-malonyl-CoA units and one acetyl-CoA unit to form an octaketide acyl chain (Fig. 2.29). Carbon dioxide is released in the final reaction step. Anthraquinones synthesized from the polyketide pathway exhibit a characteristic substitution in both A and C rings. For example, the A and C rings in chrysophanol and emodin are typically substituted with hydroxyl groups. Enzymes of the polyketide pathway have been successfully studied and documented in bacteria mostly, with rare occurrences in higher plants (Bernard, 1999).

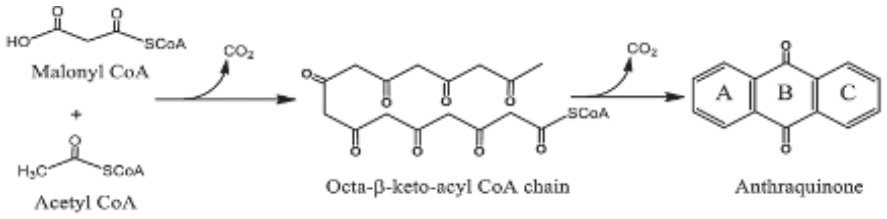

FIGURE 2.29 Polyketide pathway for anthraquinone biosynthesis.

The shikimic acid pathway for the synthesis of anthraquinones also incorporates metabolites from the mevalonic acid pathway (Fig. 2.30). Their A and B rings are derived from chorismic acid and α-ketoglutarate, while the C ring is from IPP (Leistner, 1981; Leistner, 1985) which is a key intermediate in terpenoid biosynthesis. This pathway is used to synthesize anthraquinone with substitutes such as hydroxyl or methyl groups in only one of the rings, while in the polyketide pathway, anthraquinones are synthesized with substitutes in both rings (Han et al., 2001).

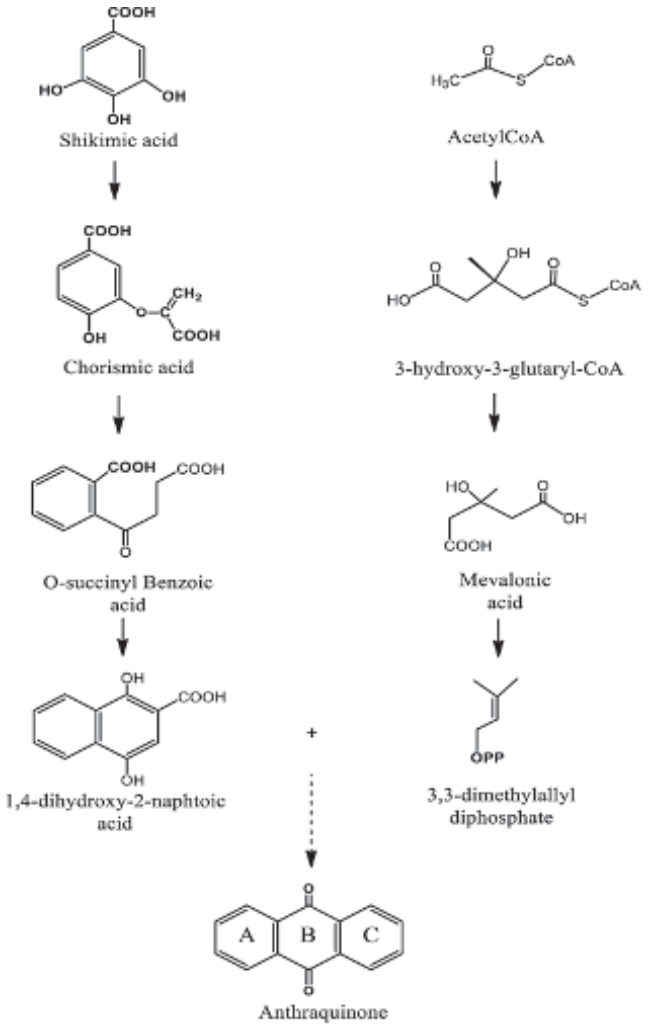

FIGURE 2.30 Shikimic acid pathway for anthraquinone biosynthesis.

2.2.10.1 BIOACTIVITY OF ANTHRAQUINONES

Anthraquinones are of importance to plants. The substituted anthraquinones such as hydroxymethyl anthraquinones protect plants against parasite invasion and protect their seeds from birds (Korulkin and Muzychkina, 2014).

2.2.11 BIOSYNTHESIS OF STEROIDS

Phytosterols are an important aspect of plant physiology and function with over 250 of such sterols and their related compounds in plants and marine materials (Akihisa et al., 1991). Their functions and structure are similar to human cholesterol. Biosynthesis of phytosterol consists of over 30 enzymes-catalyzed reactions, which have been detected in plant membranes (Nes, 1977; Benveniste, 1986) making the pathway a complex one. Their precursors are derived from the isoprenoid pathway through the synthesis of IPP, DMAP, and the C_{30} triterpenoids. The source of IPP in the pathway is argued to be either from the mevalonic acid or independent mevalonic acid pathways. They have been presumed to be derived exclusively from the mevalonic acid pathway rather than from the independent mevalonic pathway (McCaskill and Croteau, 1998). Formation of squalene is achieved by the action of the enzyme squalene synthetase followed by squalene-2,3-oxide (Fig. 2.31). In photosynthetic plants, squalene-2,3-oxide is converted to cycloartenol by cycloartenol synthase, which is, however, different in fungi/yeast where they are converted to lanosterol by lanosterol synthase (Hartmann, 1998; Ohyama et al., 2009). Advances in the studies of phytosterol synthesis have revealed the presence of lanosterol synthase genes from dicotyledonous plant species, indicating the abilities of higher plants to also biosynthesize phytosterols through lanosterol rather than through cycloartenol only. Cycloartenol can be further modified to form campesterol, sitosterol, and stigmasterol. Methylation of cycloartenol during the biosynthesis to form 24-methylene derivative is a rate-limiting step in the pathway, which serves to regulate the pathway (Nes and Venkatramesh, 1997).

2.2.11.1 BIOACTIVITY OF STERIODS

Steriods are among the naturally occurring plant phytoconstituents with great therapeutic applications. Plant steroids termed “steroid glycosides” or “cardiac glycosides” are found to be of great therapeutic applications

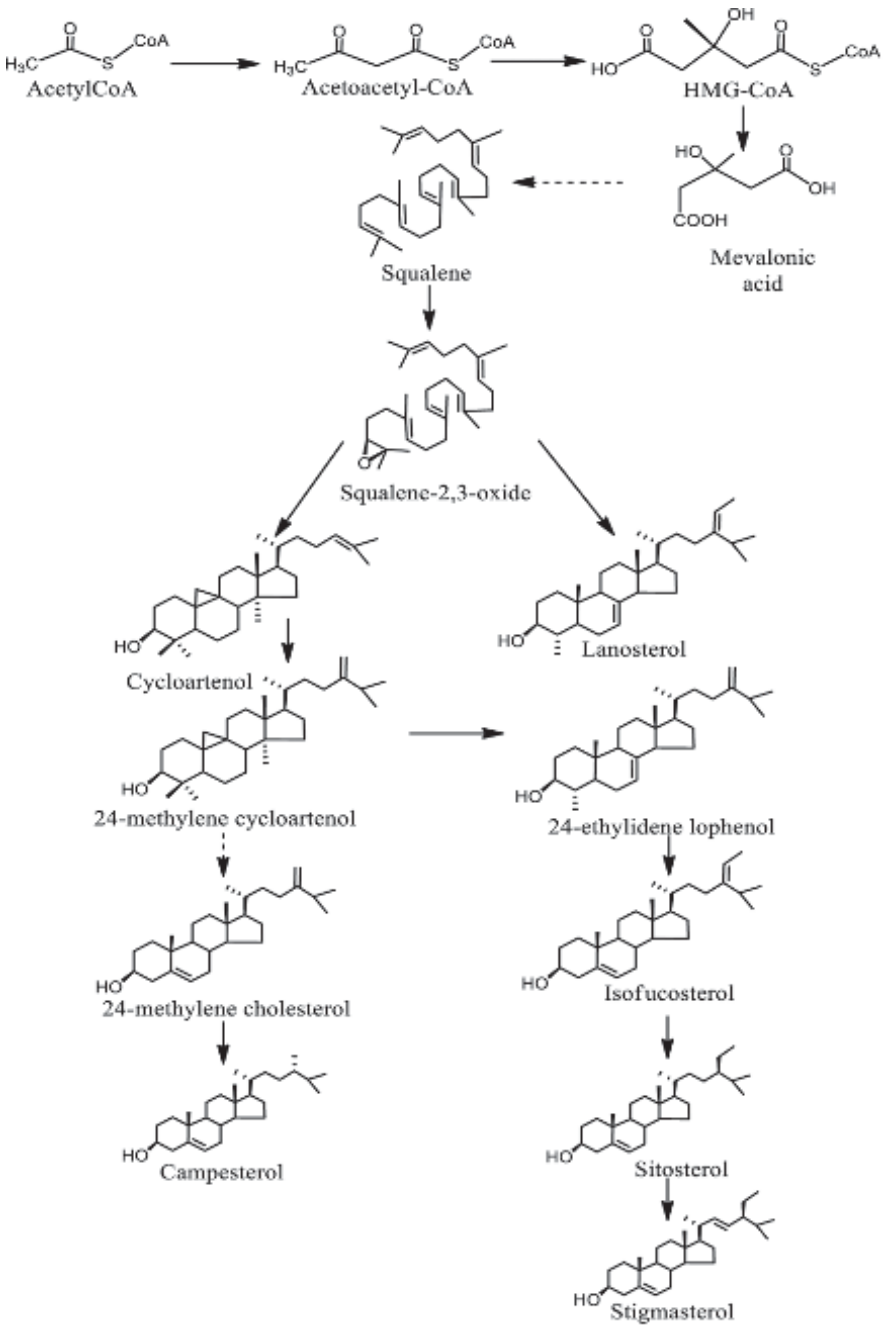

FIGURE 2.31 Biosynthesis of plant steroid.

as “arrow poisons” or “cardiac drugs” (Firn, 2010). Cardiac glycosides are known for their ability to affect the cardiac muscle after an in vivo administration through injection. Sterols possess the ability to regulate the fluidity of membranes. For example, sitosterol, stigmasterol, and campesterol are phytosterols that restrict the movement of fatty acyl chains across their membranes (Piironen et al., 2000).

2.3 SYNTHETIC APPROACHES TO PHYTOCHEMICALS

Aside from the in vivo synthesis of plant phytochemicals and the various methods of isolations and purification, the synthetic approach to the production of phytochemicals is used to prepare phytochemicals of great importance in reasonably large quantities. Several approaches have been employed to achieve this goal of phytochemical synthesis. Among these is a recent approach that involves metabolic engineering in microbes and plant cells or the in vitro reconstruction of already established natural products (Mora-Pale et al., 2013).

Plant cell cultures were established in the late 1930s after which in the year 1956 the first patent involving plant cell culture was filed by Pfizer Inc. for phytochemicals production by cell cultures (Ratledge and Sasson, 1992). This technique has been used in countries such as Germany, Japan, and the United States for commercial production of plant secondary metabolites in similar manners in which bacteria and fungi have been employed in the production of antibiotics and amino acids (Mulabagal and Tsay, 2004).

The synthetic approach has distinct advantages over the in vivo synthesis. They are independent of environmental conditions, quality fluctuation, and allows for the isolations of important metabolic pathway intermediates. However, the chemical synthesis approach has limitations as well. They are in some cases not possible, if possible, may be time-consuming or economically not feasible (Smetanska, 2008). The enzymes may require purifications, high stability, and activities (Kwon et al., 2012) and the products may need to be further purified. An example of one of the most successful conventional synthesis of plant-derived phytochemicals is the artemisinins, which were originally obtained from the plant *Artemisia annua*. Their synthesis has a large number of reaction steps and the production yield is very low (Chemler et al., 2006). Other examples of phytochemical from plant culture are presented in Table 2.1.

TABLE 2.1 Examples of Plant-Culture-Derived Phytochemicals.

Phytochemical	Plant source	References
Shikonin	*Lithospermum erythrorhizon*	Matsubara et al. (1989); Kim and Chang (1990)
Berberine	*Coptis japonica*	Fujita and Tabata (1987), Matsubara et al. (1989)
Sanguinarine	*Papaver somniferum*	Dicosmo and Misawa (1995)
Visnagin	*Chenopodium rubrum*	Berlin et al. (1986)
Diosgenin	*C. rubrum*	Berlin et al. (1986)
Ajmalicine	*Catharanthus roseus*	Lee and Shuler (2000)
Anthraquinones	*Morinda citrifolia*	Zenk (1977)
Caffeic acid	*Vanilla planifolia*	Knorr et al. (1993)
Ginsenoside	*Panax ginseng*	Matsubara et al. (1989)
Nicotine	*Nicotiana tabacum*	Mantell et al. (1983)
Rosmarinic acid	*Coleus blumei*	Petersen and Simmond (2003)
Ubiquinone-10	*N. tabacum*	Fujita and Tabata (1987)

2.4 CONCLUSION

The literature in the present work provides the necessary reaction steps in the biosynthesis of phytochemicals. The various contributing pathways to these syntheses were also reviewed up to the important phytochemicals obtained from each of them. Some biosynthetic pathways are complex and are catalyzed by several enzymes. These pathways were summarized for better presentation and understanding. These pathways have been studied, in some cases, to provide alternative means of obtaining phytochemicals in larger quantities with the aim to apply them in bioengineering, pharmaceutics, and nutraceuticals as well as in other fields.

KEYWORDS

- **phytochemicals**
- **biosynthesis**
- **bioactivities**
- **pathways**
- **enzymes**

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CHAPTER 3

MECHANISMS OF PLANT DEFENSE AGAINST PATHOGENS: PHYTOALEXINS INDUCTION

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ABSTRACT

Crops are usually attacked by many pathogens and they respond by the activation and production of defense genes, the formation of ROS, the synthesis of pathogenesis-related proteins, localized cell wall enforcement and the induction of antimicrobial compounds. Plants use innate defense mechanisms toward pathogens, including the induction of substances that have antimicrobial activity which are known as phytoalexins (PhA). PhA production is now considered as an important defense mechanism toward microbial infection through a broad spectrum of various secondary metabolites induced. Molecular approaches are helping to resolve some mechanisms and the complexity of these bioactive PhA compounds. This chapter focuses on the biosynthesis and regulation of various PhA compounds and its role in plant defense. Moreover, this chapter also discusses some of the PhA induced by plants from different families. This includes the very recently identified kauralexins and zealexins induced by maize, and the biosynthesis and regulation of PhA induced by rice.

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3.1 INTRODUCTION

Terrestrial plants produce antimicrobial compounds which are called phytoalexins (PhA) following invasion by different microbial infections. They possess an inhibitory activity against bacteria, fungi, nematodes, insects and can be toxic to the animals and to the plant itself (Braga, 1991). PhA described as "stress compounds" are stimulated by different kind of factors capable of causing damage or poison to plant tissues, such as ultraviolet (UV) light (Bridge et al., 1973), exposure to heat or cold (Rahe et al., 1975), heavy metals (Rathmell et al., 1971), fungicides (Oku et al., 1973), and antibiotics (Cruickshank et al., 1974). The synthesis of PhA in response to pathogen invasion could be influenced by so many factors such as temperature, humidity, and water availability. Several parts of the plant could produce PhA such as leaves, flowers, stems, seeds, and root tubers (Mikkelsen et al., 2003). The majority of PhA are lipophilic constituents that have an ability to pass through plasma membrane (PM) and enter the cell. Smith (1996) has reported that PhA toxicity occurs in the plant as a function of their acidic character due to the presence of hydroxyl group. The pivotal role of PhA in plant disease resistance has been described earlier (Hans and Jurgen, 1978; Hain et al., 1992).

3.2 PHYTOALEXIN (PhA) CONCEPT

The PhA concept started from ancient years from the findings that *Solanum tuberosum* (a tuber tissue of potato) had previously been infected with an incompatible race of *Phytophthora infestans* which promoted resistance to a compatible race of *P. infestans* (Muller and Borger, 1940). It was presumed that the tuber tissue of potato in response to the incompatible interaction-induced compounds that prevented the pathogen and protected the tissue against infectious attack by other compatible races of the pathogen (Muller and Borger, 1940; Coleman et al., 2011; Pedras et al., 2011). Later studies have evolved not only to investigate the roles of PhA in defense against pathogens, but also to their health improving effects (Boue et al., 2009; Holland and O'keefe, 2010; Ng et al., 2011; Smoliga et al., 2011; Jahangir et al., 2009; Yang et al., 2009). For example, indole PhA from *Brassica* vegetables possesses an antioxidant, anticarcinogenic, and cardiovascular protective activities (Jahangir et al., 2009; Pedras et al., 2011). A peanut *Arachis hypogaea* PhA have antidiabetic, anticancer, and vasodilator effects (Holland and O'keefe, 2010). The glyceollin PhA from soybean (*Glycine*

max) has many biological activities such as antiproliferative and antitumor actions (Ng et al., 2011). The sorghum PhA, (*Sorghum bicolor*), 3-deoxy-anthocyanins, may have a potential effect against gastrointestinal cancer (Yang et al., 2009). Resveratrol PhA from the grapevine, *Vitis vinifera*, has anti-aging, anticancer, anti-inflammatory, and antioxidant properties that may promote the longevity of life (Smoliga et al., 2011).

Plant pathogens have evolved various strategies for getting nutritious materials from plants and then preformed chemical and physical barriers such as the immune system to fight the pathogen invasion. Antimicrobial substances from plants are classified into two broad categories: phytoantipicins (PhT) and PhA (Mansfield, 1999). PhT is defined as "low molecular weight, antimicrobial substances that are found in plants before microorganisms invasion, or are induced after infection only, from prerequisite precursors" (which are constitutive in plants). On the other hand, PhA are described as "low molecular weight, anti-microbial substances that are both synthesized and accumulated in plants after exposure to biotic or abiotic agents" (increased markedly in response to infection attack) (Paxton, 1980; Braga and Dietrich, 1987; Van Etten et al., 1994; Grayer and Kokubun, 2001; Zhang et al., 2013). Plants usually produce more than 100,000 compounds of low molecular weight – the secondary metabolites, which differ from the primary metabolites because they are not essential to plant life (Domingo and López-Brea, 2003). Some metabolites act as antimicrobials, working in defense against plant pathogens. Although fungi are the most commonly used in the study of induction of PhA, bacteria, viruses, nematodes, and stimuli from abiotic origin might also induce the accumulation of these defense compounds in plants. PhA represents one of the potential component-induced defense mechanisms used by lytic enzymes in plants as chitinases and glucanases, oxidizing agents, cell wall lignification and numbers of pathogenesis-related (PR) proteins, and transcripts of unknown functions (Lamb et al., 1989; Dixon and Lamb, 1990).

Genetic changes in plants might enhance PhA production and thus increase its resistance to stress or diseases. These genes degradation might be exploited for transforming microbial pathogens to plants (Turgeon and Yoder 1985; Van Etten et al., 1989). However, PhA degradative enzyme inhibitors (PhA synergists) may be used to control plant diseases (Van Etten et al., 1989). It has been stated that the mechanisms of PhA on fungi may include fracture of the PM, cytoplasmic granulation, cellular contents disorganization, and fungal enzymes suppression, which leads to seed germination inhibition and germ tube elongation, and mycelial growth inhibition (Cavalcanti et al., 2005).

Numerous reports have revealed a significant role of PhA in the resistance of plants against stress/diseases. Several PhA compounds with similar structure were not produced only as a response to infection but also, on exposure to any of stress mechanisms; they are not specific toxins. Specificity is depended on the way in which the plants react (resistance or susceptibility) in certain host–parasite combinations (Hans and Jurgen, 1978).

Sometimes, the pathogenic fungi are insensitive to the PhA of their hosts, whereas nonpathogenic fungi are sensitive to the same PhA. The in vitro tolerance of fungi toward PhA might be related to the ability of the fungi to convert the PhA into nontoxic compounds. The host–parasite system is affected by many factors in vivo. This system consisting of several cultivars (varieties) of the host plant and numerous pathogenic races of microbes, therefore, some varieties of host plant are susceptible for and some are resistance to (varietal specific resistance). On the other hand, the resistance reaction in such system is usually accompanied by the hypersensitivity reaction in which many cells at the site of infected plant tissue rapidly die, necrosis cells are formed and PhA accumulates (Hans and Jurgen, 1978). The system of host–parasite combinations consisted of many species of fungus, for example, *Colletotrichum lindemuthianum* and many varieties of bean (*Phaseolus vulgaris*), fungus *Phytophthora megasperma* var *sojae* and soybean (*Glycine max*), also of the fungus *P. infestans* and potato (*S. tuberosum*) (Bailey and Deverail, 1971; Keen, 1971; Keen et al., 1971; Bailey, 1974; Cramer et al., 1985; Kessmann et al., 1990). In spite of many species of the pathogenic agents, they are almost evenly sensitive to the PhA of their host (Keen et al., 1971; Bailey, and Deverail, 1971; Bailey, 1974) and the host cultivars produced the same PhA (Keen, 1971), some cultivars of the host plant are susceptible for and others are resistance. This dissimilar behavior might be owing to the PhA synthesis commenced after varying lag periods, a conduct at different rates and which lead to different concentrations. These have shown that higher concentrations of PhA were more rapidly accumulated in incompatible combinations of host cultivars and parasite species (i.e., with resistance) than in compatible combinations (i.e., susceptible). Thereby in incompatible interactions, PhA accumulation inhibit or kill pathogen growth, thereby allowing resistance to the plant. In compatible interactions, the pathogen might either tolerate the accumulated PhA, detoxifies them, decreases PhA accumulation, or avoids eliciting PhA production (Mansfield, 1982).

Furthermore, many compounds may act as PhA in a plant organ and be substantial in another part of the same plant, for example, momilactone A, which is substantially present in rice husks, and rice stems (Lee et al., 1999),

but is a PhA in rice leaves (Cartwright et al., 1981). Moreover, it has been reported that momilactone A is substantially produced and exuded from the root. Therefore, PhA are identified as dynamics of their function and biosynthesis, not by the class of chemical structure to which they belong, or biosynthetic pathway through which they were formed (Toyomasu et al., 2008). Nevertheless, the biosynthesis of the most PhA and the regulatory networks, as well as the molecular mechanisms beyond their cytotoxicity, are largely unknown.

3.3 ELICITORS OF PhA

Enhancement of PhA level in a plant (biotic or abiotic elicitors) is utilized which always activate mRNA transcripts and enzymes translation needed in PhA biosynthesis. Moreover, genes encoding elicitor-releasing proteins such as β-1,3-endoglucanase of one plant may be transferred to another for more potent resistant. Transgenic plant shows higher β-1,3-glucanase activity than an untransformed plant. PhA biosynthetic genes might also be converted to the desired plant which will impact resistance. Besides, biosynthetic pathways of PhA can be successfully changed by recombinant deoxyribonucleic acid (DNA) technology for enhancing PhA accumulation (Purkayastha, 1995). Many studies on PhA biosynthetic genes will actively contribute to our knowledge of the molecular biology of the host cells. The techniques of plant molecular biology have been applied successfully to establishing the role of PhA in plant resistance. However, the elicitation of the marked concentration of PhA by genetic manipulation is not always useful but may be more harmful to the host plant. Therefore, the understanding of PhA inhibitor/suppressor or degradation/detoxification mechanisms will be an aid in the regulation of excess PhA accumulation in plants and to comprehend the molecular basis of pathogenicity. PhA degradation by a pathogen metabolite makes the plant more vulnerable to invasion. Therefore, PhA inhibitors detoxifying enzymes should be exploited for enzymes inactivation and for keeping PhA level at the site of infection. Genes encoding PhA detoxifying enzymes and PhA suppressors has been determined in many virulent fungal pathogens. Although, extensive work has been done on PhA during the last century (Purkayastha, 1995), but some fundamental issues has remained yet to be elucidated.

Tissues from whole parts of terrestrial plants might produce PhA in response to infection by pathogenic and nonpathogenic fungus (Keen and Harsch, 1972), bacteria (Stholasuta et al., 1971, Keen and Kennedy, 1974),

viruses (Bailey et al., 1975; 1976), and nematodes (Abawi et al., 1971; Rich et al., 1977). The production of PhA is induced "elicited" not only by living microbes but also by different extracts (Varns et al., 1971). Compounds released from microbes which stimulate the induction of PhA synthesis in plants has been claimed as elicitors (Keen et al., 1972). However, the PhA might remain ineffective if the pathogenic agent does not get in contact with them during its development in the plant. Biotic elicitors might contribute to the interaction between plants and pathogens, whilst abiotic elicitors may not contribute to these interactions. Exogenous biotic elicitors might be in the attack of the organism, whilst endogenous elicitors are of plant origin and they are induced by the interaction of both microorganism and plant (Darvill and Albersheim, 1984).

Substances with elicitor activity have been detected along with a wide range of chemical structural types including polysaccharides, glycoproteins, lipids, lipopolysaccharides, oligosaccharides, and even enzymes, though their activity can be contributed to their effect in producing elicitor-active substances from the cell walls of the host or pathogen (Anderson, 1989; Blein et al., 1991; Hahn et al., 1992; Alghisi and Favaron, 1995). Abiotic elicitors form a numerous collection of substances that are not derived from natural sources, such as the tissues of the host or pathogen. Under normal conditions, they would not be produced by the plant, this includes substances such as fungicides, heavy metals (Cu^{2+} and Hg^{2+}), detergents, polylysine, histone, and reagents that intercalate a DNA (Dixon et al., 1983; Darvill and Albersheim, 1984). The exposure the plant tissues with factors that cause stress such as freezing, wounding, or exposure to UV light can also induce PhA synthesis (Kodama et al., 1988; Kodama et al., 1992a,b,c; Mert-Türk et al., 1998).

PhA consists of various groups of natural products. The only compounds considered were those which satisfied the standard of PhA concept (compounds which only detect in high amounts after infection and which prohibit the growth of microbes). There is a relationship between the chemical nature of the PhA and plant families, such as Legumes, Solanaceae, Fabaceae, and Orchidaceae produces isoflavonoids, diterpenes, polyacetylenes, and dihydrophenanthrenes, respectively (Ingham and Harborne, 1976; Harborne, 1999; Cavalcanti, 2005; Ahuja et al., 2012; Ejike et al., 2013). Table 3.1 is a list of the major PhA of various plant families. PhA include isoflavonoids, sesquiterpenes, furanoterpenoids, polyacetylenes, dihydrophenanthrenes, and so forth. Pisatin was the first natural product PhA characterized from pea plants. After this, other PhA were isolated from various crops such as beans, rice, barley, banana (Braga, 1991), and more than 300 types of PhA have been identified. *Poaceae* plants have also been studied for the induction of

TABLE 3.1 Phytoalexin from Different Plant Families.

Plant families	Types of phytoalexins (PhA)/examples	References
Amaryllidaceae	Flavans	Coxon et al. (1980)
Brassicaceae (Cruciferae)	Camalexin sulfur-containing/indole PhA/ brassinin	Browne et al. (1991)
Compositae	Safynol/polyacetylenes	Geigert et al. (1973)
Chenopodiaceae	Flavanones/betagarin Isoflavones/betavulgarin	Pedras et al. (2000)
Convolvulaceae	Ipomeamarone/Furano sesquiterpenes	Allen et al. (1971)
Euphorbiaceae	Diterpenes/casbene	Uritani et al. (1960); Sitton et al. (1975)
Linaceae	Coniferyl alcohol/phenylpropanoids (PP)	Keen et al. (1975)
Leguminosae	Isoflavans, isoflavones, isoflavanones, coumestans, pterocarpans/pisatin, glyceollin, phaseolin, maiackiain, wyerone/furano acetylenes, stilbenes/resveratrol pterocarpens	Jeandet et al. (2013)
Moraceae	Furano pterocarpans/moracins A-H	Takasugi et al. (1979)
Malvaceae	Gossypol/terpenoids naphtaldehydes	Kumar et al. (2006)
Orchidaceae	Loroglossol/dihydrophenanthrenes	Ward et al. (1975)
Poaceae	Diterpenoids: oryzalexins; momilactones; phytocassanes; zealexins; kauralexins; apigeninidin flavanones/sakuranetin phenylamides and deoxyanthocyanidins/ luteolinidin	Poloni et al. (2014); Park et al. (2013); Jeandet et al. (2013)
Rutaceae	Xanthoxylin/methylated phenolic compounds	Hartmann et al. (1974); Harding et al. (1981)
Rosaceae	Cotonefurans/biphenyls/auarperin dibenzofurans	Kokubun et al. (1995)
Solanaceae	PP-related compounds; steroid glycoalkaloids; norsequi and sesquiterpenoids coumarins; polyacetylenic derivatives	Jeandet et al. (2013)
Umbelliferae	Falcarinol phenolics: xanthotoxin 6-methoxymellein/polyacetylenes	Johnson et al. (1973); Condon et al. (1963)
Vitaceae	Stilbenes/resveratrol	Langcake et al. (1979)

a variety of PhA (Ahuja et al., 2012). They have a great diversity of classes of chemical compounds, members of family Poaceae produces mainly diterpenoid PhA. Corn produces kauralexins diterpenoids and zealexins acid sesquiterpenoids C and D PhA. The elicitors of PhA in corn include

pathogens such as *Aspergillus flavus, Aspergillus sojae, Cochliobolus heterostrophus, Colletotrichum sublineolum, Fusarium graminearum, Ostrinia nubilalis, Rhizopus microspores*, *and Ustilago maydis* (Huffaker et al., 2011, Rejanne et al., 2016). The rate of PhA production is considered a key factor for the establishment of the pathogen infection. Therefore, in some cases, PhA could be used as taxonomic markers.

3.4 PhA IN DISEASE RESISTANCE

The regulation and mechanisms of PhA in plants defense response are important in the resistance to infection in plants. Many work has been carriedout with the aim of exploring the PhA induced in different parts of plants families in the defense against pathogenic invasion and to detect the use of substances isolated from these plants to enhance the synthesis of PhA (Ejike et al., 2013). It follows that PhA induction or accumulation in a host plant might be regulated in many ways owing to promote its resistance to diseases. The biotic elicitors, which are characterized by viable or inactivated microbes, plant extracts have been used to enhance the induction of resistance, once these compounds have the advantages of not being phytotoxic and are readily biodegradable (Devaiah, 2009). Otherwise, when they are not pathogenic organisms, their mechanism of action is enhanced systemic resistance, which not causing symptoms as necrosis and might activate the defense response of plants by induction of PhA.

The suppressor sometimes acts as both suppressor of PhA biosynthesis and a determinant of pathogenicity by suppressing other defense reaction (Oku and Shiraishi, 1995). Resistance or susceptibility of plants growing under physiological stress conditions depends on several factors and not on PhA alone. There is evidence that sometimes plants under physiological stress are unable to produce an adequate amount of PhA and become more susceptible to disease.

Moreover, the pathogenic organisms might prevent the plant from synthesizing PhA. It has been established whether the PhA produced by pathogenic microbes (Strobel, 1977) may be termed as effectors for such mechanism. Another mechanism could be by the inhibition of elicitor action by competitive factors (Ayers et al., 1976) or inactivation of the elicitors before they reach their site of action in the plant cells.

The original response of plant defense is activated through two mechanisms, which are triggered immunity (PTI) known as nonhost defense and effector-triggered immunity known as host-specific resistance. The PTI defense mechanism occurs when in contact with the pathogen, and is

determined as the first line of innate immunity in plants (Zhang and Zhou, 2010). Scientific evidence that PhA are effective in providing plants with disease resistance has been confirmed by transferring foreign PhA expression from one plant to another. Stilbene PhA, which include resveratrol (1) (Fig. 3.1) need only stilbene synthase, to link the two present precursors, malonyl-CoA and p-coumaryl-CoA, for their synthesis. Pathogen resistance has thus been engineered into tobacco plants by the transfer of stilbene synthase genes from grapevine, *V. vinifera*, a resveratrol inducer. The regenerated tobacco plant proved to have improved pathogenic resistance to *Botrytis cinerea*. It not only accumulates its usual sesquiterpenoid PhA on inoculation but also rapidly synthesizes up to 40 μg resveratrol/g fresh weight (Hain et al., 1993). According to the gene for gene theory, it depends on the highly specific interaction between the pathogen and R gene products (Boller and Felix, 2009). Based on the signaling pathway, which might lead to the expression of defenses, enhancement of resistance can be divided into induced (ISR), that is activated by nonpathogenic microbes and mediated by jasmonate and ethylene, and that acquired (SAR), which is activated by pathogenic microbes, and is a salicylate-dependent induction (Pieterse et al., 2001; Zhang et al., 2015). The understanding of these pathways is important for selecting the most suitable elicitor agent which cause the desired defense system and thus obtain the highest expression of these antimicrobial substances.

Camalexin (3-thiazol-2ʾ-yl-indole) and rapalexin A are a major PhA in *Arabidopsis* (Browne et al., 1991; Pedras and Adio, 2008), which has also been detected in *Arabidopsis* and *Brassicaceae* species (Bednarek et al., 2011). Many studies on the major pathways controlling the production of camalexin in Arabidopsis indicated that their participation may depend on the pathogenic attack. Other studies searching for the response of *Arabidopsis* jasmonic acid (JA) signaling mutants to *Alternaria brassicicola* infection led to the summary that camalexin synthesis is under the control of a JA-independent pathway (Van Wees et al., 2003; Thomma et al., 1999). Innovative studies with *B. cinerea* have concluded that JA signaling controls camalexin synthesis to a substantial extent (Thomma et al., 1999). Other studies have suggested that camalexin induction is controlled by salicylic acid (SA)-independent (Nawrath and Metraux, 1999; Roetschi et al., 2001) and SA-dependent (Denby et al., 2005) signaling pathways. Reactive oxygen species are also combined with camalexin induction, as shown by oxidative stress-inducing chemicals such as paraquat and acifluorfen (Zhao et al., 1998; Tierens et al., 2002). Nevertheless, it has been suggested that both hydrogen peroxide and SA are required for the production of camalexin (Chaouch et al., 2010).

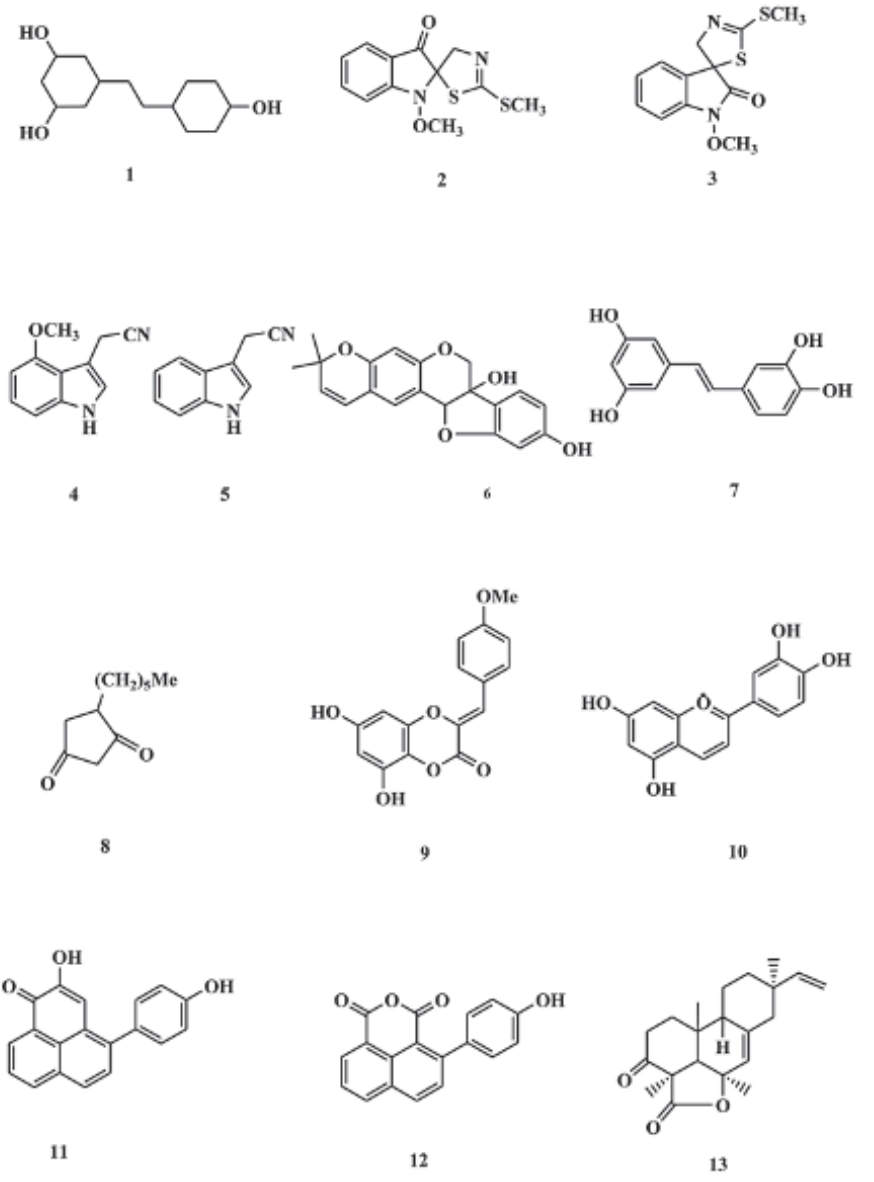

FIGURE 3.1 Chemical structures of isolated phytoalexin (PhA).

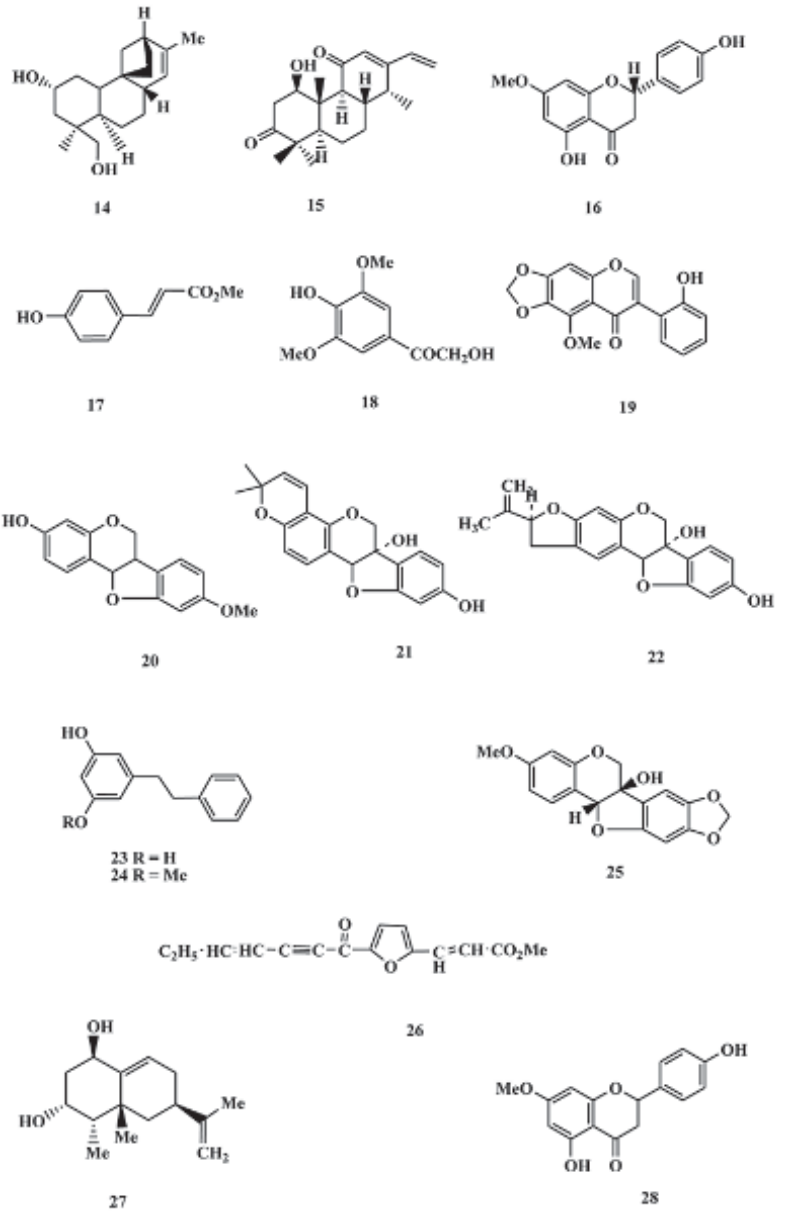

FIGURE 3.1 *(Continued)*

Erucastrum canariense Webb and Berthel F. *Brassicaceae* is a wild crucifer that grows in a drastic condition such as rocky soils, in salt, and water-stressed habitats. Abiotic stress induced by $CuCl_2$ caused accumulation of galacto-oxylipins PhA in *E. canariense*, whilst wounding produced galacto-oxylipins but not PhA. The isolation and structure determination of compounds isolated from the leaves of *E. canariense* revealed the presence of erucalexin (2), 1-methoxyspirobrassinin (3), arvelexin (4) and indolyl-3-acetonitrile (5), but no galactooxylipins were detected (Fig. 3.1).

It appears that the PhA response is well represented in gymnosperms. Many reports of PhA in gymnosperms such as the maidenhair tree *Ginkgo biloba*, has been examined for a PhA response. In fact, infection by *Botrytis allii* successfully produced substances, which inhibited fungal penetration into the leaf (Christensen, 1972). Other report about the treatment of *Pinus contorta* bark with *Ceratocystis clavigera* lead to enhanced terpenoid synthesis and accumulation of the oleoresin was observed (Croteau et al., 1987). Moreover, in pines, the PhA are mainly stilbenes, with benzoic acid and a flavanone being also detected. In *Pseudotsuga*, it is a dihydroflavonol, in *Picea* a lignan and in *Cupressus* two tropolones.

There are about 46 PhA that have been identified in 17 monocot species. Some are listed in Table 3.2. One of the major obstacles in progressing PhA research in monocotyledonous families is the difficulty of inoculating tissues of plant and getting a necrotic reaction. For example, the leaves of the rice plant are comparatively resistant to inoculation by the rice blast organism *Pyricularia sativa*, even when using a susceptible cultivar. This can be overcome by employing UV irradiation or by using punch inoculation to mimic pathogen attack (Dillon et al., 1997). Many PhA types that are present in the monocotyledons are the same as those in the dicotyledons, and this support the theory that the monocotyledons are thought to have evolved from within the dicotyledons. However, there are some astonishing equivalent in PhA production between mono- and dicots. The most noteworthy is of two characteristic legume PhA, glyceollins (GLNs) II (6) and III (Fig. 3.1) from soyabean *Glycine max*, *Costus speciosus* (Costaceae) leaves infected by *Drechslera longirostrata* (Kumar et al., 1984). GLNs are PhA produced by soy plants in response to stressful stimuli such as fungal infections, UV exposure, or changes in temperature. GLNs have demonstrated antiestrogenic activity both in in vitro and in vivo, suppressing estrogen-responsive tumors through action at estrogen receptors (ERs). Recent evidence suggests that GLNs might also possess estrogenic properties or might have exhibited their effects through non-ER-mediated mechanisms. In addition to antitumor effects, GLNs possess antimicrobial

and antioxidant activity, along with effects on glucose and lipid metabolism (Bamji, 2017).

TABLE 3.2 Phytoalexin Compounds Isolated from Monocotyledons Plant Families.

Plant families	Chemical classes	Compounds isolated	References
Alliaceae	Cyclic dione*	5-Hexylcyclopenta-1,3-dione	Tverskoy et al. (1991)
Amaryllidaceae	Flavan	7,4'-Dihydroxyflavan	Coxon et al. (1980)
Costaceae	Isoflavonoid	Glyceollin II	Harborne (1999)
Dioscoreaceae	Bibenzyl	Batatasin IV	Hashimoto et al. (1972)
Gramineae	Flavanone	Sakuranetin	Ogawa et al. (2017);
	Stilbene	Resveratrol	Shrikanta et al. (2015);
	Deoxyanthocyanidin	Luteolinidin	Viswanathan et al. (1996)
	Diterpene*	Momilactone A	
	Anthranilic acid	HDIBOA glucoside	
Liliaceae	Stilbene	Resveratrol	Shrikanta et al. (2015)
Musaceae	Phenalenone*	Musanolone C	Holscher et al. (2014)

*Chemical classes from monocotyledons plant families.

Other parallel findings are of the stilbene resveratrol (1) in *Festuca versuta* leaf (Powell et al., 1994) and of the related piceatannol (7) (Fig. 3.1) in the leaf of sugarcane, *Saccharum officinarum* (Brinker and Seigler, 1991). Resveratrol is a PhA found in the dicots of both the peanut *Arachis hypogaea* (Leguminosae) and the vine, *V. vinifera* (Vitaceae), and in the gymnosperms of *Pinus* sapwoods.

However, in many cases, PhA produced is entirely distinctive of a particular monocot genus, Table 3.2. Such as infected bulbs of the onion *Allium cepa* induce two aliphatic diones, 5-hexylcyclopenta-1,3-dione (8) (Fig. 3.1) and the 5-octyl analogue (Tverskoy et al., 1991). In spite of onion and other *Alliums* species that are rich in sulfur compounds, some of which are antimicrobial, it is remarkable that the PhA of onion is not only sulfur. It has an individual structure PhA which is yurinelide (9) (Fig. 3.1) and a benzodioxin-2-one synthesized by *Lilium maximowiczii* in the bulb (Monde et al., 1992). Moreover, monocots have the ability to synthesize PhA pigments such as red 3-desoxyanthocyanidin. Apigeninidin and luteolinidin (10) (Fig. 3.1) pigments were identified in sorghum tissues after response to infection by a fungus (Nicholson et al., 1987). Apigeninidin 5-caffeoyl-glucoside and luteolinidin 5-methyl ether have also been identified in

inoculated sorghum tissue (Lo et al., 1996). Sugarcane (*Saccharum officinarum*) also accumulates luteolinidin and piceatannol (7) in response to fungal infection (Brinker and Seigler, 1991). In the the banana plant, *Musa acuminata* (Musaceae), phenalenone-type structures are accumulated after fungal infection *Colletotrichum musae* such as irenolone (11) and naphthalic anhydride (12) (Fig. 3.1) from leaves and fruit peels (Hirai et al., 1994; Luis et al., 1993).

Sixteen (16) PhA have been reported in rice, *Oryza sativa* (Gramineae), 14 are diterpenoids, ranging from momilactones A (13) and B (Cartwright et al., 1981) and oryzalexin S (14) to phytocassanes (15) (Koga et al., 1997). Two flavanones reported are naringenin and its 7-methyl ether sakuranetin (16) (Fig. 3.1) (Kodama et al., 1992a,b,c). Experiments with susceptible and resistant rice cultivars suggest that sakuranetin, momilactone A, and oryzalexin S are produced in suitable amounts to support such resistance (Dillon et al., 1997).

Maize (*Zea mays*) is Poaceae crop plant. The PhA which accumulate in the plant in response to pathogen attack are kauralexins and zealexin (Fig. 3.2). It has recently been disclosed that maize stem invaded by fungi *Rhizopus microsporus* and *Colletotrichum graminicola* stimulates the accumulation of six ent-kaurane-related diterpenoids, termed kauralexins (Huffaker et al., 2011). A physiologically relevant amount of kauralexins inhibited the growth of these pathogens. Accumulation of the fungal-induced kaurene synthase 2 (An2) transcripts preceded highly localized kauralexin induction, and a combination of JA and ethylene application detected their synergistic role in kauralexin regulation. Other maize PhA, termed zealexins, have also been recently discovered following an attack by *F. graminearum* (Schmelz et al., 2011). The characterization of these recently discovered kauralexins and zealexins should be required in the identification of the roles of nonvolatile terpenoid PhA in maize disease resistance (Huffaker et al., 2011; Schmelz et al., 2011).

About 24 families of the dicotyledons are listed in Table 3.3. PhA production may yield one or more of the same structural class, while other species can give rise to multiple unrelated structures. PhA in the dicotyledons are classified into four main chemical classes as phenolics, acetylenics, terpenoids, and nitrogen-containing compounds. In phenolics, they have free hydroxyl groups masked by o-methylation and this might enhance their lipophilicity. Phenylpropanoids (PPs) have been reported in the Linaceae and Scrophulariaceae, Table 3.3. Moreover, p-coumaric acid methyl ester (17) (Fig. 3.1) has been isolated from cucumber leaves, *Cucumis sativus* infected by powdery mildew (Daayf et al., 1997).

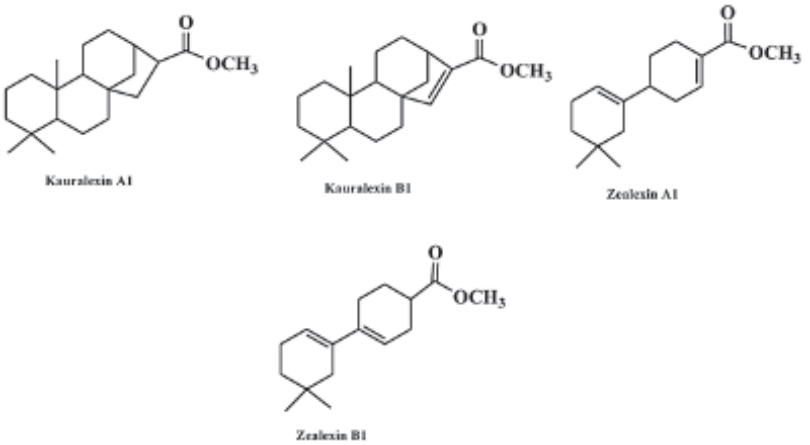

FIGURE 3.2 Structure of PhA in maize plants.

Xanthoxylin (phloracetophenone 4,6-dimethyl ether) is PhA from *Citrus limon* (Afek and Sztejmberg, 1988), while danielone (18) (Fig. 3.1) is a PhA from papaya fruit, *Carica papaya* (Caricaceae) (Echeverri et al., 1997), whereas 4-hydroxy- and 3,4-dihydroxyacetophenone, are PhA in cocoa *Theobroma cacao* (Sterculiaceae) (Resende et al., 1996). In the cocoa plant, they also accumulate arjunolic acid triterpene. Moreover, many reports of coumarin PhA such as aesculetin 6,7-dimethyl ether are found in *C. limon* (Rutaceae) (Afek and Sztejnberg, 1988) and furanocoumarin, xanthyletin from the same plant (Khan et al., 1985). Furthermore, coumarin scopoletin PhA is reportedly found in the rubber tree *Hevea brasiliensis* (Euphorbiaceae) leaves (Giesemann et al., 1986) and from a plant tree, *Platanus acerifolia* (Platanaceae) leaves (El Modafar et al., 1993). Isoflavones PhA are accumulated in legume leaves and are formed in the unrelated *Beta vulgaris* of the Chenopodiaceae, Table 3.3. Betavulgarin (19) (Fig. 3.1) a major isoflavone of *B. vulgaris*, also occurs as the 2′-xyloside and as the 2′-glucoside. It has been suggested that these glycosides have a role in promoting resistance (Elliger and Halloin, 1994). Acetylenics are presented in Umbelliferae and Compositae families. They have also been determined in the Solanaceae, a family where sesquiterpenoids are the main PhA. Occasionally, various terpenoid have been reported also from Convolvulaceae, Euphorbiaceae, Malvaceae, Phytolaccaceae, Tiliaceae, and Ulmaceae (Table 3.3). Finally, to develop disease protection strategies, plant pathogen research in the field of PhA has concentrated on interpreting their biosynthesis pathways and regulation in different crop plants by using different cultivars, transgenic

plants, and mutants, and by applying molecular biology and biochemical approaches (Ahuja et al., 2012).

TABLE 3.3 Phytoalexin Compounds Isolated from Dicotyledons Plant Families.

Plant families	Chemical classes	Compounds isolated	References
Cercidiphyllaceae	Biphenyl	Magnolol	Takasugi and Katui (1986)
Chenopodiaceae	Isoflavone	Betavulgarin	Marti (1977)
Convolvulaceae	Furanoterpenoid	Ipomeamorone	Coxon et al. (1975)
Cruciferae	Indole*	Spirobrassinin	Mezencev et al. (2009)
Compositae	Acetylenic*	Safynol	Ranga et al. (1977)
Cactaceae	Aurone	4,5-methylenedioxy-6-hydroxyaurone	Paul et al. (1991)
Euphorbiaceae	Diterpene	Casbene	Sitton and West (1975)
Linaceae	PP	Coniferyl alcohol	Hano et al. (2006)
Leguminosae	Isoflavonoid*	Pisatin	Perrin and Bottomley (1962)
Moraceae	Stilbene	Oxyresveratrol	Chung et al. (2003)
Malvaceae	Sesquiterpene	Gossypol	Stipanovic et al. (2005)
Phytolaccaceae	Saponin	Phytolaccoside	Kanzaki et al. (1999)
Papaveraceae	Alkaloid	Sanguinarine	Cline et al. (1988)
Rubiaceae	Anthraquinone	Purpurin 1-methyl ether	Wijnsma et al. (1984)
Rutaceae	Acetophenone	Xanthoxylin	Hartmann et al. (1974)
Rosaceae	Biphenyl*	Aucuparin	Widyastutfi et al. (1992).
Solanaceae	Sesquiterpene*	Rishitin	Komaraiah et al. (2003)
Scrophulariaceae	PP	Acteoside	Chun et al. (2002)
Tiliaceae	Sesquiterpene	7-Hydroxycalamenene	Burden and Kemp (1983)
Umbelliferae	Furanocoumarin*	Xanthotoxin	Al-Barwani et al. (2004)
Ulmaceae	Diterpene	Mansonone A	Meier et al. (1997)
Vitaceae	Stilbene	Pterostilbene	Langcake et al. (1979)
Verbenaceae	Naphthofuranone	Naphtho [1,2-b] furan-4,5-dione	Tsai et al. (2014)

*Chemical classes from dicotyledons plant families.

3.5 ELICITATION OF PhA BY INDUCING/STIMULATION mRNA TRANSCRIPTION AND ENZYMES TRANSLATION SOUGHT IN PhA BIOSYNTHESIS

It has been known that both biotic and abiotic elicitors give similar PhA accumulation. Addition of fungal elicitors (e.g., elicitor, heat released from cell walls of the pathogenic fungus, *C. lindemuthianum*, was added to a final concentration of 60 μg of glucose equivalent/ml) to bean cell suspension cultures results in inducing PhA synthesis (Cramer et al., 1985). The elicitors stimulate the formation of messenger ribonucleic acids (mRNAs) encoding the enzymes of PP metabolism. These enzymes are synthesized prior to PhA synthesis and might be noticed within 20 min after elicitor was added and reaches its maximum level within 3–4 h. Most of the PhA are derived from the PP biosynthetic pathways. The PPs are involved in many functions and play a pivotal role in the interaction between the plant and its surrounding. Various secondary metabolites contribute in the plant diseases resistance and enzymes that catalyze the biosynthesis of PhA are phenylalanine ammonia lyase (PAL), cinnamate-4-hydrolase, and coenzyme 4-cumaraseligase (Mateos and Leal, 2013). The understanding of the PhA biosynthetic pathways and the required enzymes has lead to the knowledge of the expression of plant response to pathogens infection.

Terpenoid PhA exists in a variety of species such as tobacco (*Nicotiana tabacum*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), and elm (*Ulmus americana*) (Harborne, 1999). In plants, the isopentenyl diphosphate and dimethylallyl pyrophosphate isoprenoid precursors can be derived from the mevalonate or metileritritol phosphate. The methylerythritol phosphate (MEP) pathway leads to the biosynthesis of geranylgeranyl diphosphate and the diterpenoid biosynthesis pathway (Okada, 2007). The diterpene cyclases catalyzes the conversion of ent-copalyl-diphosphate (ent-CDP) or syn-copalyldiphosphate (syn-CDP) in four key hydrocarbons in the PhA biosynthesis in rice, which are entcassa-12,15-diene; ent-sandaracopimara-8,15diene; 9*β*-pimara-7,15-diene; and stemar-13-ene. The geranylgeranyl diphosphate production in rice probably occurs in the plastids by MEP pathway, whereas sesquiterpenes and triterpenes are usually synthesized in the cytoplasm through the mevalonate pathway (Toyomasu 2008 , Vranova et al., 2013). Diterpenoid PhA are expected to be formed through the diphosphate geranylgeranyl methylerythritol.

In the biosynthesis of isoflavanoid PhA, medicarpin (20) (Fig. 3.1) several reactions are involved. Activation of enzymes by elicitors to enhance PhA production. Soybean plants synthesize and accumulate isoflavonoid derived

PhA, GLNs in the plant's tissues after exposure to pathogens (Bailey et al., 1975). Synthesis of GLNs can be produced in soy plants after exposure to fungal strains such as *A. sojae* (Boué et al., 2000), which elicits induction of a trace to moderate amounts of GLNs mixture, I (21), II (6) and III (22) (Fig. 3.1) in response to the pathogenic attack (Eromosele et al., 2013; Isaac et al., 2017). Many studies have been established that GLNs have antibacterial, antioxidant, antifungal, anti-inflammatory, antitumor, and insulinotropic actions both in vitro and in vivo (Salvo et al., 2006; Kim et al., 2010a,b; Park et al., 2010; Kim et al., 2011; Ng et al., 2011).

GLNs biosynthesis (Fig. 3.3) includes enzymes that are used in the synthesis of isoflavonoids and PhA (Ebel, 1986). Phenylalanine is first converted to trans-cinnamic acid by the action of enzyme PAL, followed by a hydroxylation reaction catalyzed by cinnamate 4-hydroxylase (C4H) that converts the trans-cinnamic acid to *p*-coumaric acid (Dixon and Paiva, 1995). A series of subsequent reactions involving enzymes 4CL, chalcone synthase (CHS), CHR, chalcone isomerase (CHI), and IFS, leads to the formation of 2,7,40-trihydroxyisoflavone, which then leads to the production of the isoflavonoid daidzein (Dixon and Paiva, 1995; Yoneyama et al., 2016). Daidzein undergoes cyclization and hydroxylation reactions to glycinol, (non-prenylated precursor for GLNs) (Dixon and Paiva, 1995). Two separate prenylated transferases, G4-DT and G2-DT, then prenylated glycinol to GLNs I and II, respectively (Akashi et al., 2009). The final steps of GLNs biosynthesis are the cyclization of glyceollidins by GS to convert glyceollidin I to glyceollin I, and glyceollidin II to GLNs II and III (Welle and Grisebach, 1988).

Moreover, CHS is the key enzyme of the flavonoid/isoflavonoid biosynthetic pathway. The amount of CHS mRNA increased significantly in soybean leaves when inoculated with an avirulent race of *Pseudomonas syringae* pv. *glycinea* (*Psg*) but not with a virulent race. In contrary, the increase in CHS mRNA was similar in roots inoculated with zoospores from an avirulent or a virulent race of *P. megasperma* f. sp. *glycinea* (*Pmg*) (Dhawale et al., 1989). The major elicitor releasing an enzyme in soybean is β-l,3-endoglucanase. This enzyme could release PhA elicitors from *Pmg* cell walls. Antiserum raised against the purified enzyme was detected to be specific for soybean releasing enzyme. The antiserum inhibited β-1,3-glucanase, the elicitor-releasing activity of the purified enzymes and soybean crude extracts (Takeuchi et al., 1990, Yoshioka et al., 1993). This observation indicated that host enzymes play a pivotal role in the production of PhA by releasing PhA-elicitors from fungal cell walls. It disclosed that β-l,3-endoglucanase was a key enzyme involved in elicitor signal induction. It was found that transgenic tobacco

plants, expressing the soybean glucanase gene were resistant to fungal diseases. These results interpreted that disease-resistant transgenic tobacco plants might be developed which would promote the release of active elicitor molecules from fungal cell walls (Yoshioka et al., 1993).

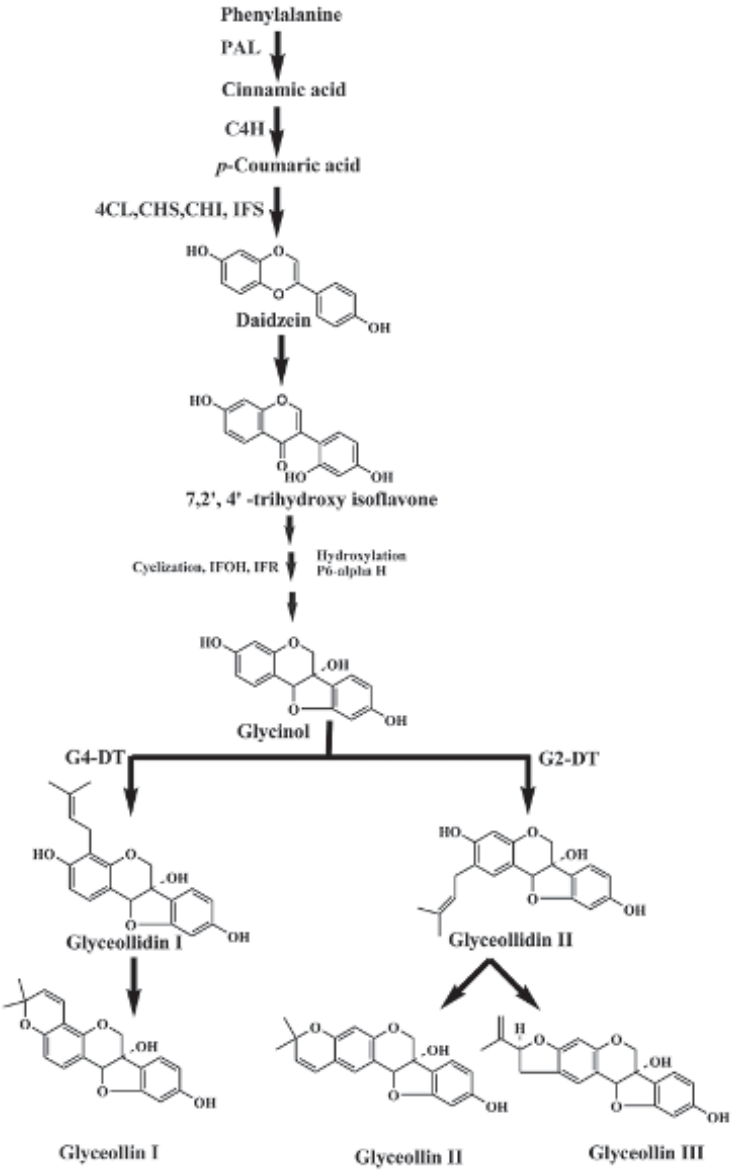

FIGURE 3.3 Biosynthetic pathway of glyceollins in soybeans.

Elicitor enhances metabolic changes in cell cultures of both resistant and susceptible chick pea (*Cicer arietinum* L.cvs) were established by Daniel et al. (1990). Cell suspension cultures of resistant (cv. ILC 3279) and susceptible (cv. ILC 1929) cultivars were compared to their elicitor enhance accumulation of pterocarpan PhA and activities of enzymes. The treatment of cell cultures with a polysaccharide elicitor from *Ascochyta rabiei* afforded 5-fold higher concentrations of PhA medicarpin and maackiain in the cells of the resistant than in the susceptible cultivar. Daniel and Barz noticed a differential accumulation of Medicarpin and Maackiain PhA in *C. arietinum* cvs. and enhance CHS-mRNA activity after implementation of an *A. rabiei* derived elicitor (1990).

The CHS involved in the first step of PhA biosynthesis showed a maximum activity between 8–12 h after elicitation in the cells of both cultivars. An approximately 5-fold higher concentration of PhA and a 2-fold increase in CHS activity was determined in the cells of the resistant cvs. The highest of the elicitor produced CHS-mRNA activity was noticed 4 h after the onset of induction in both the cultivars by mRNA activity was 2-fold higher in resistant cultivar (ILC 3279) than in the cells of susceptible cultivar (ILC 1929). Elicitation mechanism of isoflavan PhA in *Agrobacterium rhizogenes* transformed root of *Lotus corniculatus* cultures by glutathione (GSH) leads to the accumulation in both tissues and in callus medium. Nevertheless, the accumulation was preceded by a temporal increase in the activity of PAL (Robbins et al., 1991, Seifert et al., 1993). Isoflavonoid PhA was isolated from cell suspension cultures and intact plants of *Ornithopus sativus* after using of yeast or $CuSO_4$ elicitor or a spore suspension of *Colletotrichum trifolii*. Glabridin was identified by using physical and spectroscopic properties, the transient increase of this PhA was preceded by a transient increase in the activities of PAL and CHS. A study indicates that exogenous JA also enhances the accumulation of PAL, CHS and proline-rich protein (PRP) transcripts in soybean cells (Gundlach et al., 1992), as well as, it might be enhanced to activate PhA accumulation and other defense responses (Dixon et al., 1994).

CHS change in activity is relative to the time of accumulation of PhA, 3-deoxyanthocyanidin, in etiolated sorghum mesocotyls inoculated with *C. graminicola* (Wei et al., 1989). Results revealed that PhA began to accumulate in the tissue between 3 and 6 h. after the initial increase in the level of the enzyme. Sorghum PhA produces known as apigeninidin (2-(4-hydroxyphenyl) benzopyrilium chloride) and luteolinidin (2-(3,4-dihydroxyphenyl) chromenylium 5,7-diol). The biosynthesis of 3-deoxyanthocyanidin is independent of light, it happens in the dark (Wharton and Nicholson, 2000).

Huang and Backhouse (2004), have reported that the production of 3-deoxyanthocyanidin is not the first line of defense by sorghum against pathogens. They noticed that sorghum inoculated with *Fusarium proliferatum,* and *Fusarium thapsinum*, enhanced the levels of apigeninidin and luteolinidin, also the amounts of the resistance-related proteins peroxidases, β-1,3 glucanases and chitinases.

Stress responses in alfalfa (*Medicago sativa* L.) has been conducted. It was noticed that cell suspension cultures accumulated high amount of the pterocarpan PhA medicarpin after treatment with an elicitor prepared from cell walls of *C. lindemuthianum*. A maximum accumulation was detected within 24 h after treatment. This was preceded by an increase in the extractable biosynthetic enzymes of isoflavonoid for example L- PAL, Cinnamic acid 4-hydroxylase (CA4H), 4-coumarate COA ligase (4CL), CHS, CHI and Isoflavone O-methyl transferase, Isoflavone hydroxylase, Isoflavone reductase, and pterocarpan synthase. Pectic polysaccharides are weak elicitors of PAL, therefore could enhance medicarpin concentration (Dalkin et al., 1990).

Some serious diseases on rice (*Oryza sativa*) as blast, which is caused by the fungus *Pyricularia oryzae*, lead to losses in productivity (Grayer and Kokubun, 2001). In fact, studies have been done to improve more resistant cultivars. The manipulation of defense mechanisms is one of the potential strategies to impart resistance in rice plants, which can be induced by a variety of biotic and abiotic factors (Li et al., 2014). Several diterpenoids, Momilactone A and B and Oryzalexins A–D (Fig. 3.4) were detected in rice tissue infected by *Pyricularia oryzae* and casbene was extracted from *Rhizopus stolonifera*, infected castor bean seedlings. Seven enzymes were found to be involved in casbene (from mevalonate to casbene) biosynthetic pathway, enhanced activities of casbene synthetase and farnesyl transferase were noticed in cell free extracts of castor bean seedlings after infection with *R. stolonifera* (Charles et al., 1990). The changes in enzymes activities after fungus exposure indicate that the elicitors released by the fungus are recognized by the host cells to trigger the defense response of the host.

Ajuha *et al*, have been also indicated that many diterpenoids such as momilactone A, B, and phytocassane A-E as well as flavonoids such as sakuranetin (5,4'-dihydroxy-7-methoxyflavanone) were isolated from infected rice (2012). The sakuranetin is an antifungal agent present in the rice leaves. it is produced at the moment of the pathogen invasion (Kodama et al., 1992a,b,c). Okada (2007) has been reported that the diterpenoid PhA are classified into four distinct types of polycyclic diterpenes based on the structure of their hydrocarbon diterpene precursors: phytocassanes A-E, A-F oryzalexins, momilactones A and B and oryzalexin S (Fig. 3.4). Two

diterpenoids PhA has been reported; phytocassane F and ent-10-oxodepressin (Inoue, 2013; Horie, 2015). The Momilactones A and B are potent antifungal agents against *Magnaporthe oryzae*, *Botrytis cinerae*, *Fusarium solani*, *Fusarium oxysporum*, *Colletrotichum gloesporides*. These PhA have potential antibacterial activity against *Escherichia coli*, *Pseudomonas ovalis*, *Bacillus pumilus* and *Bacillus cereus* (Fukuta et al., 2007).

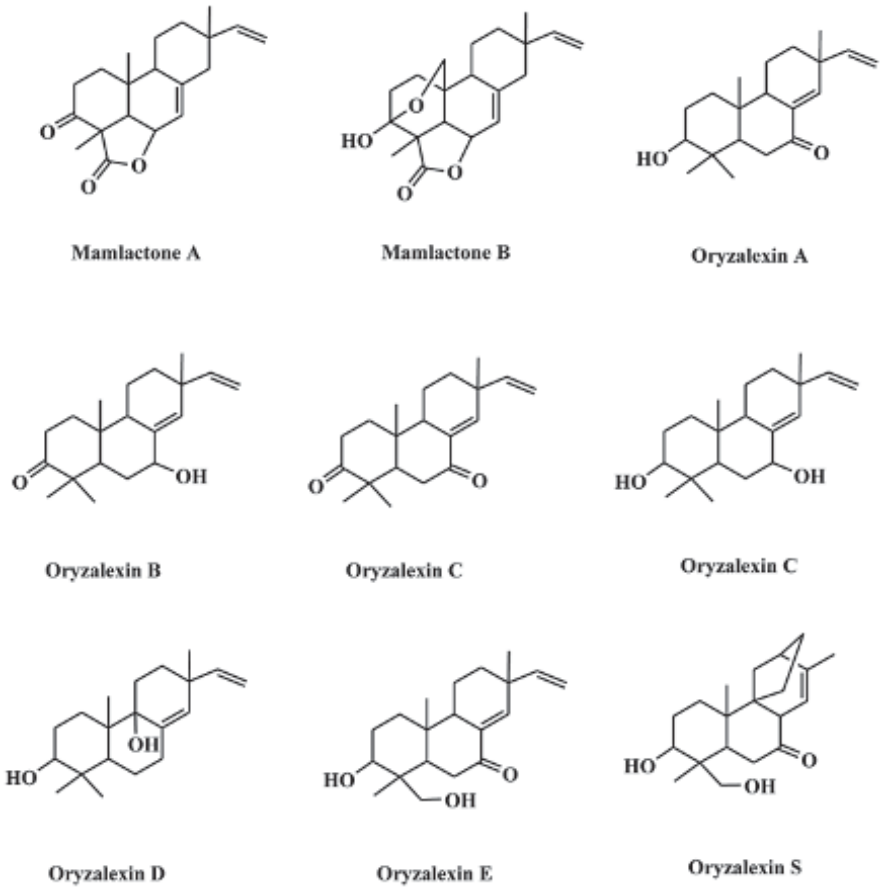

FIGURE 3.4 Chemical structure of PhA isolated from rice plants.

Diterpene PhA biosynthesis pathway of Momilactone and Oryzalexins in rice was postulated by Charles et al. (1990). Many authors suggested that geranylgeranyl-PP is considered to be an acyclic precursor of all cyclic diterpenes. Two modes of cyclization of geranylgeranyl-PP have been operated to afford bicyclic pyrophosphorylated intermediates 9βH-labdadienyI-PP

and copalyl-PP as precursors of the Momilactone and Oryzalexin respectively. The cyclization activity was enhanced greatly by UV-irradiation (an abiotic PhA elicitor) of rice leaves. Diterpene products are accumulated in UV-irradiated rice leaf extract from geranylgeranyl-PP and copalyl-PP as substrates (Charles et al., 1990).

The production of stilbene PhA in various plants has also been reported. The heartwood of pine tree contains large amounts of stilbene, pinosylvin (23) and pinosylvin monomethyl ether (24) (Fig. 3.1). In young plants of *Pinus sylvestris*, the concentrations of these stilbenes were difficult to detect. However, at stress conditions, stilbene content is dramatically increased. Pinosylvin was considered to be the predominant PhA formed by pines and may be responsible for resistance. Only synthase acting upon cinnamoyl-CoA was the key enzyme for the formation of stilbenes. Pinosylvin was formed by Stilbene synthase which was isolated from UV light-treated 4 weeks of the old plants, purified and identified by Gehlert et al. (1990). The inoculation of pine seedlings with *B. cinerea* owing to a 38-fold high in stilbene synthase activity within a day. The rapid response might be measured prior to any detectable lesion, sclerotia or blight. Melchior and Kindl have also studied the mechanisms controlling the production of stilbene synthase and PAL "key regulatory enzymes of the biosynthetic pathway of stilbene PhA." The enzymes were induced in cell suspension cultures of grape (*Vitis cv* optima) by fungal (*Phytophthora cambivora*) cell wall. Both PAL and stilbene synthase mRNA were produced within 1 h by adding fungal cell wall preparations to the cell cultures. The majority of activity was reached within a maximum of 6 h and then declined (1991).

Similarly, the addition of *Phytophthora* sp. cell wall fragments or *Trichoderma viride* cellulase to tobacco cv. KY 14 cell suspension cultures enhanced the accumulation of the extracellular sesquiterpenoid PhA "capsidiol" and also enhance activities of both 3-hydroxy-3-methyl glutaryl coenzyme A reductase and sesquiterpene cyclase. These enzymes were involved in sesquiterpene biosynthetic pathway (Chappell et al., 1991). It appears from the above information that many key enzymes of PhA biosynthetic pathways played a pivotal role in PhA-regulation mechanisms.

3.6 INHIBITION/SUPPRESSION OF PhA PRODUCTION BY DIFFERENT MICROBIAL METABOLITES

It has been reported that infection of two carnation varieties, highly and moderately resistant to *Fusarium wilt* (caused by *F. oxysporum* f. sp. *dianthi*)

stimulate the accumulation of PhA and aromatic acids. Pretreatment of plants with phenyl serine or SA diminished the PhA production and their resistance to infection (Niemann and Baayen, 1989). Etiolated hypocotyls of a resistant soybean cv. Harosoy 63 were susceptible to *P. megasperma* sp. *glycinea* race I after treated with abscisic acid. The susceptibility was manifested by increments in lesion size and a dramatic decrement in the accumulation of isoflavonoid PhA glyceollin. Nevertheless, PAL activity and accumulation of mRNA of this enzyme rapidly increased after infection in untreated hypocotyls. These increases were suppressed after treating the hypocotyls with abscisic acid. It has been suggested that the possibility of glyceollin biosynthesis in the resistance response of soybean may be controlled at the transcriptional level by changes in abscisic acid amount (Ward et al., 1989). Johal and Rahe noticed that *P. vulgaris* (bean plants) treated with glyphosate (2.5 μg), decreased the accumulation of PhA. The spreading lesions caused by *C. lindemuthianum* coalesced and rolled the entire hypocotyl. In contrast, the normal levels of PhA have been detected at infection sites in bean plants pretreated with a PAL. In addition to, lesions didn't spread. These results support the interpretation of the ability of glyphosate to decrease the accumulation of PhA that depends on the availability and demand for precursors such as PAL (1990).

Yoshioka et al., have been studied the effects of elicitor and suppressor of pisatin (25) (Fig. 3.1) released by *Mycosphaerella pinodes* (pea pathogen) on ATPase activity in the PM of etiolated pea epicotyls. They noticed that pisatin concentration induced by the elicitor was delayed for 3–6 h in the presence of orthovanadate or verapamil (100 PM). It was unaffected by the elicitor but was dramatically inhibited by suppressor from *M. pinodes* or verapamil. This result suggests that the ATPase played a pivotal role in pisatin accumulation (1990).

Yoshioka and Sugimoto (1993) demonstrated that soybean pathogen might induce an intracellular, branched β-1,3-glucan with suppressor activity with the cell wall-associated elicitors. This is a unique interaction at the site of receptor binding. The intracellular branched β-l,3- glucan claimed "mycolaminaran" was isolated and purified from the cytosol of *P. mega sperma* f. sp. *glycinea* which acted as a suppressor of glyceollin production in soybean cotyledons. The assumed mechanism of suppression of the PhA accumulation by mycolaminaran was suggested that the possibility to compete for the elicitor binding to the putative receptor in soybean membranes. Pisatin biosynthesis was also determined in pycnospore germination fluid of *M. pinodes*. The elicitor was detected as a polysaccharide and two suppressors were found to be glycopeptides. Eight species of pea (non-pathogens)

established infection on pea leaves in presence of suppressor (Oku and Shiraishi, 1995).

A model system has been established to detect suppression of defense responses in bean by the compatible bacterium *P. syringae* pv. *phaseolicola*. Bean plants infiltrated with *P.s. phaseolicola* failed to induce transcript for a PAL. CS or CHI up to 120 h after infiltration and CHT (chitinase transcript) concentration was also significantly delayed when compared to the incompatible *P. syringae* strains. Bean plants infiltration with 10^8 cells/ml of *P.s. phaseolicola* NPS 3121, 8 h precede to infiltration with an equal amount of incompatible *P.s. pv. taba* Pt 11,528 significantly decreased the typical profile of defense transcript accumulation when compared to plants infiltrated with Pt 11,528 alone. Suppression of PhA concentration was also noticed. NPS 3121 suppressed PAL, CS, CHI, and CHT transcript concentrations and PhA accumulation induced by *Escherichia coli* or by the elicitor GSH. Heat-killed cells of both cells treated with protein synthesis inhibitors or NPS 3121, lost the suppressor activity. These findings suggested that NPS 3121 had an active mechanism to suppress the production of defense transcripts and PhA biosynthesis in bean (Jakobek et al., 1993).

Specific biosynthesis suppression of PhA (shikimate pathway in *Cassia obtusifolia*) by a sublethal dose (50 mM) of glyphosate has been shown to increase the susceptibility to the *Alternaria cassia* (mycoherbicide). Glyphosate utilized the conidia suppressed PhA synthesis at 12 h. Five times less inoculum was needed for the induction of disease symptoms after used with glyphosate. The PhA synthesis elicited by fungal inoculation was suppressed also by darkness (Sharon et al., 1992.

3.7 DEGRADATION OF PhA BY MICROBIAL ENZYMES

Virulence of organisms may depend upon its ability to degrade PhA (Desjardins et al., 1989; Miao and Van Etten, 1992). It is obvious that the control of degradation by using PhA-detoxifying enzymes inhibitors could promote plant disease resistance. Wyerone (26) (Fig. 3.1), is the predominant PhA induced by *Vicia faba*. Wyerone degradation by *Rhizobium leguminosarum* has been reported by George and Werner (1991). HPLC analysis of the medium during bacterial growth obviously indicated that *R. leguminosarum* was able to metabolize Wyerone, identified as hydroxyester wyerol.

N. haematococca on pea, virulence of *Gibberella pulicaris* on potato tubers and its association with a gene for rishitin metabolism were studied by Desjardins and Gardner (1991). The ability of field strains of *G. pulicaris*

to trigger dry rot of potato tubers is relevant to their ability to metabolize rishitin (potato PhA). Genetic analysis of one potato pathogenic field strain R-6380 postulated that multiple loci may counter rishitin metabolism. More studies has disclosed that rishitin metabolism in str. R-6380 is controlled by genes at two or more loci but high virulence of *G. pulicaris* on a potato is related to only one of these loci, as Rim I (Desjardins and Gardner 1991). The ability of *B. cinerea* to degrade stilbenes produced by grapes has also been discussed in detail by Pezet et al. (1992).

3.8 PhA DETECTION METHODS

Shimizu et al., have been explored a protocol for precise quantification of phytocassanes and momilactones PhA compounds in rice, using HPLC and mass spectrometry (MS) with electrospray ionization as an analytical method. These PhA are commonly analyzed by using gas chromatography (GC). The authors confirm that use of HPLC method is more benefit than GC especially when HPLC coupled with MS in tandem which does not require sophisticated methods for purification and also no required high temperatures (2008).

Nugroho et al. (2002) have been investigating the accumulation of PhA produced in tobacco plants and they have shown that an efficient analytical method in the determination of sesquiterpene PhA, Capsidiol (27) (Fig. 3.1) is the use of a GC equipped with an autosampler and flame ionization detector. The detection of these PhA was done after the extraction process using ethanol, MeOH and chloroform and liquid nitrogen for plant maceration. Sakuranetin compound (28) (Fig. 3.1) was analyzed by injecting the leaf extract of rice using HPLC (RP-18 column) eluted with benzene-ethyl acetate-formic acid (10:1:1). The sakuranetin was detected by UV at λ 285 nm (Kodama et al., 1992a,b,c). Pedras et al. (2008) reported the identification of 23 cruciferous PhA by HPLC and MS using diode array detection.

Detection of PhA in a specific plant species supports the prospective findings of elicitors of active substances in plant defense against biotic stresses. The search for potential molecules might be extracted in large scale and be used to enhance other plants producing these substances, which is an effective mechanism that can give high protection to these plants. Plant extracts contain a diversity of secondary metabolites, which act synergistically to protect the plant against pathogens and can be used as a matrix for the isolation and synthesis of selective potent molecules, facilitating enormous amounts used. The synthesis of PhA mechanism has not been established

yet and therefore the means of obtaining this is restricted to the process of isolation and determination, which requires a time, effort and lower yield, due to the lack of specific standards for such determination. The research also need to find the best elicitors for each species studied.

3.9 CONCLUSION AND SUMMARY

PhA are considered important for plant resistance toward pathogens, although the PhA of most plants are yet to be characterized. Recent studies on the PhA of some crop plants from different families have created information on basic parts of plant defenses, including how to improve control of the diseases. More importantly, efforts put in studying the regulation of PhA compound biosynthesis which led to the new description of possible signaling pathways. Nevertheless, the roles of each of these pathways under specific inducing conditions, as well as their interactions, needs to be well understood and one which requires more investigation. Moreover, the ways in which compounds act upon pathogens and the different mechanisms that some pathogens have developed to detoxify other compounds are also still poorly understood. PhA study has focused on both dicot species (e.g. Arabidopsis, peanut, and grapevine) and monocots (e.g. rice, maize, and sorghum), which has promoted our understanding of plant resistance mechanisms.

Moreover, PhA are components of the multiple mechanisms for disease resistance in plants. Studies on PhA have contributed to molecular biology and plant biochemistry. The study on the PhA should be integrated with genetical analyses in which the biochemical and physiological evidence are a key role for PhA in resistance. The study of molecular biology is a strategy that has been strongly analyzed due to the understanding of expressed genes which facilitates the elucidation of species with similar defense response characteristics that allows the extraction and elucidation of these PhA through a bank of genomic information. The use of more advanced techniques such as metabolomics and proteomics which are intended to qualify and quantify the set of metabolites induced by plant, are tools available that might be relevant to a complete process of the analysis of PhA. Research to detect the different mechanisms regulating PhA in various crop plants should have high potential in developing strategies to enhance and manipulate disease resistance in these plants. Novel approaches, such as genome-wide analyses, should start for studies of the regulatory networks controlling the metabolism of PhA and support for a better knowledge of the role of PhA in defense toward pathogens.

Prospects for further studies to evaluate the discovery of novel PhA identification mechanisms, and research that prove their activities and the application process for this mechanism to reduce production losses of important crops for human consumption. Obviously, future studies on these compounds will permit the researcher to investigate and evaluate plant-pathogen interactions and provide new techniques to control the diseases. All efforts in molecular biology and biotechnology should be geared toward the introduction of novel approaches to disease control that are environmentally friendly.

KEYWORDS

- **phytoalexins**
- **plant defense**
- **pathogens**
- **antimicrobials**
- **disease resistance**

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CHAPTER 4

BIOLOGICAL ROLES OF PHYTOCHEMICALS

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ABSTRACT

Globally, there is a growing need for the search of chemical compounds from natural sources for the treatment of human chronic diseases such as diabetes, microbial infections, cancer, and so forth. Over the years plants have become a major source of such chemical compounds known as phytochemicals with different medicinal and biological activities. The aim of this topical study is to provide a succinct knowledge on the biological roles of phytochemicals. This would serve as a veritable resource in the area of phytochemistry. There are many phytochemicals that can be found in plants. This chapter focuses on eight major phytochemicals found in most plants; tannins, saponins, alkaloids, flavonoids, glycosides, oxalates, protease inhibitors, and amylase inhibitors. Most phytochemicals are obtained from the seeds, fruits, bark, and leaves of different medicinal plants or legumes. Their different biological roles such as antioxidant, antibacterial, antitumor, antidiabetic, antiviral activities, and so forth, have been discussed.

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4.1 INTRODUCTION

Generally, phytochemicals are plant-derived chemical compounds that thrive or thwart predators, competitors, or pathogens (Kaufman et al., 2000). They are broadly categorized into primary and secondary metabolites. The secondary metabolites function in a defensory capacity against herbivores, insects, pathogens, or adverse growing conditions. They are present naturally in plants and are necessary for its conventional cellular and physiological functions (Horvath, 1981), hence they must be supplied from the diet (Ashutosh, 2008; Evans, 2009).

These phytochemicals can be obtained from different sources of plants and plant parts such as bark, leaves, fruits, seeds, and so forth. They are known to have various biological roles in man and animals. This ranges from antibacterial, antioxidant, anticancer effects, and so forth (Ashutosh, 2008).

Due to the diverse chemical properties of individual compounds, the biological effects of the various phytochemicals differ remarkably (Kaufman et al., 2000). The diverse structures and properties and their essential biological roles are discussed in this chapter.

4.2 MAJOR PHYTOCHEMICALS OF PLANT ORIGIN

The major phytochemicals mostly found in plant sources are listed below: tannins, saponins, alkaloids, glycosides, oxalates, protease inhibitors, and amylase inhibitors.

4.2.1 TANNINS

Tannins (Fig. 4.1) are complex substances that are ubiquitously distributed among plants and localized in the leaves, fruits, barks, or stems of plants. They combine with protein and precipitate them out of solution which is their main characteristic property. They are also complex with organic compounds such as amino acids, alkaloids, and some essential metals like iron and calcium to form insoluble tannates which are extremely valuable in the antidotal treatment of alkaloid, iron, and calcium poisoning. Tannins have a bitter taste which helps prevent damage to plants by insects and fungi (Ashutosh, 2008).

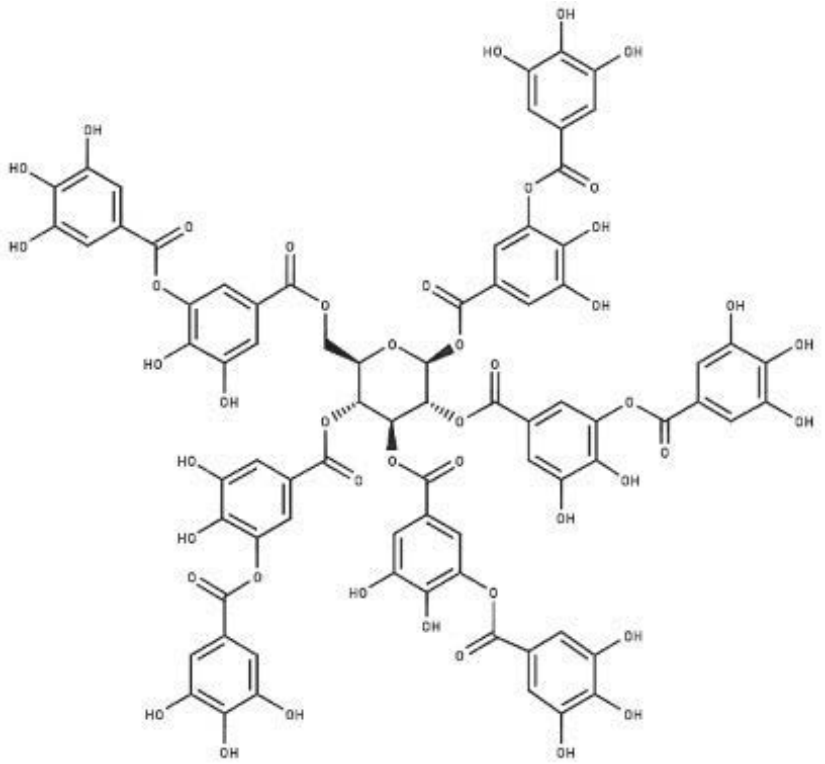

FIGURE 4.1 Structure of tannic acid.

4.2.1.1 THE CHEMISTRY OF TANNINS

Tannins are a unique group of water-soluble, polyphenol compounds with molecular weights between 500 and 30,000 Da widely distributed in plants as defined by Bate-Smith and Swain (1962). They can complex strongly with carbohydrates, proteins, and alkaloids. Some of its physical and chemical properties are:

1. Tannins appear as light yellow or white amorphous powders or shiny, nearly colorless masses.
2. Tannins are known to have characteristic bitter (astringent) taste and strange smell.
3. Tannins are freely soluble in water, glycerol, alcohol, acetone, and dilute alkalis. They are sparingly soluble in chloroform, ethyl acetate, and other organic solvents.

4. They give dark-blue color or greenish-black precipitate with iron compounds (Agrawal and Paridhavi, 2012).
5. Tannins produce a deep red color with potassium ferricyanide and ammonia and are precipitated by metallic salts of copper, lead, tin, and also by strong aqueous potassium dichromate solution (Agrawal and Paridhavi, 2012).
6. They combine with skin and hide to form leather and with gelatin and isinglass to form an insoluble compound. They combine with alkaloids to form the insoluble tannates, most of which are insoluble in water.
7. Chemically, tannins are amorphous compounds which in the presence of water cannot crystallize out hence, they form colloidal solutions. They have molecular weights ranging from 500 to over 3000 like the gallic acid esters and up to 20,000 like the proanthocyanidins (Teng et al., 2013).

Tannins can be divided either by their chemical structure or their solubility and extractability.

a) **Hydrolyzable tannins:** This can be hydrolyzed into simpler molecules upon treatment with mineral acids or enzymes such as tannase. It can further be subdivided into gallotannins which yields sugar and gallic acid on hydrolysis and ellagitannins which produces not only sugar and gallic acid but also ellagic acid. Plants like rhubarb, clove, and nutgall contain gallic acid while ellagic acid is found in eucalyptus leaves, oak bark, pomegranate bark, and myrobalans (Khanbabaee and van Ree, 2001).
b) **Condensed tannins**: Condensed tannins also called proanthocyanidins, yield anthocyanidins when depolymerized under oxidative conditions. They are, however, resistant to hydrolysis and do not contain sugar moiety (Khanbabaee and van Ree, 2001). Their building blocks include catechins and flavonoids which are esterified with gallic acid. This class of tannins differs from the hydrolyzable tannins in that on treatment of acids or enzymes, they are converted to red complex insoluble compounds called phlobaphenes, which give a characteristic red color to many drugs such as red cinchona bark that contains phlobatannins and their composition products. They are also sometimes called catechol tannins because on dry distillation, they yield catechol as the end product. Examples of plants containing condensed tannins are: cinchona bark, male fern, tea leaves, wild cherry bark, and cinnamon bark.

c) **Complex tannins:** This is a combination of either gallotannin or ellagitannin unit from the hydrolyzable tannins and the catechin unit of condensed tannins. Monomers of this class have been isolated in some plant families like Combretaceae, Fagaceae (e.g., *Quercus robur*, *Castanea sativa*) Mytarceae, Polygonaceae, and Theaceae (Ashutosh, 2008).
d) **Pseudotannins**: These are phenolic compounds of lower molecular weight than the true tannins. They differ from the hydrolyzable and condensed tannins since they do not change color during the Gold-beater's skin test and so cannot be used as tanning compounds. Some examples of pseudotannins and their sources are gallic acid found in rhubarb crystallizable catechins found in catechu, cutch; chlorogenic acid found in cocoa, coffee, nux vomica, and ipecacuanha tannins found in ipecacuanha root (Ashutosh, 2008).

4.2.1.2 SOURCES OF TANNINS

Food rich in tannins include:

1. Beverages such as red wine, tea, apple juice, beer, and so forth.
2. Chocolates and coffee powder which have the high-tannin content unlike milk and white chocolate that have less.
3. Legumes such as chickpeas, beans, black-eyed peas, and lentils that have high protein and low-fat content also contain high levels of tannins. Those with darker colors such as red or black beans are known to contain more tannin as compared with light-colored legumes like white beans (Horvath, 1981).
4. Fruits such as apples, grapes, pomegranates, and berries (blueberries, blackberries, cherries, and cranberries) have tannins concentrated in their peels.
5. Vegetables like squash, rhubarb, and herbs and spices such as cinnamon, thyme, cumin, tarragon, cloves, and vanilla (Ashutosh, 2008).

4.2.1.3 BIOLOGICAL ROLES OF TANNINS

A number of observable biological actions and pharmacological activities have been attributed to tannin-containing food and plant extract due to their astringent, hemostatic, and antiseptic properties (Ashutosh, 2008):

1. **Antimicrobial activity:** Tannins are capable of disrupting the extracellular microbial enzymes, thereby depriving the microbes the necessary substrates required for microbial growth. Tannins also act directly on the microbial metabolism through inhibition of oxidative phosphorylation (Akiyama, 2001; Scalbert, 1991).
2. **Anti-inflammatory activity:** Tannins are useful remedies for toothache, pain, and inflammation. Food rich in tannins has been helpful in controlling all indications of gastritis, esophagitis, enteritis, and irritating bowel disorders (Chamakuri, 2015).
3. **Antioxidant activity:** Both hydrolyzable and condensed tannins are potentially very important biological antioxidants as they scavenge free radicals in the body. The polyphenolic nature of tannic acid, its relatively hydrophobic "core" and hydrophilic "shell," as well as the tannin-protein complexes formed, are the features responsible for its antioxidant action (Gunnars and Lars, 2009; Parmar, 2015).
4. **Anticancer activity:** The ability of tannins to inhibit active oxygen, scavenge free radicals, and inhibit the growth of tumors has made it a useful compound in cancer therapy (Ashutosh, 2008; Parmar, 2015; Yamada, 2012).
5. **Anti-allergic activity:** Studies have shown that tannins are capable of inhibiting allergic reactions and may be useful for the treatment or prevention of allergic diseases (Gunnar and Lars, 2009; Kojima, 2000).

4.2.2 SAPONINS

Saponins are structurally complex glycosidic compounds with diverse physical, chemical, and biological functions. They are amorphous in nature with high-molecular weight and are widely recognized for their soapy lather when agitated in water, hence, the name "saponins." This ability to froth is a distinguishing feature for their identification in plant species. They have other properties that are peculiar to particular types of saponins such as their hemolytic activity, bitterness, and cholesterol-binding properties.

The hydrolysis of saponin yields an aglycone (sapogenin) and aglycone (sugar). Saponins are classified based on the nature of their aglycone, which includes those bearing a steroidal aglycone and those bearing a triterpenoid aglycone. Steroidal saponins are present in vast amounts in nature especially among plants such as tomato, yam, pepper, oat, allium, and so forth (Oakenfull, 1981). Triterpenoid saponins are present in *Solanum* spp., potato, peppers,

and tomatoes and have been reported to have toxic effects. The structural and unique diversity of saponins is reflected in their diverse biological and physicochemical properties which have been harnessed for a number of traditional applications such as the production of soaps, molluscicides, and fish poison. There had been limited application of saponins in food due to its bitter taste which erroneously leads to its removal by early researchers in a bid to facilitate human consumption. However, recent research highlighted the increasing health benefits of saponins in lowering cholesterol as well as its anticancer properties. Also, the health benefits of foods such as soybeans and garlic have been attributed to their saponin content (Guclu-Ustundag and Mazza, 2007).

4.2.2.1 CHEMISTRY OF SAPONINS

Saponins (Figs 4.2 and 4.3) are glycosidic compounds that contain one or more sugar chains (pentoses, hexoses, or uronic acid) on either a triterpenoid or a steroidal aglycone backbone (He et al., 2010). The aglycone nature, the functional group on its backbone, and the number and nature of its sugar varies greatly; hence, it is widely diverse (Guclu-Ustundag and Mazza, 2007).

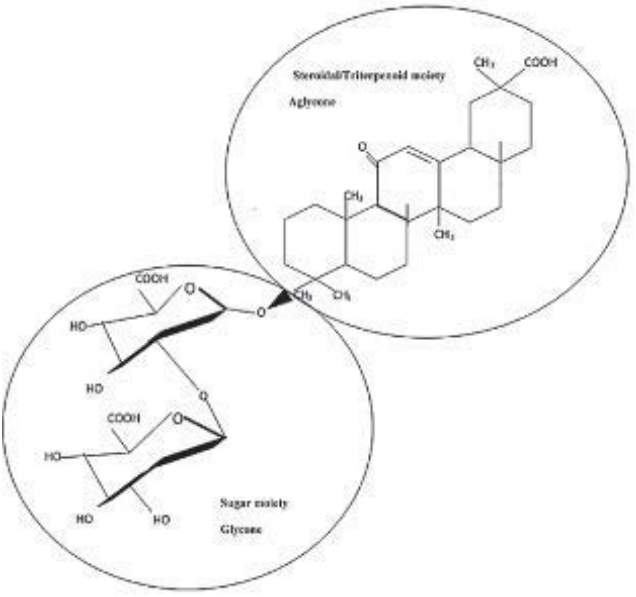

FIGURE 4.2 General structure of saponins showing its steroidal and sugar moieties.

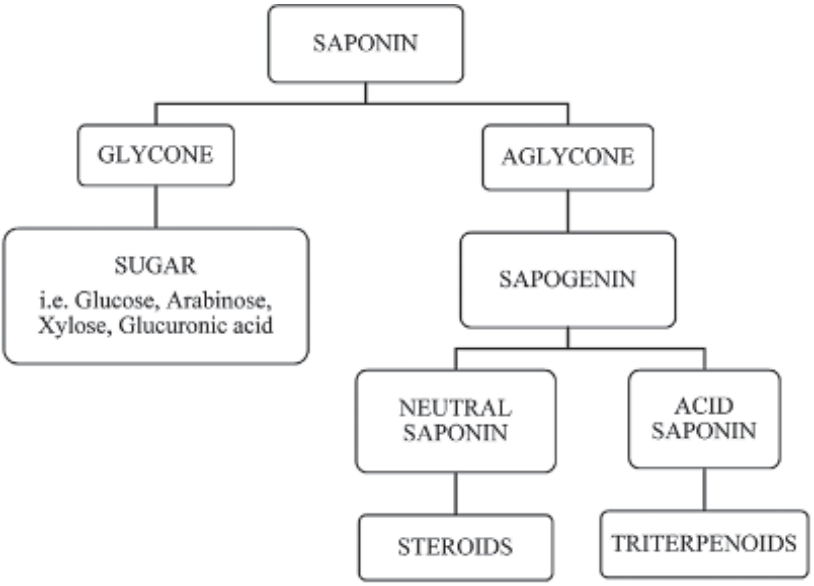

FIGURE 4.3 Diagrammatic representation of saponins.

The physical and chemical properties of saponins are as follows:

1. Saponins are amphiphilic in nature due to the presence of a lipid-soluble aglycone and a water-soluble sugar chain in their structure. Their amphiphilic attribute is responsible for its diverse physical properties such as its ability to act as surface active agents/surfactants, emulsifying agents, and form stable foams (He et al., 2010).
2. They also have a strong hemolytic activity and can form micelles just like detergents. This diversity of physical properties has been exploited technologically in the manufacturing of carbonated drinks and shampoo (Negi et al., 2013).
3. The physicochemical and biological properties of saponins are modified by their interaction with sterols, proteins, and minerals. The interaction of sterols (cholesterol and phytosterols) with steroid (saponins, alfalfa saponins and triterpenoid saponins) form water-insoluble additional products (He et al., 2010).
4. Saponins undergo chemical transformations during storage and processing, which has a significant effect on their pharmacological properties. Heating in the presence of water, acids/alkali, enzymes, and microbial activity causes hydrolysis of the glycosidic (bond

between the sugar chain and aglycone) and interglycosidic bonds (bond between the sugar residues) leading to the formation of aglycones, sugar residues, monosaccharides, and prosapogenins (Negi et al., 2013). Acid hydrolysis yields aglycone and monosaccharides while alkaline hydrolysis yields prosapogenins (Guclu-Ustundag and Mazza, 2007).

4.2.2.2 SOURCES OF SAPONINS

Foods rich in saponins include the following (Oakenfull, 1981):

- Legumes and beans such as kidney beans, chickpeas, soybeans, navy beans, and so forth, are very rich in saponins. Soybeans are actually one of the major sources of dietary saponins and can be prepared in various ways. Another notable source of dietary saponins is chickpeas.
- The rhizome and root of liquorice (*Glycyrrhiza glabra*) are important sources of saponin and thus used as a condiment/flavoring agent in cooking. They are also used in the tobacco industry.
- Oats, quinoa, and amaranth are rich sources of saponins.
- Garlic and vegetables such as alfalfa sprouts, peas, yucca, and asparagus are rich sources of saponin.
- Red wine is also a rich source of saponin, although the type of wine determines the quantitative content of the saponin. The saponin content of wine is derived from the coating on the skin of the grapes that are used in wine production.
- The bark of *Quillaja saponaria* (soap tree) are rich sources of saponin (about 10%) and are used as a medium for extracting saponin for commercial purposes. The saponin from *Quillaja* is used as foaming agent in the food industry.
- Animals like sea cucumber (marine invertebrate) also contain saponins; however, the amount of saponin depends on the type of the animal. These animals can be either be eaten raw, for example, sushi or cooked in different ways (Sparg et al., 2004).

4.2.2.3 BIOLOGICAL ROLES OF SAPONINS

Saponins possess an extremely diverse range of biological activities ranging from its role as an adjuvant, anti-inflammatory agent, antioxidant,

antiparasitic, antispasmodic, antiphlogistic, antipsoriatic, antiprotozoal, antipyretic, antimutagenic, anti-allergic, anti-edematous to its effect on absorption of minerals and vitamins, cognitive behavior, animal growth, and reproduction (Das et al., 2012; Egbuna and Ifemeje, 2015)

a) **Hemolytic activity:** Saponins are capable of lysing erythrocytes. They do this by forming pores on the cell membrane thereby increasing its permeability and subsequent bursting. This has been attributed to the affinity between the aglycone moiety (in the saponin) and the phospholipids on the cell membrane which leads to the formation of insoluble complexes. The deleterious effect of saponin on the lipid bilayers is irreversible as saponin-lysed erythrocytes are incapable of resealing. Escin saponins present in *Aesculus hippocastanum* L. (Hippocastanaceae) and jujuboside saponins from *Zizyphus jujuba* Mill. (Rhamnaceae) have strong hemolytic activity.
b) **Immunological adjuvant:** Saponins improve the effectiveness of vaccines administered orally by enhancing the immune response to antigens. They also enhance assimilation of large molecules and have immunostimulatory effects. *Q. saponaria* is mainly used for the industrial production of saponin adjuvants (Das et al., 2012).
c) **Cholesterol-lowering effect:** The cholesterol-lowering effect of saponin has been observed in human and animal studies. Animals fed with saponin-rich diets (soybean, chickpea, alfalfa, etc.) had lower plasma and liver cholesterol levels. This attribute is based on the ability of saponin to inhibit the absorption of cholesterol from the small intestine and the reabsorption of bile acids (Guclu-Ustundag and Mazza, 2007).
d) **Anticancer effect:** Triterpene and steroidal saponins have been reported to have anticancer activities. The National Cancer Institute has identified protoneodioscin, protodioscin (furastanol saponins isolated from the rhizomes *of Dioscorea collettii*) methyl protoneogracillin, and methyl protogracillin (steroidal saponins extracted from the rhizomes of *D. collettii*), as potential anticancer agents (Thakur et al., 2011).
e) **Antimicrobial effects:** Oleanolic acid (the most common triterpene saponin aglycone) has been reported to possess antiviral and antibacterial activities. When surgical site infections were stimulated in a wound model using BALB/c mice, Diosgenyl 2-amino-2-deoxy-β-D-glucopyranoside was reported to be effective against

Enterococcus faecalis and *Staphylococcus aureus.* Saponins from *Q. saponaria* have been reported to have antiviral activity against the rhesus rotavirus in BALB/c mice. CAY-1 (a steroidal saponin) extracted from the fruit of *Capsicum frutescens* L. (Solanaceae) was observed to be a potent antifungal agent as well as having anti-yeast properties (Das et al., 2012).

f) **Tumor-suppressive effects:** Saponin has been observed to have anti-tumor suppressive effects. They are capable of enhancing the specificity of target cells such as the reported observation in increasing the toxicity and synergism of specific ribosome-inactivating proteins at sub-micellar levels (Das et al., 2012).

4.2.3 ALKALOIDS

Alkaloids are an enormously large and diverse family of naturally occurring nitrogen-containing compounds. They are secondary metabolites produced by a wide variety of plants (i.e., potato, tomato, etc.), animals (i.e., shellfish), and even microorganisms such as mushroom and bacteria. They have diverse pharmacological applications and can be used as recreational drugs, medications (i.e., quinine, the antimalarial drug), as well as an analgesic. Some alkaloids are bitter, that is, caffeine and can be purified from crude extracts by simple acid–base extractions (Edeoga and Eriata, 2001; Babbar, 2015).

Alkaloids can be broadly classified according to four different schemes namely:

1. **Taxonomy:** Alkaloids are classified exclusively based on taxa such as family, genus, specie, subspecie. Examples of plant families (i.e., natural order) and their associated species are:
   - Solanaceous alkaloids, for example, *Atropa belladonna*, *Mandragora officinarum*, *Capsicum annuum* L., *Solanum dulcamara,* and so forth.
   - Cannabinaceous alkaloids, for example, *Cannabis sativa*.
   - Rubiaceous alkaloids, for example, *Mitragyna speciosa*, *Cinchona* sp.
   - Based on genus, we have *Ephedra*, *Cinchona*, coca, and so forth.
2. **Pharmacology**: Alkaloids are classified based on their pharmacological activity as they exhibit a wide range of pharmacological

characteristics such as analgesics (morphine), antimalarial (quinine), oxytocic (ergonovine), neuaralgia (aconitine), choleretics, and laxatives (boldine), reflex excitability (lobeline), antiglaucoma agent (pilocarpine), bronchodilator (ephedrine), and so forth (Babbar, 2015). The alkaloids in this group do not have any chemical or structural similarity.

3. **Biosynthesis:** Here, alkaloids are classified based on the precursors utilized by plants to synthesize the compound regardless of their pharmacological characteristics and taxonomic distribution. Examples include:
   - Indole alkaloids from tryptophan.
   - Imidazole alkaloids from histidine.
   - Piperidine alkaloids from lysine.
   - Phenylethylamine alkaloids from tyrosine.
   - Pyrrolidine alkaloids from ornithine.
4. **Chemical classification:** Alkaloids are classified into two major categories based on their basic ring structure, that is, the presence or absence of a heterocyclic nucleus.

4.2.3.1 NON-HETEROCYCLIC ALKALOIDS

As the name implies, they lack a heterocyclic ring in their nucleus. It includes:

- Phenylethylamine: The alkaloids contain phenylethlamine as their basic ring structure (Fig. 4.4). Examples are narceine (*Papaver somniferum*), ephedrine (*Ephedra vulgaris*), mescaline (*Laphophora williamsii*), hordenine (*Hordeum vulgare*), capsaicin (*Capsicum annunum*), and so forth.

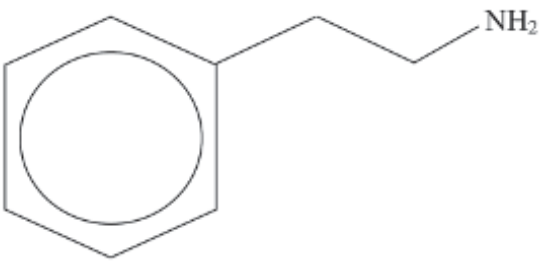

FIGURE 4.4 Phenylethylamine.

4.2.3.2 HETEROCYCLIC ALKALOIDS

Heterocyclic alkaloids (Fig. 4.5) contain a heterocyclic ring in their nucleus (Babbar, 2015). They include:

- Pyrrolidine: They contain pyrrolidine as their basic ring structure. Examples are hygrine (*Erythroxylon coca*), stachydrine (*Stachys tuberifera*).
- Pyridine: They contain pyridine as their basic ring structure. Examples are arecoline (*Areca catechu*), ricinine (*Ricinus communis*).
- Piperidine: They contain piperidine as their basic ring structure. Examples are coniine (*Conium maculatum*), lobeline (*Lobelia inflata*).
- Tropane (piperidine–pyrrolidine): Tropane is their basic ring structure. Examples are atropine (*Atropa belladonna*), cocaine (*E. coca*) (Griffin and Lin, 2000).
- Quinoline: Here, quinoline is the basic ring structure. Examples are quinine and quinidine (*Cinchona officinalis*), cuspareine (*Cusparia trifoliata*).
- Isoquinoline: The basic ring structure is isoquinoline. Examples are berberine (*Hydrastis canadensis*), Papaverine (*P. somniferum*).
- Aporphine (reduced isoquinoline): Here, the alkaloids contain a reduced isoquinoline ring in their nucleus. Example is boldine (*Peumus boldus*).
- Norlupinane: They contain norlupinane as their basic ring structure. They are present in leguminosae plants. Examples are sparteine (*Lupinus luteus)*, Lupinine (*Anabasis aphylla*).
- Indole (benzopyrrole): They contain an indole ring in their nucleus. Examples are ergotamine (*Claviceps purpurea*), reserpine (*Rauvolfia serpentina*).
- Imidazole: They contain an imidazole basic ring structure. Examples are pilocarpine (*Pilocarpus jaborandi*).
- Purine: These contain a purine ring in their nucleus. Examples are caffeine (*Thea sinensis, Coffea arabica, Theobroma cacao*) (Zrenner et al., 2006).
- Tropolone: They contain a tropolone ring as their basic structure. Examples are colchicine (*Colchicum autumnale*).
- Steroid: They contain a steroidal ring in their nucleus. Examples are funtumine (*Funtumia latifolia*), solanidine (*Solanum sp.*).
- Pyrrolizidine: Alkaloids in this group contain a pyrrolizidine ring as their basic structure. Examples are Senecionine (*Senecio vulgaris*) (Mattock, 1986).

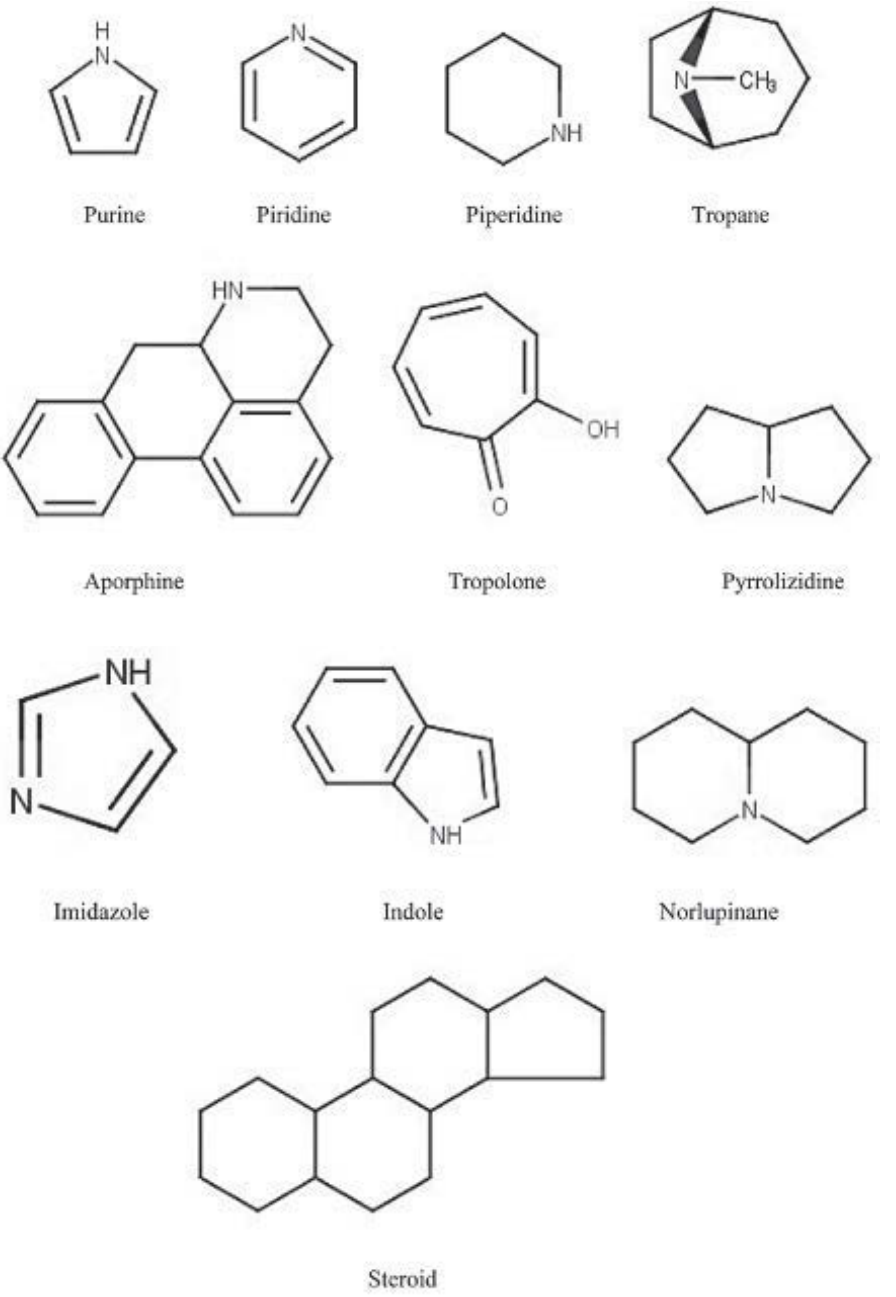

FIGURE 4.5 Basic structures of some alkaloids.

4.2.3.3 CHEMISTRY OF ALKALOIDS

The physical and chemical properties of phytochemicals are as follows:

Physical properties

- Alkaloids are solid crystalline compounds except for coniine and nicotine which are liquid. Emetine is amorphous.
- They are colorless with the exception of berberine and betaine, which are yellow and red, respectively.
- The presence of a nitrogen atom makes them basic which depends on the availability of free electrons, as well as the type of hybridization and aromaticity.
- They are insoluble in water except for liquid alkaloids like nicotine that are soluble in water.

Chemical properties

- They are present in plants in diverse forms such as esters, quaternary compounds, salts, N-oxide, and so forth.
- They are unstable when exposed to heat or light.
- They have a characteristic bitter taste.
- They are soluble in an aqueous solution of sodium carbonate or ammonia on treatment with alcohol, thus forming an ester.

4.2.3.4 SOURCES OF ALKALOIDS

Alkaloids are found in the tissues (i.e., stems, leaves, roots, seeds, and barks) of a vast variety of higher plants such as Apocynaceae (the richest source by a large margin), Papaveraceae (a particularly rich source though less than the Apocynaceae), Rubiaceae, Asteraceae, Rutaceae, Boraginaceae, Berberidaceae, Ranunculaceae, Fabaceae, Rutaceae, and Solanaceae. Foods such as tea, coffee, cocoa, honey, as well as nightshade fruits like potatoes, eggplant, honey, hot peppers are sources of alkaloids (Ranjitha and Sudha, 2015).

4.2.3.5 BIOLOGICAL ROLES OF SOME SELECTED ALKALOIDS

Alkaloids have diverse pharmacological and biochemical properties. For instance; ajmaline is reported to be antiarrhythmic. Codeine is used as cough medicine and an analgesic. Colchicine is used as a remedy for gout, ergot alkaloids serve as vasodilator and antihypertensive drugs. Quinidine is antiarrhythmic, quinine antipyretics is useful as an antimalarial agent. Reserpine is also antihypertensive. Tubocurarine serves as a muscle relaxant. Vinblastine and vincristine are antitumor agents. Yohimbine is a stimulant and an aphrodisiac (Hesse, 2000; Babbar, 2015).

Atropine

Biological function

- Ophthalmic functions: The application of atropine topically aids in dilating the pupils by temporarily paralyzing the accommodation reflex. Hence, it serves as a therapeutic mydriatic.
- Resuscitation: Atropine injections are useful in treating extremely low heart rate conditions as well as pulse-less electrical activity in patient under cardiac arrest.
- Secretions and bronchoconstriction: The action of atropine on the parasympathetic nervous system prevents the formation of secretions such as saliva, sweat, mucus, and so forth. This is particularly useful in treating hyperhidrosis.
- Treatment of chemical poisoning: Due to its ability to block acetylcholine receptors, atropine is given as a prophylactic for the salivation, lacrimation, urination, diaphoresis, gastrointestinal motility, emesis symptoms that occur as a result of poisoning by organophosphate-containing insecticide.

Cocaine

Biological functions

- It is a potent central nervous system stimulant and an appetite suppressant.
- It is applied topically as anesthetic in ophthalmology as it is quickly absorbed from the mucous membrane.

Purine

Biological functions

- Paraxanthine, a caffeine metabolite helps in elevation of plasma levels of glycerol and free-fatty acids.
- Theophylline, another metabolite of caffeine is used to treat asthmatic patients as it relaxes the smooth muscles of the bronchi.
- Caffeine consumption increases vigor and mental alertness while reducing the sensation of tiredness.
- Theobromine, a caffeine metabolite facilitates dilation of the blood vessels.
- Caffeine has been shown to increase one's capacity for mental and physical labor.
- Caffeine citrate is reported to be invaluable in treating breathing disorders and bronchopulmonary dysplasia in premature babies (Zrenner et al., 2006)

Pyrrolizidine

Biological functions

- They are used as a cough suppressant.
- They can be used in treating obstructive lung diseases such as asthma, bronchitis, and emphysema.
- Pyrrolizine alkaloids have been observed to have a significant antimicrobial activity such as its capacity to inhibit the growth of *Escherichia coli, S. aureus, Bacillus subtilis, Bacillus anthracis.*
- Saturated pyrrolizidine alkaloid (PA) has spasmolytic, antihistaminic, anti-HIV, antiviral activities, as well as acting as an inhibitor of glucosidase (Mattocks, 1986).

4.2.4 GLYCOSIDES

Glycosides are chemical organic compounds that on hydrolysis by mineral acids or enzymes yield sugars. They are usually of plant origin and composed of sugar portion linked to a non-sugar portion via a glycosidic bond. The sugar portion is known as the glycone while the non-sugar moiety is referred to as the aglycone or genin of the glycoside. The aglycone portion may either be a flavonoid, saponin, terpene, coumarin, or any other natural product.

Glycosides are stored in the vacuole, protected from hydrolytic enzymes of the cytoplasm. They exist as solid bitter compounds and belong to different secondary metabolites.

4.2.4.1 CHEMISTRY OF GLYCOSIDES AND SOURCES

The general chemical properties of glycosides are:

- Glycosides are colorless, crystalline amorphous, nonvolatile chemical compounds (except flavonoid which gives yellow color and anthraquinone with red color)
- Except for populin, glycyrrhizin, and stevioside, others exist as bitter compounds.
- They are insoluble in organic solvents but soluble in water.
- They can easily be hydrolyzed by mineral acids, water, or enzymes except for C-glycosides that require ferric chloride for hydrolysis.
- On hydrolysis, glycosides give positive test to Molisch's test and Fehling's solution.
- In their pure state, they are optically active (levorotatory) in nature.
- The aglycone portion is responsible for the biological or pharmacological activity of the glycosides.

Glycosides are classified on the basis of the type of aglycone linked to them and the type of glycosidic bond involved in the bond formation.

Table 4.1 shows classification of glycosides on the basis of the chemical structure of the aglycone or pharmacological activity and their sources:

TABLE 4.1 Classification of Glycosides and Various Sources.

Aglycone portion	Sources
Anthraquinone glycosides	Aloe, buckthorn, senna, rhubarb
Anthocyanosides	Blueberry, currant
Cardiac glycosides	Digitalis, strophanthus, lily of the valley, squill
Coumarin glycosides	Bergamot, sweet woodruff
Cyanogenic glycosides	Almond, cherry, apricot
Flavonoid glycosides	Hyssop, liquorice
Hydroquinone glycosides	Arbutus
Saponin glycosides or saponosides	Liquorice, horse chestnut
Iridoid glycosides	Plantain, devil's claw
Salicylic glycosides	White willow, poplar

Based on the nature of the glycone; if it is a glucose, then the molecule is a glucoside; if it is fructose, then the molecule is a fructoside. Hence, the terminal -e- of the name of the corresponding cyclic form of the monosaccharide is replaced by -ide-. Also, if it is glucuronic acid, then the molecule is a glucuronide.

Chemically, the sugar moiety of a glycoside can be joined to the aglycone via a nucleophilic atom such as (Fig. 4.6):

- Oxygen atom (O-glycosides)
- Carbon atom (C-glycosides)
- Nitrogen atom (N-glycosides)
- Sulfur atom (S-glycosides)

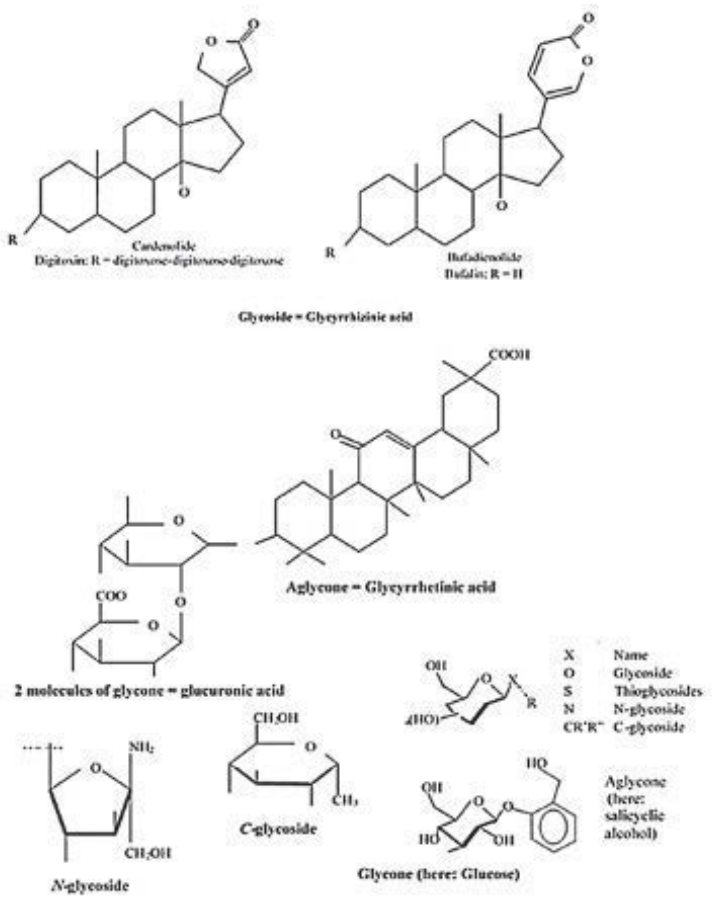

FIGURE 4.6 Chemical nature of glycosides.

4.2.4.2 BIOLOGICAL ROLES OF GLYCOSIDES

- Glycosides-containing plants defend themselves against herbivores becoming less attractive or palatable.
- Anthracene glycosides are used as cathartics and exert their action by increasing the tone of the smooth muscle in the wall of the colon and stimulate the gastric secretion of water and electrolytes into the large intestine.
- Cardiac glycosides, for example, digitoxin from *Digitalis purpurea* are known to exert powerful effect on the heart muscle and heart rhythm leading to myocardial contraction.
- Many glycosides are extremely active compounds but toxic at the same time (e.g., digitoxin)
- Saponin glycosides are employed in the making of soaps and detergents because of their foaming ability. Their hemotoxic nature and hemolytic ability makes it possible for them to be employed as fish poisons.
- The flavonoids glycosides are known for their antioxidant effect hence find therapeutic applications as anti-cancer, antiasthmatic, antispasmodic, antimicrobial, fungicidal, and estrogenic activities. They also have the ability to decrease capillary fragility.

4.2.5 FLAVONOIDS

Flavonoids are secondary metabolites present virtually in all plants. They are the most important color pigment for the leaves, fruits, and flowers of plants designed to attract pollinator animals. Examples of flavonoids are the yellow color of chalcones, purple color of anthocyanins, and so forth. When not visible, they act as copigments, for example, colorless flavones. They are hydroxylated polyphenolic compounds with a 2-phenylbenzopyranone ring as the core structure and are synthesized through the phenylpropanoid pathway (Corradini et al., 2011).

Foods and vegetables, as well as wine and tea, are among the major dietary sources of flavonoids. However, they have a relatively low bioavailability as they are not easily absorbed from the digestinal tract and thus are quickly eliminated from the body (Kumar and Pandey, 2013). The main flavonoids in plants are β-carotene, rutin, catechin, α-carotene, lycopene, capsanthin, cryptoxanthin, anthocyanins, quercetin, hesperidin, and resveratrol (Tapas et al., 2008).

Flavonoids have versatile physiological and biochemical functions in all forms of life. In higher plants, they serve as combatants of oxidative stress, growth regulators, and inhibitors of the cell cycle as well as chemical messengers amongst others. In humans, they can scavenge free radicals and chelate metal ions due to the presence of functional OH groups (i.e., anti-oxidation activities). They play roles in protecting the body against cardio-vascular diseases, cancer, as well as other age-related diseases. They are anti-inflammatory, hepatoprotective, and have antiviral attributes (Ghasemzadeh and Ghasemzadeh, 2011, Øyvind and Markham, 2006; Ververidis et al., 2007).

4.2.5.1 CHEMISTRY OF FLAVONOIDS

The general structure of flavonoids is based on a 15-carbon skeleton made up of two benzene rings connected by a heterocyclic pyrane ring (Fig. 4.7). Flavonoids can exist both as glycosidic conjugates and free aglycones (the basic structure). The former increases polarity of the flavonoid as well as decreasing its reactivity, which is vital for preventing cellular cytoplasmic damage (Kumar and Pandey, 2013). C-linkage bond between aglycone and sugar is quite strong; therefore, a mixture of concentrated HCl and acetic acid (Killian's agent) is used to carry out the hydrolysis of C-glycosides (Corradini et al., 2011).

There are eight major classes of flavonoids based on the degree of unsaturation and oxidation of the three-carbon segment (Kumar and Pandey, 2013). They are:

- Flavones, for example, luteolin, apigenin, tangeritin, and so forth.
- Isoflavones, for example, genistein, daidzein, glycitein.
- Anthocyanidins, for example, cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin.
- Flavanones, for example, hesteretin, naringenin, eriodictyol.
- Flavanols, for example, catechins and epicatechins.
- Flavonols, for example, quercetin, kaempferol, myricetin, isorhamnetin, pachypodol.
- Chalcones, for example, butein.
- Aurones, for example, isoliquiritigenin and hispidol.

Flavonoids are crystalline in nature. Flavones, chalcones, aurones are bright yellow while catechins, flavanones form colorless crystals. Generally,

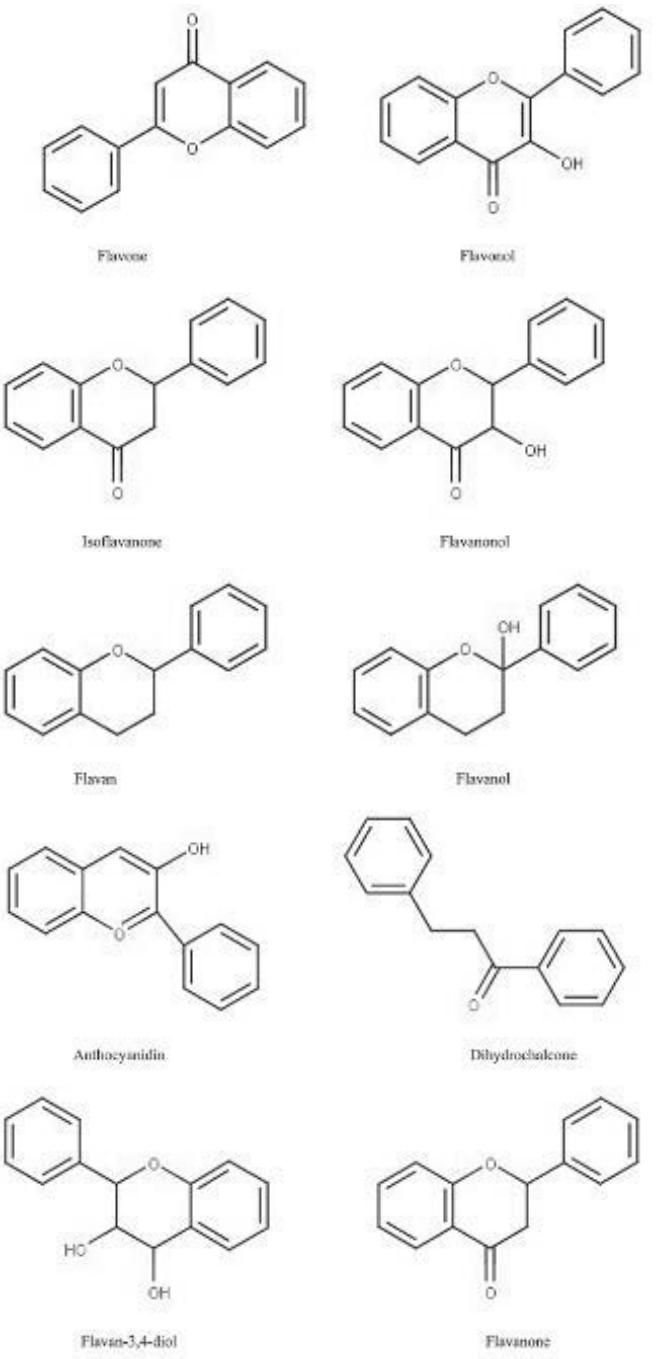

FIGURE 4.7 Flavonoid subgroups

glycosides are soluble in water and alcohol; however, flavonoid glycosides are soluble in hot water and diluted alcohols. Aglycones are soluble in alkaline hydroxide solutions and apolar organic solvent due to the presence of a free phenolic group; however, flavonoid aglycones are soluble in diethyl ether, alcohols, acetone, but practically insoluble in water (Kumar and Pandey, 2013).

The color of sap pigments (which are anthocyanins) is determined by the pH of the sap, that is, in acid medium, they turn red while in alkaline medium, they turn blue. Also the red color of roses and blue color of cornflower is as a result of the presence of the same glycosides (Corradini et al., 2011).

Catechins are optically active. When flavononones and flavanones are treated with oxidants, they yield leucocyanidins and chalcones, respectively. Hence, they are very unstable compounds.

4.2.5.2 SOURCES OF FLAVONOIDS

All fruits and vegetables contain one or more flavonoids. Common food sources for anthocyanidins are red, blue, and purple berries and grapes as well as red wine; for flavanols, teas, chocolate, grapes, berries, apples, red grapes, red wine, and so forth are the major dietary sources. Citrus fruits (oranges, grapefruits, lemons) are the major sources of flavanones. Onions, scallions, broccoli, teas, apples, berries, and kales are the major food sources. For flavones, parsley, thyme, hot peppers, and celery are the sources while soybeans, legumes, and soy foods are the major sources (Tapas et al., 2008; Kumar and Pandey, 2013).

4.2.5.3 BIOLOGICAL ROLES OF FLAVONOIDS

Flavonoids are known for their prominent antioxidant functions as they help in preventing coronary heart diseases as well as cancer. They also have antimicrobial, antidiabetic, antihistamine, mood boosting, as well as memory-enhancing properties (Agrawal, 2011; Friedman, 2007; Tapas et al., 2008). Some specific biological roles of flavonoids are highlighted below:

- **Effect on cholesterol:** Flavonoid-containing chocolate/cocoa helps in reducing blood pressure. They also have significant positive effects on low-density lipoprotein and high-density lipoprotein cholesterol

levels in the body (Ghasemzadeh and Ghasemzadeh, 2011; van Dam et al., 2013).

- **Anticancer agents:** Flavonoid-containing celery, artichokes have cytotoxic effects on cancer cells. However, they should not be administered with the chemotherapeutic drug as it may be counterproductive but instead should be given as pretreatment.
- **Effect on menopause**: Isoflavones in soybean reduces the sensation of hot flash that is so common among perimenopausal and postmenopausal women. It also helps in increasing the bone mineral density thereby reducing the risk of bone fracture as well as postmenopausal osteoporosis.
- **Therapeutic effect on rheumatoid arthritis**: The antioxidation activity of grape seed proanthocyanidin extract scavenges the hydrogen peroxide product of oxidative stress thereby reducing the destruction of collagens by the hydrogen peroxide. This aids in the treatment of collagen-induced rheumatoid arthritis.
- **Prevention of gum disease:** Proanthocyanidins in cranberry has anti-inflammatory activity thereby preventing the development of gum disease. It can also be used to cure the disease.
- **Improved cognitive functions**: Anthocyanidins in blueberries and strawberries improves cognitive functions of elderly people as it effectively slows down the rate of cognitive decline. It also improves body movements and slows down irritability, fatigue, and depression (Spencer, 2008).

4.2.6 OXALATES

Oxalates (ethanedioate) is a dianion salt with a molecular formula $C_2O_4^{2-}$ (Fig. 4.8). It is a salt of oxalic acid a strong dicarboxylic acid found in many plants; oxalate is produced in the body by metabolism of glyoxylic acid (from glycine) or ascorbic acid; it is not metabolized but excreted in the urine.

Oxalate is a bidentate ligand (having two teeth) and can form complexes with metal ions and some nutrients. A high intake of oxalate can sometimes cause a deficiency, since calcium oxalate is insoluble. Some disease states are associated with oxalate metabolism, for example, urolithiasis, nephrolithiasis; hyperoxaluria is a primary risk factor of urolithiasis because virtually all oxalate consumed is excreted via urine.

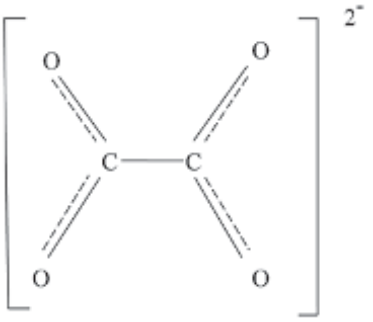

FIGURE 4.8 Structure of the oxalate anion.

4.2.6.1 CHEMISTRY OF OXALATES

Physical properties:

- Oxalate is an odorless white solid with a sour taste and a density of 1.9 g/cm^3.
- Oxalate has a melting point temperature of 189.5°C.
- Oxalate is soluble in ethanol but slightly soluble in ether and insoluble in benzene, chloroform, and petroleum ether.

Chemical properties:

- Oxalate is a strong reducing agent.
- Oxalic acid can bind with minerals as iron and calcium to form iron oxalate and calcium oxalate, respectively.
- Oxalate forms acid chloride (oxalyl chloride).
- Oxalate is a conjugate base of oxalic acid and a ligand (bidentate) for metal ions.
- Oxalate undergoes autocatalytic reaction in which it is oxidized by permanganate.

4.2.6.2 SOURCES OF OXALATES

Oxalate is found in many food substances; oxalate is also present in the body as metabolites of glycine and ascorbic acid metabolism.

Foods rich in oxalates includes nuts, cranberries, coffee, chocolate, spinach, berries, oranges, rhubarb, sesame seeds, millet, soybeans, black tea, wheat bran, and beans.

4.2.6.3 BIOLOGICAL ROLES OF OXALATES

- Oxalate (calcium oxalate) regulates calcium (Ca) and aids protection against herbivores.
- Oxalates help to confer tolerance to heavy metals on plants.
- Oxalate (oxalic acid) is required in the formation of orotic acid and uracil: a component of ribonucleic acid.
- Oxalate helps decrease the risk of cancer: oxalate is a competitive inhibitor of lactate dehydrogenase (an enzyme that catalyzes the conversion of pyruvate to lactic acid). Cancer cells use anaerobic metabolism hence, inhibition of lactate dehydrogenase leads to the inhibition of tumor formation and growth.

4.2.7 PROTEASE INHIBITORS

Proteases are important enzymes that aid the regulation of cellular processes, they are ubiquitous and present in all cells and tissues. Upon lysing of the cell, cell proteases are released into the lysate. Proteases catalyze the hydrolysis of peptide bonds. Release of these enzymes in large quantities may impede biochemical analysis and may be potentially destructive to cells and tissues, hence the need for its regulation by the body. Protease inhibitors are a group of low-molecular-weight protein present in cells and tissues that regulate the effects of proteases; these enzymes may act endogenously or exogenously as defense mechanisms against proteolytic degradation and regulation of many biological processes (Vijaya and Gandreddi, 2014). They do this by forming inactive complexes or complexes with lesser activity when they bind to cognate enzymes; the mode of this inhibition may be direct blockage of the active site, indirect blockage of the active site, adjacent or exosite binding, and allosteric inhibition (Huma and Khalid, 2007). Protease inhibitors occur widely in nature and are found in plants, animals, and microbes where they regulate biological processes; some other functions other than regulation of proteases have been observed for protease inhibitors, functions as regulation of growth activities, receptor clearance signaling, regulate inflammation, extracellular matrix synthesis, and tissue repair (Huma and Khalid, 2007).

4.2.7.1 CHEMISTRY OF PROTEASE INHIBITORS

There has been an initial classification of protease inhibitors and this classification was based on the protease inhibited but more recently classification is based on the similarities of primary amino acid sequence and based on the nature of binding of the inhibitor to its enzyme. The classification given below is based on primary amino acid sequence (Aberoumand, 2012; Meenu and Murugan, 2016; Vijaya and Gandreddi, 2014).

1. Serine protease inhibitors (Serfins) pathogenesis-related proteins
    - Bowman–Birk (trypsin/chymotrypsin)
    - Kunitz (trypsin; others)
    - Potato I (trypsin; chymotrypsin)
    - Potato II (chymotrypsin; trypsin)
    - Curcubit (trypsin)
    - Cereal superfamily (amylase, trypsin)
    - Ragi I-2 family (amylase, protease)
    - Maize 22 kDa/thaumatin/Pathogenesis related proteins (amylase, trypsin)
2. Cysteine protease inhibitor (cystatins and stefin)
    - Cystatin superfamily
    - Cystatin family
    - Stefin family
    - Fitocystatin family
3. Metalloprotease inhibitor
    - Carboxypeptidase

4.2.7.2 SOURCES OF PROTEASE INHIBITORS

Protease inhibitors are found occurring naturally in fungi, bacteria, viruses, Kingdom Animalia and Kingdom Plantae. It is found in a wide variety of edible plant (Vijaya and Gandreddi, 2014).

Soybeans and other legumes: These are abundant source of protease inhibitors and this is reported to be the reason for its anticancer activity. Two types of protease inhibitors found in soy are Kunitz trypsin inhibitor which is present in large concentration and Bowman–Birk inhibitor.

Potatoes: Protease inhibitors are present as small proteins in the tubers and stem of potato where they are induced in response to attack or injury caused by pathogenic microorganisms or insects. Protease inhibitors suppress tumor growth in clinical setting.

Green tea: This contains epigallocatechin gallate a catechin that possesses antioxidant properties and inhibits proteases. It also contains vitamins, minerals, theanine, and saponins.

Blue-green algae: The blue-green algae are rich in nutrients and supplements. It is also a rich source of protease inhibitors.

4.2.7.3 BIOLOGICAL ROLES OF PROTEASE INHIBITORS

Protease inhibitors have been shown to elicit antimicrobial, antiviral, antineoplastic, insecticidal, and defense mechanism and anti-inflammatory properties (Hiestra, 2002; Whitaker, 1997).

a) **Antiviral property:** Viruses replicate by proteolytic cleavage of protein precursors essential for the production of viral particles; protease inhibitors bind to viral proteases thereby preventing the proteolytic cleavage of protein precursors necessary for the production of infectious viral particles.
b) **Antimicrobial property:** Certain phytopathogenic fungi are known to produce proteases extracellularly; these proteases play an important role in the pathogenesis of diseases. Plant protease inhibitors suppress these enzymatic activities in response to attack by proteases produced by the fungi.
c) **Insecticidal property and defense mechanism:** It has been observed that protease inhibitors are capable of inhibiting the growth of insect larvae; wounded plants produce protease inhibitors; hence, the presence of these enzymes prevents absorption of proteins and disrupts the activities of some essential proteases in the insect thus discouraging the insect from consuming them.
d) **Antineoplastic agent:** Protease inhibitors affect the expression of certain oncogenes, hence suppression of carcinogenesis.
c) **Anti-inflammatory property:** Protease inhibitors regulate the proteolytic activity of proteases implicated in inflammation and it also modulates signal transduction, cytokine expression, and tissue remodeling thus preventing inflammation (Hiestra, 2002).

4.2.8 AMYLASE INHIBITORS

Amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) catalyze the hydrolysis of the α-1,4 glycosidic linkages in oligosaccharides, that is, starch. Amylase inhibitors are proteinaceous molecules that delay the breakdown of these oligosaccharides. They are often referred to as "starch blockers." The necessity of delaying the breakdown is to allow for a consistent level of glucose in the blood. This controlled kinetics in the breakdown and absorption of carbohydrate is invaluable in controlling disorders associated with carbohydrate uptake such as obesity, periodontal diseases, diabetes, as well as hyperlipidemia and hyperproteinemia (Alagesan et al., 2012).

The smaller the molecular weight of the amylase inhibitors, the more effective the compound as they can be easily absorbed into the bloodstream from the gut. They are generally heat labile and are active over a pH range of 4.5–9.5.

4.2.8.1 SOURCES OF AMYLASE INHIBITORS

Amylase inhibitors are found in over 800 genera of plants, all of which are relevant in treating type II diabetes. Amylase inhibitors are either of plant (legumes and cereals) or microbial origin (*Streptomyces* spp.). Plant sources are widely considered to be safer and more effective compared to microbial sources. Also, spices such as chili, garlic, rosemary, clove, sage, basil, parsley, and onion have significant amylase inhibition activities. Pigeon pea also contains amylase inhibitors. Catechins from black teas have also been implicated in the inhibition of salivary amylases (Sales et al., 2012).

4.2.8.2 BIOLOGICAL ROLES OF AMYLASE INHIBITORS

The aqueous extracts of *Syzygium cumini* seeds and *Psidium guajava* leaves are reported to show a dose-dependent inhibitory effect on the activity of amylase. *S. cumini* extract was notably reported to have significantly reduced the blood glucose levels in diabetic rats. Also, the hexane, methanol, and ethyl acetate extracts of the seeds of *Amaranthus caudatus* were reported to have significant amylase inhibition activity (>80% inhibition rate at dose concentrations of 0.25–1 mg/mL) (Archana and Jeyamanikandan, 2015).

In a cohort study with healthy and type II diabetic subjects, it was observed that natural amylase inhibitors of plant origin (wheat and white bean) markedly reduced the peak of postprandial glucose (Alagesan et al., 2012).

Quite a number of natural compounds also have excellent amylase inhibitor activities such as flavonols, flavanone, proanthocyanidin, tannins, cinnamic acid derivatives (chlorogenic acids, rosmarinic acids, isochlorogenic acids), and terpenes. Tannins carry out their inhibitory effect due to their ability to bind strongly to carbohydrates and proteins as well as its free OH groups that facilitate hydrogen bonding.

Acarbose is a product of the microbial fermentation of *Actinoplanes* spp. It is an oligosaccharide inhibitor and is commonly used for treating type II diabetes (diabetes mellitus) (Khacheba et al., 2014). It competitively inhibits amylase due to its unsaturated cyclohexene ring as well as its glycosidic nitrogen linkages that mimics the transition state for the enzymatic cleavage of the glycosidic linkages. Acarbose is hence metabolized by the intestinal carbohydrases to yield acarviosine glucose and glucose (Elya et al., 2015; Prabhakar et al., 2013).

4.3 CONCLUSION

Phytochemicals found in plants and other food sources contain essential substances that exert various biological functions in the human body. Alkaloids, saponins, tannins, and flavonoids are among the major sources of essential phytochemicals. The tannins and flavonoids due to their polyphenolic nature serve as antioxidants, antimutagenic, and anticholesterol activities. It is important to know that the biological roles of the different phytochemicals are linked to their plant sources, structural properties, as well as the severity of the diseased state, the age of the patient, and the quantity taken of the phytochemical.

KEYWORDS

- **phytochemicals**
- **flavonoids**
- **alkaloids**
- **saponins**
- **tannins**

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CHAPTER 5

PHYTOCHEMICALS AS IMMUNOMODULATORS

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ABSTRACT

Immunomodulators are substances capable of weakening or suppressing the immune system. They are considered to have fewer side effects than existing drugs, including less potential for creating resistance when treating microbial infections. Immunomodulators could be classified as either immunostimulants, immunoadjuvants, or immunosuppressants. Some plant-derived chemicals such as those from alkaloids, phenolics, flavonoids, anthocyanins, terpenoids, saponins, sterols, polysaccharides, and lectins have been established as having an immunomodulating property. Phytochemicals are good immunomodulatory candidates as they are considered to be safer than synthetic drugs. This chapter details the immunomodulating functions of plant-derived chemicals with supporting evidence. Background information was laid on the phytochemicals discussed and plant families and extracts confirmed to have immunomodulating properties were presented in tables.

5.1 INTRODUCTION

Homeostasis is a well-organized feature of a healthy living organism in which the immune system maintains a stable internal environment. Numerous endogenous and exogenous factors affect the immune system's function, which by some mechanism results in either immunostimulation

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