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Medicinal Plants

From Farm to Pharmacy

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Foreword

Plants have been used to satisfy a wide variety of human needs since the dawn of civilization, especially those that contribute towards meeting the increasing demand for food. A number of plant species that provide health benefits in addition to meeting the dietary needs have been discovered and used for the prevention and treatment of ailments. While such plant species were primarily collected from the wild, processed locally, and used in conjunction with local traditions, there has been an increasing trend in the commercial cultivation of medicinal and aromatic plants, large-scale extraction of the active compounds, along with their processing and marketing as dietary supplements. Furthermore, several chemical compounds with specific mode of action in the human body have been discovered, tested, and produced as drugs. This trend has been catalyzed by sharing of information across the globe, providing impetus to research towards discovering potential life-saving drugs. This has also facilitated regulations to ensure generation of accurate, science-based information that can be disseminated in an unbiased manner for the benefit of humankind.

The book *Medicinal Plants: Farm to Pharmacy*, edited by Drs. N. Joshee, S. Dhekney, and P. Parajuli, is an important treatise that covers topics providing an excellent, in-depth review of the pipeline starting from the production, mechanistic studies, and efficacy testing, up to the time a plant-based product reaches the market.

The editors have assembled experts in the field of medicinal plants cultivation, chemistry, and biomedical research to effectively highlight the production, testing, and development of plant-based medicines. I am confident that the book will be very useful to researchers and readers interested in the areas related to medicinal plants cultivation and processing. It will also be useful to students at both the undergraduate and graduate level who wish to pursue a career in the field of medicinal plant chemistry and cultivation. The book comes at a time when interest in the use of herbal medicines is growing at a rate that has never been witnessed before. The contributing authors are spread across several countries, which testifies global recognition of research and regulatory framework on medicinal and aromatic plants. I believe the authors have addressed an area that covers a vast amount of knowledge in a very effective manner through the selection of relevant topics. I commend the editors and authors for their efforts in this excellent compilation and am confident that the book will be useful to a wide spectrum of people in the research community as well as the general population interested in knowing more about the effects of plants on human health and well-being.

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Pramod Tandon

Preface

This book is an extension of the idea that was presented as a workshop "From Farm to Pharmacy" during the American Society for Horticultural Sciences annual meeting, August 8–11, 2016, Atlanta, Georgia. The session was conceived by Dr. Changbin Chen, University of Minnesota, and Dr. Hideka Kobayashi, Kentucky State University; and Dr. Nirmal Joshee served as the moderator.

Having spent some momentous years of life in the Indian and Nepal Himalayas where a vast majority of the world's medicinal herbs are found, the three editors of this book share personal connections with traditional medicinal plants. This book was motivated by a shared love and respect for medicinal herbs. The editors have tremendous amount of shared expertise on a number of plant species with medicinal properties, commercial or otherwise, and this experience led them to compile information on the usages of various plants in alternative and complementary medicinal practices. They are also driven by a deep desire to generate awareness and dissipate knowledge about the agricultural/harvesting practice and current research on biological activities and medical usages of some popular and some relatively unknown traditional herbs from different parts of the world. Overall, this book is a celebration of the vast plant wealth around us and their tremendous benefit to both humans and animals.

An increasing interest in plants as a source of medicine along with an awareness of the side effects of synthetic drugs has led to a rapid increase in the utilization of several plant species for medicinal purposes. Several medicinal plants are grown commercially to meet the demand for supplemental plant products. This book provides timely information on the techniques for cultivation of plants with medicinal properties, in vitro studies detailing the effect of bioactive molecules from various plant species using human/animal cell culture system as well as in vivo disease models and the processing of various plant parts for formulation into medicines.

We expect this book will attract attention of a large cross section of people who are interested in plants and their healing properties. This book can be read on two different levels. First, it may be read by ordinary people with a limited scientific background. Most chapters of the book have been written with this audience in mind. The book offers initial few chapters that provide a rich heritage and practice

of ethnobotanical systems still adopted in different parts of the world. There are other chapters that deal with specific crops, their commercial potential, agronomic practices, distribution in different ecological conditions, and molecular mechanisms of bioactive compounds present therein. Some chapters may contain somewhat overwhelming Latin names, while some chapters, especially the ones detailing the mechanisms of action, may include intimidating chemical names and busy schemes. These scientific details are meant for the scholarly readers and can be avoided by others without diluting the overall message. Nonetheless, the scientific evidence supported by citations from original documents and the inferences as well as recommendations made can be easily comprehended by readers from all background. This book will be particularly useful for people seeking to optimize production and post-harvest practices for medicinal and aromatic plants, students and researchers interested in elucidating the effects of plant metabolites on cell/animal disease models, and individuals in the private sector/industry who utilize plants for the development of herbal medicines/supplements.

There are 17 chapters in the book. It opens with a commentary on the increasing role of medicinal plants as a reservoir of many bioactive compounds from a practicing physician's perspective. The book is broadly categorized in sections that briefly talk about the use of plants for drug discovery and development, cultivation/production practices for medicinal plants, studies detailing the effects of plant extracts and phytochemicals on in vitro/in vivo disease models, and the various bioactive molecules used for the development of plant-based medicines.

There is a need to conduct systematic research on numerous plants that have been used in traditional medical systems—to scientifically test their efficacy against various diseases and to isolate and characterize therapeutically active molecules. The book strives to drive the point that a lot of fruits, vegetables, ornamental plants, and other herbs that are part of our daily consumption come with beneficial medicinal properties. In our daily life, plants are often content being in the background. It is about time that we appreciate them, treat them with proper respect, and spread awareness with thorough conservation programs. We hope this book will instigate some level of consciousness, enthusiasm, and gratitude towards the tremendous health benefits that are hidden in the flora surrounding us.

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Chapter 1
The Evolution of Modern Medicine: Garden to Pill Box

Tejas S. Athni and Sudhir S. Athni

1.1 Introduction

To first explore how natural substances have been used to treat humans, one can look to Colombia and Peru over 8,000 years ago. Historical evidence suggests that these ancient foraging societies chewed on the leaves of the cocoa tree to keep warm, to battle altitude sickness, and to provide a quick source of instant energy (Mortimer 1974). As history progressed, the cocoa leaf became an integral panacea medicine for nearly all illnesses and diseases in Andean Incan society. This is the first documented example of the utilization of biologically-active, plant-derived substances—referred to as phytochemicals—to treat human ailments. Over the last few millennia, thousands of other herbal substances have been used by various cultures to combat a myriad of sicknesses. Human society has consistently been able to harness the power of phytochemicals, even though at times the exact mechanisms of action were not well understood. In the contemporary age, naturally derived medicine has allowed society to tackle diseases in numerous areas of healthcare. Although there are too many such drugs to discuss in this chapter, a few medications in select healthcare categories will be highlighted.

From an economic standpoint, the medicinal plant industry has been equally productive. Global imports and exports (2000–2008) of medicinal plants were worth USD $1.59 and $1.14 billion/year, respectively, with a >40% growth rate per annum (Rajeshwara Rao and Rajput 2010). As is evident in this statistic, the medicinal plant industry is growing at an ever-increasing rate. However, it is important to note that out of the 3000 medicinal plants traded internationally, only 900 are

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under cultivation, and the vast majority of exported biomass is harvested from the wild (Rajeshwara Rao and Rajput 2010). Hence, this implies that more than two-thirds of the medicinal plants currently exported have yet to be cultivated commercially, signaling an area of huge economic potential.

1.2 Types of Drugs

In medicine, drugs are often classified based on their functional use in the human body. In this chapter, four major categories of drugs are explored: cardiovascular, oncologic, neurologic, and pain-suppressants. Pertinent plant-derived medications, including their history, botanical origins, chemical properties, and mechanism of action, will be discussed in each category.

1.2.1 Cardiovascular Drugs

1.2.1.1 Atropine

Imagine a patient feeling lightheaded and confused Drugs, experiencing symptoms Cardiovascular drugs such as fainting and shortness of breath. After visiting the clinician, the patient finds out that he or she has been diagnosed with bradycardia, a medical condition where the electrical impulses in the heart don't fire as normally as they should, resulting in an abnormally low heart rate (Kounis and Chopra 1974). A medication that is used every day in the hospital to revive a patient's abnormally slow heart rate and to treat conditions such as bradycardia is a drug known as atropine (Kounis and Chopra 1974).

Before delving into the specifics of this medication, some foundational human physiology will first be discussed. The human nervous system is broken down into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS includes the brain and the spinal cord. The PNS contains all other nerves outside of the CNS. More specifically, the PNS can be categorized into the somatic nervous system and the autonomic nervous system. The somatic nervous system controls voluntary muscle movements (e.g., raising an arm, moving a leg), and the autonomic nervous system is in charge of functions that are not consciously controlled (e.g., breathing, heart beat). Within the classification of the autonomic system, there are two main types of nerves which work antagonistically with each other: the sympathetic and parasympathetic nerves (Langley 1921). In simple terms, the sympathetic nerves are activated during times of stress ("fight" response), causing an increase in heart rate and rise in blood pressure (Jänig 2006). The parasympathetic nerves slow down the heart rate and lower the blood pressure ("flight" response). There is a push-pull balance between these nerves to maintain homeostasis.

Atropine works as an anticholinergic or anti-parasympathetic medication through competitive inhibition (Satake et al. 1992), causing decreased activity of the parasympathetic nerves and leading to an increase in sympathetic nerve activity. In the clinical setting, an increase in sympathetic activity coupled with a decrease in parasympathetic activity leads to an elevation in heart rate and a rise in blood pressure (Jänig 2006). For critically ill hospitalized patients, atropine is used to chemically stimulate the heart when the heart rate drops significantly, such as in cases of poisoning (McDonough and Shih 2007). This medication is a crucial aspect of all crash carts in the hospital and ICU settings. Atropine is even on the World Health Organization's List of Essential Medicines, which is a comprehensive compilation of the most safe and effective medicines (WHO 2011).

While atropine is a critical, lifesaving medication, its origins are quite humble. The drug is naturally derived from various plants found in nature, such as the deadly nightshade (*Atropa belladonna*, found mainly in Europe, North Africa, and Western Asia), henbane (*Hyoscyamus niger*, found mainly in Eurasia), thorn apple (*Datura stramonium*, found mainly in Central America), and mandrake (*Mandragora officinarum*, found mainly around the Mediterranean coast) (West and Mika 1957). The medicinal properties of the mandrake plant were first described in fourth century B.C. by Theophrastus, a Greek plant biologist, who described the chemical as an ideal treatment for wounds, sleeplessness, and love (Hamilton and Baskett 2000). Extracts from the henbane plant were used in the last century B.C. by Queen Cleopatra of the Ptolemaic Kingdom of Egypt as a pupil dilation agent to appear more alluring (Hocking 1947). During the Renaissance period of Europe, many women also used the juice of the deadly nightshade plant in order to enlarge their pupils.

These plants contain both hyoscyamine and hyoscine, which are two closely related alkaloid compounds (Pearse 1876). Specifically, atropine is a mixture of two different forms of the hyoscyamine alkaloid. As technology advanced, atropine itself was isolated in the year 1833 (West and Mika 1957). Since that point, through extensive research and scientific testing, atropine is now one of the most widely available generic medicines found in hospitals around the world, with a price tag less than USD $0.50 for 1 mg vials. Derived from plants and first used over 6,000 years ago, atropine has sealed its place as a crucial drug in the modern medical system.

1.2.1.2 Digoxin

There are other cardiac arrhythmias (i.e., abnormal cardiac rhythms) and heart conditions (e.g., congestive heart failure) which can be disabling and sometimes fatal (Domanski et al. 1994). Some of these arrhythmias include atrial fibrillation and atrial flutter. For these conditions, the plant-derived drug digoxin can help regulate the heart rhythm and improve cardiac function during heart failure (Hollman 1996). Digoxin's ability to inhibit the sodium potassium adenosine triphosphatase enzyme (Na/K pump), mainly in the myocardium, helps the heart to beat more regularly and with stronger force (Schwartz et al. 1968).

Like atropine, digoxin is on the World Health Organization's List of Essential Medicines (WHO 2011). Unlike intravenous atropine used in the hospital setting, however, digoxin is used as a cardiac medication consumed daily in the form of a tablet. As of 2019, monthly prescription of digoxin costs less than USD $10. Digoxin was first derived from the foxglove plant, also known as *Digitalis lanata* (Hollman 1996). Digoxin has also been extracted from other plants from the same genus *Digitalis*. The foxglove plant is part of the plantain family, originally coming from the continent of Europe. However, as colonization occurred, the plant was domesticated and brought to North America. The slightly acidic soils of the continent helped foster an environment suitable for optimal plant growth (Allen 1987). The foxglove plant can be found in a wide variety of geographic locations, ranging from woods and cliffs to grassy meadows and wastelands.

In 1785, the English physician William Withering first described the medicinal practicalities of *Digitalis* derivatives in his book, "An Account of the Foxglove and Some of its Medical Uses with Practical Remarks on Dropsy and Other Diseases" (Withering 2014). In his descriptions, Withering talks about *Digitalis* extract's ability to fight dropsy, which is the former name for congestive heart failure and the associated edema (i.e., abnormal accumulation of fluid within certain tissues of the body) (Withering 2014). Based on these accounts, digoxin and digoxin-related substances have been used to treat congestive heart failure for almost 250 years.

1.2.1.3 Warfarin

Warfarin is one of the most popular anticoagulants (i.e., blood thinners) available on the drug market (Pollock 1955). Today, warfarin is commonly used to treat conditions such as blood clots and deep vein thrombosis (i.e., a blood clot in a deep vein, usually within the legs), as well as to prevent strokes in people who have artificial heart valves, atrial fibrillation (i.e., irregular heart rhythm), and valvular heart disease (i.e., damage or defect in one or more of the four heart valves) (Pollock 1955). Warfarin can also help prevent future blood clots and embolism, a condition in which a blood clot migrates through vasculature and physically blocks blood supply to vital organs or tissue.

This drug's origins can be traced back to a very unusual disease that afflicted cattle in the 1920s—one that resulted in sudden and fatal bleeding after minor injuries. Investigation of this mysterious illness concluded that these cattle had consumed a plant known as the sweet clover (*Melilotus alba* and *M. officinalis*) (Kresge et al. 2005). The sweet clover, part of the family Fabaceae, is a part of the common grassland plants and often known as the "weed of cultivated ground." These plants originally are from Asia and Europe, but they are now found all throughout the world. Intrigued by this plant, scientists found that it contained a hemorrhagic factor that reduced the activity of prothrombin, a protein present in the plasma of blood. Researchers were determined to find the identity of this unknown hemorrhagic factor, and eventually identified the active compound as coumarin. These coumarin compounds can also be found in other plants, most notably the sweet-scented bedstraw

(*Galium odoratum*, family Rubiaceae) and lavender (*Lavandula angustifolia*) (Pollock 1955). However, the coumarin compounds themselves do not exert any effect on clotting. Rather, they must be metabolized into compounds such as 4-hydroxycoumarin by various fungi, and then into a compound called dicoumarol, which is the actual active component (Bye and King 1970).

Physiologically, dicoumarol works as an anticoagulant by acting as a vitamin K depleter, functioning to reduce the metabolism of vitamin K in the blood and effectively reduce the clotting of blood cells (Bye and King 1970). Mechanistically, dicoumarol acts to competitively inhibit vitamin K epoxide reductase, the enzyme responsible for vitamin K recycling (Patel et al. 2019). After extensive research into these naturally derived compounds and their medicinal properties, Karl Link and fellow scientists at the University of Wisconsin decided to produce the pharmaceutical drug known today as warfarin (a name which is the combination of the acronym WARF, which stands for the Wisconsin Alumni Research Foundation, and the suffix "-arin" from its coumarin components). Today, warfarin is on the World Health Organization's List of Essential Medicines, yet interestingly, was first approved as a rat poison before its use on humans (WHO 2011).

1.2.2 Oncologic Drugs

1.2.2.1 Paclitaxel

Breast cancer is the second most common type of cancer in the United States (second only to skin cancer). Each year, approximately 266,000 women are diagnosed with invasive breast cancer and an additional 65,000 women are diagnosed with noninvasive breast cancer in situ (Levi et al. 2002). Research into finding effective treatment options for breast cancer has been unrelenting. Currently, treatment options include surgical resection, chemotherapy, radiation therapy, immunotherapy, and hormonal therapy (Levi et al. 2002). One of the more effective chemotherapy options is a medication known as paclitaxel, another member of the World Health Organization's List of Essential Medicines (WHO 2011).

Paclitaxel is a microtubule-stabilizing drug which interferes with the arrangement of microtubules during the process of mitotic cell division, ultimately impeding the proliferation of cancer cells and inducing cell death (Long and Fairchild 1994). As the most well-known naturally derived antitumor drug in the United States, paclitaxel's history is quite unique. In 1962, samples of the Pacific yew tree, also known as *Taxus brevifolia*, were sent by the US Department of Agriculture to the National Cancer Institute (NCI) to aid in their objective of finding natural products that could potentially help treat and cure cancer (Stierle et al. 1994). Scientists from the Research Triangle Institute in North Carolina soon discovered that extracts from the bark actually caused cytotoxic activity on cancer cells (Stierle et al. 1994).

Following this stunning discovery, additional samples of bark were collected and extracts produced for further testing to identify the most bioactive component within

the samples (Stierle and Stierle 2000). After several years, paclitaxel in its pure form was finally discovered as the main bioactive constituent within the Pacific yew tree's bark. Then began the process of testing paclitaxel's biological mechanisms. In-vitro biological mechanistic studies eventually led to in-vivo trials against the mouse melanoma B16 model (Holmes et al. 1991). Finally, paclitaxel was selected to be further developed in the clinical pipeline after extensive testing, both in-vitro and in-vivo.

The wealth of compounds found in an extract such as the bark of the Pacific yew is quite remarkable. Before the evolution of modern paclitaxel, native peoples in North America used the needles and twigs of the tree in order to brew homeopathic teas for various ailments (Wilson and Hooser 2012). However, many traditional shamans were careful in using the tree's medicinal properties, as an excess amount could lead to devastating toxico logical consequences for the human body, including yew poisoning. Interestingly, the yew tree was known as the "tree of death," whose extracts were used to murder ancient kings such as Catuvolcus, the king of the Gallic-Germanic tribe known as the Eburones (Panzeri et al. 2010).

1.2.2.2 Vinblastine

Although some chemotherapeutic agents are used to treat one specific type of cancer, other agents have been used for the treatment of many. An exemplar drug is vinblastine, a common chemotherapeutic used in the field of oncology. Typically utilized as an adjuvant treatment in conjunction with other medications, vinblastine is effective in treating various forms of cancer. For example, the drug is known for its ability to fight non-small cell lung cancer, brain cancer, testicular cancer, melanoma, bladder cancer, and most notably Hodgkin's lymphoma (Ratain et al. 1987). Vinblastine is also used to treat non-malignant conditions, such as histiocytosis and other blood disorders (Tennant Jr 1969).

Currently, vinblastine is on the World Health Organization's List of Essential Medicines and has been recognized as one of the most effective chemotherapeutic substances (WHO 2011). Chemically, vinblastine is an alkaloid compound. It works to inhibit cancer growth by specifically targeting metaphase in the mitotic process. Normally during metaphase, each chromosome lines up in the center of the cell, with every sister chromatid being attached to a respective spindle fiber. Vinblastine binds to tubulin—the protein which is the main constituent of cellular microtubules—in order to prevent the cell from creating spindle fibers in the first place (Jordan et al. 1992). This ultimately interferes with the cell division process and inhibits tumor cell growth by stopping proper mitosis. Vinblastine is similar to paclitaxel in that it interferes with tumor cells during the cell cycle. However, while paclitaxel targets the arrangement of microtubules, vinblastine targets the creation of microtubules altogether (Long and Fairchild 1994).

With all of its intricacies and molecular charm, vinblastine derives its origins from a simple plant known as the Madagascar periwinkle (*Catharanthus roseus*), which is in the dogbane family Apocynaceae (Iskandar and Iriawati 2016). Also

called the "old maid" and the "Cape periwinkle," this plant is native and endemic to Madagascar. However, the periwinkle is grown in various places around the world as a medicinal and ornamental plant. For example, the extract of the roots and shoots of *Madagascar periwinkle* has been used for numerous centuries as an Ayurvedic (i.e., traditional Indian medicine) agent against several diseases (Iskandar and Iriawati 2016). In traditional Chinese medicine, the plant was used to battle various other diseases ranging from malaria to diabetes.

Having such an extensive history of use among various cultures, it was only recently that drug development of vinblastine took place. In 1958, Robert Noble and Charles Thomas Beer at the University of Western Ontario isolated the vinca alkaloid vinblastine from the periwinkle plant (Ratain et al. 1987). Shortly thereafter, vinblastine's potential use as a chemotherapeutic agent was advanced after a successful decrease in infected rabbits' white blood cell count—an indication of the effectiveness of the compound.

1.2.2.3 Etoposide

Besides paclitaxel and vinblastine, another common chemotherapeutic agent is etoposide. Primarily used to treat testicular cancer, leukemia, neuroblastoma, ovarian cancer, lymphoma, and lung cancer, etoposide can either be ingested orally or be injected directly into the bloodstream through an intravenous injection (Van Maanen et al. 1988). The drug is also on the World Health Organization's List of Essential Medicines (WHO 2011).

The mechanism of action of etoposide is very different from other chemotherapeutic substances of its sort. Instead of inhibiting the microtubule processes during mitotic cell division like paclitaxel and vinblastine, etoposide targets the actual DNA strands of the tumor cell. The drug forms a ternary complex with the topoisomerase II enzyme on the DNA (Hande 1998). Topoisomerase II is a protein which helps with DNA unwinding and is crucial for cancer cell function since tumor cells divide so rapidly. When etoposide latches onto the topoisomerase II enzyme, it prevents the DNA strands from religating, ultimately causing errors in DNA synthesis and causing the DNA strands to break (Van Maanen et al. 1988).

While etoposide was approved for medical use in the United States in 1983 and eventually received a designation on the World Health Organization's List of Essential Medicines (WHO 2011), the plant the compound is derived from, known as the wild mandrake (*Podophyllum peltatum*), has a long and extensive history of medicinal use (Hande 1998). Also known as the mayapple, the wild mandrake is a herbaceous perennial plant in the family Berberidaceae, first described as a genus by the Swedish botanist Carl Linnaeus in 1753 (Springob and Kutchan 2009). The wild mandrake is found all across the eastern United States and southeastern Canada. Interestingly, every single part of the plant is poisonous, including its green fruit.

The plant has been used for many centuries by American Indians as an emetic (i.e., vomit inducing), antihelminthic (i.e., expelling parasitic worms), and cathartic

(i.e., psychological relief) agent (Hamilton and Baskett 2000). By boiling the poisonous roots of the wild mandrake, the Native Americans were able to create a natural tonic water in order to treat stomach aches and other gastrointestinal problems. As further research was done into the plant, historians found that the rhizome of the plant was used by settlers in the New World to treat ailments. Creating a semisynthetic derivative of a compound known as podophyllotoxin from the rhizome of the wild mandrake, scientists were able to produce the chemotherapeutic drug known today as etoposide (Hande 1998).

1.2.3 Neurologic Drugs

1.2.3.1 Scopolamine

The brain is regarded as the control center for volitional activities. The brain is also the command module for involuntary activities, such as breathing, heartbeat, bowel and bladder activities, and much more. A critical neurotransmitter, acetylcholine, is involved with the transmission of the brain's signals to the various muscles of the body, both voluntary and involuntary (Perry et al. 1999).

The type of receptor on which acetylcholine interacts is labeled as either muscarinic or nicotinic (Leprince 1986). Nicotinic receptors are usually found on muscles over which the body has volitional control. Muscarinic receptors typically control involuntary function. Chemicals or medications which block the muscarinic receptors prevent acetylcholine from reaching its post-synaptic target site which, in essence, cause malfunction of the end organ (Perry et al. 1999). These chemicals are said to have anticholinergic properties. When one consumes medications or substances with such properties, numerous effects are observed, such as dry mouth, constipation, urine retention, and sleepiness, to name a few.

One commonly used anticholinergic chemical is scopolamine, which is derived from various genuses in the Solanaceae (nightshade) plant family, most notably *Scopolia* and *Hyoscyamus* (Phillipson and Handa 1975). Scopolamine is also found in the secondary metabolites of other plants, such as jimson weed (*Datura stramonium*) and corkwood (*Duboisia myoporoides*) (Phillipson and Handa 1975). For many centuries, the effects of scopolamine were observed and used for various medicinal and divine purposes. Starting from the Neolithic period (from 10,200 to 2,000 BCE), the henbane plant (*Hyoscyamus niger*) has been harnessed for human benefit (Hocking 1947). The ancient Egyptians, Celts, Germans, and Greeks all sought use of the plant as a sacred healing agent as well as an alcoholic enhancer. For example, the Oracle of Delphi inhaled the burning smoke of the henbane plant before forecasting prophecies and divinations (Hocking 1947). After a short period of disappearance from historical records, the drug reappeared during the Middle Ages (4,000–1,400 CE) as a topical ointment. Since strong doses of the medication caused hallucinations and delirium, many regarded topical henbane ointment as the "witch's herb" (Müller 1998).

In the modern era, scopolamine is a common medication in the antimuscarinic family. Used to treat conditions including postoperative nausea, vomiting, motion sickness, and intestinal and bladder cramps, scopolamine can help alleviate the spasmodic pain that is associated with such problems (Hardy and Wakely 1962). Scopolamine is also known for its effects targeting other gastrointestinal problems such as irritable bowel syndrome and diverticular disease. The drug works by blocking the effects of acetylcholine, ultimately reducing the spasmodic contractions of the smooth muscles in the walls of the gastrointestinal tract (Hardy and Wakely 1962).

Interestingly, scopolamine was initially used in conjunction with opioids such as morphine and oxycodone in order to put mothers in labor to a deep sleep (Phillipson and Handa 1975). Scopolamine's analgesic properties when combined with opioids are strong enough to be used as a form of anesthesia. Since scopolamine was first isolated and tested, extensive research has been done on the specific properties of the drug. It is now a key member of the World Health Organization's List of Essential Medicines (WHO 2011).

1.2.3.2 Levodopa (L-Dopa)

Another neurotransmitter, dopamine, is a critical chemical used in the control of body movements, along with many other functions (Hornykiewicz 2010). When there is a loss of dopamine activity in parts of the brain known as the substantia nigra and the basal ganglia, a condition known as Parkinson's Disease (PD) ensues due to impaired neuron-to-neuron communication and firing (Hornykiewicz 2010). This neurodegenerative disorder can be quite disabling without treatment.

A phytochemical compound, levodopa (also known as L-dopa), was first isolated in the years 1910–1913 using the seeds of the broad bean plant (*Vicia faba*) (Tomita-Yokotani et al. 2004). Functionally an amino acid, levodopa is a precursor to dopamine but is not an active compound itself. A few years after the initial discovery of L-dopa, however, scientists discovered in 1938 that an enzyme called dopa-decarboxylase was able to enzymatically convert L-dopa into the neurotransmitter dopamine (Blaschko 1942). This finding set the basis for further research into dopamine replacement therapy, a type of treatment which increases levels of dopamine in affected brain regions to optimal levels for proper neuronal functioning (Kebabian and Calne 1979).

A member of the World Health Organization's List of Essential Medicines (WHO 2011), L-dopa is now a critical component of the modern health system's toolkit to battle CNS diseases including PD (Rodnitzky 1992). In 1967, a levodopa drug regimen was introduced to help treat PD and has been used extensively ever since. Mechanistically, L-dopa crosses the blood-brain barrier—a highly selective and semi-permeable border that serves to separate circulating intravascular blood from cerebrospinal fluid—and is enzymatically decarboxylated into dopamine. The blood-brain barrier is comprised of a close packaging of endothelial cells that line the blood vessels of the CNS, astrocyte end-feet, and pericytes and their basal

lamina (Daneman and Prat 2015). It is also comprised of tight junctions of protein complexes, which serve to bolt the endothelial cells together and restrict movement of ions, molecules, and cells into and out of the CNS (Luissint el al. 2012). Since dopamine itself is too large to cross the blood-brain barrier, levodopa is a smaller, more permeable molecule that is an ideal alternative to get dopamine into the brain (Hornykiewicz 2010). Physiologically, this levodopa-induced spike in dopamine concentrations helps to fight many of the motor symptoms caused by PD, such as bradykinesia, tremors, and impaired gait (Zach et al. 2017).

Historically, levodopa has been used for thousands of years in order to treat PD-like symptoms. For example, ancient civilizations in India used extracts of a therapeutic legume known as the velvet bean (*Mucuna pruriens*) as a psychospiritual and purifying herb in Ayurvedic medicine (Tomita-Yokotani et al. 2004). More specifically, the velvet bean is an annual climbing shrub that is of the family Fabaceae and can grow to over 50 feet in length. Interestingly, in addition to its use as a treatment for neurological diseases, the plant also was used as an antagonist to fight the toxins of various snake bites (Tomita-Yokotani et al. 2004). Other than the velvet bean, L-dopa can also be derived from natural sources such as the broad bean (*Vicia faba*) and plants in the *Cassia*, *Dalbergia*, *Piliostigma*, *Phanera*, and *Canavalia* genera.

1.2.4 Pain Suppressants

1.2.4.1 Aspirin

Fever? Headache? Reach for the aspirin: one of the most widely used medications found in households all over the world (Miners 1989). For everyday aches, aspirin has consistently been the go-to pharmaceutical agent of the past century. A member of the World Health Organization's List of Essential Medicines, aspirin acts in numerous pain suppressant roles and is used immediately after a heart attack in order to reduce the risk of death post-cardiac arrest (WHO 2011). The medication is also used as a preventative medication for ischemic strokes, heart attacks, and blood clotting (Reed 1914). Mechanistically, aspirin inhibits the activity of cyclooxygenase (COX), an enzyme which functions to produce prostaglandins—a specific group of lipids. Physiologically, when aspirin is specifically used as a pain reliever, COX inhibition reduces swelling, inflammation, and pain.

Before the development of the pharmaceutical drug aspirin, its precursor was the medicinal usage of bark from various species of the willow tree family, such as the white willow (*Salix alba*), the black willow (*Salix nigra*), the weeping willow (*Salix babylonica*), and the crack willow (*Salix fragilis*) (Norn et al. 2009). These trees are native to Asia, Europe, and some parts of North America. Since 2,000 B.C., the willow bark's medicinal potential has been recognized and widely used for its anti-inflammatory effects and ability to treat conditions such as headaches, muscle pains, menstrual pains, and arthritis (März and Kemper 2002). For example, about

4,000 years ago, the Sumerian culture described the pain-relieving properties of willow bark on clay tablets (Norn et al. 2009). Ancient Mesopotamian civilizations utilized this willow bark's extracts to treat the daily pains and inflammations of citizens. For more than 2,000 years, traditional Chinese medicine has also made use of willow bark and the bark of the poplar tree in order to help treat colds, goiter, and fever. Additionally, around 400 B.C., during the time of the Greek physician and ancient founder of medicine Hippocrates, citizens were advised to chew on the bark of the willow tree and drink teas derived from the willow tree to relieve fever and pain (Norn et al. 2009).

Despite such long historical usage of the willow bark, it wasn't until 1763 that Edward Stone, member of the Royal Society of London, conducted the first clinical study using an extract of the willow bark on patients affected with ague (i.e., a fever which many believed to be caused by malarial agents) (Stone 1763). By the nineteenth century, many chemists and pharmacists were experimenting with various chemicals found in the willow bark extract. In 1829, a French pharmacist by the name of Henry Leroux isolated a pure crystalline form of a compound known as salicin—one of the primary active components in the willow bark (Norn et al. 2009).

After identifying this salicin compound, in 1853, a French chemist by the name of Frederic Gerhardt combined sodium salicylate (i.e., a sodium salt of salicin) with acetyl chloride to produce the novel compound of acetylsalicylic acid (Norn et al. 2009). Over the next few decades, various chemists worked to determine the exact chemical structure and properties behind this molecule. Eventually, in 1899, scientists at the Bayer company manufactured the drug known as aspirin derived from acetylsalicylic acid (Sneader 2000).

1.2.4.2 Morphine

Morphine is one of the most popular and most commonly used pain medications in the world. Classified as an opiate, morphine works to decrease the feeling of pain by acting directly on the CNS. The drug can be taken for both chronic and acute pain, with morphine being used to treat pain from conditions such as bone fractures, cancer-associated pain, and postsurgical pain (Hamilton and Baskett 2000).

Mechanistically, morphine works by binding to μ-opioid receptors in the CNS. The G-protein in the opioid signaling chain increases the conductivity of potassium channels and inhibits adenylyl cyclase (i.e., the enzyme which synthesizes cyclic AMP from ATP) (Brook et al. 2017). Together, all of these biochemical changes dampen the effect of the nervous system signaling systems which transmit pain.

Morphine is derived from a plant called *Papaver somniferum*, commonly known as the opium or breadseed poppy, which is a species of flowering plant in the family Papaveraceae (Miller et al. 1973). The plant was traditionally grown in the eastern Mediterranean, but is now found all over the world. Primarily, morphine is isolated from the poppy straw portion of the opium poppy. First isolated by the German pharmacist Friedrich Serturner in 1804 (Lockermann 1951), the alkaloid

compound was named after Morpheus, the Greek god of dreams, because of its tendency to cause sleep (Hensel and Zotterman 1951). Before the actual isolation of the compound, the opium plant has had a long and extensive history of use. Traditionally, an opium-based elixir was brewed by alchemists in the Byzantine era as a potent painkiller. In the year 1522, the Swiss physician and astrologer Paracelsus referenced an opium-based pain medication called laudanum (from the Latin "laudare," to praise) in his texts, but stated that it should only be used sparingly (Miller et al. 1973). In the late eighteenth century, laudanum reappeared and became popular among the East India Trading Company, which had a direct interest in the opium trade in India. Fast forward to the nineteenth century—a few years after Friedrich Serturner's isolation of the alkaloid compound of morphine in 1804—the pharmaceutical company Merck began marketing morphine commercially (Brook et al. 2017). Within a few decades, pharmaceutical production of morphine and other opioid-based medications had become a huge industry.

Currently, morphine is classified as a schedule II drug in the United States and is on the World Health Organization's List of Essential Medicines (WHO 2011). However, unfortunately, the abuse of and addiction to prescription opioids such as morphine is one of the most rampant drug epidemics that the United States and other countries have ever seen (Brook et al. 2017).

1.2.4.3 Menthol

Cough drops. Soothing tea. Chapstick. What key ingredient do all three of these day-to-day, winter-time items have in common? The answer is menthol, a drug produced from the mint plant that provides a cooling sensation when ingested or applied. Besides helping to relieve sore throats and chapped lips, menthol is also used to treat minor pains and aches of muscles and joints (Hensel and Zotterman 1951). For example, menthol is commonly used to treat conditions such as arthritis, backache, and sprains.

How exactly does menthol work? The drug has a natural analgesic (i.e., pain relieving) property. Mechanistically, the menthol compound acts as a ligand and binds to the κ-opioid receptor, effectively producing a numbing effect in the target location (Hensel and Zotterman 1951). Additionally, when menthol is applied onto a sore or an aching muscle, the blood vessels in that location are dilated, increasing blood flow to the area and bringing necessary nutrients faster (Hensel and Zotterman 1951). Menthol also acts by stimulating thermoreceptors within the skin cells themselves, tricking the brain into thinking that the temperature in that area has decreased drastically (a process known as counterirritation) (Yosipovitch et al. 1996). In reality, this sense of cooling distracts the brain from the uncomfortable, hot pain at the inflammation site.

For its vast plethora of properties, menthol is derived from a very simple plant: the wild mint, a group of 15-20 species found in the *Mentha* genus (Iqbal et al. 2013). These plants are part of the family Lamiaceae and are found in nature as

perennial herbs. Having originated in the Mediterranean region and Asia, the plant has been known to possess beneficial medicinal effects throughout history. For example, the ancient Greeks added mint into their baths to help stimulate their bodies, and the ancient Romans included mint in certain sauces as a digestive aid and mouth freshener (Iqbal et al. 2013).

Medieval monks also used the mint plant, especially in their cooking, in order to help ward off illnesses. In the seventeenth century, an English traveler by the name of John Josselyn chronicled in his writings that the pilgrims brought mint to the New World and included botanical information on the plant (Josselyn and Tuckerman 1865). Currently, mint has a wide distribution all around the world, including in five continents.

1.3 Conclusion

Although this chapter details just a few of the countless naturally-derived drugs used to treat human ailments, the full list of medications is far more extensive. Mankind has harnessed thousands of compounds, but the wealth of undiscovered medicinal gems in nature is unfathomable. The focus of modern pharmaceutical research has been toward the direction of lab-synthesized drugs and compounds, yet many of these molecules unfortunately have undesirable side effects. Rather, society should look to discover the true potential of phytochemicals in nature—an option that is both scientifically and economically rewarding. Medicinal plants also provide an opportunity to train young minds in the fields of botany, plant conservation, and natural product chemistry (https://www.youtube.com/watch?v=0YqEPDAYsWQ). Time after time, nature has shown to be the master craftsman in creating an inexhaustible array of therapeutic molecules, and carries infinite potential for future drug discovery and treatment of human diseases.

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Chapter 2
Bioprospecting for Pharmaceuticals: An Overview and Vision for Future Access and Benefit Sharing

Danielle Cicka and Cassandra Quave

2.1 Introduction

Before the advent of synthetic chemistry, plants were well known as a primary source of medicine. Still today, medicinal plants are used for healing around the world; in some regions, up to 80% of the population relies on plants as primary sources of medicine (WHO 2002). Conservative estimates show that at least 28,187 plant species are currently recorded as being of medicinal use (RBGK 2017). Though drug discovery has tended toward synthetic compounds, almost half of the drugs approved since 1994 are based on natural products (Harvey 2008). These medicines include some of the world's most essential medicines including acetylsalicylic acid, dihydroartemisinin, pilocarpine, and warfarin (WHO 2017). With the increase of microbial resistance, chronic disease, and heavy burden of communicable disease, new medications are in high demand. Plants can help meet this demand by their unparalleled array of untapped complex chemical diversity.

This chapter gives an overview of the role of bioprospecting in the drug discovery process, the unique regulatory environment for such drugs, challenges in bioprospecting, and a vision for navigating these processes for future discovery.

2.2 Plants in Drug Discovery

Plants play several critical roles in the development of pharmaceuticals. Their unique chemical compounds may be developed into novel drugs as well as become the basis for novel drug classes. Further, the accessibility of plants throughout

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history has allowed for much investigation by local communities and this knowledge can be used to assist targeted drug development.

2.2.1 The Story of Paclitaxel

The discovery of bioactive compounds from natural products has had important contributions in several aspects of drug development. The story of the discovery of the natural product paclitaxel, a widely used cancer chemotherapy, exemplifies the contribution of natural product research throughout the drug discovery process.

In 1962, the National Cancer Institute and the United States Department of Agriculture conducted a collection of plants for screening. Through this untargeted mass screening of plant extracts for bioactivity, 4% of extracts were found to have anticancer activity (Suffness and Douros 1982). The Pacific yew (*Taxus brevifolia* Nutt., Taxaceae) was found to have cytotoxic effects. The compound paclitaxel was eventually isolated from the Pacific yew via bioassay-guided fractionation and underwent clinical trials for ovarian cancer (McGuire et al. 1989) and breast cancer (Holmes et al. 1991). Paclitaxel stabilizes microtubules, preventing their disassembly during cell division and is used especially for ovarian tumors, representing a novel mechanism of action at the time (BMS 2011; Schiff et al. 1979).

However, *T. brevifolia* yields a small quantity of paclitaxel, just 0.02% from the bark (Wani et al. 1971). Further, stripping the bark for collection of paclitaxel is detrimental to the survival of the tree and therefore is not a sustainable resource for anticancer treatment. The structure of paclitaxel is so complex that the process of total synthesis is neither time nor cost efficient. To preserve the species, structure-activity relationship (SAR) studies were undertaken to determine the active structural components of paclitaxel necessary for the preservation of therapeutic effect. A related species, English yew (*Taxus baccata* L., Taxaceae) was screened for its microtubule effects (Guenard et al. 1993). A compound in the species, baccatin, was found to be a precursor of paclitaxel, but did not exhibit the anticancer properties. The difference between baccatin and paclitaxel resides in a specific side chain, which was then deemed essential for paclitaxel's bioactivity. Baccatin as well as 10-deacetylbaccatin III is found in higher quantities than paclitaxel in *T. baccata*; 10-deacetylbaccatin III can then be synthesized into paclitaxel (Fig. 2.1). These compounds are found in high quantities in the needles of the tree, providing a renewable resource (van Rozendaal et al. 2000). Paclitaxel can also be more sustainably synthesized from plant cell cultures (Malik et al. 2011) or from fungal endophytes (Stierle et al. 1993). These options allow the sustainable production of paclitaxel in higher quantities.

Paclitaxel is just one example of a lifesaving, natural product pharmaceutical. The structural complexity and diversity of natural products derived over years of selective pressure of plant defenses can not only offer novel treatments, but also add to the repertoire of chemical structures from which drugs can be modeled.

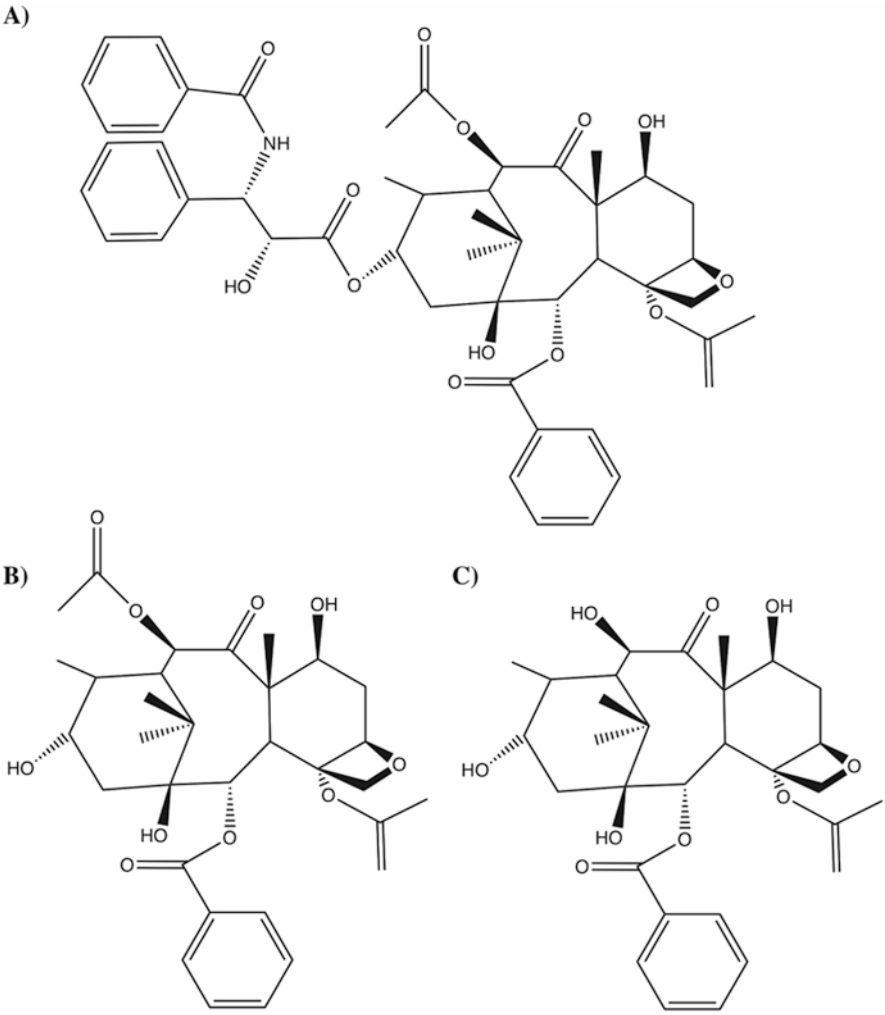

Fig. 2.1 Structure of paclitaxel and its precursors. (**a**) Paclitaxel. (**b**) Baccatin III. (**c**) Deacetylbaccatin III

2.2.2 Structure-Activity Relationship Studies

Bioactive compounds from plants can be used as scaffolds for structure-activity relationship (SAR) studies. These analyses identify the active groups of the compound to determine how modifications can maximize the effect of the compound. As discussed previously, SAR studies were crucial for the discovery of a sustainable source of Taxol.

As natural compounds have greater complexity and diversity than synthesized libraries, the potential for novel structures is great (Camp et al. 2012). There has

been continued use in recent years of SAR studies of natural products in search for treatments for diseases such as malaria, breast cancer, and Alzheimer's (Aratikatla et al. 2017; Lee et al. 2017; Robles et al. 2017). The more natural product structures that are elucidated, the more SAR studies can be completed by comparing the activity of various molecules. Screening large natural product libraries has many challenges compared to synthetic compounds; however, natural products likely already have bioactivity for the plant, and thus may have a high likelihood to be active in other biological systems (Atanasov et al. 2015; Hunter 2008).

2.2.3 Ethnobotanical Approach to Drug Discovery

An ethnobotanical approach to drug discovery is one in which the traditional uses of a plant are taken into consideration when looking for potential therapies in nature. In fact, natural products are more likely to be bioactive when based on ethnomedical data rather than random screens (Elvin-Lewis 2011). Though random screens such as the National Cancer Institute's search for anticancer compounds had viable yields, a more strategic approach can increase prospects of finding bioactive compounds.

The discovery of artemisinin particularly exemplifies this strategy and its necessity for effective drug discovery. Dr. Youyou Tu is credited with the discovery of artemisinin from sweet wormwood (*Artemisia annua* L., Asteraceae). This compound and its derivatives have been widely used as an antimalarial drug, rendering Dr. Tu a winner of the 2015 Nobel Prize in Physiology or Medicine. Artemisia was screened for antimalarial properties in the 1960s as part of a study of traditional Chinese medicine (TCM), but was found to have fluctuating effects on the parasitic infection. However, when an extraction method was developed that incorporated its traditional preparation, the active compound was isolated (DeNicola et al. 2011). Artemisinin-based combination therapies are now recommended for treatment of *Plasmodium falciparum* infections worldwide (WHO 2016).

2.3 Framework for Managing Intellectual Property

Given the successes of bioprospecting, it is essential to preserve natural biodiversity and the capacity of corporations and local communities to continue to develop the potential of natural resources. Care must be taken to ensure the protection of knowledge and maintenance of ethical standards to avoid exploitation of natural products and traditional knowledge while recognizing the financial incentive for companies and local communities to develop lucrative pharmaceuticals. Though this section is not all encompassing, discussion of the regulatory environment is necessary to paint a picture of the hurdles and protections involved in bioprospecting. Even though a

framework exists for navigating the regulatory environment, there is a lack of clarity for its implementation. It is a problem for all parties involved when intellectual property is not protected. This is especially clear when natural products and their indications for use are traditionally used by local communities.

2.3.1 Protecting Intellectual Property of Researchers: Patenting of Natural Products

The process of drug discovery is long and arduous; however, as stated at the start of this chapter, patience is rewarded as there have been many invaluable medicinal discoveries from natural resources. To market active compounds as pharmaceuticals in the United States, the product must meet standards for approval by the Food and Drug Administration as a pharmaceutical. While drugs are being developed and undergoing clinical trials, protection of the investigators' rights to the compound must be maintained. Investigators may seek to secure patents on the active compound while bringing the use of the natural product to commercialization to the public domain. The FDA also grants market exclusivity separately from patents that may have a different time course than the patent for certain pharmaceuticals (FDA 1999). Without patents, discoveries would become public knowledge much sooner, which may inhibit a discoverer from recouping discovery costs as soon as another party is able to market and sell the compound.

There are specific patenting issues to address when working with natural products. Patent laws differ by country and a product can be patented in multiple countries (Worthen 2004). Focusing on the United States in this chapter, patenting natural products has recently been put into the spotlight. In 2013, the United States Supreme Court ruled against patenting of naturally occurring entities such as genes in *Association for Molecular Pathology* vs. *Myriad Genetics Inc*. Due to this case and others before it, the court issued a memorandum on patent eligibility for natural products (Wong and Chen 2014). This document states that a patentable product must be significantly different from its naturally occurring form (Hirshfeld 2014). Therefore, a biologically active natural product likely will have to be modified if it is to be patentable. This lends to the following possibilities for patentable entities. One could possibly patent the altered product itself, the method of production, or a novel use of the product. If the constituents of the product cannot be fully determined, a product-by-process patent may be considered. This does not include products synthesized from a new process that are identical to a naturally occurring product. For one to patent the method of production, the method cannot simply be common knowledge or a general application of the product. The specific dosage, regime, and disease target must be stated (Hirshfeld 2014; Wong and Chen 2014). Though the barriers to patenting drugs may be stringent, they provide some hope for stability and protection in a competitive market in which investment of time and capital is extraordinary.

2.3.2 Protecting Intellectual Property of Local Communities and Biodiversity

Exploitation of natural resources and their guardians is a concern in the pursuit of lifesaving medications. In 1993, the Convention on Biological Diversity (CBD) established rules regarding the protection of biodiversity. This agreement laid the groundwork for fundamental principles of intellectual property rights to protect both economic incentives and maintain biodiversity. The convention established that prior informed consent must be obtained from informants and mutually agreed terms determined in order to achieve adequate benefit sharing (UN 1992). Around the same time, in 1991, the International Cooperative Biodiversity Groups Program (ICBG) was established and provided for benefit-sharing projects. The CBD was an important step; however, it was criticized for its lack of specific requirements, leaving interpretation to individual countries. Thus, the 1995 Agreement on Trade-Related Aspects of Intellectual Property (TRIPS), for members of the World Trade Organization, attempted to fill some of the gaps left by the CBD, particularly by requiring minimum patent laws of member nations (WTO 1995).

Further, the Nagoya Protocol, implemented in 2014, was developed as a supplement to the CBD. Its aim is to ensure benefit sharing when using genetic resources (CBD 2011). It currently has 93 member parties. Most members of the UN have ratified the Nagoya Protocol, the notable exception being the United States (CBD 2017). The Nagoya Protocol aimed to address specific benefit-sharing goals not explicitly addressed by the CBD including promotion of the role of women as stakeholders of traditional knowledge and necessity of explicit capacity-building agreements with developing countries.

The Nagoya Protocol and the TRIPS agreement, though both aimed at the protection of knowledge, highlight different aspects of the protection. TRIPS does not state the importance of benefit sharing, but rather focuses on the importance of maintaining intellectual property rights, often in the form of patents. The Nagoya Protocol focuses more on access and benefit and places intellectual property rights as one of the possible benefits. Various other legal documents to clarify access and benefit sharing have been developed, though mostly by developing nations (Medaglia and Silva 2007). However, these major legislative pieces highlight the different aspects of post-CBD agreements between researchers and local communities. Implementation of the Nagoya Protocol and CBD may be improved by having an overarching regulatory body to help navigate adherence to the protocol.

2.3.3 Organizational Regulations

Various international professional societies have delineated standards for members' publications which help to accomplish the goals of the international regulations. These standards include obtainment of informed consent and engagement of

communities throughout the process. For example, the Society for Economic Botany adheres to the International Society of Ethnobiology Code of Ethics (ISE 2006), which expects its members to uphold communities' rights as historical protectors of the genetic resources and requires informed consent. The American Society of Pharmacognosy also mandates its members to try to protect communities and obtain prior informed consent (Flamini et al. 2002). Though these statements err on the side of vagueness, this may allow each agreement to be shaped to fit the needs of the project and the parties involved.

2.3.4 Managing Expectations

Ethical standards are critical; however, those standards can only be maintained if both parties agree that the standards have been met. Managing expectations of the outcome of bioprospecting endeavors is vital to this mutual understanding of realistic results. With the hope of a blockbuster drug on the horizon, source countries may have unreasonable expectations about the financial gains of partnering with a drug discovery team. Expectations on both sides need to be transparently outlined initially. A typical marketable drug costs $2.558 billion to develop (DiMasi et al. 2016) and can take at least 10 years (NCCIH 2015). Local communities and partnering countries need to be aware that there is no guarantee that a blockbuster drug will be discovered from the partnership. On the other hand, researchers face expectations for publication and revenue generation from their employers. Provisions for conservation and benefit sharing at the initiation of the project can protect against pressure-causing parties to default on vague promises or act on unfounded fears.

Disparities in expectations can arise from differences in the information to which each party has access. Countries supplying natural products may not know how much the products are worth and overestimate their value. They also may not be able to follow the product throughout the research process and therefore enact stringent access regulations. Researchers may distrust their in-country partners since the quality of the natural products may not be transparent and they may not know how their benefits are being utilized (Richerzhagen 2011). Maintaining realistic expectations is difficult but necessary in a field of uncertainty and diversely specialized players.

2.4 Challenges in Bioprospecting

Though there is much promise in bioprospecting for the discovery of novel compounds and uses, there are significant hurdles to overcome in the process. Strides have been made toward improving access and benefit sharing, but the potential ben-

efit of these agreements is still greatly untapped. An interdisciplinary approach may be necessary to harness the potential of these regulatory environments to better serve all parties.

2.4.1 Biopiracy

The fine line between bioprospecting and biopiracy has been a constant debate despite regulations (Rose et al. 2012). The term "biopiracy" is credited to the Rural Advancement Foundation International (RAFI) and generally refers to the use of intellectual property systems to legitimize the exclusive ownership over biological resources and processes that have been historically used in nonindustrialized nations (RAFI 1993).

International cooperative biodiversity groups (ICBGs) were developed in 1991 to be the model for cooperation between drug discovery and protection of knowledge. They supported the ideals of prior informed consent, benefit sharing, local infrastructure development, and biodiversity management (Rosenthal 1997). RAFI's critiques, along with protests from COMPITCH, the State Council of Organizations of Indigenous Traditional Healers and Midwives, against ICBG-Maya caused the project to fold in 2001 (ETC 2001). Critiques also came from the International Society of Ethnobiology for failure to achieve adequate consent and debate ensued over the criteria of such consent. The conflict that arose over ICBG Maya reveals the complexities of remaining an unbiased researcher while navigating a the historical and political landscape as well as managing external pressures and competing voices (Hayden 2003). This case highlights how the very fine line between biopiracy and bioprospecting is subject to interpretation by different participating members.

Biopiracy is still a major concern, though. At a 2002 meeting in Cancun, 17 member nations of Like-Minded Megadiverse Countries made it a priority to ensure the careful protection of Latin America's and South America's unparalleled diversity of plants. Commenting on the status of bioprospecting in this region, Fernando Quezada, Consultant of the Sustainable Development and Human Settlements Division of the Economic Commission for Latin America and the Caribbean, acknowledged that the megadiverse countries have a fear of being taken advantage of and this has resulted in a hindrance to drug development (Quezada 2007).

Stringent legislation regarding bioprospecting stems partly from fear of biopiracy and exploitation of endangered species. This fear is not unfounded for cases such as Brazil where the illegal trade of wildlife is a massively profitable and rampant industry (Rocha 2003). The illegal trade is detrimental to biodiversity and to indigenous communities as protocols for benefit sharing and conservation are not adhered to.

Though the CBD established states' control over access to their genetic resources, these agreements will require careful planning (UN 1992). The Nagoya Protocol combats this problem of biopiracy by providing more specific international require-

ments for research endeavors. Promoting transparency, ensuring accountability, and establishing legal frameworks will need to occur in order for adherence to this protocol (Kursar 2011).

2.4.2 Conservation

The Nagoya Protocol encourages conservation efforts when bioprospecting. Enforcement of these measures is crucial as nonadherence can have devastating effects. For instance, it was found that parts of Bushman's hat (*Hoodia gordonii* (Masson) Sweet ex Decne., Apocynaceae) have dramatic appetite-suppressant effects. The San people, an indigenous group in South Africa, used the plant to suppress appetite while hunting. Phytopharm filed for patents on *H. gordonii* active components; however, this was done without prior informed consent or benefit sharing with the San people. The South African Council for Scientific and Industrial Research intervened to establish an agreement between the two parties (Robinson 2010). Though it is not currently marketed as a pharmaceutical, the publicity generated led to its collection and marketing as a health food supplement by other companies not a part of the agreement (Wynberg et al. 2009). Now, *H. gordonii* is included in Appendix II of the Convention on International Trade and Endangered Species of Wild Fauna and Flora to protect it from unsustainable collection (CITES n.d.). This case highlights the importance of up-front consent and benefit sharing and the importance of ensuring biodiversity within those agreements.

Furthermore, the World Health Organization issued regulations on the collection of medicinal plants for the safety of the species and the users. The regulations prevent overharvesting by prohibiting collection of plants that are scarce and mandating that the source country ensure that the plants do not become endangered. Further, these regulations ensure that plants are identified correctly and are not exposed to large amounts of pesticides or chemicals (WHO 2003). Adherence to the Nagoya Protocol and WHO policies is one step toward the prevention of the detrimental effects of the commercialization of medicinal plants, as seen with the collection of *H. gordonii*.

2.4.3 Sharing of Intellectual Property

There are two intellectual property components at stake—the property of the local communities that provide ethnobotanical knowledge and the property of the researchers that develop the plant extracts into single-compound pharmaceuticals or refined formulations of botanical drugs. Without protected intellectual property, the economic incentive for developing pharmaceuticals could diminish for all parties involved. However, the intellectual property used in the discovery process must be shared respectively among all parties involved in the development of the pharma-

ceutical and contracts must be negotiated fairly and clearly across national and political boundaries. Tangible benefits of sharing intellectual property can take various forms and will be discussed in the future direction portion of this chapter.

There are inherent general challenges when sharing intellectual property. First, determination of who is the rightful owner of the ethnobotanical knowledge can be difficult. Different communities may have similar ethnobotanical knowledge of how to use a plant for medicinal purposes. Determining who should be given benefits and a share in the intellectual property is challenging and is best determined early on in the process of bioprospecting. Researchers have used a variety of definitions to guide them. In ICBG-Peru, it was discussed that all Aguaruna communities would receive long-term benefits and those directly involved with research would receive more immediate benefits. Sometimes, royalties are shared among all communities a company has worked with, a stance taken by Shaman Pharmaceuticals (King 1994; Rosenthal 1997). Naming individuals on patents is a more guaranteed way to ensure that benefits are distributed to locals, but such individual inventors must be defined (USPAT 2015).

2.4.4 Access to Resources

Before the bioprospecting research even begins, there are hurdles to overcome, specifically in obtaining permits to access the plants of interest. Access permits are administered by each individual country. These permits are difficult to obtain in certain countries, to the extent that researchers have been dissuaded from collecting in some regions. For example, Brazil has a multitude of agencies with the power to approve a permit (Silva and Espindola 2011), rendering the permit process unclear and decentralized (Danley 2011). Additional permits may also be needed after access is granted. Permits may be needed to export materials from the country and for additional uses than originally intended (Medaglia and Silva 2007; Silva and Espindola 2011).

2.5 Future Directions in Bioprospecting

The future of bioprospecting will necessitate addressing some of the challenges associated with bioprospecting, especially regarding adequate access and benefit-sharing agreements. To accomplish this, several models of access and benefit sharing have been employed around the world. However, there is no streamlined process for formulating these complex agreements. Ensuring that interdisciplinary players and representatives of all parties are party to forming these agreements can help mitigate some of the difficulties of bioprospecting.

2.5.1 Access and Benefit Sharing

A practical way to ensure protection of intellectual property of host countries is through adequate access and benefit sharing. Though access and benefit sharing have been required by the Nagoya Protocol, there is much room for interpretation. The benefits have taken a variety of forms suited to the project's capabilities and the communities' needs.

Benefit sharing can take two forms: monetary or nonmonetary. For monetary benefit sharing, different payment schedules can be devised, including payments for the plants collected, research using the plants, and royalties from any product developed. Advance payments can also be used, as in the form of trust funds, which can help communities up front during the long drug discovery process (Guerin-McManus et al. 2002; Rosenthal 1997). Patents may provide some monetary benefits, but are generally not considered the best mechanism for benefit sharing with local communities (Greaves 1994) and require that all parties to receive monetary benefit be individually named on the patent for effective distribution of monetary compensation (Rose et al. 2012).

Access and benefit sharing do not solely imply monetary compensation for gains from a blockbuster drug. Monetary compensation may never be a reality as many potential pharmaceuticals fail clinical trials, and many products collected and tested never make it past the laboratory bench. Access and benefit sharing require open communication with the host country and groups throughout the process.

Perhaps equally constructive for local communities are nonmonetary benefits. Maintaining close connections with the host country and the local communities may help mitigate some of uncertainty of loose ties between parties in the lengthy research and development process. These benefits may be in the form of training for the project, capacity building, commitment to research local diseases, or provision for conservation needs. Several ICBGs enacted advance payments for conservation and development projects. In fact, each ICBG had elements of capacity building in the form of community health clinics, herbaria, or equipment for research (Rosenthal 1997).

Collaborations with host country universities can support worldwide research connections. For example, the National Cancer Institute established a partnership with a Panamanian research institute to build local capacity and relations (Rose et al. 2012). Additionally, researchers can assist in the preservation of knowledge by reporting back collected data in a manner that is accessible to the local communities. For instance, books in the local language recording uses of local plants can be gifted to the community. Lastly, there is an untapped market for the pharmaceutical industry to contribute to combating diseases that primarily affect the source countries of their partnerships (Rose et al. 2012). Encouraging pharmaceutical companies to invest in those disease areas could increase the industry's involvement in the neglected disease sector.

Outside organizations such as governmental institutions or NGOs may be necessary to ensure conservation of natural resources long term. Benefits may be given to

the government or NGO as well as in the case of Suriname ICBG and the African ICBG, respectively (Rosenthal 1997). To further ensure adequate benefit sharing, local organizations can empower local communities to become knowledgeable about the contracts they are signing.

Benefit sharing is often undefined, causing remunerations to be forgotten or lost in the discovery process. Because research is often not carried out by the same people for the entirety of the project, original informants or terms of agreement may be ignored (Rose et al. 2012). Further, as stated earlier, determining the recipient of such benefits is important and can take several forms.

2.5.2 The Role of Ethnobotanists

Ethnobotanists provide a crucial link between the protection of indigenous knowledge and the advancement of scientific discoveries due to their close relationship with communities and their knowledge of botany. Ethnobotanists are those generally equipped with botanical and anthropological knowledge, though there are few degree-awarding programs in ethnobotany. While ethnobotanists may seem like the perfect marriage of anthropology and botany, they are still an underutilized source for medicinal plant collections. In a 2010 editorial, the Journal of Ethnopharmacology highlighted the underutilization of interdisciplinary research, though it is a popular buzzword (Heinrich 2001).

Ethnobotanists have the skillset to address the social aspect as well as the botanical aspect of bioprospecting. For example, it is important to address the distribution of botanical knowledge and whether the knowledge differs between lay person and healer in order to correctly choose informants for the project. Knowledge of techniques such as cultural consensus analysis, an anthropological technique to determine the accurate descriptions of local knowledge, can be employed in determining the trustworthiness of information from single informants. Further, a researcher who knows how to correctly collect anthropological data will be better suited to classify illnesses where terminology may differ from Western medicine (Reyes-García 2010).

Botanical knowledge of an ethnobotanist is vital as well since the researcher must have the skills to correctly prepare a botanical voucher and obtain the necessary information on how a plant is prepared for usage (Elvin-Lewis 2011). The ability to integrate anthropological and botanical knowledge will not only provide a robust study of medicinal plants, but also build strong relationships with the community.

Furthermore, registering knowledge in a database may be useful for communities who wish to provide regulated access to their knowledge for data mining of the information collected by researchers (Ningthoujam et al. 2012). Ethnobotanists would be a vital asset in helping to set up such databases in a fashion suited for the community. Countries have already begun to establish databases, but some have expressed concerns about their release until there is a system of international

protection for the information (Elvin-Lewis 2007). The regulation of such databases will be further discussed in the following section.

2.5.3 A Vision for an Interdisciplinary Bioprospecting Strategy

The crux of successful bioprospecting hinges on access and benefit sharing between all parties involved. Partnerships between industrialized and developing nations through bioprospecting have the potential to play a vital role in fostering trust and teamwork. Bioprospectors and local communities both recognize the value in the natural world and can unite around their common respect for nature and its potential to fight illness. Few areas of science incorporate both the traditional and modern views of healing and this can be a major point of dialogue.

These terms of benefit sharing must be clearly delineated before research has begun. While there may not be a standard template for all bioprospecting endeavors, flexibility will allow each project to reach terms that are mutually agreeable to the parties involved. The terms must include provisions for plasticity if needed (Rosenthal 1997). This may necessitate the involvement of an unbiased third party to negotiate the terms. Ethnobotanists could be an important part of that team since they are familiar with the challenges of obtaining knowledge and protecting it.

Public registration of traditional knowledge in databases would allow for large searches for information; however, this would strip the knowledge of some ability to generate revenue. If the knowledge is available to all, it also becomes prior art and cannot be patented according to US law (35 U.S.C. § 102). However, much of the medicinal plant knowledge today is already public. On the other hand, databases with selective access may allow communities to generate revenue from access fees imposed on knowledge for research (Elvin-Lewis 2007). Additionally, knowledge from multiple communities could be registered in a database in which communities only have access to their own information. When there is a question as to whether certain information is unique to a community, software may be used to search the whole database for similar information (Sampath 2005). Elvin-Lewis describes the complexities of establishing databases and defining ownership (Elvin-Lewis 2007).

In an ideal world, a third-party or academic organization would establish an independent organization that could negotiate between local communities, their databases, researchers, and governments (Fig. 2.2). This would ensure adequate benefit sharing, minimizing lawsuits post-discovery. This organization would compile a regulatory board of reviewers for each bioprospecting venture. The board could be based on permanent or long-term representatives from academia representing the areas of anthropology, ethnobotany, law, and basic science. Academics are in a unique position to help subdue biases of supplier countries and researchers, with their general commitment to furthering of scientific knowledge and access to disciplines that work closely with local communities. Others on the board would change for each case, with the minimum following categories being represented: a pharmaceutical company representative, a source country governmental

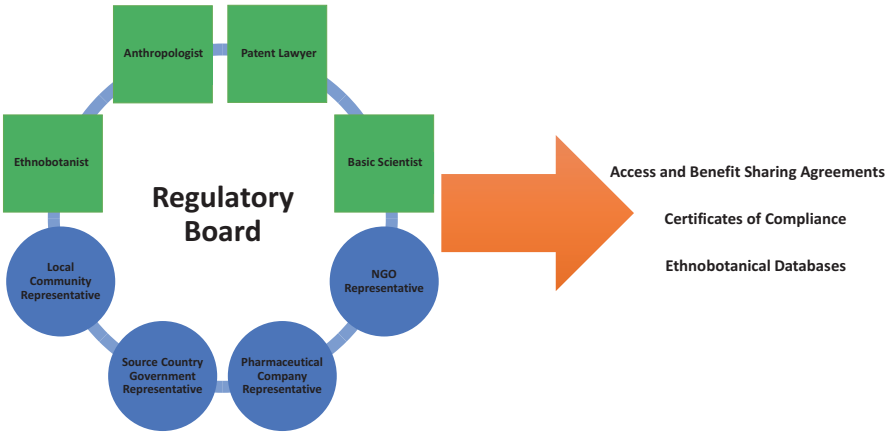

Fig. 2.2 Model for a third-party regulatory board. The board would serve to check the interests and biases of other board members while taking into account each member's expertise. Issues and pressures would be brought to the board to protect the active participants in the agreements. Green squares: long-term members. Blue circles: project-specific members

representative, and a local community representative. NGOs supporting the rights of local communities may be involved as well. The long-term members should be trained in the intricacies of bioprospecting negotiations.

Importantly, this third-party organization could supply certificates of compliance (Richerzhagen 2011) to each party, certifying that each has met the standards of the CBD and Nagoya Protocol. This international indicator of compliance would diminish some of the mistrust and increase transparency in negotiations between parties involved.

Ethnobotanists on the team could assist communities in establishing databases of their traditional knowledge since there is danger of its loss through generations (Benz et al. 2000; Krauss 1992; Ramirez 2007; Reyes-García et al. 2013). They would also be in a position to encourage governments to conserve their biodiversity. When negotiations are made, benefits could be given to the local people and to the governments, to avoid dissemination of benefits through corrupt governmental infrastructure.

The organization could be the mediator between researchers and local databases of knowledge so that local people can effectively capitalize on their knowledge while avoiding pressures from large pharmaceutical companies. It is imperative that local communities and their advocates be vital members of any discussions.

A third party would allow for centralization of negotiations. Many projects have received criticism for failing to interact with communities or provide adequate benefits. Further, researchers have become leery of the difficult legal processes involved in each country. This would take some burden off the researchers and communities and allow centralization of regulatory processes.

2.6 Conclusion

If bioprospecting practices are not established in an easy-to-navigate and enforceable format, they will continue to be circumvented for exploitation of local communities and the environment. Ethical players will be discouraged from entering the bioprospecting business.

Though there is much room for improvement, it is clear that nature's chemistry is complex and rich in diversity and should be utilized to maximize its benefits, especially for those that already use natural products for treatment. This area of scientific endeavor has the opportunity to unite efforts across economic borders if this turbulent area of work can be navigated and result in the centralization of negotiations of individualized agreements that adhere to the ethical and equitable principles of the Convention on Biological Diversity and Nagoya Protocol.

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Chapter 3
Nepal: A Global Hotspot for Medicinal Orchids

Brajesh Nanda Vaidya

3.1 Introduction

Throughout the human history, there has been fascination towards orchids, and this is primarily due to attractive flowers, challenges in cultivation, and the therapeutic qualities that it carries as potent medicinal plant. Orchids are one of the most traded and cultivated plants in the world because of its geographical and taxonomic diversity (Hinsley et al. 2017). Not only they are known for their horticultural and cut flower trade, many orchid species have been used all over the world for their medicinal properties using traditional knowledge. Ancient ethnobotanical practices like *Ayurveda* and Traditional Chinese Medicine (TCM) have used orchids for their therapeutic properties. It is a well-established fact that the orchids contain various phytochemicals such as alkaloids, flavonoids and glycosides that make them medicinally important. Numerous controlled preclinical studies on laboratory animals establish the efficacy of the active ingredients present in many orchids (Bulpitt et al. 2007). Throughout the world, orchids have been used as medicine.

In this chapter, while the importance of medicinal orchids of Nepal is discussed and described, it is worthwhile to touch upon orchid usage by various ethnic systems all over the world. Much of the information on ethnomedicinal uses by native tribes was passed on through generations by word of mouth. The Native Americans used different orchid species, mostly terrestrial, to treat many ailments ranging from convulsion to urinary problems. These tribes, native to Alaska to southern tip of Florida, used local plants and plant parts including orchids for ethnobotanical and ethnomedicinal uses (Moerman 1998; Wilson 2007). The details of species, their vernacular English names, tribes that use them, and parts that are used, are presented in Table 3.1. Roughly about 21 species from North America have been cataloged so far

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Table 3.1 Orchids species used by Native Americans (Moerman 1998; Wilson 2007)

#	Scientific name	English name	Native tribes	Uses	Plant part and their use
1	*Calypso bulbosa*	Fairyslipper orchid	Nlaka'pamux	Anticonvulsive	Orchid bulbs are chewed, and flowers are sucked for mild epilepsy
2	*Coeloglossum viride*	Bracted orchid/ large-bracted orchid	Iroquois	Gynecological aid	Used in removing placenta after childbirth as decoction mixed with other plants
			Ojibwa	Love medicine	Roots used as aphrodisiac
3	*Cypripedium sp.*	Lady's slipper orchid	Cherokees, Iroquois, Menominee, MicMac, Penobscot	For insomnia, anxiety, fever, headache, neuralgia, emotional tension, palpitations, tremors, irritable bowel syndrome, delirium, convulsions and to ease menstruation and childbirth pain	Root powder is prepared as tincture
4	*Epipactis gigantea*	Stream orchid, Giant Helleborine	Navajo	Ceremonial medicine	Used in puberty rites for girls, general body disease
			Kayenta	Pediatric aid	New born infants are purified using the plant
5	*Habenaria bracteata*		Ojibwa	Love charm	Whole plants used
6	*Habenaria odontopetala*	Longspur orchid	Seminole	Strengthener	Used in making body strong
7	*Liparis loeselii*	Yellow widelip orchid	Cherokee	Urinary problem	Roots infusion to fix urinary problem
8	*Malaxis uniflora*	Green adder's mouth orchid	Ojibwa	Diuretic	Roots are used for diuretic compounds
9	*Piperia elegans*	Hillside bog orchid	Pomo, Kashaya	Food	Bulbs are baked as food
10	*Piperia unalascensis*	Alaska rein orchid	Pomo, Kashaya	Food	Bulbs are baked as food
11	*Piperia* sp.		Mahuna	Dermatological aid, eye medicine, snakebite	Whole plant used for black widow spider and rattle snakebite. Also used for eye trachoma.

(continued)

12	*Plantanthera ciliaris*	Yellow-fringed orchid	Cherokee	Analgesic, antidiarrheal	Infusion (cold) used in headache. Root infusion is taken to stop diarrhea
			Seminole	Snakebite	Roots are used
13	*Plantanthera dilatata*	Rein orchid	Micmac	Urinary aid	Root juice is used
			Shuswap	Poison	Leaves are poisonous
14	*Plantanthera grandiflora*	Greater purple-fringed orchid	Iroquois	Protection	Root decoctions are used to ward off evil
15	*Plantanthera leucostachys*	Bog orchid	Nlaka'pamux	Analgesic, disinfectant, orthopedic aid	Plant used against rheumatism, joint and muscle aches; whole plant is used after sweat bath to disinfect, used in sweat bath for sprain, stiff and aching muscles and joints
16	*P. x media*	Intermediate bog orchid	Potawatomi	Love medicine	
17	*Platanthera orbiculata*	Round-leaved orchid	Iroquois	Dermatological aid, tuberculosis	Leaf poultice used on scrofula and cut sores
			Montagnais	Dermatological aid	Leaf poultice to treat blisters on feet and hand
18	*Platanthera psycodes*	Lesser purple-fringed orchid	Iroquois	Analgesic, gynecological and pediatric aid	Root infusion is given to children for cramps; root infusion taken as a parturition medicine
19	*Platanthera sparsiflora*	Bog orchid	San Felipe	Food	Used as food during food shortage
20	*Platanthera stricta*	Bog orchid/Modoc bog orchid	Kwakiutl	Love medicine	Used as love charm
21	*Zeuxine strateumatica*	Soldier's orchid	Seminole	Gynecological, reproductive aid	Used against impotency

that have been in use by the tribes (Moerman 1998; Wilson 2007). Some orchids are used as substitute for food during scarcity; either specific parts, such as leaf and roots, or whole plants are used as cold infusion or decoctions.

3.1.1 General Botany

Orchids (Family *Orchidaceae*) constitute one of the largest families of flowering plants, comprising 28,484 species and 763 genera spread all over the world (Govaerts et al. 2017). It is considered as one of the most evolved monocot families within the superorder *Liliiflorae* (Dresseler 1982). These are herbaceous perennials that grow as shrubs, and vines. Orchids generally have three types of growths, monopodial, sympodial, and diapodial (Dresseler 1982). The monopodial growth has the main axis growing with a single shoot apex; in sympodial growth, the main axis terminates, but horizontal growth continues in forming successive bulbs; and the diapodial growth is very much like sympodial, but without the bulbs. Some of the stems are modified into rhizomes (except in monopodial) and pseudobulbs. The pseudobulbs have different functions, one of them being moisture conservation and act as water reserve. Most of the flowers in this family have radial symmetry, fused stamen, and cymose inflorescence. The most striking features of the flower are that the stamens are symmetrically arranged on one side of the flower, have one fertile stamen, one fused structure for pistil and stamen, and a petal is modified into a labellum. The flowers in *Orchidaceae* contain three alternating petals and three sepals (trimerous), and an unpaired petal modified into a labellum or lip, an adaptation for the insects to facilitate pollination. The pistil and the stamen are fused into a distinct modification to make a single reproductive organ called gynostemium (Arditti 1992). The pollinia (mass of pollen grains attached together by an adhesive substance called viscin) are stored at the tip of the gynostemium beneath rostellum (a tissue separating the stigma and the anther) near the stigmatic surface. Pollinia are transported by insect vectors from one flower to another while visiting for nectar secreted by flowers, aiding in cross-pollination (Darwin 1862). Once the flower is fertilized, a thousand to four million minute seeds known as microspermae are formed and stored within the seed capsules. These microspermae consist of simple undifferentiated embryo inside a transparent integument without an endosperm (Banarji 1978). Survivability of seeds in nature is low after the mature pods dehisce, but symbiotic mycorrhizal association and in vitro tissue culture can ensure higher percentage of germination (Arditti 1992, 1993). All orchid blooms go through the process of resupination. Many of the species drop their leaves and go through dormancy when the conditions are unfavorable for growth such as in winter.

3.1.2 Why Orchids of Nepal?

There are many scientific and nonscientific publications covering various facades of medicinal orchids of Nepal. This chapter is probably the first attempt to encompass, in one place, the history, ethnobotany, scientific validations, and local uses that have been passed on for generations.

Current work documents 130 orchid species, extending an earlier list of 90 (Pant 2013) that are used as traditional medicine in Nepal and Asia. Out of these, 34 species have been tested in laboratory for the bioactive compounds and their therapeutic potential so far. Phytochemicals such as terpenoids, stilbenoids, flavonoids, saponins, tannins, steroids, mucilage, starch, glucoside, amino acids, carbohydrates, and coumarins are present in orchid tissues. These compounds are known to have healing properties and may have synergistic activities when mixed or taken with other bioactive compounds (Teoh 2016). Extracts derived from orchids growing in Nepal have shown positive results against specific bacteria and fungi (Marasini and Joshi 2012; Rashmi et al. 2015; Shweta et al. 2015; Paul et al. 2013; Bhatnagar and Ghosal 2018).

3.2 Orchids in Written History

Earliest written records of orchids as medicine are from China (Bulpitt 2005). Chinese medicine is based on the concept of Yin and Yang, balancing them in the body is a way of using preventative medicine. The Chinese philosopher Confucius described orchids as the king of fragrant plants. There is a written record of orchids [known as Lan (兰) in Chinese] in the TCM from eighth century BCE (Bulpitt et al. 2007). Chinese folklore mentioned the term *Shên-nung* in *Materia Medica* describing orchids such as *Bletilla striata* and *Dendrobium* species. In the year 1233, *Chao Shig-Kêng* wrote detailed description of twenty orchids, and in 1247, a book called *Lan Pu* (*Treatise on Chinese Orchids*) written and published by *Wang Kuei-hsueh* described 37 orchid species (Berliocchi 2004). Three species found in Nepal that are used heavily in TCM are *Shi-Hu* (various species of *Dendrobium*), *Tian-Ma* (*Gastrodia elata*), and *Shan-Ci-Gu* (*Cremastra appendiculata*), though other species were also used as replacement in the *Materia Medica* of *Shen Nong* (Teoh 2016).

The earliest written record of orchid is in the Book of Songs (ca. tenth to sixth century BCE) in China (Arditti 1992). Confucius mentioned *Che Lan* (Cymbidium) in his book around 500 BCE (Bulpitt 2005). The scientific research on orchids began when in 1753, Swedish botanist Carl Linnaeus adopted the name orchid in his famous book *Species Plantarum* but the family was named *Orchidaceae* only in 1836, by John Lindley, a British botanist. Even before then, the Dutch explorer Georg Eberhard Rumphius described orchids in his books *Herbarium Amboinense* written during 1660–1690, comprising description of 1300 flowering plants mostly from Southeast Asia. He had two volumes out of twelve dedicated only to the orchids (Baas and Veldkamp 2013). In seventeenth century, Jacob Breynius, a Polish naturalist, wrote *Exorticum aliarum que minus cognitium plantarum*, which detailed orchids.

3.3 Orchid in Mythologies and Mysteries in Various Cultures

In one of the chapters of the ancient Hindu epic the *Ramayana*, a description of Lord Rama's wife Sita mentions orchid flowers adorning her hair. The ancient Greek believed that the orchid bulbs had magical power that did extraordinary

things to the human body. The orchid flower in English is sometimes called Satyrion, as orchid plants were thought to be originated from the semen of Satyrs, a mythical creature in ancient Greece and Rome. Medieval European herbalists knew that orchids had medicinal properties but thought that orchids grew out of the semen dropped from birds and beasts and therefore used for potions with aphrodisiac properties. Even until 1600s, it was believed that orchids rose from animal semen (Arditti 1992).

3.4 Etymology and Origin of Word for Orchid in English and Nepali

The Greek philosopher Theophrastus (372–286 BCE) coined the word *Orchis* or *Orkhis* (Barnette 1992). The terrestrial orchids have underground tubers that have resemblance to a pair of testicles [*Órchis* (όρχις)] is the Greek word for testicles). The origin of the word "*orkhis*" is thought to be from Indo-European root: *ERGH*, which means to mount (Barnette 1992).

The vernacular Nepali name for orchid is *Sunakhari* (सुनाखरी) that has *Sanskrit* origin. *Sunakhari* is composed of two separate words, *SÚn* and *Khari*, where *SÚn* means gold, and *Khari* denotes a fodder and timber tree (European Nettle tree; *Celtis australis* L.) found at 500–2400 m ASL (Campbell 1983). As per the Nepali dictionary, *Nepali Birhat Sabdakosh* (Anonymous 1983), *Sunakhari* has two meanings: (1) a small type of yellow flowering weed found on *Khari* tree and (2) a type of flora growing on tree crevices with multitude of colors. For the first meaning, it is possible that a common orchid of mid-hills in Nepal, *Dendrobium densiflorum* is described and presented as orchid in general, since it has bright golden yellow-colored flower. The second descriptions for orchids are due to the fact that in many places different colored orchid species blooms are prominent in trunks and tree crevices. Though *D. densiflorum* has also been referred to as *Sungava* in Nepali and it should be noted that both common vernacular names, *Sunakhari* and *Sungava* are exchanged and overlap when orchids are described. There are many other vernacular and indigenous tribal common names for orchids in Nepal such as *Bankera*, *Jiwanti*, *Thurjo* and *Thur* depending on the species and the location where they are found (Subedi et al. 2013; da Silva and Acharya 2014). A detailed list of vernacular names is presented in the Table 3.3.

3.5 Flowering Plants and Orchids in Nepal

Nepal has high biological diversity and is considered as an ecological and biodiversity hotspot due to varied physiographic zones and phytogeographic regions (GoN 2008). The flowering plants from Nepal consist of 6501 taxa with 5636 species, 206 subspecies 599 varieties and 60 forma (DPR 2001). Out of these plants, 396 species

are endemic to Nepal. Most of the plant species are used in traditional medicine in different forms such as in raw paste, dried powder, mixed decoction and raw juice from fresh and dried plants.

According to a recent report Nepal has 107 genera with 451 species of orchids (Rajbhandary 2015). An updated online list mentions 481 species of orchids from Nepal (Acharya 2018). To understand the diversity of orchids found in Nepal, one must understand natural vegetation and climatic conditions that change in different altitudinal range. Banarji (1978) defined Nepal's phytogeography in relation to the distribution of orchids. Entire area is divided into Western Nepal, Central Nepal, and Eastern Nepal. Based upon this phytogeographical division, distribution of orchids was studied (Banarji 1978). It was interesting to note that, there were 55 species that were restricted to Central Nepal, 49 species restricted to East Nepal, 19 species extended from West to Central Nepal, and 24 species were distributed all over the Nepal, West to the East (Banerji and Pradhan 1984). Eastern Nepal has abundance of epiphytic and terrestrial orchids and there is gradual decline in the number of both types of orchid species when we move westward (86°E to 83°E) due to drier conditions. Moving west of 83°E, there is a drastic decline in the number of epiphytic orchid species, although the decline in terrestrial orchids is not as extreme. Moreover, there are species with a wide distribution within various altitudinal ranges, while some other species have a limited distribution; the distribution pattern of orchids ranges from subtropical to warm temperate and finally alpine when we move from south to north Nepal (Vaidya et al. 2002).

3.6 Early Botanical Expeditions and Work on Orchid in Nepal

Francis Buchanan-Hamilton, a Scottish medical doctor, employed by the East India Company of British Raj (Empire) visited the Kingdom of Nepal in 1802 with the Royal British mission of Captain W.O. Knox (Hara et al. 1978). He was the first westerner to describe orchids and other flora from Nepal (mostly in Kathmandu valley and adjoining places) using John Lindley's nomenclature. The first recorded botanical exploration to Nepal was by Hamilton and Captain Charles Crawford from 1807 to 1814 (Sutton 1978). Hamilton mentions that most of the plants from alpine region were collected for him by the locals, due to travel restrictions imposed on outsiders by the Nepalese government (Hamilton 1819).

In 1824, David Don and Hamilton, as members of Linnaean Society, published *Prodromus Florae Nepalensis* in London which they compiled Francis Hamilton's (Buchanan) work from 1802 to 1803. This was the beginning of orchid study in Nepal with a detailed description of 51 species (Hamilton and Don 1824). Nathaniel Wallich, a British naturalist, was in Nepal from December 1820 to November 1821 and published *Tentamen Florae Nepalensis* (1824), describing many orchids. Sir Joseph Dalton Hooker, one of the famous explorers and an avid plant collector, studied flora of Tamur valley in Eastern Nepal in the year 1848. He later became the

director of Royal Botanic Garden, Kew and contributed to describing the orchid flora of Nepal. It is interesting to note that during that period, in many occasions, orchids were described as parasitic plants growing on tree trunks (Hooker 1855). Schlangintweit brothers in 1857, J. Scully in 1876, and I. H. Burkill in 1907 published reports on flora including orchids found in Nepal (Banerji and Pradhan 1984). *Landon's Nepal* mentioned 148 orchid species (Landon 1928). Orchids of Nepal (Banerji and Pradhan 1984) became the first book fully dedicated to orchids, based on the research he carried out during 1969–1975, describing 194 species (Banarji 1978). Soon after, orchid flora with complete enumeration and a key was published using herbarium specimens (Banerji and Pradhan 1984).

3.7 Ethnobotany and Medicinal Orchids of Nepal

The presence of phytochemical compounds such as alkaloids, flavonoids and glycosides in orchid make it an important medicinal herb. Acharya and Rokaya in 2010 reported 82 orchid species in Nepal with medicinal properties. As per Pant and Raskoti (2013) and Pant (2013), 90 orchid species from Nepal contain medicinal properties and Rokaya et al. (2013a, b) listed 16 orchid species that were used specifically to treat gastrointestinal disorders as part of traditional medicine.

In Nepal, the Tibetan medicine men (*Amjis*), or priests (*Lamas*), *Newar* medicine men (*Vaidyas* and *Guvajus*), *Tamang* medicine men (*Lamas*) and healers (*Jhankri*), and *Tharu* priests (*Guraun*) are among those who use wild plants in their medicine making. These medicine men include orchids in their medicinal concoction (Pohle 1990). The number of ethnic groups using orchids as medicine is not known but it is estimated that almost all ethnic groups (18 major ethnic groups and many small tribal groups) of Nepal use them in one way or the other (Pant 2013). Depending on the type and availability, orchids are used in minute amounts, as major components or substituted with closely related species. Some of the orchid species that are used in traditional medicine in Nepal are described below:

Coelogyne flaccida Lindl.: Pseudobulb juice/paste is used to treat headache and indigestion (Manandhar 2002). The pseudobulb paste of *C. stricta* (D. Don) is applied on forehead to reduce headache and fever (Baral and Kurmi 2006). The pseudobulb paste and juice of *C. fuscens* Lindl. are used for abdominal pain and skin burns (Pant and Raskoti 2013).

Cymbidium aloifolium (L.) Sw.: *Cymbidium* species exhibit purgative and emetic properties (Dash et al. 2008). The leaves and pseudobulbs have been used as tonic, and leaf/pseudobulb paste is topically applied on bone fractures and dislocations. Inflammations including fever are also treated using orchid leaves in traditional medicine. Juice extracted from the leaves of *C. iridoides* is applied to the wounds and cuts to prevent blood loss (Subedi 2002).

Dactylorhiza hatagirea (D. Don) Soo: This highly sought-after and valuable terrestrial orchid is found in the higher reaches of Western Nepal (2800–3400 m ASL). It has been extensively used in *Ayurveda* medicine to treat many conditions rang-

ing from diarrhea to strengthening of uterus (Kizu et al. 1999). In Middle East and Turkey, a beverage made from *D. hatagirea* tuber extracts called *Salep* is known to have aphrodisiac properties and used as a sexual stimulant. The word *Salep* originates from the Arabic expression *ḥasyu al-tha'lab* (سحلب) or fox testicles, a graphic description of the appearance of orchid tubers. The ancient Romans used terrestrial orchid bulbs to make aphrodisiac drinks called *Satyrion* and *Priapiscus*.

Various species of *Dendrobium*: *Dendrobium candidum*: The pseudobulb pulp of this species is used to prevent pimples (Pant and Raskoti 2013). Dried pseudobulbs of *D. primulinum* are known to boost immune system (Pant and Thapa 2012). *Dendrobium macraei* is one of the notable *Dendrobium* that is used in *Ayurvedic* medicine. As per Khasin and Rao 1999, this *Dendrobium* species is used in medicine called *Jivanti* and has Jebantine alkaloid. The whole plant of *D. fibriatum* has been used for liver problem as well as for nervous breakdown (Baral and Kurmi 2006; Parmar and Acharya 2016).

Rhyncostylis retusa (L.) Blume: An ethnobotanical study conducted in Northeast India found that the roots of *R. retusa*, a species with wide distribution from the Central Himalayas, including Nepal, to Far East Asia (Philippines and Indonesia), are used for preparing medicines for rheumatism, asthma, tuberculosis, nervous twitching, cramps, infantile epilepsy, vertigo, palpitation, kidney stone, and menstrual disorders (Saklani and Jain 1994).

3.8 Orchids Are More than Medicine

Most of the orchid species that have been used in traditional medicines are edible. Orchids have been used as food, additive, flavoring agents, and herbal tea in many parts of the world (Singh et al. 2016). Orchids are taken orally in the form of powder, decoction, tincture, juice, either alone or mixed with other ingredients such as honey to make them more palatable. Salep, a drink made using *Orchis* and *Dactylorhiza* tuber powder, is popular in Turkey and in Middle East. Dried *Dendrobium* pseudobulbs are used as ingredient for Chinese tea which is said to improve eye health. *Dendrobium* flower (Shi hu 石斛) is used in many cuisines (China Tea Review 2012). In Bhutan, *Cymbidium hookerianum* is used in traditional dish called *Ola she* (ཨོལ་ཞེ) and *Ola choto* (ཨོལ་ཆོད) and the flowers impart characteristic bitterness to the dish (Thapa 2009).

In rural Nepal, orchids have been used by local people to prepare various dishes. The leaves of *Cypripedium cordigerum* and the leaves as well as shoot of *D. hatagirea* are eaten as vegetables (Raskoti 2009). On the other hand, pseudobulbs of *Coelogyne ovalis* and *Peristylus constrictus* are eaten to quench the thirst (Raskoti 2009). The *Chepang* community from Makawanpur district eat leaves of *Habenaria intermedia* and roots of *Epipactis royleana* either raw or after cooking. Similarly, boiled leaves and pseudobulbs of *Satyrium nepalensis* are eaten in the villages of western Nepal (Raskoti 2009).

3.9 Traditional Medical Systems in Nepal

There are four major Hindu scriptures or *Vedas*: the *Rigveda*, the *Yajurveda*, the *Samaveda*, and the *Atharvaveda*. "*Ayurveda*," known for its teachings in ancient medicine, falls under *Rigveda* and *Atharvaveda* with its center of origin in India more than 5000 years ago (Lad 1996). *Ayurvedic* teachings flourished in Indus Valley civilization around 3000–1500 BCE (Morningstar 1994). *Rig Veda* is considered about 4500 years old and 128 hymns describe 67 herbs with medicinal use (Malla and Shakya 1984). The first *Ayurvedic* text *Charaka Samhita* is thought be written two to four centuries before the birth of Christ (400–200 BCE) (Dash and Sharma 2015), and *Susrut Samhita* was written much later in Banaras, India (Rao 1985; Loukas et al. 2010). Besides, *Charaka* and *Susrut*, use of orchid as *Ayurvedic* medicine has been described in other ancient medical textbooks such as *Astanga Hridayam Samhita (*written by *Vagbhata)*, *Sarangadhara Samhita*, *Bhava Prakash Samhita*, and *Madhava Nidanam Samhita* (Kaushik 1983).

Ayurvedic medicines contain ingredients such as minerals, powder of precious and semi-precious stones, and ash residue of these materials after incineration, in addition to the plants that have healing properties. Many orchids have been used as ingredients in various formulations, including a popular tonic paste called *Chyawanprash*. *Chyawanprash* consists of eight ingredients known as *asthavargha*, and four of these are orchids. The orchids present in *Asthavargha* are: *Malaxis muscifera* (*Jivak*), *M. acuminata* (*Rishbhak*), *Habenaria intermedia* (*Riddhi*) and *H. edgeworthii* (*Vriddhi*) (Singh and Duggal 2009). A Sanskrit verse (*Shloka*) from *Nighantu* on *Vanda* describing its medicinal properties is presented in Fig. 3.2a. *Vanda* (Sanskrit *Banda*) in *Ayurveda* is known to alleviate *kapha* (watery bio-element) and cures *vāta rakta* (Gaut), *śopha* (Oedema), *vrana* (ulcer) *sula* (colic pain), and *udara* (obstinate abdominal diseases including ascites) and *visha* (poisoning) (Vaidya 1991). In Sanskrit nomenclature for *Vanda,* besides *Rāsnā*, many other synonyms have been used (Vaidya 1991). The taste for *Vanda* has been described as bitter, with heavy attributes and potency as hot. The specific action of this orchid as known in Sanskrit is *Amapacani* which means that with the help of which *āma* (undigested food) undergoes the process of cooking or digestion and then reduction of *Kapha* and *Vayu*. *Charaka Samhita* (volume 6, plate 40) describes vandoid group of orchids which are called *Ruha* and 36 Sanskrit synonyms have been assigned (Kaushik 1983). Fourteen major synonyms are described in Table 3.2. *Dendrobium macraei*, known as *Jivanti* in Sanskrit, has been used as a stimulant and a demulcent, and has properties of lowering blood pressure. *Vanda roxburghii* (Sanskrit: *Rāsnā*) is used as an anodyne, a carminative agent, an expectorant, and a nervine tonic, which stimulates cholinergic nerve endings, and it lowers blood pressure, reduces rheumatism, and works against syphilis and otitis media (Fig. 3.2b, c) (Karnick 1996).

The Traditional Tibetan Medical (TTM) system, also known as *Sowa-Rigpa* medicine is based on four *tantras* (*rgyud bzhi*) and is one of the oldest system comprising of holistic and naturopathy system (Kumar and Choedon 2013). Medicines consist of dried herbs, tree parts, and other metallic elements that are combined to create formulations for patients. Many of the high-altitude orchids have been

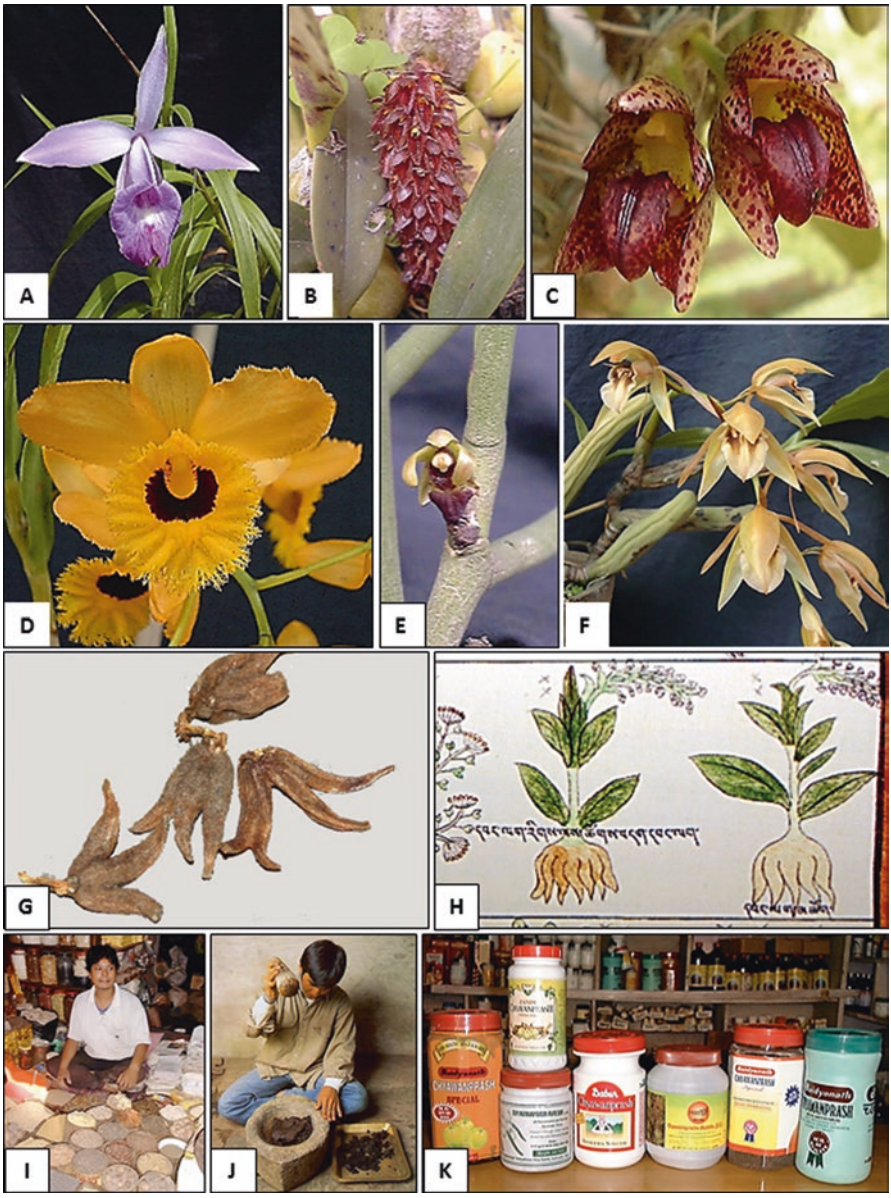

Fig. 3.1 (**a**) *Arundina graminifolia* flower. (**b**) *Bulbophyllum caryanum* flower. (**c**) *Bulbophyllum leopardinum* flower. (**d**) *Dendrobium fimbriatum* flower. (**e**) *Luisia trichorhiza* flower. (**f**) *Otochilus porrectus* flower. (**g**) Dried Dactylorhiza hatagirea tuber. (**h**) Dactylorhiza hatagirea tuber in Tibetan thangka painting describing its medicinal value. (**i**) Typical baniya shop selling medicinal plants including orchid parts. (**j**) Preparation of Ayurvedic medicine in a traditional method. (**k**) Different brands of Chyawanprash which uses orchid parts

Table 3.2 Specific description of orchids in Sanskrit with its literal translation

Sanskrit vernacular name	English	Description
गन्धमादनी	Ghandamadini	Extremely sweet smelling
परपुष्टा	Parapushtha	Brought up by somebody else
पराश्रया	Parashrata	Having taken shelter from someone (epiphytic)
वृक्षरूहा	Brichshyaruha	Grows on a tree
जीवन्तिका	Jivantika	Vitalizer that is pertaining to its medicinal value
शेखरा	Shekhara	Thick flower garland, pertaining to its inflorescence.
परवासिका	Parawasika	Inhabiting on someone else (epiphytic)
पाद्पररूहा	Padraparuha	Can climb a tree (epiphytic)
तरूरोहणी	Tarurohani	Capable of climbing a tree
केशरूपा	Keshrupa	Like a hair on the head. Pertaining to its orderly arranged leaves like two combed locks
तरूरूहा	Taruruha	Grass of a tree
कामीनी	Kamini	Extremely pleasing
वृक्षभक्षा	Brikshyabhachhya	That eats a tree
नीलभर्णा	Neelvarna	Pertaining to a blue color

The orchids with such physical properties are used in *Ayurveda* for their medicinal qualities (Kaushik 1983)

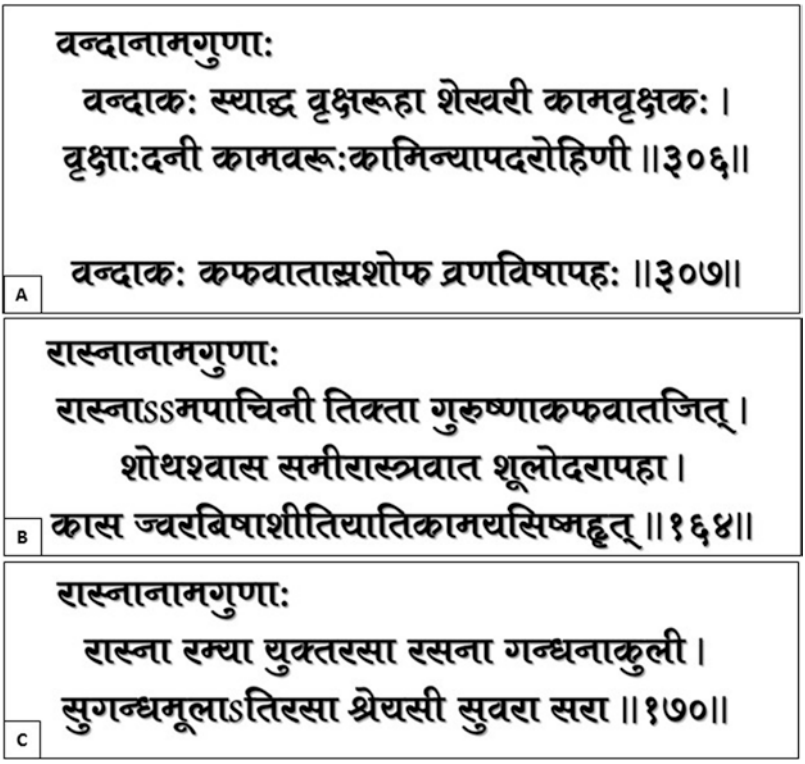

Fig. 3.2 (**a**) Sanskrit sloka describing medicinal properties of Vanda in *Ayurveda*. (**b**, **c**) Ancient Sanskrit verse from *Bhaba Prakasha Shamhita* describing usage of *Acampe papillosa* in *Ayurveda*

described in TTM (Mikage et al. 1988) and have also been depicted in traditional paintings called Thangka (Fig. 3.1h).

The *Amchis*, traditional Tibetan healers, mostly in the high mountainous regions of Nepal use fresh as well as dried plants/herb for medicinal concoction. Because of geographical location of Tibet and Tibetan plateau (just north of the Himalayan range), rainfall and forest vegetation is nonexistent. Therefore, terrestrial plants such as shrubs and small trees make up a major part of this landscape. Most of the area is covered by alpine tundra like vegetation or montane grassland while other parts are dry and barren steppe and meadows. Therefore, most of the orchids used in Tibetan medicine are the high-altitude terrestrial orchids and dried orchids imported from adjoining regions. One of the orchids that have been used extensively is *Dactylorhiza hatagirea*, locally known as *Wang-lak* (ཝང་ལག་) in Tibetan or *Panchaunle* in Nepali. This orchid, as mentioned in Tables 3.3 and 3.4 and in Fig. 3.1g, is known to be used against many different types of ailments and diseases. *Dendrobium moiliforme*, *pu-shel-rtse* (སྤུ་ཤེལ་རྩེ་) and *mun-se-shing* (སྨན་སེ་ཤིང་) *Gymnadenia conopsea*, *dbang-lag* (དབང་ལག་) are some of the orchids commonly used in TTM (Molvray 1988).

Though Traditional Nepali Medical System (TNMS) is not directly influenced by TCM, there are orchid species used in both Nepal and China with wide distribution and population in both countries. It is worthwhile to note that about 300 orchid species are used in TCM (Long and Long 2006), out of which, five *Dendrobium* species have been described in Chinese Pharmacopoeia, including *D. nobile* (*Jin chai shi hu*) used in clinical medications (Yang et al. 2013; Wang et al. 2017). Another species, *D. candidum*, has long been used in TCM to treat cataract, throat inflammation, gastritis and for improving overall human health (Bao et al. 2001; Zha et al. 2009).

3.10 Current Research Based on Traditional Knowledge

Alkaloids, bibenzyl derivatives, flavonoids, phenanthrenes, triterpenoids, stilbenoids, steroids, tannin, reducing sugars, anthraquinones, phabatanins, and saponins are some of the phytochemicals isolated from orchids (Table 3.4). These bioactive compounds have antimicrobial, anti-inflammatory, anticancer, antioxidative, and antiviral properties. The extracts have been used against convulsive diseases, and for antioxidant, diuretic, hepatotoxic, antidiabetic, neuroprotective, antipyretic, analgesic, immunomodulatory, antimutagenic, and antiallergic properties (Gutiérrez 2010; Ramesh and Renganathan 2016).

A study conducted with 525 orchids from around the world found that 53 species found in Nepal contained alkaloids on dry weight basis (Lüning 1964). Since then extensive research has been done in many more species which have been detailed in the Table 3.4.

Table 3.3 Ethnobotanical uses of Nepali Orchids

#	Scientific name	Common name	Parts used	Disorder	Author
1	*Acampe carinata* (Griff.) Pantl.	Kano-Kato (O); Sonmunda (H)	Root, leaf	Root paste applied on scorpion bites and snakebites; leaf paste consumed to get relief from chest pain and stomach disorder during hyperacidity	Dash et al. (2008)
2	*Acampe papillosa* (Lindl.) Lindl.		Root	Treatment of rheumatism	Pant (2013)
3	*Acampe praemorsa* (Roxb.) Blatt. & McCann (syn. *Acampe papillosa* (Lindl.) Lindl.)	Parajivi (N), Rasna (S), Kano-Kato (O), Sonmunda (H)	Root powder; seed; leaf, whole plant	Used to treat rheumatism and for cooling and soothing effect; against arthritis; against cough; seed applied on wound as antibiotic, leaf juice applied on chest for stomachache, leaf juice applied for earache, whole plant is used as tonic	Subedi et al. (2013); Dash et al. (2008); Tiwari et al. (2012); Shanavaskhan et al. (2012); Chopra et al. (1956); Kirtikar and Basu (1935)
4	*Aerides multiflora* Roxb.	Parajivi, Thuur (N); Maana (H); Draupadi puspa (S)	Leaf powder; tuber; pseudobulbs	For tonic preparation; antibacterial; leaf paste used for cuts and wounds; has antibacterial property	Subedi et al. (2013); Singh and Duggal (2009); Joshi et al. (2009)
5	*Aerides odorata* Lour.	Parajivi (N); Hameri (O) Pargasa	Leaf paste; Roots	For treating wounds; To reduce joint pain and swellings, leaf juice is taken against tuberculosis	Subedi et al. (2013); Dash et al. (2008)
6	*Anoectochilus roxburghii* (Wall.) Lindl.		Whole plant	For treatment of tuberculosis, fever, pleurodynia, snakebite, liver and lung disease, hypertension, and malnourishment; cancer; as an antiviral drug; as an antidiabetic drug; treatment for cardiovascular disease; for treatment for nephritis	Zhang et al. (1999); ASP (2013)
7	*Anthogonium gracile* Lindl.		Tuber	Paste is used as a glue for nonmedical purposes	Yonzone et al. (2012b)
8	*Arundina graminifolia* (D. Don.) Hochr.	Bamboo orchid	Root	Used to relieve body ache; treatment for bacterial infection of wounds; bulbous stems are applied on heels to treat cracks	Yonzone et al. (2012a)
9	*Brachycorythis obcordata* (Lindl.)	Gamdol (N)	Tuber powder	For tonic preparation	Balami (2004)

10	*Bulbophyllum careyanum* (Hook.)	Banharchul, Thuur, Parajivi (N)	Leaf powder; fresh pseudobulb pulp	LP mixed with honey to stimulate abortion within first trimester and to help child birth recovery; fresh pseudobulb pulp applied on burned skin	Subedi (2002); Subedi et al. (2013)
11	*Bulbophyllum cariniflorum* Rchb. F.	Sumura (O)	Root	Used for inducing abortion	Dash et al. (2008)
12	*Bulbophyllum leopardinum* (Wall.)	Thuur (N), Parajivi 12(N)	Leaf and pseudobulb juice; whole plant	Application on burn using fresh juice	Subedi et al. (2013)
13	*Bulbophyllum odoratissimum* (Sm.) Lindl. ex Hook. f.	Thurjo	Whole plant	Treatment against tuberculosis, chronic inflammation as well as bone fractures	Chen et al. (2007a, b)
14	*Bulbophyllum sterile* (Lam.) Suresh		Pseudobulb	Applied to cure rheumatism	Shanavaskhan et al. (2012)
15	*Calanthe plantaginea* Lindl.		Rhizome	Powder used as tonic and aphrodisiac.	Pant (2013)
16	*Calanthe puberula* Lindl.		Rhizome	Dry powder us mixed to drink as tonic	Pant (2013)
17	*Calanthe sylvatica* (thou.) Lindl.		Flower and leaf	Used to stop nasal bleeding and gum bleeding; leaves are used in cold and cough	Yonzone et al. (2012b)
18	*Calanthe tricarinata* Lindl.		Leaf, pseudobulb	Leaf paste applied on skin sores and eczema, leaves and pseudobulbs are aphrodisiac	Joshi et al. (2009)
19	*Cephalanthera longifolia* (L.) Fritsch.	Narrow-leaved helleborine; Lampatter, sword-leaved helleborine	Rhizome, roots	For wound healing, taken as tonic; used during weakness for vigor	Yonzone et al. (2012b); Shapoo et al. (2013)
20	*Coelogyne corymbosa* Lindl.		Pseudobulb juice	Paste applied on forehead to reduce headache	Jana et al. (1997)

(continued)

Table 3.3 (continued)

#	Scientific name	Common name	Parts used	Disorder	Author
21	*Coelogyne cristata* Lindl.	Gondya (H), Harjojan (H); Swarna jibanti (S)	Pseudobulbs	Taken during constipation. Used as aphrodisiac. Juice is applied on wounds and boils. Pseudobulb gum is used for sores	Pant and Raskoti (2013); Joshi et al. (2009); Mitra et al. (2017)
22	*Coelogyne flaccida* Lindl.	The loose Coelogyne Pseudobulb	Pseudobulb paste and juice	Indigestion	Manandhar (2002)
23	*Coelogyne fuscescens* Lindl.	Ocher yellow Coelogyne; ban kera (N)	Pseudobulbs	Indigestion	Manandhar (2002)
24	*Coelogyne nitida* (Wall. ex Lindl) D. Don.		Pseudobulbs	Headache and fever	Pant (2013)
25	*Coelogyne ovalis* Lindl		Pseudobulbs paste; whole plant	To relieve fever and headache; aphrodisiac; used for urinary tract problems	Yonzone et al. (2012b)
26	*Coelogyne prolifera* Lindl.		Pseudobulbs paste	To reduce fever and headache and also applied on burnt skin; used for boils and backache	Pant (2013)
27	*Coelogyne punctulata* Lindl.		Dried pseudobulbs	Dried powder is applied on burn spots to heal and reduce pain	Yonzone et al. (2012b)
28	*Coelogyne stricta* (D. Don)	The rigid Coelogyne Pseudobulb	Pseudobulb paste	Headache and fever	Baral and Kurmi (2006)
29	*Conchidium muscicola* (Lindl.) Lindl.		Whole plant	Used during respiratory, cardiac, and nervous disorder.	Pant (2013)
30	*Cremastra appendiculata* (D.Don) Makino		Root paste, tuber	Paste is used for toothache; tuber is used for abscesses, skin problems, and as antidote for snakebites. Used against tonsillitis and hypertension.	Yonzone et al. (2012b); Bulpitt et al. (2007)
31	*Crepidium acminatum* (D. Don) Szlach.	Gachno, Gavndamala	Roots and pseudobulbs; stem	Root powder used against burning sensation, used to stop bleeding and fever; used as male aphrodisiac	Subedi et al. (2013); Panda and Mandal (2013)

32	*Cremastra appendiculata* (D.Don)	Rsabhaka (S)	Stem and root	Root powder is used in snakebite, stem powder is used for dental caries	Panda and Mandal (2013)
33	*Cymbidium aloifolium* (L) Sw. *Cymbidium aloifolium* D. Don	Banharchul, Kamaru, Harjor; Supurn (O); Manu vajinika	Whole plant; Pseudobulb; leaves; seeds	Leaf juice extract; leaves as tonic and paste; dried powder for diarrhea, paste used for bone-related ailments such as dislocation and fracture; during fracture and dislocation of bones; fever and inflammations; to clot blood; paste as tonic; root powder mixed with spices and taken orally to reduce paralysis; ripe seeds are used for curing wounds, heated leaf juice used for treatment of otitis	Subedi (2002); Gewali (2008); Dash et al. (2008); Subedi (2002); Shanavaskhan et al. (2012)
34	*Cymbidium devonianium* Lindl. ex Paxton		Whole plant	Root paste is applied to treat boils; concentrated decoction is taken in cough and cold	Pant (2013)
35	*Cymbidium eburneum*		Seeds and leaves	Leaves are used in ptosis, facial paralysis, headache, and in tumors. Seeds are used in diabetic foot ulcers	Panda and Mandal (2013)
36	*Cymbidium elegans* Lindl.	Thuur	Leaves, roots, and pseudobulbs	Pseudobulb juice taken orally against fever; Root juice given to livestock to relieve from cold symptoms	Gewali (2008); Thakur et al. (2010)
37	*Cymbidium ensifolium* (L.) Sw.		Rhizome, flowers	Decoction is used against gonorrhea; flowers are used against eye sores.	Hossain (2011)
38	*Cymbidium hookerianum* Rchb. F.		Seeds	Applied on cuts as hemostatic.	Yonzone et al. (2012b)
39	*Cymbidium iridioides* D. Don	Thuur	Pseudobulbs and leaf	Leaf juice extract; pseudobulb powder used as tonic; to clot blood;	Vaidya et al. (2002); Subedi (2002)
40	*Cymbidium macrorhizon* Lindl.		Rhizome	Used in boils.	Hossain (2011)
41	*Cypripedium cordigerum* D. Don	Lady's slipper orchid	Roots	Used as tonic, eaten as vegetable	Pant (2013)

(continued)

Table 3.3 (continued)

#	Scientific name	Common name	Parts used	Disorder	Author
42	*Cypripedium elegans* Reichenb .f. Nep	Lady's slipper orchid	Roots	Tonic used in hysteria, spasm, epilepsy and rheumatism	Pant (2013)
43	*Cypripedium himalaicum* Rolfe	Khujukpa (T); Lady's slipper orchid	Entire plant	Plant juice and powder are used for renal activity, heart problems and coughs	Lama et al. (2001)
44	*Dactylorhiza hatagirea* (D.Don) Soo	Panchaunle, Hatajadi; Salampanja, marsh orchis, salep orchid. Munjataka. Hatapanja (U)	Tuber; entire plant	Paste is used to reduce fever; mixed with honey and milk, powder is used as aphrodisiac; decoction drank against intestinal pain; used against diabetes, diarrhea, impotence, and malnutrition; nerve tonic; for treating weakness in children and women; root powder is used to control wound bleeding; whole plant is used for ailments caused by bacteria; aerial part works against *E. coli* and rhizome works against disease caused by *Shigella flexineri*; used in healing fracture, flower and tuber used during cough and cold, headache, used as vermifuge; used during diarrhea	Manandhar (2002); Gewali (2008); Khory (1982); Chauhan (1999); Kizu et al. (1999); Pant and Raskoti (2013); Watanabe et al. (2005); Baral and Kurmi (2006); Thakur and Dixit (2007); Giri and Tamta (2010); Shapoo et al. (2013)
45	*Dendrobium amoenum* Wall. ex Lindl.	Thuur	Pseudobulb, leaves	Paste applied on burnt skin and dislocated bones	Subedi et al. (2013)
46	*Dendrobium aphyllum* (Roxb.) C.E.C. Fisch	Fasia mach (Chakma)		Leaf paste is used on newborn's deformed body parts to reshape into normal	Hossain (2011)
47	*Dendrobium candidum* Wall ex. Lindl.		Leaves	Curing cataract as a therapeutic agent; during throat inflammation; for gastritis; used for improving the overall human health	Bao et al. (2001); Zha et al. (2009)
48	*Dendrobium chrysanthum*		Leaves	Antipyretic, skin diseases	Li et al. (2001)
49	*Dendrobium crepidatum* Griff.		Pseudobulb	Paste used on fractured bones	Pant (2013)

50	*Dendrobium densiflorum* Lindl.	Sungava (N)	Pseudobulb	Fresh pulp paste is used; tonic to strengthen stomach, against cataract development, to relieve fatigue, and overall to boost body immune system; to prevent pimples in Nepal	Pyakurel and Gurung (2008); Thakur et al. (2010); Bao et al. (2001); Pant and Raskoti (2013)
51	*Dendrobium denudans* (D.Don)		Stem	Stems are used for cough, cold, nasal block and tonsillitis. Also used as a tonic	Panda and Mandal (2013)
52	*Dendrobium eriiflorum* Griff.	Thurjo	Pseudobulb	Used during bone dislocation and fracture. Tonic is made from powder	Subedi et al. (2013)
53	*Dendrobium farmeri* Paxton		Whole plant	Antibacterial property	Hossain (2011)
54	*Dendrobium fimbriatum* Hook.	Fringe-lip dendrobium	Whole plant	Liver problem; nervous breakdown	Baral and Kurmi (2006)
55	*Dendrobium heterocarpum* Wall. ex Lindl.	Thuur	Pseudobulb	Paste used in bone dislocation and fracture	Subedi et al. (2013)
56	*Dendrobium longicornu* Lindl.	Kause	Pseudobulb and root	Root juice taken against fever; livestock are fed boiled roots to reduce cough	Manandhar (2002)
57	*Dendrobium macraei* Auct. (Syn. *Ephemerantha macraei* (Lindl.) Hunt et Sunmeh	Jivanti (S); Jevajevaniya, sakashreshtha, yasasvini, jiva bhadra.	Tuber	Stimulant, demulcent, lowers the blood pressure; against general debility; cures skin allergy	Karnick (1996)
58	*Dendrobium macrostachyum* Lindl.	Radam	Leaf, tender shoot tip	Juice is used for earache	Hossain (2011)
59	*Dendrobium moniliforme*		Dried pseudobulb	Used as tonic and aphrodisiac	Gutiérrez (2010)
60	*Dendrobium monticola* Hunt & Summerh.	Jiwanit (S)	Whole plant	Pseudobulb pulps used in boils and pimples.	Singh (2001)
61	*Dendrobium moschatum* Lindl.		Pseudobulb	Paste used in bone dislocation and fracture	Pant (2013)

(continued)

Table 3.3 (continued)

#	Scientific name	Common name	Parts used	Disorder	Author
62	*Dendrobium nobile* Lindl.	Shi-Hu (C)	Pseudobulb; seeds	Used in treatment of pulmonary tuberculosis, general debility, flatulence, dyspepsia, reduced salivation, night sweats, fever, and anorexia; seeds used to treat wounds	Nguyen and Nhu (1989)
63	*Dendrobium primulinum* Lindl.	Primrose yellow dendrobium	Dried pseudobulb	Boosting immune system	Pant and Thapa (2012)
64	*Dendrobium transparens* Wall.	Parajivi, Thur	Pseudobulb	Paste used in bone dislocation and fracture	Subedi (2002)
65	*Dienia cylindrostachya* Lindl. (syn. *Malaxis cylindrostachya* (Lindl.) Kuntze)		Pseudobulb	Tonic is made from powder	Subedi et al. (2013)
66	*Epipactis helleborine* (L.) Crantz	Sabazl poosh-e-panja; broad-leaved helleborine	Roots; leaf, rhizome; tuber	Juice taken for gout problem, and for insanity; Leaf infusion given during fever, Rhizome has aphrodisiac properties; Taken during boils, and as tonic	Vaidya et al. (2002); Joshi et al. (2009); Shapoo et al. (2013)
67	*Epipactis royleana* Lindl.	Wazul poosh-e-panja, red flowered helleborine	Rhizome	Powder used in wounds; general weakness, and boiled rhizome taken during seminal debility	Shapoo et al. (2013)
68	*Eria spicata* (D.Don) hand.-Mazz	Parajivi	Pseudobulb	Powder used for reducing stomachache, and paste applied on forehead for headaches	Vaidya et al. (2002)
69	*Eulophia dabia* (D.Don) Hochr.	Hatti paila (N); Salam mishri (H)	Rhizome	Powder used against coughs, heart ailments, also used as tonic and appetizer	Vaidya et al. (2002); Lawler (1984); Joshi et al. (2009)
70	*Eulophia herbacea* Lindl.	Bilakand (H);	Tuber	Against rheumatism; considered tonic	Tiwari et al. (2012)
71	*Eulophia graminea* Lindl.	Dudhiya	Tuber	Tuber is used as vermifuge	Chauhan (1990)
72	*Eulophia herbacea* Lindl.	Dudhiya	Tuber	Used as tonic also made into Salep	Singh and Duggal (2009)

73	*Eulophia nuda* Lindl.	Amarkand (H)	Tuber	Used in increasing appetite, used against tuberculosis and glands in neck, tumors and bronchitis	Tiwari et al. (2012)
74	*Eulophia spectabilis* (Dennst.) Suresh (syn. *Eulophia nuda* Lindl.)	Amarkand; Bonga taini (O)	Tuber; leaf	Powder used against worm infestation, scrofula, blood disorders, bronchitis and appetizer; mixed with spices and taken orally against aphrodisiac; leaf decoction used as vermifuge.	Vaidya et al. (2002); Dash et al. (2008)
75	*Flickingeria fugax* (Rchb.f.) Seidenf.	Jiwanti	Whole plant	Powder used in tonic against weakness and as stimulant	Subedi et al. (2013)
76	*Flickingeria macraei* (Lindl.) Seidenf.	Jiwanti; Sakar (O)	Whole plant	Paste for snakebites, for weakness, as stimulant and as demulcent; root paste mixed with spices applied to cure skin diseases and eczema.	Lawler (1984); Dash et al. (2008)
77	*Galeris strachaeyi* (Hook. f.) P. F. Hunt		Tuber	Used as tonic and to reduce headache	Pant (2013)
78	*Gastrodia elata* Blume	Chih Chien, Tian-Ma (C)	Tuber	Dried powder used for tonic and for treatment of headaches	Vaidya et al. (2002)
79	*Geodorum densiflorum* (Lam.) Schltr.	Kukurmuria (O); Donthula gadda	Roots, tuber	Fresh root paste used to regularize menstrual cycle; impotency in men; tuber paste powder is used against dysentery	Dash et al. (2008); Tiwari et al. (2012); Hossain (2011)
80	*Goodyera repens* (L.) R. Br	Girwara (H); Meend (U); Rattlesnake plantain (E)	Whole plant	Tuber paste applied in syphilis, extract is used in purifying blood; leaves used in toothache, whole plant used in wound, loss of appetite, urinary irritation, roots and leaves are used for irregular menstruation, and insect bites	Joshi et al. (2009); Shapoo et al. (2013)
81	*Goodyera schlechtendaliana* Rchb. F.		Whole plant	Tincture mixed with rice wine taken to improve blood circulation including internal injuries and as tonic;	Yonzone et al. (2012b)
82	*Gymnadenia conopsea*		Tubers	Tincture is used as aphrodisiac	Gutiérrez (2010)

(continued)

Table 3.3 (continued)

#	Scientific name	Common name	Parts used	Disorder	Author
83	*Gymnadenia orchidis* Lindl.		Tuber	Powder used for treatment of headaches and to be supplemented as tonic. Edible tuber made into Salep; root is used in gastric and urinary complications.	Vaidya et al. (2002); Yonzone et al. (2012a); Hossain (2011)
84	*Habenaria commelinifolia* (Roxb.) Wall. ex Lindl.	Devsunda (O); Vanpyazi (H); Jadu	Roots	Dried root decoction is taken to cure spermatorrhea; leucorrhoea	Dash et al. (2008); Tiwari et al. (2012)
85	*Habenaria furcifera* Lindl.	Nela jimmi gadda	Tuber	Paste is used as ointment for cuts, bites and wounds.	Roy et al. (2007)
86	*Habenaria intermedia* D. Don	Riddhi; Riddhi, Laksmi, Mangala, Rathanga, Risisrista, Saravajanpriya, siddhi, Sukha, Vasu and Yuga	Roots, leaf	Powder used for disease related to blood; has cooling and spermopiotic effect.	Singh and Duggal (2009); Singh (2006a, b); Singh and Sandhu (2005)
87	*Habenaria marginata* Coleb.	Humari (O)	Tuber	Tuber decoction is taken for treatment of malignant ulcer; against mental deficiency	Dash et al. (2008); Tiwari et al. (2012); Hossain (2011)
88	*Habenaria pectinata* D. Don	Seto Musli (N); Jivaka, Chiranjivi, Dirghayu, Harsanga, Ksveda, Kurchasira, Pranda, Sringaka and Svadu	Tuber, leaf	Juice extracted from leaf used against snakebites and tuber used for arthritis pain.	Singh and Duggal (2009); Singh (2006a, b)
89	*Habenaria plantaginea* Lindl.	Jhulukia (H)	Tuber	For menstrual cycle	Tiwari et al. (2012)
90	*Herminium lanceum* (Thunb.) Lindl.	Kusum gadda; Jalya	Whole plant	Plant extract used during urinary problem	Joshi et al. (2009)
91	*Herminium monorchis* (Linn.) R. Br.		Roots	Used as tonic	Pant (2013)

92	*Liparis nervosa* (Thunb) Lindl.		Tuber	Used in stomachache, and some ulcers	Pant (2013)
93	*Liparis odorata* (wild.) Lindl.		Leaf; pseudobulb	Fresh juice is used for burns, ulcers, and gangrene.	Yonzone et al. (2012b)
94	*Liparis rostrata* Rchb. f.		Tuber	Used in stomach problem	Hossain (2011)
95	*Luisia trichorhiza* (Hook.) Blume	Arjona (N); Koira (O)	Whole plant	Plant paste used for muscular pain; dried plant paste is taken orally to cure jaundice; root extract is used to treat diarrhea in cattle.	Vaidya et al. (2002); Dash et al. (2008)
96	*Luisia tristis* (G. Forst.) Hook.f. (syn *Luisia zeylanica* Lindl.)	Bori jhaar (N)	Whole plant	Juice applied on wounds	Vaidya et al. (2002)
97	*Malaxis acuminata* D. Don	Rishbhaka, Bandhura, Dhira, Durdhara, Gopati, Indraksa, Kakuda, Matrika, Visani, Vrisa, and Vrisnabha (S); Lasuni (H)	Pseudobulb	Cooling, febrifuge, and spermopiotic; bleeding diathesis, burning sensation, fever and phthisis; used during bronchitis	Singh (2006b); Singh and Sandhu (2005); Joshi et al. (2009)
98	*Malaxis muscifera* (Lindl.) Kuntze	Jivaka (S); Adder's mouth orchid	Pseudobulb	Paste used on skin and on sore muscles during burning sensation, to reduce fever, and as tonic	Subedi et al. (2013); Joshi et al. (2009)
99	*Neottianthe calcicola* (W.W. Sm.) Soo.		Rhizome	Tonic	Pant (2013)
100	*Nervilia aragoana* Gaudich.	Van Sighada	Whole plant	Used in renal problem, cough, asthma, vomiting and diarrhea; blood dysentery	Tiwari et al. (2012)
101	*Nervilia plicata* (Andr.) Schltr.	Satram dumpa	Tubers	Paste used against insect bites	Roy et al. (2007)

(continued)

Table 3.3 (continued)

#	Scientific name	Common name	Parts used	Disorder	Author
102	*Oberonia caulescens* Lindl.		Tubers	Used in liver problems	Pant (2013)
103	*Oberonia falconeri* Hook f.	Banda (H)	Leaves	Used during bone fractures	Tiwari et al. (2012)
104	*Oberonia pachyrachis* Rchb. f. ex Hook. f.	Rat tailed orchid	Leaf	Has antibacterial property	Hossain (2011)
105	*Otochilus albus* Lindl.	Aankhle laharo	Whole plant	Powder used to make tonic	Subedi et al. (2013)
106	*Otochilus lancilabius* Seidenf.	Aankhle laharo	Whole plant	Paste applied during bone fracture and dislocation	Raskoti (2009)
107	*Otochilus porrectus* Lindl.		Whole plant	Used as tonic, against sinusitis and rheumatism	Pant (2013)
108	*Papilionanthe teres* (Roxb.) Schltr.	Harjor, Thurjo	Pseudobulb, leaf	Paste applied during bone dislocation	Manandhar (2002)
109	*Pectilis susannae* (L.) Rafin.		Tuber	Used against boils	Jalal et al. (2008)
110	*Peristylus lawii* Wight		Tuber	Used against insect bites	Roy et al. (2007)
111	*Peristylus plantagineus* Lindl.	Kachari (H)	Tuber	Dried tuber powder is used against cough mixed with honey	Tiwari et al. (2012)
112	*Phaius tankervilliae* (banks) Blume.		Tubers, pseudobulb	Tonic; paste is used to reduce leg/hand swelling and bone fracture; taken orally to stop dysentery. And to reduce skin infection	Yonzone et al. (2012b)
113	*Pholidota articulata* Lindl.	Hadjor; Jeevanti (H); rattlesnake orchid	Whole plant	Paste applied during bone fracture and dislocation. Juice used as tonic	Joshi et al. (2009); Subedi et al. (2013)
114	*Pholidota imbricata* Lindl.	Thurjo, Patharkera	Pseudobulb	Paste eaten to reduce fever. Powder taken as tonic; crushed pseudobulbs are used in expelling spines	Subedi et al. (2013); Shanavaskhan et al. (2012)

115	*Pholidota pallida* Lindl.	Thurjo, Patharkera; Akongtong (H); Greater butterfly orchid	Rhizome, pseudobulb	Paste eaten to reduce fever; powder used against sleep apnea, and to reduce stomach pain, juice used to reduce navel pain; pseudobulb is used against intestinal worms and roots are used against rheumatism	Subedi et al. (2013); Hossain (2011)
116	*Platanthera edgeworthii* (Hook.f. ex Collett) (syn. *Habenaria edgeworthii*)	Riddhi	Rhizome, leaf	Powder and paste used against blood disease, and for body cooling effect	Singh and Duggal (2009)
117	*Platanthera sikkimensis* (Hook. f.) Kraenzlin.		Pseudobulb	Juice relieves naval pain, abdominal pain, and rheumatic pain	Pant (2013)
118	*Pleione humilis* (Sm.) D.Don	Shaktigumba;	Pseudobulb	Paste applied on cuts and wounds, powder used in tonic	Manandhar (2002); Pyakurel and Gurung (2008)
119	*Pleione maculata* (Lindl.) Lindl.		Rhizome	Used for liver and stomach problems	Pant (2013)
120	*Pleione praecox* (Sm.) D. Don	Shaktigumba; Lasun pate	Pseudobulb	Powder consumed as tonic mixing with milk. Paste used on cuts and wounds	Lawler (1984); Manandhar (2002); Subedi (2002); Thakur et al. (2010); Manandhar (1993)
121	*Poneorchis chusua* (D. Don) Soo		Tuber	For treating diarrhea, dysentery and chronic fever	Yonzone et al. (2012b)
122	*Rhyncostylis retusa* (L.) Blume	Kopu phool (A); Chadephuul, Dhogegava; Pumam (O); Band (H); Kucharl (H); Fox tail orchid (E)	Roots; whole plant	Rheumatism, asthma, tuberculosis, nervous twitching, cramps, infantile epilepsy, vertigo, palpitation, kidney stone, and menstrual disorders, dried flower used as insect repellent; and to induce vomiting; root paste is used to cure blood dysentery; leaf paste is used externally to wounds; against malarial fever; leaf paste is applied externally to reduce throat inflammation. Plant is emollient.	Medhi and Chakrabarty (2009); Saklani and Jain (1994); Manandhar (2002); Thakur et al. (2010); Dash et al. (2008); Tiwari et al. (2012); Shanavaskhan et al. (2012)

(continued)

Table 3.3 (continued)

#	Scientific name	Common name	Parts used	Disorder	Author
123	*Satyrium nepalensis* D. Don	Hathjadi (N); Mishri, Thamni; Satyion, ban alu	Roots; tubers	Aphrodisiac, and as a tonic; reduces symptoms of diarrhea, malaria, and dysentery, juice used to reduce fever, and applied on cuts and wounds.	Mishra and Saklani (2012); Joshi et al. (2009); Manandhar (2002); Pyakurel and Gurung (2008)
124	*Smitinandia micrantha* (Lindl.) Holttum		Whole plant	Root powder is used as tonic; pseudobulb is antibacterial	Pant (2013)
125	*Spiranthes sinensis* (Pers.) Ames	Phirtya (H); Masti-loth (U); Lady's tresses (E)	Tuber; whole plant	Powder used as tonic and against headache and during fever; flowers used during skin eruption, tubers used during sore throat, and swelling; and whole plant used during cough and cold; tubers used during swelling, applied on wounds	Balami (2004); Tezuka et al. (1990); Joshi et al. (2009); Shapoo et al. (2013)
126	*Thunia alba* (Lindl.) Rchb. f.	Golaino	Whole plant	Paste used on bone fractures	Pant (2013)
127	*Vanda cristata* wall. ex Lindl.	Vhagute Phul, Thuur	Roots, leaf	Paste from roots applied on boils and dislocated bones. Leaf powder used as expectorant, and paste applied on skin cuts	Manandhar (2002); Pyakurel and Gurung (2008)

128	*Vanda tessellata* (Roxb. f.) Hook. ex D. Don (syn. *Vanda roxburghii* R.Br.)	Vandāka, vrksaruhā, Ŝekhari, kāmavrksaka, vksādani, kāmataru, kāmini, apadarohini (S) Rāsnā, ramya, yuktarasa, rasana, gandhanakuli, sugandhamula, atirasa, sreyasi, suvara, sara (S); Parajivi (N); Banki (O)	All plant parts	Cures gout, edema, ulcer, dyspnea, colic pain, obstinate abdominal diseases including ascites. It also acts as anodyne, carminative, expectorant, nervine tonic; Stimulates cholinergic nerve endings, lowers blood pressure rheumatism, syphilis and otitis media; antidote for scorpion sting and medicine for bronchitis and rheumatism made from root paste. leaf paste used against high fever; root powder is made into decoction for treatment of sexually transmitted diseases; root paste is also used to cure nervous disorder; aphrodisiac, analgesic, and nervine tonic; leaf juice is applied for earache; used as alexiteric, antipyretic, sexual stimulant, for muscle sprains, lumbago, back pain, fever, rheumatism, bronchitis, inflammation, hiccups, piles, boils on the scalp	Vaidya (1991); Karnick (1996); Singh and Duggal (2009); Dash et al. (2008); Shanavaskhan et al. (2012)
129	*Vanda testacea* (Lindl.) Rchb.f.	Malanga (O)	Leaves and roots	Leaf paste is applied on broken bones of cattle; root decoction mixed with other plants is taken to cure asthma; paste applied on cuts and wounds; used in malaria, rheumatism, and nervous disorders	Dash et al. (2008); Tiwari et al. (2012); Shanavaskhan et al. (2012); Khasin and Rao (1999)
130	*Zeuxine strateumatica* (L.)	Kansjhar; Piljari (H), Shwethili (B)	Root	Tonic made from dry powder; root paste is taken against fever	Subedi et al. (2013); Tiwari et al. (2012)

Nepali (N), Sanskrit (S), English (E), Hindi (H), Assamese (A), Oriya (O), Chinese (C), Bengali (B)

Table 3.4 Specific phytochemical found in Nepali orchids

#	Name	Compound	Author
1	*Agrostophyllum callosum*	Triterpenoids: agrostophyllinone; isoagrostophyllol stilbenoids: orchinol, 6-methoxycoelonin, imbricatin, flaccidin, oxoflaccidin, isooxoflaccidin, flaccidinin, agrostophyllin, callosin, callosinin, callosumin, callosuminin and callosumidin. Callosumin, callosuminin, callosumidin, 4-hydroxy-3,5-dimethoxybenzoic acid, orchinol, 6-methoxycoelonin, imbricatin, flaccidin, oxoflaccidin, isooxoflaccidin, flaccidinin, agrostophyllin, callosmin, callosinin; agrostonin, agrostonidin, agrostophyllol	Singh and Duggal (2009); Majumder et al. (1995); Majumder et al. (1996); Arora et al. (2017); Majumder et al. (1999)
2	*Anoectochilus roxburghii*	Flavonoids: Quercetin-7-O-β-D-[6″-*O*-(transferuloyl)]-glucopyranoside; 8-C-*p*-hydroxybenzylquercetin; isorhamnetin-7-O-β-D-[glucopyranoside; isorhamnetin-3-O β-D-[glucopyranoside; kaempferol-3-O-β-D-[glucopyranoside; kaempferol-7β-D-[glucopyranoside; 5-hydroxy-3′,4′,7-trimethoxyflavonol-3-β-D-[rutinoside]; isorhamnetin-3-β-D-[rutinoside]	He et al. (2006)
3	*Arundina graminifolia*	Stilbenoids: Arundinan; Benzyldihydrophenanthrene: arundinaol, stilbenoid: arundinan. Phenanthrenes: 7-hydroxy-2, 4-dimethoxy-9, 10-dihydrophenanthrene; 4, 7-dihydroxy-2-methoxy-9, 10-dihydrophenanthrene; 2, 7-dihydroxy-4-methoxy-9, 10-dihydrophenanthrene; 7-hydroxy-2-methoxyphenanthrene-1,4-dione 7-hydroxy-2-methoxy-9, 10-dihydrophenanthrene-1,4-dione	Liu et al. (2005a); Liu et al. (2004); Liu et al. (2005b)
3	*Bulbophullum leopardium*	Bulbophyllanthrin	Majumder et al. (1985)
4	*Bulbophyllum odoratissimum*	Phenanthraquinone, bulbophyllanthrone, and dimeric phenanthrenes bulbophythrin A and B are reported. Other compounds are moscatin; 7-hydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene, coelonin; densiflorol B; gigantol; batatasin III; tristin; vanillic acid and syringaldehyde; 3,7-dihydroxy-2-4-6-trimethoxyphenanthrene; bulbophyllanthrone; coelonin	Majumder and Sen (1991); Xu et al. (2009)
5	*Bulbophyllum triste*	Tristin	Majumder and Pal (1993)
6	*Coelogyne cristata*	Coelogin, coeloginin, coeloginanthridin, and coeloginanthrin; phytoalexins; combretastatinC-1	Majumder et al. (2001)
7	*Coelogyne flaccida*	Flaccidin (9,10-dihydrophenanthropyran derivative)	Majumder and Maiti (1988)

8	*Coelogyne ochracea* Lindl.	Ochrone-A	Shimura et al. (2007)
9	*Coelogyne ovalis*	Bibenzyl: 3′-0-methylbatatasin	Sachdev and Kulshreshtha (1986)
10	*Cremastra appendiculata*	Phenanthrene, bibenzyls; cirrhopetalanthin; cremastrine; homoisoflavanone; 2,7,2′,7′,2″-pentahydroxy-4,4′,4″,7″-tetramethoxy-1,8,1′,1″-triphenanthrene; flavanthrinin; gastrodin	Wang et al. (2013); Xue et al. (2006); Ikeda et al. (2005)
11	*Cymbidium aloifolium*	Flavonoids, Saponins, Tannins, Terpenoids and Steroids	Marjoka et al. (2016)
12	*Dactylorhiza hatagirea* (D.Don) Soo.	Mucilage, starch, glucoside: loroglossin, albumen, volatile oil, and ash. Dactylorhins A–E; dactyloses A and B	Khory (1982); Chauhan (1999); Kizu et al. (1999)
13	*Dendrobium amoenum* Wall. ex Lindl.	Isoamoenylin, amoenylin, moscatilin, 3,4′-dihydroxy-5-methoxybibenzyl	Venkateswarlu et al. (2003); Lam et al. (2015)
14	*Dendrobium aphyllum*	Aromatic compounds: Flavanthrin, coelonin, iusianthridin, moscatin, gigantol, batatasin III, dibutyl phthalate, diisobutyl phthalate, P-hydroxyphenylpropionic methyl ester	Lam et al. (2015)
15	*Dendrobium candidum* Wall ex Lindl.	Dendrocandin a, dendrocandin b, dendrocandin c, dendrocandin d, dendrocandin e, dendrocandin f, dendrocandin g, dendrocandin h, dendrocandin i; amotin, amoenin, flaccidin. Bibenzyls derivatives: 4,4-dihydroxy-3,5-dimethoxybibenzyl, 3,4-dihydroxy-5,4 dimethoxybibenzyl, 3-*o*-methylgigantol, dendrophenol, gigantol	Lam et al. (2015); Yan et al. (2008)
16	*Dendrobium crepidatum* Lindl. & Paxton	Crepidine, crepidamine; dendrocrepine	Arditti (1992); Majumder and Maiti (1988)
17	*Dendrobium cumulatum*	Tristin	Lam et al. (2015)
18	*Dendrobium densifiorum* Lindl. ex Wall.	Homoeridictyol; scoparone; bibenzyl, densiflorol a, phenanthrenedione, densiflorol b, dendroflorin, dengibsin, cypripedin, gigantol, moscatilin, tristin, naringenin, homoeriodictyol, moscatin, 2,6-dihydroxy-1,5,7-trimethoxyphenanthrene, 4,7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene, scoparone, scopoletin, ayapin	Fan et al. (2001); Lam et al. (2015)

(continued)

Table 3.4 (continued)

#	Name	Compound	Author
19	*Dendrobium longicornu*	Monoaromatic compounds: Bis(2-ethylhexyl)phthalate, dibutyl phthalate, ethyl haematommate, methyl B-orcinol carboxylate, N-docosyl trans-ferulate, ferulaldehyde	Lam et al. (2015)
20	*Dendrobium macraei* Lindl	Alkaloids: Jebantine, jibantic acid	Khasin and Rao (1999); Khory (1982); Lam et al. (2015)
21	*Dendrobium monoliforme* Sw.	Dendromoniliside A, dendromoniliside B, dendromoniliside C; moniliformin (2,6-dimethoxy-1,4,5,8-phenanthradiquinone); 7-hydroxy-5,6-dimethoxy-1, 4-phenanthranequinone; (+)-syringaresinol, aloifol I; daucosterol	Gutiérrez (2010); Arora et al. (2017)
22	*Dendrobium moschatum*	Phenanthrenes: rotundatin, moscatin; moscatilin (4,4-dihydroxy-3,3,5-trimethoxybibenzyl)	Miyazawa et al. (1997)
23	*Dendrobium nobile*	Bibenzyls: gigantol, moscatilin Alkaloids: dendrobinae; mucilage, alkaloid: dendrobine, 1–4: phenanthrenequinone: denbinobine, gigantol bibenzyl compound: moscatilin; (−)-dendrobine; denbinobin (5-hydroxy-3,7-dimethoxy-1,4-phenanthraquinone); dendroside A, dendroside D; dendroside E; dendroside F; dendroside G; dendronobiloside A; 4,7-dihydroxy-2-methoxy-9, 10-dihydrophenanthrene; nobilin D, nobilin E; nobilone;	Lee et al. (1995); Miyazawa et al. (1997); Khory (1982); Arditti (1992); Matsuda et al. (2004)
24	*Dendrobium plicata*	Ephemeranthoquinone	Yamaki and Honda (1996); Arora et al. (2017)
25	*Dendrobium primulinum* Lindl.	Dendroprimine; hygrine	Luning and Leander (1965)
26	*Eulophia nuda* Lindl.	Nudol	Bhandari et al. (1985)
27	*Gastrodia elata* Blume	N^6-(4-hydroxybenzyl)adenine riboside; gastrodamine; gastrodin; bis(4-hydroxybenzyl)sulfide	Zhan et al. (2016)
28	*Gymnadenia conopsea* (L.) R.Br.	Dihydroxy-2,6-*bis*(4-hydroxybenzyl)-5-methoxybibenzyl; gymconopin A; gymconopin B; Gymconopin D	Matsuda et al. (2004)

29	*Pleione maculata*	Alkaloids, flavonoids, steroid, tannin, reducing sugars, triterpenoids, anthraquinones, and phlobatannins	Bhatnagar and Ghosal (2018)
30	*Satryium nepalense*	Quercetin, alkaloids, flavonoids, steroids, reducing sugars, cardiac glycosides, terpenoids, anthraquinones, phlobatannins, saponins	Mishra and Saklani (2012); Mishra et al. (2014); Bhatnagar et al. (2017)
31	*Spiranthes sinensis*	Sinensol H, sinetirucallol; sinensol A, sinensol B, sinensol C, sinensol D, sinensol E, sinensol F, spirasineol B, spiranthol C, spiranthoquinone	Lin et al. (2001)
32	*Rhynchostylis retusa*	Alkaloids, flavonoids, steroids, reducing sugars, cardiac glycosides, terpenoids, anthraquinones, phlobatannins, saponins; tannins, coumarins, amino acids, and carbohydrates	Bhatnagar et al. (2017); Bhattacharjee and Islam (2015)
33	*Thunia alba*	Lusianthridin	Majumder et al. (1998)
34	*Vanda tessellata* (Roxb.) Hook. Ex Don	Alkaloid, glucoside, bitter principle, tannins, resin, saponin, sitosterols (40) Glycoside (melianin), complex withanolide (44, 45)	Kumar et al. (2000); Basu et al. (1971); Prasad and Achari (1966); Nayak et al. (2005); Ahmed et al. (2001)

Crude ethanolic extracts of ten orchids, namely, *Bulbophyllum affine*, *Coelogyne cristata*, *Coelogyne stricta* leaf, *Dendrobium amoneum*, *Dendrobium nobile*, *Eria spicata*, *Pholidota articulata*, *Pholidota imbricata*, *Rhyncostylis retusa*, and *Vanda cristata* have been found to have specific antibacterial activity against *Vibrio cholera*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and antifungal activities against *Candida albicans*, *Rhizopus stolonifera*, and *Mucor* spp. (Marasini and Joshi 2012). It is interesting to note that 5% ethanolic extracts of all ten orchid species were effective against *S. aureus*, while all but *B. affine* worked against *K. pneumoniae*. As per anti-fungal activities, extracts of nine species, but not of *C. stricta,* were effective (Marasini and Joshi 2012). A phytochemical analysis of 61 orchid species found in Tirubelveli Hills in South India, containing 13 species common to Nepal, exhibited the presence of flavonoid, reducing sugars, cyanogenic glycosides, terpenoids and tannin (Maridassa et al. 2008). A petroleum ether extract of *Vanda tessellata* leaves in the dosage of 200 and 400 mg/kg showed anti-diarrhoeal activity in adult albino rats (Teja et al. 2012). Oral administration of the *C. cristata* extracts showed that the spontaneous locomotor movement improved in aged wistar rats expressing chronic fatigue syndrome (CFS), possibly due to antioxidant activity of the extracts (Mitra et al. 2017). These results show promising therapeutic potentials using orchid extracts. The ethanolic extract of *Pholidota pallida* showed different zone of inhibition as a proof of antibacterial property. For *Bacillus coagulans*, *B. subtilis*, *Salmonella typhi*, and *E. coli,* the zone of inhibition were 1.5 cm, 1.8 cm, 1.5 cm, and 1.6 cm, respectively (Rashmi et al. 2015). The radical scavenging activity for 6.25 μg/mL was 8%, for 12.5 μg/mL was 8%, for 25 μg/mL was 10%, for 50 μg/mL was 21%, and for 100 μg/mL, it was 40%. The total polyphenol content of *P. pallida* was 12.33 mg/g and the scavenging potential as indicated by IC_{50} was 128.31 μg/mL, the lowest among the four orchids tested at that time (Rashmi et al. 2015). *Pholidota pallida* also showed considerable antifungal activity against *Bipolaris sorokiniana, Curvularia* sp., *Fusarium oxysporum*, and *Colletotrichum capsici* (Shweta et al. 2015).

Orchid extracts have also been shown to have antimicrobial efficacy against different strains of *E. coli*. Paul et al. (2013) reported that *Aerides odorata* leaf extracts (water) had zone of inhibition of 5 mm, and 7 mm and the zone of inhibition was 5 mm for *E. coli* nonresistant strain, *E. coli* ampicillin-resistant strain and *E. coli* kanamycin-resistant strain in nutrient agar using modified paper discs. Similarly, for the acetone extracted *A. odorata*, zone of inhibition for *E. coli* ampicillin-resistant strain was 6 mm in ampicillin suspended LB agar. As for the water extracted *A. odorata*, the zone of inhibition was 6 mm for *E. coli*, kanamycin-resistant strain suspended in LB agar using modified paper disc method (Paul et al. 2013).

Vanda tessellata is known to have aphrodisiac properties (Kumar et al. 2000). An experiment using aqueous, alcohol, and heated alcohol extract of *V. tessellata* leaf, flower, and root revealed that the flower extracts enhanced mating behavior, especially with heated alcohol extract, in adult Swiss mice (Kumar et al. 2000). Depending on the extraction solvent type, the efficacy of the antibacterial and antimycobacterial activity of orchid extracts varies. One of the experiments conducted with different solvents, that is, ethanol, butanol, and water for *Pleione maculata*

orchid, all extracts showed positive response against bacteria *E. coli*, *Staphylococcus* sp., *Serratia* sp., and mycobacteria *Mycobacterium tuberculosis* strain H37Rv (Bhatnagar and Ghosal 2018). The ethanolic extract had the minimum inhibitory concentration (MIC) value of 104.16 μg/mL against the *M. tuberculosis* strain H37Rv. The extracts contained alkaloids, flavonoids, steroid, tannin, reducing sugars, triterpenoids, anthraquinones, and phabatanins (Bhatnagar and Ghosal 2018). *Rhyncostylis retusa*, a common epiphytic orchid from mid-hills of Nepal, has antibacterial and antifungal activities (Bhattacharjee and Islam 2015). The extracts were prepared with four solvents, methanol, ethanol, chloroform, and hexane, and were tested on both gram-positive (*S. aureus* and *B. subtilis*) and gram-negative (*V. cholerae*, *E. coli*, and *K. pneumonia*) bacteria. The extracts were also used on three fungus species *Penicillium* sp., *Rhizopus* sp., and *Aspergillus niger*). Except for hexane extracts, all extracts showed activities against bacteria. For three fungi, all four extracts did have an inhibition zone (Bhattacharjee and Islam 2015).

Dendrobium amoenum, a common species of *Dendrobium*, has been described and used extensively as one of the ingredients in TCM, folk medicine and *Ayurveda*. In a work described by Paudel et al. 2015, quantification of biologically active compounds has been done using Folin-Ciocalteu reagent method for total polyphenol (TPP) content measurement, estimation of flavonoid content (FC) by aluminum chloride method and determination of antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. It was found that acetone-based extract had the highest TPP (134.34 gallic acid equivalent [GAE]) and flavonoid content (FC) of 115.73 μg/mg quercentin equivalent [QE]). As for the antioxidant activity through DPPH, IC_{50} was shown to be the lowest in the chloroform extract at 36.48 μg/mL, while the highest activity was in the ethanol extract at 334.50 μg/mL (Paudel et al. 2015).

Nine species of orchids found in Nepal were studied for antioxidant activity in different plant parts such as leaves, roots, and pseudobulbs based on FC, TPP, and DPPH free radical scavenging assay, expressed in terms of IC_{50} potency (Chand et al. 2016). The highest total FC was measured in *R. retusa* leaves (110.68 mg/g), total polyphenol content was measured in *V. cristata* stem (69.68 mg/g GAE), and the highest DPPH IC_{50} was found in *Gastrochilus acutifolius* leaves (341.79 μg/mL) (Chand et al. 2016).

Aqueous and methanolic leaf extracts of *Vanda tessellata* have been found to have antinociceptive properties that could be used in inflammatory problems (Chaudhary et al. 2015). Studies conducted in Swiss albino mice showed that at a dose of 400 mg/kg, pain was reduced to 42.37% by the aqueous extract and to 45.08% by the methanolic extract.

3.10.1 Anticancer Properties

Orchids contain many different bioactive compounds such as alkaloids, anthraquinones, cardiac glycosides, flavonoids, glycosides, phlobatanins, quercetin, reducing sugars, saponins, steroids, and terpenoids. Some of the specific compounds from

Table 3.5 Nepali orchids with anti-tumor and anti-cancer properties

SN	Species	Specific Compound	Specificity	Author
1	*Bulbophyllum odoratissimum*	Phenanthrene derivative: 3,7-dihydroxy-2,4,6-trimethoxyphenanthrene	Cytotoxicity against human leukemia cell lines (K562) and (HL60), human lung adenocarcinoma A549, human hepatoma (BEL7402) and human stomach cancer cell lines (SGC7901)	Chen et al. (2007a, b)
		Gigantol	Anticancer activity against MDA-MB-231 and MDA-MB-468 breast cancer cells	Yu et al. (2018)
2	*Bulbophyllum sterile*		Anticancer activity through apoptosis against HCT-116, MDA-MB-231 and A549 cell lines	Biswas et al. (2016)
3	*Cremastra appendiculata*	Cirrhopetalanthrin and 2,7,2′,7′,2″-pentahydroxy-4,4′4″7″-tetramethoxy-1,8,1′,1″-triphenanthrene	Cytotoxicity against human breast cancer (MCF7), human colon cancer (HCT8), human lung adenocarcinoma (A549), human stomach cancer (BGC823), human hepatoma (BEL7402), and human ovarian cancer (A2780) cell lines	Xue et al. (2006, 2006)
		Homoisoflavanone5,7-dihydroxy-3-(3-hydroxy-4 methoxybenzyl)-6-methoxychroman	Inhibitor of angiogenesis	Xia et al. (2005)
4	*Dendrobium amoenum*	(E)-13-docosenoic acid; oleic acid; 11-octadecenoic acid, methyl ester; and hexadecanoic acid, 2,3-dihydroxypropyl ester	Cytotoxic activity against cervical carcinoma and glioblastoma cell lines	Paudel and Pant (2017)
5	*Dendrobium aphyllum*	Gigantol	Anticancer activity against MDA-MB-231 and MDA-MB-468 breast cancer cells	Yu et al. (2018)
6	*Dendrobium candidum*	Gigantol	Anticancer activity against MDA-MB-231 and MDA-MB-468 breast cancer cells	Yu et al. (2018)
7	*Dendrobium chrysanthum*	Erianin	Inhibitor of proliferation of HL60 cells tumor necrosis, growth delay and rapid vascular shutdown in hepatoma Bel7402 and melanoma A375	Li et al. (2001); Gong et al. (2004)
		Dendrochrysanene	Suppress the mRNA level of TNF-alpha, IL8, IL10, and iNOS in murine peritoneal macrophages	Yang et al. (2006)
			Cytotoxic effect in T-cell lymphoma	Prasad and Koch (2016)

SN	Species	Specific Compound	Specificity	Author
8	*Dendrobium Crepidatum*		Cytotoxic effect in T-cell lymphoma	Prasad and Koch (2016)
9	*Dendrobium densifiorum*	Gigantol	Anticancer activity against MDA-MB-231 and MDA-MB-468 breast cancer cells	Yu et al. (2018)
10	*Dendrobium fimbriatum*	Fimbriatone	Inhibitor of BGC cell line	Bi et al. (2003)
11	*Dendrobium Formosum*		Anticancer property on Dalton's lymphoma (T-cell lymphoma)	Prasad and Koch (2014)
12	*Dendrobium nobile*	Denbinobin and 4,7-Dihydroxy-2-methoxy-9,10-dihydrophenanthrene	Human lung carcinoma (A5490), human ovary adenocarcinoma (SKOV3) and humanpromyelocytic leukemia (HL60) cell lines	You et al. (1995)
		Dendroside A and dendronobiloside A	Stimulatory effect proliferation of murine T and B lymphocytes	Zhao et al. (2001)
		Gigantol	Anticancer activity against MDA-MB-231 and MDA-MB-468 breast cancer cells	Yu et al. (2018)
13	*Eulophia nuda*	Phenanthrene derivative: 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol	Antiproliferative activity and cytotoxic potential against human breast cancer cell lines MCF-7 and MDA-MD-231	Shriram et al. (2010)
14	*Gastrodia elata*		Apoptosis suppression of JNK activity	Miyazawa et al. (1999)
			Antitumor activity	Heo et al. (2007)

flowers, leaves, pseudobulbs, roots, and seed pod extracts have been used in many different antitumor/anticancer studies. This work lists 14 different species found in Nepal with known bioactive compound and their specificity (Table 3.5). Most of the laboratory studies measured cytotoxicity against specific cancer cell lines. One example is extracts from *Eulophia nuda*. The pure compound 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol was isolated from crude methanolic extract and the fractionate when used for cytotoxic activities against human breast cancer cell lines MCF-7 and MDA-MB-231. The active molecule showed antiproliferative activities, and as a result tumor growth was suppressed (Shriram et al. 2010).

3.11 Threats to Medicinal Orchids in Natural Habitats

An inventory effort on wild orchids of Panchase Forest stretching 500 sq. km in Mid-Western Nepal reported 112 species under 44 genera (Subedi et al. 2007). It is noteworthy that the number of species found in such a small area has earned the designation of an orchid hotspot. Several key threats to the natural habitats of orchids have been described: (1) uncontrolled grazing by domestic animals, (2) logging of *Quercus* and *Rhododendro*n tree species hosting epiphytic orchids, and (3) orchids being used as animal fodder during winter season when green feeds are not available.

It is common to see community led conservation programs where the locals initiate to save the wild population. These groups of people or the local community identify the orchids which have traditionally been harvested from the wild to be sold in the local market for medicinal use. There are many programs that have been initiated in Nepal with Forestry Users Groups in community forestry (Pant 2015), by donor agencies and the nongovernmental organizations. However, without scientific data and validations, it is difficult to ascertain the success of these programs in terms of sustainability. According to the reports from the Government of Nepal (GoN 2008) there are 4.27 million hectares of forest cover out of 15 million hectares of total land area, comprising 29% of total land area under forest cover. Every year, on average about 1.98% of forest was cleared in Nepal during the period 1978–2000 (DFRS 1999). There are several reasons for deforestation and destruction of the primary habitats of orchids in Nepal:

(a) **Deforestation due to population growth:** Rapid population growth has put severe strains on natural resources in Nepal. The annual population growth rate in 2011 was 1.40% (Chaudhary et al. 2015). About 400,000 ha. of land was cleared between 1964–1979 in southern Nepal, an area known as *Terai,* to create additional agricultural land for migrating population from the hills (Chaudhary 1998). Due to the scarcity of arable land, people have resorted to reckless clearing of forest to be used as agricultural land to ever increasing population.

(b) **Dependency on fuel wood as a major source of energy:** As the population increased, the subsequent demand for fuel wood also increased. Research has

shown that even until 2013, 64% of the population depended in fuel wood for primary source of energy and wood is the principal source of fuel, where 87% of the nation's energy requirement is met from the forest (WECS 2013). Therefore, cutting of trees and branches for firewood rather than buying kerosene is prevalent in remote areas of Nepal. Cutting trees destroys the epiphytic habitats of orchids and cover for terrestrial orchids provided by the tree canopy filtering excess sunlight.

(c) **Illegal logging for timber industry:** Tropical hardwood has dominated the trade based on natural resources in Nepal for more than a century. The forest trees are protected by the Forest Act of 1993 with the first amendment of 1999, but for financial gains, trees are cut for timber and sold illegally for higher price.

(d) **Overgrazing:** The shortage of fodder and other feeding materials has resulted in overgrazing in the pastures and over lopping of fodder trees in the forests. About three- quarters of the fodder (out of a total of 5.6 million tons per year) is sourced from the forest and grasslands in Nepal. This aspect was described by Subedi et al. 2007 to be one of the prominent threats to the orchid hotspot mentioned earlier.

(e) **Shifting cultivation:** Shifting cultivation, which starts by clearing and burning of a patch of climax forest for agriculture, has resulted in immense habitat destruction because after every few years, farmers shift to a new forest area. In many places in Souteast Asia, increase in agrobiodiversity has been linked to shifting cultivation by indiginous farmers (Kafle 2011), but in Nepal's context, it is metioned that conservation and biodiversity is affected by shifting cultivation in marginal lands for subsistence farming (Kafle 2011).

(f) **Indiscriminate collection of orchids for export and for local sale:** Due to anthropogenic interference, there has been extensive misuse of orchids all over the world. The local newspapers and news magazines in Nepal are rife with articles published almost every month stating that truckloads of dried orchids have been seized from the adjoining borders to China and India. Nepal is a signatory nation on Convention of International Trade in Endanged Species of Wild Fauna and Flora (CITES), but illegal collection of orchids from the wild is still continuing. Commercial exploitation and illegal trade of orchids, especially of medicinal orchids are flourishing. In a survey by Bailes (1985), it was estimated that about 1000 truckloads, each with 8 ton capacity, of orchids (*Flickengeria macraei*) were shipped to India in one consignment for making *Ayurvedic* medicine. In 1979, 5 tons of *Dactylorhiza hatagirea* tubers were exported from Nepal at a price of US$900 per ton (Arditti 1992). Forest Act of 1993 protects one of the orchid species *D. hatagirea* (Panchaunle) from collection, use, sale, distribution, transportation and export, among other protected species of plants. Nurseries involved with orchid trade depend solely on wildcrafting to meet domestic and international demands. The government data and the statistics show only a fraction of total trade (Shakya et al. 1994).

(g) **Natural calamities like soil erosion and heavy landslides due to human created problems:** Uncontrolled slash and burn as well as destruction of natural

forest has caused heavy casualty during the monsoon seasons. Every year in many parts of Nepal, landslides and erosions take place which wash away fertile topsoil.

3.12 Conservation Strategy, Sustainable Production and Cultivation

Before the advent of modern medicine and allopathy, traditional medicine based on local plants, herbs and elements have been used for centuries (Medhi and Chakrabarty 2009). The complementary and alternative medicine (CAM) based system have been used by the traditional medicine practitioner (TMP) such as Tibetan *Amji*, or *Lama,* Newar *Vaidya* and *Guvaju*, Tamang *Lama* and healers *Jhankri*, Tharu priests *Guraun* and other traditional healers. They are the ones who use wild plants in their preparations. They still provide 80–85% of the population in Nepal with primary health care (Manandhar 2002), and these TMPs reliance on plants and plant product to cure medical ailments are widespread due to easy access and affordability. Recent research shows that only 28.1% of the rural population in Nepal have access to medical facility (Paudel et al. 2012), and one of the main reasons for not accessing the health care was lack of medicine and their quality (Garha 2017).

Orchids, known for their flowers, are important horticulturally as one of the most traded plants (Hinsley et al. 2017). Some species are known as the ecological indicators of changes taking place in their habitats (Joshi et al. 2009). The entire orchid family is placed in Appendix II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) restricting international trade. A rich diversity of orchid flora has contributed to orchid usage in traditional medicines in many forms.

These are the major points for conservation:

(a) Promotion of education among the local/indigenous people: Unless and until the local indigenous people are sensitized, any conservation effect to protect wild orchid species will be futile. Hence, a sound program in teaching and educating the locals should be institutionalized so that they understand the importance of these plants and participate in the conservation process.
(b) In situ conservation: The aspect of conservation in their natural habitats can only be emphasized once the local people understand the importance of conservation and are willing to protect the area so that the orchids can thrive in their natural habitats without any man-made disturbances.
(c) Habitat protection and restoration projects: Restoration projects in protected habitats with stewardship of local people should be initiated for reintroduction of species. These types of projects on orchids take decades, for the wild population has to stabilize; hence, participation from local communities is crucial.
(d) Establishment of tissue culture labs and germplasm banks in a regional level: As a concerted effect to protect and promote medicinal orchids, establishment of tissue culture lab and germplasm bank should be promoted. Initiation of new

in vitro cultures aid in germplasm collection and propagation. The propagated orchids could be relocated during in situ restoration projects.

(e) Sustainable production and cultivation: Once the orchids produced through tissue culture are established in a nursery and in natural habitats, sustainable production and harvesting can be initiated for production of traditional medicine or extracts for such.

3.13 Conclusions

There have been efforts to document TMP based knowledge so that the knowledge does not die with the individual that possesses the wisdom. For many generations, traditional knowledge on medicinal plants including orchids has been passed through word of mouth without much any evidence of written records. Information collected from traditional healer can go on a database that can be validated using scientific experiments and procedures.

The conservation strategy is directly linked not only to human activities but climatic factors such as climate change. Orchids have a highly specialized behavior that depends on microclimatic factors. Survival of orchids, beginning from germination of seeds to pollination, depends on a delicate balance of environment in their habitats (Liu et al. 2010). Soil type, soil moisture, humidity and transpiration rate, light penetration, availability of symbiotic fungi, specialized pollinators, diurnal and nocturnal temperatures are important; a slight change in one of the factors can lead to loss of an entire population over a time period. In many ways, laymen and farmers have treated orchids as grass and weeds, and a source of animal fodder during dry season. And this situation is aggravated by climate change, increasing the rate of extinction and loss of species. Therefore, to address the bigger picture on medicinal orchids, a holistic approach encompassing local knowledge, science, changing climatic factors, and sustainable production should be emphasized.

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Chapter 4
Current Status and Future Prospects for Select Underutilized Medicinally Valuable Plants of Puerto Rico: A Case Study

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4.1 Introduction

Underutilized or neglected species may also be referred to as orphan, abandoned, new, lost, underused, local, minor, traditional, forgotten, alternative, niche, promising, or underdeveloped species (Padulosi and Hoeschle-Zeledon 2004). They are also considered minor crops because they are not as important as staple crops as well as agricultural commodities in terms of global production and market value (IPGRI 2002). These species are no more considered a competition with the new crops that have taken charge of world food supply and receive aid from seed supply systems, production as well as postharvest technologies and extension services (Padulosi and Hoeschle-Zeledon 2004). With the declining value of these crops, the genetic base may be eroded and distinctive as well as valuable traits might be prevented from being utilized in crop adaptation and improvement (IPGRI 2002).

Apart from competitiveness, geographical, social, and economic reasons are other factors responsible for the decline of crops. For example, considering geographical distribution of plants, some species may be neglected in some areas while not in others. For instance, cowpea (*Vigna unguiculata*) was once widely used in Mediterranean countries and is now underutilized, but it is a staple food for sub-Saharan Africa. Chickpea (*Cicer arietinum*) is underutilized in Italy but not in Syria and West Asia (Padulosi and Hoeschle-Zeledon 2004).

Similarly, in one part of the world, a plant species may be popular but at the same time in another part of the world it could be poorly researched, marketed or managed. An interesting example of this is the dark green salad vegetables also referred to as

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rockets (*Eruca sativa*, *Diplotaxis tenuifolia*, and *D. muralis*). With the help of innovative cultivation and various commercial practices being carried, rockets have become an expensive vegetable in Europe while in Egypt, it is one of the cheapest sources and a rich source of micronutrients for the poor (Padulosi and Hoeschle-Zeledon 2004).

Several underutilized species have numerous uses and do not belong to a particular category of crops such as food, medicinal, ornamental. The analyses of useful nutritional and medicinal traits present in underutilized species are very important and must be investigated. It is also important to note that some underutilized species are adopted by the markets, whereas other such species are essential for subsistence farming. Cash income will be generated by species which are geared toward the market.

Moreover, the possibility of underutilized species to become commodity crops should not be underrated. It is not mandatory for the commodity to be a global commodity, but economic returns for the species at national, regional, and international levels can be raised if sufficient investment in research and development and in marketing and commercialization is deployed. For example, hulled wheat (*Triticum monococcum*, *T. dicoccum*) appreciates the processing technologies for allowing the usage of flour in making biscuits and pasta, and these species have regained cultivation in Italy. Owing to the simple marketing strategies, Roselle (*Hibiscus sabdariffa*) of Sub Saharan Africa has become a well-known beverage in Europe. A traditional African vegetable, okra (*Abelmoschus esculentus*) has been accepted in most markets around the world. This was not achieved with huge investments but with interests of the consumers and appropriate commercialization strategies.

However, it is also important to bear in mind that the task of promotion of underutilized species is fraught with challenges. The good news is that with growing realization about the value of many of these underutilized species, many of these challenges are being tackled (Padulosi et al. 2002). Globalization of agricultural market favors the improvement of use of underutilized species. Globe-trotting chefs and social media travel gurus bring exotic ingredients to the attention of a large number of people and consequently the demand for these products begins to rise on global platform. The question that arises now is, when underutilized species are characterized by regional national or local importance then what is the need of promoting them in the global world? The response to the question is that the key to retain the "safety net" of diversified food and natural products is protecting the resource base of underutilized or neglected species especially in developing countries (Eyzaguirre et al. 1999). For the weak social groups unable to afford certain products, diversification in agricultural systems is beneficial. For these groups the diversification in the portfolio provides them more self-sustainability and self-reliance. They live in tough areas which are not fit for cultivation of improved variety of commodity crops. The basic natural resource available to poor is plants and the part played by underutilized plant species is of utmost importance in reducing poverty and empowering the poor. This allows the poor to follow resource-base over commodity-based development (Blench 1997; Burgess 1994). However, it is not only the poor who benefit from the underutilized species, they can be shared by the entire mankind. Some of these benefits include a more balanced diet, better mainte-

nance of agroecosystems, significant use of marginal lands and increased conservation of cultural identity (Padulosi et al. 2002).

In extolling the virtues of underutilized plant species, it is also important to understand why they remain underutilized or slip into that status. The need to fulfil growing demands for food by a growing world population led to the Green Revolution and high-yielding staple crop cultivars developed by modern breeding programs slowly replaced the local traditional crop species. In contrast, the traditional crops fail to meet the modern standards for various reasons such as a lack of uniformity and other attributes and this led to them being progressively neglected by breeders from both public and private sectors (Stamp et al. 2012). Consequently, also in the marketplace, these traditional varieties are less competitive than commercial cultivars. The review by Padulosi and others presents a comprehensive account of the challenges to promoting underutilized species and the remedial measures for meeting these challenges (Padulosi et al. 2002).

4.2 Geography, Climate, and Vegetation

With regard to potentially useful underutilized plants, small islands that are rich in biodiversity are a veritable treasure trove. The unincorporated US territory of Puerto Rico includes the main island known as Puerto Rico and two other smaller islands known as Vieques and Culebra. The island covers an area of 8950 km^2 and is roughly rectangular in shape (160 km long and 56 km wide). A map showing the islands of Puerto Rico, Vieques and Culebra along with the land cover and forest formations in the year 2000 can be accessed at https://data.fs.usda.gov/geodata/rastergateway/caribbean/Puerto_Rico/IITF_GISciRS44_puertorico_landcov2000_map.pdf (USDA Forest Service, Kennaway and Helmer 2007).

The island has a climate that is tropical and predominantly maritime (Daly et al. 2003). Puerto Rico experiences the Atlantic Hurricane season which stretches from June to November, peaking from mid-August through mid-October. The main island lies between about 17°45′ and 18°30′N, and longitudinally stretches from 65°45′ to 67°15′W (Daly et al. 2003). In isolation, these coordinates may not convey the strategic location of Puerto Rico with regard to its multiple political and cultural influences. The island lies in the Western Caribbean Sea, at a distance of little over 1000 miles from Florida in the United States (North America) and less than 1000 miles from Caracas in Venezuela (South America). This proximity to two large landmasses, and also being located at about 5500 miles from the coast of Northwestern Africa has brought Puerto Rico under the influence of three politically and culturally important regions of the world at various points during the course of its history. In addition to these, Puerto Rico was under Spanish colonial rule from 1508 to 1898, before it was formally transferred to the United States of America at the end of the Spanish American war. As for the natives, the earliest inhabitants of precolonial Puerto Rico were people of the Ortoiroid culture from the Orinoco region in South America. In the centuries after the arrival of Ortoiroids, the island was successively

populated by the Igneri, the Arawak Indians and the Tainos. Even though conflicts with raiding Carib Indians and eventually the Spanish conquest nearly wiped out the Tainos, Puerto Rican culture of today has a strong representation of Taino elements (Van Middeldyk and Brumbaugh 1903). In terms of natural vegetation, tropical moist forests dominate the island. The mountainous central cordillera region has wet forests whereas dry forests are limited to the southern part of the island.

In Puerto Rico, there are a number of plant species that were important in the diversified and locally sourced diet and traditional medicine of the inhabitants of the island. This was quite unlike the situation today, when nearly in all parts of the world, our energy and nutrient needs are met by a narrowing number of plant species. Moreover, many plants that were a part of the diet of our forefathers are hard to find in the markets today. Not just the minor crops, but even some traditional staples have been lost along the way. Whereas the consumption of crops such as rice, wheat and maize continues to rise, the use of many complementary sources of energy and nutrition has drastically declined. Interestingly, in some parts of the world, the reverse trend of diversification of diets and agricultural products is also emerging with a view to promote healthy diets and sustainable food production (Dwivedi et al. 2017).

One such example of an underutilized crop on the island is *Arracacia xanthorrhiza* which is locally known as apio (Fig. 4.1a, b). The apio plant belongs to the family Apiaceae (celery family) and the part consumed are the swollen tubers (Hermann 1997). According to Hermann, apio is the most promising among the nine minor Andean root and tuber crops due to its wide culinary uses, palatability and superior quality of processed products made from it (Hermann 1997). In Puerto Rico, apio is grown in the mountainous municipalities of Barranquitas and Orocovis. An Apio Festival is also organized in Barranquitas every year in the month of April. Even though the demand for apio is high during times of availability, it has not succeeded in being established as a major crop. Also, there are about 30 species in the genus *Arracacia*, but *A. xanthorrhiza* remains as the only cultivated species. Apio is rich in calcium and some other vitamins and minerals such as ascorbic acid, Vitamin A and phosphorus. The yellow fleshed cultivars are particularly rich in beta-carotene. The starch from apio is known for its easy digestibility and is used in food preparations for convalescents and babies. In one study, flour made from noncommercial parts of apio (shoots and noncommercial roots), was successfully used to replace corn meal in semisweet biscuits. The substitution led to a superior product with lower calorific value (Gassi et al. 2016). A wild variety collected in Ecuador, which bears striking similarities to the cultivated *A. xanthorrhiza*, is used by the local people in the regions of Chimborazo and Bolivar to induce postpartum placental elimination. Notably, these potions are used on both humans and domestic animals (Hermann 1997).

Calathea allouia, known as Guinea arrowroot in English and locally as lerenes in Puerto Rico, is another such crop which has experienced a steady decline in production and consumption (Fig. 4.1c, d). Guinea arrowroot has in fact failed to establish itself as a crop of economic importance in any of the countries in which it was introduced, even though it found acceptability in various parts of the world. Traditionally, it has been grown on small scale by indigenous people in their vegetable gardens or on small farms as part of subsistence agriculture. High levels of

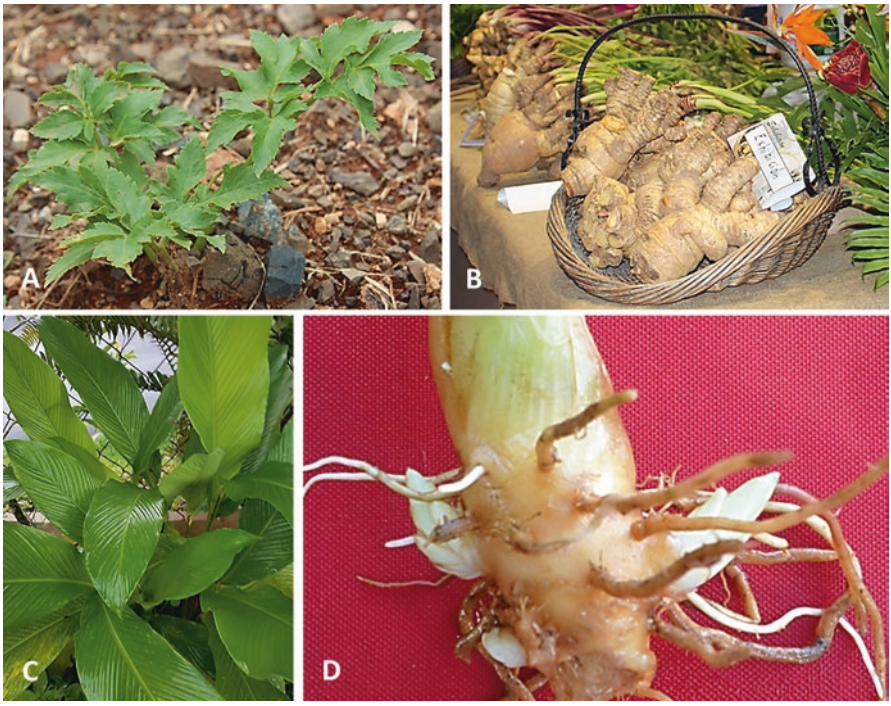

Fig. 4.1 (**a**) Young plant of Apio growing in Barranquitas, (**b**) Harvested Apio tubes on display at the Apio Festival in Barranquitas, (**c**) Leren plant, and (**d**) Leren rhizome cleaning and sterilization for ex situ conservation by tissue culture

nearly all amino acids including the essential ones (except cysteine) have been measured in the tuberous roots. Aside from culinary uses of its tuberous roots, leaves of Guinea arrowroot are used to make a traditional medicine to treat cystitis in South America (Hernandez Bermejo and Leon 1994). The leaves also have a diuretic action (Martin and Cabanillas 1976). Lerenes is also interesting from an agroeconomic standpoint as plantations at National Institute of Amazonian Research (Brazil) have shown very little damage to the plants from any pest or disease during a period of over a decade (Hernandez Bermejo and Leon 1994). And even though lerenes is difficult to grow due to its requirement of a particular combination of climatic and soil conditions, once established, these plants do not require much attention (Martin and Cabanillas 1976).

Jicama (*Pachyrhizus erosus*) is another such crop which has received little attention from growers and scientists but is full of beneficial properties. The napiform roots are edible, raw or cooked. Jicama root is a good source of minerals such as iron and magnesium and is particularly rich in Vitamin C (Duke James 1992). Jicama is also known to be rich in fructooligosaccharides including inulin. Inulin is a soluble fiber which is sometimes referred to as "natural insulin." In one study, the authors investigated the effect of jicama extract in streptozotocin (STZ)-induced

diabetic mice (Park and Han 2015). They specifically looked at the effect of jicama extract on α-glucosidase activity, α-amylase activity, and postprandial hyperglycemia in diabetic mice and found this effect to be inhibitory. In another study, the fiber extract of jicama (referred to as bengkoang in this study) was found to have immunomodulatory activity that worked through stimulating the innate and acquired immune responses (Kumalasari et al. 2014). This effect was evident in vitro (human and mouse cells) as well as in vivo (mice) (Kumalasari et al. 2014).

Special mention should be made of the fact that the "neglected and underutilized" status of the above-mentioned crops became amply clear to us during the writing of this book chapter. Very few peer-reviewed sources of information were available for each of these species and in all cases, we found ourselves depending on only one or two published articles or reports. This is as clear an indication as any that these species have not adequately been studied and investigated from a scientific standpoint. And in the absence of such studies, it is hardly surprising that these species are largely limited to cultivation in vegetable gardens in homes or as part of subsistence farming activities and no effective management practices have been developed. Apart from the above-mentioned cultivated species, Puerto Rican forests include a number of plant species that remain underutilized and neglected with regard to their medicinal properties. High biodiversity and endemism in the forests of Puerto Rico and the Caribbean in general could mean that the numbers of such useful plants could be very high and that many of these may not be found anywhere else on the planet.

There were also many other plant species that fit into the neglected category, if not from the utilization point of view, then from the fact that how little attention they have received from scientists and conservationists. Some examples of these are *Varronia rupicola*, *Bixa orellana*, *Tabernaemontana oppositifolia* and others. Some such plants are discussed below.

Varronia rupicola is native to the islands of Puerto Rico and Anegada (British Virgin Islands). In Puerto Rico, it is mainly found in dry forests along the southern part of the island and also in the isolated areas of western Puerto Rico and southern Vieques (Hamilton et al. 2015). It is a critically endangered woody shrub belonging to the Boraginaceae family and has a very restricted range of distribution. There are no studies on the habitat requirement, phenology, pollination and seed dispersal of this species (Hamilton et al. 2015). The species is facing many threats which include sea level rise, habitat loss, and attack and competition from invasive species. In 2014, The US Fish and Wildlife Service (USFWS) listed the species as "Threatened" under Endangered Species Act. It has also been designated critical habitat in US territory (Hamilton et al. 2015). Although there are no studies on phytochemistry and pharmacology of *Varronia rupicola,* studies conducted on the related species *Varronia curassavica* have demonstrated anti-inflammatory, analgesic, wound-healing and antimicrobial activities related to the presence of flavonoids, phenols and essential oils (Mota et al. 2017). Another study demonstrated that the essential oil extracted from *V. curassavica* is a potential raw material for the formulation of products to mitigate freshwater white spot disease in fish. Similar studies are required to be conducted on *V. rupicola* (Nizio de Castro et al. 2018). With regard to conservation, the US Fish and Wildlife Service have proposed seven critical habitat units for *V. rupic-*

ola. Habitat and biological features necessary for the survival of *V. rupicola* have been established. The Royal Botanical Garden (Kew) has developed a germination and cultivation protocol for *V. rupicola* and studies to determine genetic variation, reproductive biology and population ecology are being undertaken at Kew so that a management plan for the recovery of the species can be developed. *Varronia rupicola* is considered a critical element by the national heritage program of Puerto Rico Department of Natural and Environmental Resources (PRDNER). As defined by the US Fish and Wildlife Service, a critical element is a species that is under consideration for conservation because of its contribution to diversity and national heritage.

Bixa orellana L., popularly known as "urucum," is among underutilized medicinal plants in many countries, despite evidences of its ethnomedicinal uses in many communities. In Puerto Rico, it is known with the names such as chiote, achote, anatto, bija, and santo-domingo. It is native to Brazil, but it also grows in other regions of South and Central America. *B. orellana* is a small shrub or tree that grows about 3–5 m tall and about 20–30 cm in diameter. Traditionally, seeds of *B. orellana* are used in laxative, cardiotonic, hypotensive, expectorant, and antibiotic preparations. In addition, it has also been used for the treatment of wounds. An infusion of the leaves has been found to be effective against bronchitis, sore throat, and eye inflammation. Among different cultures across Central and South America, the majority of uses of *B. orellana* remain the same, as antipyretic, aphrodisiac, antidiarrheal, antidiabetic, and insect repellent. It is also used in food industry to color sauces, sausages, margarine, soup, cheese, and so on (Vilar et al. 2014). The major chemical compound present in the aril of seeds of *B. orellana* is bixin, a red-colored carotenoid. It is the main pigment responsible for the dyeing characteristics of seeds. Besides bixin, many other compounds have been isolated from its seeds such as norbixin, beta-carotene, isobixin, bixol, crocetin, bixein, bixol, ishwarane, ellagic acid, salicylic acid, threonine, tomentosic acid, tryptophan, and phenylalanine (Vilar et al. 2014). Therapeutic properties of *B. orellana* can be attributed to the high levels of carotenoids present in it. The presence of carotenoids also makes it a commercially important plant. Extracts of *B. orellana* have demonstrated biological activities such as antioxidant, hypotensive, antifungal, and insect repellent. Some of these activities are in match with traditional uses of this species (Vilar et al. 2014). There is need for more studies to confirm the biological activities of this plant. The presence of a wide array of chemical compounds and lack of mutagenic and cytotoxic activity associated with it makes it a potential source of several valuable phytopharmaceutical products (Vilar et al. 2014).

Tabernaemontana oppositifolia is a shrub or small tree restricted to forest on limestone hills. It belongs to the family Apocynaceae and is one of the potential medicinal plants native to Puerto Rico. It has been declared "vulnerable" according to The IUCN Red List of Threatened Species (World Conservation Monitoring Centre 1998a). Being an underutilized plant species, there are not many studies on the medicinal aspects of this plant. However, studies conducted on other plants of the same genus have demonstrated high medicinal potential. The genus *Tabernaemontana* has been known to produce indole alkaloids which are important bioactive molecules. The stalks of the *Tabernaemontana australis* produce coronaridine and voacangine and roots of *T. divaricate* produce bisindole alkaloids which are known to be

the inhibitor of acetylcholinesterase enzyme. Inhibition of acetylcholinesterase is one of the important therapeutic strategies for Alzheimer's disease (Murray et al. 2013). Similar studies are required to be conducted on *T. oppositifolia* before this potential medicinal plant belonging to a promising genus becomes extinct.

Magnolia portoricensis is endemic to Puerto Rico where it is found in the upper region of the mountain range. It is classified as "Endangered" by The IUCN Red List of Threatened Species. A member of the Magnoliaceae family, the distribution and population of this plant have shown dramatic decline due to selective harvesting for timber (Global Tree Specialist Group 2014). The wood of this tree species is majorly used in Puerto Rico for making furniture. Although *Magnolia portoricensis* has not been studied for medicinal potential, but studies conducted on other species of this genus have shown remarkable medicinal qualities. *Magnolia grandiflora* which is one of the most studied members of this genus is reported to possess anti-inflammatory activity which has been attributed to the presence of sesquiterpene lactones. The bioactive seeds of the *M. grandiflora* are known to contain honokiol and magnolol. The lignans honokiol and magnolol have a variety of pharmacological effects such as antibacterial, anti-inflammatory, and antioxidant (Lee 2011). *Magnolia portoricensis* has been the subject of very few scientific studies and there are no conservation measures in place in Puerto Rico with respect to this species. As the species is threatened in its natural habitat, ex situ conservation strategies should be put in place.

Ternstroemia subsessilis is native to Puerto Rico and occurs principally in the Caribbean National Forest. It has been listed as a "Critically Endangered" by The IUCN Red List of Threatened Species 1998 (World Conservation Monitoring Centre 1998b). It belongs to Pentaphylacaceae family and is a small tree of wet montane forests. There are only three small subpopulations left in the Caribbean National Forest and few specimens are scattered throughout the forest in Carlos Rivera. Efforts are being made to save this plant from extinction. The draft recovery plan for this species was proposed and cites low population densities, limited distribution, and vulnerability to habitat disturbances as principle threats to its existence. Another major threat to the existence of this species is hurricanes. The recovery plan for this species includes actions like habitat protection, protection of existing population, establishment of new populations and continuous research and monitoring.

There has been no study on the medicinal aspect of this plant species. Other species of this genus namely, *Ternstroemia oocarpa*, *Ternstroemia pringlei*, *Ternstroemia sylvatica* are used in the treatment of the anxiety and depression in the Mexican traditional system (Gutiérrez et al. 2014). A scientific study conducted on *Ternstroemia sylvatica* demonstrated it as a plant with inflammatory and analgesic properties, which is in harmony with its use in traditional medicine of Mexico (Moreno-Quirós et al. 2017).

Pleodendron macranthum is rare evergreen tree in the Canellaceae family. It is endemic to Puerto Rico and can grow up to 10 m tall and 20 cm in diameter. This species is federally listed among endangered species of the United States. There are only three tiny populations of this species remaining. It is limited in distribution because of deforestation and habitat modifications in the forests of Puerto Rico. A recovery plan was proposed in 1998 by The U.S. Fish and Wildlife Service. The

objective of the recovery plan was to direct the reversal of the decline of this species and to restore it to a self-sustaining status. Very little is known about the reproductive biology of *Pleodendron macranthum* because of the low numbers and isolated locations (US Fish and Wildlife Service 1998). There are no direct studies on the genetic, reproductive or medicinal aspects of this tree. But some other species of this genus, for example, *Pleodendron costaricense* is known to produce essential oils containing α-pinene, β-pinene, β-myrcene, β-thujene, and β-caryophyllene as their major constituents. Phytochemical assays conducted on *P. costaricense* also resulted in the isolation and identification of δ-tocotrienol, β-sitosterol, and four known drimane-type sesquiterpenes. The compounds isolated from *P. costaricense* were tested for antifungal activity and the investigation yielded positive results (Amiguet et al. 2006).

Maytenus ponceana is a species of plant endemic to Puerto Rico. It belongs to the Celastraceae family. There is no research specific to this species and there is a need to investigate its medicinal properties as its close relatives have shown immense medicinal potential. In fact, the genus *Maytenus* is known for its pharmacological potential and various biologically active compounds. A number of species in this genus are used in traditional medicine in South America. The leaves of many species are used to prepare an infusion to treat gastric conditions such as hyperacidity, gastric ulcers and chronic gastritis. Various studies conducted on other members of the genus *Maytenus* have revealed medicinal activity. Ethanolic extracts of the roots of *M. putterlickoides* demonstrated antileukemic activity and the root extracts of *M. senegalensis* were found to be antibacterial. *Maytenus emarginatus* also known as *kankero* has been traditionally used in India to cure diabetes and jaundice, and to heal sores (Veloso et al. 2017).

Picrasma excelsa is a member of family Simaroubaceae. It is known by many common names which include bitterwood, amargo, and quassia wood. It is native to South America and found in Brazil, Bolivia and Peru, the Caribbean and through Central America to Mexico. In Puerto Rico, it is a rare tree found at low to middle elevations. There are populations in the southeastern foothills, Central Cordillera and in the northern limestone hills (Gann et al. 2015–2018). It has been listed as "Vulnerable" under The IUCN Red List and the major threat to this plant species is deforestation (Areces-Mallea 1998). It has been part of traditional medicine for hundreds of years. The bark of this plant contains quassinoids, alkaloids, a coumarin, scopoletin, and vitamin B1. It has been traditionally used to lower fever, increase appetite and to improve digestion. The presence of strongly bitter principle in the bark improves digestion by increasing bile flow and secretion of salivary juices and gastric acids. Traditionally, it has also been used for its antiparasitic properties to rid the body of nematode worms. It is also used in the treatment of malaria. The bark is the most commonly used part of this plant and it can be harvested as per requirement throughout the year and can be dried for later use.

Petiveria alliacea L., a member of Phytolaccaceae family, is popularly known as "mucuracaa," "guine," and "pipi" in the tropical regions of the Americas like the Amazon forest, Central America, Caribbean, and Mexico where it is commonly found. It has been used in traditional medicine to treat ailments related to central nervous system (e.g., anxiety, pain, memory problems, and seizures). It was also used for its sedative properties. Additionally, it was also used in religious ceremo-

nies. Further studies must be conducted on this plant to identify and estimate its bioactive compounds and apply it to modern medicine (Luz et al. 2016).

Genipa americana is another plant species which is commonly known as Genipa and is commonly found in South America, Central America and Caribbean islands. It has been traditionally used to treat diarrhea, ulcers, anemia and liver impairments, jaundice and for its antibiotic and antiseptic properties. Being a highly medicinal plant, *Genipa americana* needs to be explored further and to be used in modern medicine (Fern 2018).

In addition to those discussed above, there are many other exemplary plants found in the forests of Puerto Rico which require the attention of scientific community to further strengthen and diversify the pill box of modern medicine. All of the above examples serve to bring to light the great importance of underutilized and neglected plant species, particularly those with potential medicinal value. At the same time, it is also amply clear that in Puerto Rico, as well as in other parts of the world, underutilized medicinal plants are at a great risk due to certain anthropogenic and natural factors. The major anthropogenic factors which affect these plants are uncontrolled deforestation, encroachment, overgrazing, and lack of awareness among local people (Roberson 2008; Shedayi et al. 2016).

4.3 Anthropogenic Factors

Anthropogenic factors have been major drivers in the decline of biological diversity as human pressures on ecosystems across the globe continue to rise (Geldmann et al. 2014). Below is an account of the major anthropogenic factors threatening underutilized medicinal plants.

4.3.1 Destruction of Natural Habitats

Habitat loss can be divided in three categories:

- Mass deforestation in which trees and plants are completely removed to change the landscape,
- Habitat fragmentation which involves alteration of the land to make roads and attractions for tourists in the middle of the forests which leads to an environmental chaos, and
- Habitat degradation which is caused by pollution by altering the quality of air, water and soil which causes invasion of some nonnative species which eventually hinders the growth of native species by increasing competition (Rinkesh 2018).

Agricultural industry runs on a business model which requires mass production of food leading to the alteration/destruction of natural habitats. In order to produce large amounts of food and cash crops, more agricultural land has to be prepared by

clearing the natural vegetation (Rinkesh 2018). However, it is noteworthy that some developed countries have shifted their focus to set aside a percentage of their agricultural land to grow novel/underutilized crops. Attention to high value products especially for medicinal purposes has led to this step. But poorer and developing countries have not been able to give much attention to this due to over reliance on the staple crops to feed the increasing population (Williams and Haq 2000).

Another crucial aspect related to habitat destruction is reduction in the populations of pollinators and seed-dispersal agents. Plants heavily depend on pollination for reproduction. Bees, butterflies and many other insects and birds are means of pollen transfer from one flower to another. Habitat destruction causes reduction in the numbers and diversity of these pollinators, which in turn affects growth in plant numbers. Similarly, many plants are aided by birds and animals in seed dispersal. Reduction in the populations of such agents of dispersal can also lead to a decrease in the associated plant species. Habitat destruction also negatively impacts natural assemblages of plants through reduction in plant diversity. This decrease in the number of species in a given area implies more numbers of one (or only a few) kinds of plants. When the buffer provided by one or more nontarget species between plants of the target species is lost, diseases and pests spread faster and farther. Plant diversity provides habitat to a large variety of insects and other organisms that include the natural enemies of plant pests. When diversity is reduced, populations of such natural enemies decline, and this allows pests to multiply uncontrollably. Also, it has been found that in agroecosystems where a single type of crop is cultivated, fungal diseases of plants are more severe as compared to the areas with greater diversity (Datta 2018). Additionally, the loss of certain plant species makes it difficult for other kind of plants to grow as it changes the composition of soil and alters its quality.

4.3.2 Encroachment

The expansion of residential, retail and industrial development from urban and suburban areas is referred to as sprawl and this has deeply affected the population of underutilized plants over the years. The need for more housing and business and industrial complexes has led to vast areas of natural vegetation being destroyed for the construction of buildings. Natural habitats of wildlife are destroyed to fulfill business purposes. Moreover, even unique ecosystems such as wetlands, where soil is covered with water, are filled up to make buildings (Rinkesh 2018). Due to these encroachment activities, natural habitats get affected in many ways and this in turn negatively impacts various underutilized plants with potential medicinal value. The explosive growth of human population in the past few decades has resulted in an increase in agricultural encroachment over forest lands and high sloppy lands. The cultivation practices followed on high sloppy areas often involves felling of trees and clearing of vegetation. This in turn leads to soil erosion and landslides which bury and destroy small, vulnerable populations of medicinally valuable plants. Also, encroachment into forest lands increases runoff of nutrients and other ions into

surface waters which, in turn, alters the vegetation and biodiversity of the forest (Zedler and Kercher 2005). For instance, in the southern portion of the Boreal Plains Ecozone in Canada, such processes have greatly reduced and degraded the forest cover due to agriculture and industrial encroachment (Bayley et al. 2013).

4.3.3 Siltation of Water Bodies

Siltation of water bodies is one of the major causes of deterioration of underutilized medicinal plant species. Silt is a material formed by rock or soil which usually occurs as suspended sediment on the surface of water bodies. It may also be present as soil deposition on a river bed (The Wye and Usk Foundation 2012). Siltation of water bodies in the forests results in decrease in water holding capacity of soil which drastically causes depletion of underground water. Thus, siltation of water bodies makes the nearby land unfavorable to the growth of various types of plants including those with medicinal value (Ramakrishnappa 2002).

4.3.4 Uncontrolled Deforestation

Irrespective of the cause of deforestation, it is the most severe threat to underutilized medicinal plants. In fact, deforestation remains the main factor behind extinction of many useful plant species in last decades. Puerto Rican forests are no exception to this. Caribbean forested zones have been drastically reduced due to human activities. The main factors for deforestation in Caribbean include urban development, converting land for agricultural economy, industrialization and tourism. Moreover, natural disasters are one of the chief reasons for deforestation which causes soil erosion, resulting in landslides and inundating floods. Global warming is further exacerbating these phenomena and contributing to deforestation even more. Similarly, greenhouses gas emissions are further increasing which again contributes to the warming effects on the earth, so it has become a complete chain of negative impacts of one climatic phenomenon over the other, all arising from human activities and lack of awareness among people (Maysonet 2011). The agricultural practices and techniques used also caused a huge amount of deforestation. Accelerated rates of deforestation have threatened the natural habitat of about 3000 native Puerto Rican plant species. This is a subject of worry for many plant species because many medicinal plants are not easy to propagate, especially the plant species which are monoecious (bearing flowers of one sex) that requires the opposite sex flowers from the other plant in order to reproduce.

4.3.5 Overgrazing

Overgrazing is another major threat to the numbers and distribution of medicinal flora. Grazing animals influence soil properties, plant species composition and alter biomass and distribution of biodiversity. Overgrazing reduces the ground cover vegetation and productivity. Overgrazing of plant species can cause genetic depletion resulting in rapid downward trend of their populations (Sher et al. 2010).

4.3.6 Shift in People's Interest from Ancient to Modern Medicine

The tropical forests of Puerto Rico are home to many medicinal plant species and ethnobotanical evidences show that they were extensively used by the local people in the past to treat various ailments. However, with advances in medical science, people's interests have shifted toward modern medicine, rather than relying on their local medicinal flora. Gradually, with the passage of time, traditional knowledge related to such plant species eroded and such uses of many plants were abandoned and forgotten. This loss of ethnobotanical knowledge is also a blow to modern medicine because over 50% of drugs used clinically today were originally derived from naturally occurring compounds (Vilar et al. 2014). Therefore, it cannot be stressed enough that there is a strong need for looking into the ethnobotanical aspects and traditional usage of underutilized plants in traditional medicine.

In addition to these human factors, natural factors have always been major threats to plant populations. Climate change is considered to be the biggest reason for the gradual decline and extinction of many medicinal plants. In the recent past, various natural disasters have enormously impacted the flora of North America, Central America and the Caribbean which is a clear indication that the underutilized medicinal plants are at a higher risk in these areas. The chief natural calamities which have jeopardized these plants are droughts, floods, hurricanes, extreme heat, extreme freezes, strong winds and tornadoes, and sudden climate change (Shannon and Motha 2015).

4.3.7 Natural Factors

In recent past, climate has drastically changed due to global warming and various ecosystems are struggling with the variabilities in environment caused by global warming (Kaneryd et al. 2012). In fact, it would not be incorrect to say that coping with the changing climate has become the biggest challenge for mankind and other life forms. For medicinal plants, a change in environmental factors such as temperature and precipitation greatly affect the levels and types of secondary metabolites (Das et al. 2016). Therefore, even though the adverse impacts of a changing climate

will have deleterious effects on plant diversity in general, these effects will be particularly significant in case of underutilized medicinally active plants.

The flora of the Caribbean region has been tremendously affected by climatic phenomenon in the recent past. North America and the Caribbean experiences a wide range of weather conditions and including extratropical cyclones, mesoscale convective systems and invasion of hot and cold air in northern areas. Similarly, tropical disturbances and mild to hot air incursions are experienced in southern areas. Among the individual locations, weather varies significantly depending upon the elevation, latitude, and proximity to ocean which eventually contribute to climatic anomalies such as droughts, floods, hurricanes, freeze, heat waves, blizzards, storms, and tornadoes (Shannon and Motha 2015).

Drought has always been a constant issue which adversely affects the flora of the United States and the Caribbean. The drought of 1930s in the United States and Canada impacted a wide range of plants and crops and caused an extensive crop loss. The flora of the United States Caribbean including Puerto Rico has not been spared by the negative effects of droughts. A huge loss in agriculture occurred in the Central America during the droughts of 1988, 2000, 2009 and 2012. Caribbean endemic flora has fallen prey to drought years and many plant species became endangered, many of them extremely underutilized. It is expected that in future, climate change may cause more droughts in the Caribbean region and therefore affect the already endangered and underutilized medicinal plants (Das et al. 2016; Shannon and Motha 2015).

Flash floods are a regular problem in Caribbean and Virgin Islands of the United States which are the regions vulnerable to flooding. Lately, the frequency of floods has only gone up due to climate change. Heavy local floods are common in Puerto Rico, and these not only destroy the agricultural production but also have devastating effects on the natural plant populations (Shannon and Motha 2015).

Extreme heat occurs regularly in Central America and the Caribbean; therefore, heat stress causes the death of many plant species and reduces the population of many medicinal herbs and shrubs. A drought in central and eastern Cuba occurred in the year 2003 and 2004 along with extreme heat conditions which led to the scarcity of water and death of many plants. In the year 2007, extreme hot weather in the United States, Canada and Mexico resulted in loss of agriculture production (Shannon and Motha 2015).

Earth's climate is continually changing at a rate faster than ever due to the elevated levels of carbon dioxide and other greenhouse gases. Since the late 1700s, the amount of carbon dioxide has increased by a whopping 40%. Climate change and the increasing frequency and intensity of extreme weather events pose grave danger to underutilized plants. In recent times, the impacts of these events have been felt across multiple scales of biodiversity including genus, species, communities, and ecosystems (Bellard 2012; Parmesan and Hanley 2015). As per the 2016 Royal Botanic Garden Kew State of the World's Plants Report, 3.96% of the vascular plant species assessed on the IUCN Red List are threatened by climate change and severe weather (RBG Kew 2016). Small islands, such as Puerto Rico and many others in the Caribbean region, are especially vulnerable to effects of climate change. For example, since 1901, ocean water around the island of Puerto Rico has warmed by around two degrees and the sea level has been rising almost an inch every 15 years.

Sea level close to Puerto Rico has risen by around 4 in. since 1960s. Due to this, storms and hurricanes are becoming more frequent and severe in intensity and heat levels are rising every year. Tropical storms and hurricanes have gotten more intense and catastrophic in last 20 years. Increasing temperatures negatively affects the forests of Puerto Rico which leads to expansion, shrinkage or shifting of ranges of many plants. If a plant species which optimally survives in drier areas is pushed toward the wet forests dominating zones, it may not necessarily tolerate the changes and could eventually die out. All these factors collectively affect the medicinal flora of Puerto Rico adversely which includes many underutilized plant species which are in fact, endemic to Puerto Rico (EPA 2016).

Storms and hurricanes are other major agents of destruction in the Atlantic and Caribbean zones. Hurricanes forming near the African coast intensify as they reach the Atlantic Ocean. These result in enormous environmental chaos almost every year (Miller and Lugo 2009). A number of hurricanes have cause wide spread damage in the Caribbean in recent years. Very recently, in the year 2017, two major hurricanes hit many Caribbean islands including Puerto Rico (Hillman 2018). Puerto Rico experienced a natural disaster of catastrophic proportions in the form of hurricane Maria in September 2017. Maria made landfall as a Category 5 hurricane and ravaged the island. The hurricane was followed by days of torrential rainfall that led to flash floods and landslides. Not only was the infrastructure of the island destroyed almost in its entirety, but natural vegetation and agriculture also experienced incalculable loss. El Yunque National Forest of Puerto Rico which is the only tropical rain forest in the US national forest system suffered destruction on a massive scale. Apart from reports from the ground which indicated near-total defoliation and extensive breakage and uprooting of trees, some of the first evidence for large scale destruction of vegetation and natural environments came from the first natural-color satellite images of Puerto Rico released by NASA's Earth Observatory. These post-Maria images taken by Operational Land Imager (OLI) on the Landsat 8 satellite when compared to images of the same areas of the island as imaged in 2016 revealed an unmistakable "browning" of the surface of the land due to loss of green plants and trees. It is expected that millions of trees have died and millions more will eventually die as a result of the effects of Maria. According to an article that discusses rapid remote sensing assessment of Puerto Rico following Maria, around 23–31 million trees could have been destroyed and severely damaged (Feng 2018). In another study that used multispectral remote sensing, it was shown that the extent of hurricane-induced damage in Puerto Rico was dependent on type of vegetation cover, elevation, and distance from the path of the hurricane (Hu and Smith 2018). Unfortunately, the cloud forest formations and evergreen forests which are centers of diversity and endemism were found to be among the most sensitive vegetation types (Helmer 2002; Hu and Smith 2018). As for agriculture, over 80% of the crops growing on the island were lost to Maria resulting in economic losses to farmers and across the agricultural industry. It is estimated that these losses run well over a billion dollars.

Aside from the financial impacts of the disaster, the damage to vegetation and natural environments has been immense and it is near impossible to put a number to the short and long-term loss caused in this respect. Unfortunately, for species-rich

and underexplored ecosystems such as the rainforests of Puerto Rico, it is likely that a few plant species, as-yet-unknown to science have been lost. Also, species with a narrow distribution across the island might find their very survival to be threatened by loss of habitat. It is also important to understand that underutilized plant species for which we have limited, or no germplasm collections combined with little knowledge about pests and pathogens are at particular risk. Underutilized species that are wild are at an even higher risk compared to their cultivated counterparts.

However, increasing frequency and intensity of natural disasters and other threats posed by humans to underutilized plants is a harsh reality of living in an ever-changing world. The responsibility of conserving the fauna and flora of the planet essentially falls on us humans, because there is no doubt that our activities in the last hundred or so years have greatly contributed to the ecological imbalance we are witnessing today. However, with careful thought and planning and scientifically backed conservation and cultivation programs, we could enhance the survival and utilization of underutilized plants. On the other hand, for plants that are already well known for their useful properties, harvesting such resources from the wild populations is a continuous practice that is increasing with the increasing demands of medicinal plants. The demand for medicinal plant resources is increasing at the rate of 8–15% per year in Europe, North America and Asia. There is a threshold, below which, reproductive capacity of the species starts declining irreversibly. Various sets of recommendations regarding the conservation of the medicinal plants have been developed which encompass both ex situ and in situ conservation (Chen et al. 2016).

4.4 In Situ Conservation

In situ conservation involves the maintenance or recovery of the viable populations in their natural environment and in case of the domesticated species, in the surrounding where they have developed their distinctive properties. The goal of in situ conservation of the target species is protection, management and monitoring of the selected populations in their natural habitat without disrupting their natural evolutionary processes (Heywood 2014). This allows new variations to be generated in medicinal plants that are mostly endemic in nature and their medicinal properties can be attributed to the presence of secondary metabolites which are a product of their interaction with the natural environment. The medicinal properties of these plants may not be expressed under culture conditions. Natural reserves and wild nurseries are examples of in situ conservation whereas botanical gardens and the seed banks are the examples of ex situ conservation (Chen et al. 2016).

In situ conservation is also promoted because landraces cultivated by local farmers are essential components of indigenous cultures. Also, the cost of in situ conservation is low, and it is the main form of conservation for wild relatives of crops, while allowing evolutionary forces to continue acting on populations (Gepts 2006).

One of the fundamental components of in situ conservation is the involvement of local communities. The reasons for this is that it is not difficult for collectors of medicinal plants to harvest medicinal plants unsustainably or illegally, if the only regulations in place are those which are associated with government officials. But if local communities participate in conservation, such illegal activities can be greatly reduced. This makes formation of relationship between scientists, government officials, local communities, and other stakeholders at the onset of the conservation projects as critical (Hamilton 2004).

Natural reserves are those protected wild areas that are created for the preservation and restoration of biodiversity. There are more than 12,700 protected areas that have been established accounting for 8.81% of the earth's land surface. However, it is not possible to designate every natural habitat a protected area because of cost restrictions and increasing land uses. In such cases, wild nurseries are established in a place that is only a short distance away from the natural habitat and this involves species-oriented cultivation of endangered medicinal plants. Wild nurseries can be a particularly efficient method for the conservation of endemic, endangered plants (Chen et al. 2016).

4.5 Information and Assessment

Quantitative assessment of the distribution and status of the underutilized medicinal plants is indispensable for the proper management of such plant resources. A lack of information or unreliable information can cause various inconveniences which may lead to mismanagement of medicinal plants. At the same time, it is also critical to identify various genetic variants of medicinal plant species among the wild and cultivated populations (Manivannan 2010).

4.6 Species Management Plans

To achieve effective action in the in situ conservation process, there should be some kind of plan of action. These are generally known as conservation plan, management plan or recovery plan. It usually involves the range of action including both in situ and ex situ techniques and the plan can be focused on one single species or multiple species. The common features of the species management plan include description of the species, ecogeographical information, nature of threats, existing conservation action, management objectives, required actions, and a statement about how the plan will be implemented, cost, budget, and so on. Species management plans should complement the protected area management plans. Preparing a species management plan is a time taking process and it requires approvals and financing before the start of implementation (Heywood 2014).

4.7 Ex Situ Conservation

Ex situ conservation effectively complements in situ conservation and is not sharply separated from it, especially in case of endangered medicinal plants, plants with low growth rate and small population. It aims at cultivating and naturalizing threatened species to ensure their continued survival and sometimes to produce high quantities of superior quality drug material. Many wild species of medicinal plants can retain their medicinal potency when grown in gardens far away from their habitats and can also have their propagules stored in seed banks for future plantations. Botanical gardens and seed banks are two major methods of ex situ conservation.

Botanical gardens can also maintain ecosystems that can enhance the survival of endangered or rare species. However, botanical gardens maintain only few individuals of each species, so their role in genetic conservation is rather limited. On the other hand, botanical gardens can play an increasing role in the conservation of underutilized medicinal plants by developing cultivation and propagation protocols and by undertaking programs involving domestication and variety breeding (Chen et al. 2016).

Seed banks are facilities for storage of seeds under cold and dry conditions with the aim of prolonging seed viability and preserving plant material for future use. Seed banks are gaining popularity as a tool for the conservation of wild species of medicinal plants and this rise in popularity can be attributed to a number of factors. Seed banks allow quick access to the plant samples to evaluate their properties so that helpful information for the conservation of remaining natural population can be generated. Also, plants conserved in the seed banks are protected from problems like pests, predators and habitat destruction which can plague both wild and cultivated populations (Schoen and Brown 2001). However, reintroduction of plant species back into the wild and assisting in the restoration of the wild populations is still a challenging task for seed banks (Chen et al. 2016).

4.8 Good Agricultural Practices (GAP) and Organic Farming

Good agricultural practices need to be formulated, as this approach ensures the production of high-quality, safe, and contamination-free herbal drugs. GAP apply available knowledge to address various problems in the production of herbal drugs. GAP encompasses production sites, germplasm, cultivation, collection and various quality aspects. China has actively promoted GAP to enhance the growth of commonly used herbal drugs at the sites where those medicinal plants are traditionally cultivated (Chen et al. 2016).

As medicinal plants are required to be free from harmful residues, modern agricultural methods and techniques that utilize a large amount of chemical inputs cannot be adopted for the cultivation of medicinal plants. There have been instances of detection of harmful chemicals in some herbal products. Therefore, to ensure safe

and reliable raw material for herbal products, there is an urgent need to implement cultivation practices that are consistent with principles of good agricultural practices (Malik et al. 2013).

Organic farming is seen as a route to developing integrated, humane, and environmentally and economically sustainable production methods for cultivation of medicinal plants. Organic farming prohibits the use of synthetic fertilizers, pesticides, and herbicides. Organic fertilizers such as compost, bone meal, and fish meal used in organic farming are typically not harmful to the environment. Organic fertilizer significantly affects the growth of medicinal plants and synthesis of bioactive compounds by supplying essential nutrients and by improving soil stability. For example, the application of the organic fertilizer in *Chrysanthemum balsamita* increased its biomass yield and essential oil content. Therefore, organic farming has an important role to play in sustainability cultivation of medicinal plants (Chen et al. 2016).

4.9 Sustainable Use of Underutilized Medicinal Plants

To prolong the advantages and effectiveness of medicinal plants, sustainable use and good harvesting practices are of also of great importance. Good harvesting practices need to be formulated as some harvesting practices are more harmful to one species of medicinal plants than to others. Root or whole plant harvesting is more damaging to the medicinal plant than collecting leaves and flowers or buds. Some herbal drugs are required to be made up of whole plants or roots, and in such cases testing their leaves as a source of the drug can be a good approach toward more sustainable harvesting practices (Chen et al. 2016).

4.10 Micropropagation: Synthetic Seeds and Somatic Embryogenesis

Micropropagation via tissue encapsulation of propagules not only plays role in the storage and transportation but also gives high regeneration rates. Production of synthetic seeds can be effectively utilized for the conservation of rare and endangered underutilized plants which are difficult to regenerate through conventional methods. It can help to overcome problems like low seed set and poor germination rate. Synthetic seed technology is an alternative method for production of seeds by using various plants parts like shoot apices, nodes, and somatic embryos. Many medicinal plants do not produce viable seeds and sometimes seed set is very low. For example, in *Cineraria maritime* synthetic seeds produced by using micro shoots, stored at 25 ± 2 °C have yielded regeneration rate of 82.35% (Kumar et al. 2014).

Somatic embryogenesis involves the induction of embryos from the somatic cells or tissues of the plants. Somatic embryos are bipolar structures capable of forming

shoots and roots from their apical and basal meristematic regions respectively. After excision from the parent tissues, they can be induced to germinate in tissue culture media under aseptic conditions. It is a desired method of plant regeneration and these embryos can further be encapsulated to form synthetic seeds. Somatic embryogenesis, alone or in combination with synthetic seed technology, provides an alternative method of conservation of medicinal plants (Kumar et al. 2014).

4.11 Conversation Through Cryopreservation

Cryopreservation of in vitro cultures of medicinal plants is a useful, long-term conservation technique. It involves storage of propagules in liquid nitrogen (−196 °C) in which cell division and metabolic and biochemical process are arrested. As whole plants can be regenerated from the frozen propagules, cryopreservation can be used for the conservation of endangered medicinal plants (Sharma and Dubey 2011).

In case of Puerto Rico, a key aspect of conservation and enhancing the use of underutilized medicinal plants would be the participation of traditional healers and practitioners of natural, plant-based medicine. It has been widely accepted that our limited economic and scientific resources for plant-based drug discovery need to be channeled through an ethnobotanical approach. In fact, one of the most productive approaches for screening plants for medicinal and nutraceutical properties is through ethnobotany (Albuquerque et al. 2012; Cox and Balick 1994). Practitioners of traditional medicine have valuable information which may have been passed down verbally, often with few or no written records. Even though this knowledge is not scientifically tested or validated, it provides information that could be a narrower starting point as compared to random bioprospecting. Particularly for biodiversity rich zones like Puerto Rico, it would be practically impossible to screen all of plant species for their medicinal and nutraceutical properties! And faced with the vagaries of climate and negative impacts of human activities, it is even more important to proceed in a targeted manner and identify and conserve the species that could be most useful to mankind. This way, instead of having to undertake the impossible task of screening thousands of plants in a biodiversity-rich zone, we will find ourselves devoting the available resources toward thoroughly investigating a much smaller number of candidate species. With the growing number of emerging diseases and resistance of pathogens to our current repertoire of drugs, the need to identify novel drug compounds is dire and time is of the essence.

Also, of primary importance is the collection and conservation of such plants. The locations of different wild populations of a candidate species should be identified and collections should be made in such a way that there is minimal disturbance to the habitat and the remaining members of the population. Preservation of germplasm should be carried out where such facilities are already available on the island (research institutes and universities) or it may be an attractive option to establish a germplasm conservation center for one or more such species. However, with the limited resources of the island, a more practical alternative could be to bring such

wild species into cultivation. The cultivation could be undertaken in two ways. Either it could be completely in the hands of trained personnel at scientific institutes and universities or another way could be to solicit the support of local farmers whose efforts would be overseen by scientific personnel. Community participation in such conservation-through-cultivation programs could be a good, long-term strategy. At the same time, the introduction of underutilized medicinal plants into domestic cultivation will allow the recovery of wild populations of medicinal plants, some of which may be overharvested for their medicinal or other uses. Also, as a result of availability from cultivation, nonsustainable harvesting methods will be discouraged. It is also notable that any form of cultivation or harvesting comes with some selective practices on the part of the grower or harvester. In case of wild populations, such selective harvesting practices might erode genetic diversity within the species and threaten its survival. But in case of cultivation, such selection practices could actually help enhance the medicinally important aspects of a plant species. Cultivation also opens doors for opportunities like yield optimization, uniform, high quality product and control at various stages of the production process (Amujoyegbe et al. 2012). In addition to the cultivation, there are various other approaches that can be adopted to modify the traits of underutilized medicinal plants in Puerto Rico.

4.12 Study of Reproductive Biology of Underutilized Plants

Medicinal plants, especially flowering plants show great floral diversity and confusing array of reproductive adaptations. One of the notable features of this diversity is that even related species often differ in mating and pollination methods (Barrett 2010). Reproductive biology studies encompass sexual and asexual reproduction in plants which further includes pollination, gene flow, propagule dispersal and genetic variation between and within populations. To develop effective strategies for the conservation and sustainable use of the underutilized plants detailed information on the reproductive biology is a prerequisite. About 87.75% of the flowering plants are pollinated by animals. Plants and pollinators have coevolved to develop mutualistic adaptations through their 100 million years old relationship. Human induced environmental and anthropogenic disturbances have severely reduced the number of pollinators globally. Seed dispersal is one of the important factors affecting the population size in different favorable habitats. Seed dispersal and availability of pollinators heavily affect the success of sexual reproduction in plants. Studies on the diverse aspects of the pollination biology and seed dispersal ecology are must to understand plant pollinator and seed dispersal system (Sreekala 2017).

Experimentation and field observations on the various aspects of the pollination biology and seed dispersal ecology must be conducted in Puerto Rico to develop strategies for the use and conservation of underutilized plants. For example, Royal Botanical Garden (Kew) has developed a protocol for the germination and cultivation of *V. rupicola*. Currently studies on the reproductive biology and population ecology are being conducted to develop management plan for the recovery of this

an endangered underutilized plant (Rivera and Mackenzie 2014). Reproductive biology of the *Jatropha curcas* was studied in a site of tropical southeastern Mexico, within its center of origin. Study demonstrated female flowers open before the male flowers favoring xenogamy which could be the reason behind high genetic variability found in *J. curcas* for this region of the world (Rincón-Rabanales et al. 2016). To establish any underutilized plant as a new crop, reproductive aspects which include knowledge about flowering, phenological behavior, sexual system, and fruit and seed production should be studied.

4.13 Development of Suitable Propagation Methods

An understanding of suitable methods of propagation is pivotal for cultivation and commercial success of underutilized plants. Many medicinal plants are cross-pollinated and maintaining uniform yield by propagating through seedlings is not possible. In such cases, in-vitro propagation methods are preferred. For example, *B. orellana* is traditionally raised through seedlings, but it leads to nonuniform yield because of the cross-pollinating nature of *B. orellana*. In such cases tissue culture techniques can play significant role to generate plants of uniform yield and toxicity, and to maintain genetic fidelity (Siril and Joseph 2013). Development of the suitable propagation method not only helps to maintain the demand of the products derived from plants but also helps in conservation and sustainable use of it. *Pityopsis ruthii* is an endangered plant endemic to Tennessee. Efforts to recover this plant mainly focused on the development of the vegetative propagation, in vitro multiplication and seed germination (Wadl et al. 2014).

Tissue culture can be used to generate fertile haploid plants and to overcome the reproductive barriers between the distantly related plants. Micropropagation, somatic embryogenesis, or organogenesis can be used to clone large number of plants from genotypes having desirable characteristics allowing these elite plants to be more widely distributed (Dawson et al. 2009).

4.14 Application of Conventional Breeding Techniques

In the cultivation of medicinal plants, both traditional and biotechnological plant-breeding principles cam be applied to improve yield and uniformity and to alter toxicity or potency. The selection of the vigorous and fertile genotypes can play considerable role in the improvement of traits like seed production and seed viability. Although artificial selection is a time-consuming process, but it can be accelerated by developing reliable methods for the detection of the desired traits at an early

stage of the reproductive cycle. For instance, laser speckle technology distinguishes between viable and nonviable seeds based on the differences in the speckle activity when seeds are illuminated by laser (Braga Jr et al. 2003).

Marker(s)-assisted selection is a way to recognize desirable genotypes at an early stage to accelerate the selection process. These markers identify alleles that are directly concerned with a desired trait or genes very close to that. Although identifying functional genes and useful marker sequences is a time-consuming and costly process, progress in this area of molecular biology has been accelerated by availability of whole-genome sequences of model species such as *Oryza sativa* and *Arabidopsis thaliana*. The DNA probes from one species can be used to identify homologous DNA sequences in closely related species, as there is high similarity in the DNA sequences of the functional genes between different species. In *Cannabis*, amplified fragment length polymorphisms and microsatellites molecular markers have been developed for breeding as fiber crop and for forensic use (Canter et al. 2005).

4.15 Genetic Transformation of Medicinal Plants

Genetic transformation involves direct manipulation of the DNA sequences to alter gene expression. Although content of the active compounds is the primary target for trait manipulation in medicinal plants, for the development of the medicinal plant species as a crop, traits related to agronomic value should be improved. This includes uniformity, stability, growth and development, and resistance to biotic and abiotic factors (Canter et al. 2005). One of the common approaches to increase the product output of the medicinal plants is genetic transformation of cultures. In this, bacterial vectors are used to transfer the desired genes into the cultured plant material. For example, hairy roots transformed with *Agrobacterium rhizogenes* have been suitable to produce secondary metabolites in hormone-free conditions with stability and high productivity. Many medicinal plants have been successfully transformed with *Agrobacterium rhizogenes* (Sevon and Oksman-Caldentey 2002). Biosynthetic pathways of *Mentha* spp. have been engineered to modify essential oil production in the trichomes and to increase resistance to fungal pathogens and abiotic stresses. Agrobacterium-mediated gene transfer systems are already in place for *Taxus* (yew), *Echinacea*, *Scrophularia* (figwort), *Artemisia*, *Thalictrum* (meadow rues), and *Digitalis* (foxglove). Tissue culture and regeneration are not only essential elements of the manipulation of the genetic makeup of the medicinal plants, but they can contribute to other aspects of plant breeding (Canter et al. 2005). In addition to technologies for genetic transformation, it is also important to develop "omics" tools for crops of interest. The chloroplast genome of Apio was recently sequenced by researchers at the Barranquitas campus of Universidad Interamericana de Puerto Rico (Alvarado et al. 2017).

4.16 Pathway Engineering in Medicinal Plants

There are many examples of utilization of pathway engineering in the improvement of medicinal plants. In transgenic henbane (*Hyoscyamus niger*) hairy root cultures, simultaneous introduction and overexpression of genes encoding the rate limiting upstream and downstream enzymes of scopolamine biosynthesis resulted in nine times higher amount of scopolamine than that obtained with the wild type plants (Zhang et al. 2004). Hyoscyamine 6 beta-hydroxylase is an enzyme that converts hyoscyamine to scopolamine through an oxidative reaction in the biosynthetic pathway. Hydroxylase gene was introduced into hyoscyamine-rich *Atropa belladonna* through *Agrobacterium*-mediated gene transfer. Scopolamine was the only alkaloid present in the leaves and stem of the transgenic plants thus obtained. Such metabolically engineered plants can also serve as good breeding material to obtain improved medicinal compounds (Yun et al. 1992).

4.17 Improving Agronomic Traits in Medicinal Plants

Much like other agricultural crops, the key agronomic targets for improvement of medicinal plants have been resistance to pests, diseases and herbicides. Transgenic *Atropa belladonna* with resistance to the herbicides bialaphos and phosphinothricin have been developed using Ri binary vector and plant regeneration from hairy roots (Saito et al. 1992). Herbicide resistant transgenic *Panax ginseng* was produced through *Agrobacterium tumefaciens* cocultivation for the introduction of phosphinothricin acetyl transferase gene that provides resistance to the herbicide bialaphos (Choi et al. 2003). Herbicide-tolerant *Solanum nigrum* has been regenerated using somatic hybridization from tissue culture and atrazine selection (Canter et al. 2005). A reliable system of polyploidy induction was developed in *Anoectochilus formosanus,* a medicinal orchid. The resulting tetraploid demonstrated a significant enhancement of various agronomic traits such as shoot length, leaf width, root length, and size of stoma (Chung et al. 2017). The application of biotechnology for tolerance to abiotic stresses is not common for medicinal plants, but it is believed to have great potential.

4.18 Identification of Disease and Pests and Development of Management Strategies

In case of medicinal plants, quality and quantity of active compounds is of prime importance to industries that process these crops. But from the growers' point of view, performance of the plants in the field is key to a successful harvest. A major goal of conserving and cultivation underutilized medicinal plants is to identify diseases and pests associated with these plant species. In case of underutilized plants,

very little information about these aspects is available and knowledge must be generated to ensure success in the field. A crop that is extremely vulnerable to large losses from disease and pests will not find favor with growers. For example, in case of apio in Puerto Rico, a parasitic nematode causes rot of the corm which is the commercially important part of the plant. A team of researchers from University of Puerto Rico has worked on developing treatments and integrated pest management approaches for corm rot disease of Apio. The group has also developed in vitro protocols with the goal of providing disease-free apio propagation material to Puerto Rican farmers (Giraldo and Sanchez 2017).

4.19 Establishment of Facilities for Processing and Marketing of Products

Deriving the benefits of underutilized medicinal plants also depends on the availability or establishment of facilities for processing agricultural output. Farmers will only grow crops that they can find a market for. For small islands like Puerto Rico, if it such processing facilities are not available and difficult to establish, a system should be put in place through which the agricultural products can be transported to the nearest appropriate processing facilities.

In conclusion, it is very important to identify, conserve, and utilize neglected and underutilized plant species of medicinal value. But humankind seldom strikes a fair balance between utilization and conservation of natural resources. In case of plants of medicinal value, the imbalance is impossible to ignore. On the one hand, there are medicinal plant species that have been overharvested and dragged to the brink of extinction. On the other hand, there are plant species whose status is such that "neglected" and "underutilized" are words that can be aptly used to describe their status. The key to long term benefits from such plants lies in the sustainable utilization of such resources. In addition, it is important to ensure that farmers who adopt and nurture these valuable species and their communities receive fair benefits. The scientific community needs to investigate the medicinal properties of such species and also to gear up to play a role in improving the public understanding of the value of these underutilized species. For small, biodiversity rich islands such as Puerto Rico, the utilization of such underutilized medicinal plants can have several benefits. On the one hand, the scientific identification and validation of medicinal properties of such species will give the necessary impetus to their cultivation and conservation, and on the other hand, the communities that adopt these crops for cultivation can generate employment opportunities and improve their economic situation.

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Chapter 5
Black Pepper: Health Benefits, In Vitro Multiplication, and Commercial Cultivation

Virendra M. Verma

5.1 Introduction

Black pepper (*Piper nigrum* L.) belongs to the family Piperaceae. It is a perennial woody climbing liana. The plant is native to India, Indonesia, Malaysia, South America, and the West Indies but is also widely cultivated in tropical regions worldwide. Black pepper is a universal table condiment used to flavor all types of cuisines worldwide. It is considered as the "King of Spices" (Nair 2004; Srinivasan 2007). The spicy taste is mainly due to the presence of the compound piperine. Piperine is a pungent alkaloid (Tripathi et al. 1996) that enhances the bioavailability of various structurally and therapeutically diverse drugs (Khajuria et al. 2002). Increasingly popular modern-day uses of piperine, the active principal of black pepper, are to stimulate metabolism, aid in absorption of nutrients, and boost the efficacy of drugs (Szallasi 2005).

In most pepper-producing countries, black pepper is a smallholders' crop and many farmers depend on it for their livelihood. Cultivation varies from intensive monoculture to extensive homestead gardens. The use of reliable standards (supports) for the successful establishment of black pepper plantations is a common practice in producing countries. Standards are of two types: living and nonliving. The use of nonliving (dead) standards (reinforced cement-concrete posts, granite pillars, and teak poles), though often resulting in higher black pepper yields, is less widely practiced by smallholders, mainly due to the high capital investment required (Dinesh et al. 2005). Nonliving standards have been used in Malaysia, Vietnam, Brazil, Thailand, and Indonesia, facilitating closer spacing and higher yields (Menon et al. 1982; Kurien et al. 1985; Reddy et al. 1992).

Black pepper is considered as an important cash crop in the Pacific, specifically in Micronesia. The Micronesian islands lying just above the Equator enjoy a tropical

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climate, with relatively even, warm temperatures throughout the year. The Federated States of Micronesia (FSM) is made up of 607 small islands spread over a million square miles of the Western Pacific Ocean with a total land area of only about 271 square miles. Rainfall is generally plentiful, and Pohnpei, the capital state, is reputedly one of the wettest places on Earth, with up to 330 in. of rain per year. Nevertheless, drought conditions do occur periodically throughout FSM, especially when the El Niño condition moves into the Western Pacific. At these times groundwater supplies dwindle to emergency proportions. Tropical typhoons constitute an annual threat, particularly to the low-lying atolls of FSM (Government of the Federated States of Micronesia 2014).

Agriculture is an important industry and it could greatly help in economic development and growth, and in bringing food self-sufficiency in Micronesia. However, current agricultural programs in the country are mostly on a subsistence level, and economic development is largely dependent on the outside world. Serious damage caused by natural calamities such as wave surges, saltwater flooding, and drought continually pose challenges for the local farmers. Moreover, a lack of technical know-how and changing lifestyle and food habits of the islanders are causing an increase in the consumption of imported foodstuff, leading to an overall decline in local agricultural production.

One recent example of such decline in agricultural production is the ceasing of local production of black pepper in Pohnpei, Micronesia. Black pepper from Pohnpei, Micronesia, is regarded as a relatively rare commodity of exceptionally high quality. The "Pohnpei Pepper," a pepper product that was unique to Pohnpei, was marketed successfully from Pohnpei for a short time. The product was admittedly a high-end, niche product without a large volume of sales, but the potential of the product was barely tapped before its production ceased (Cheshire 2003). Therefore, to promote sustainable black pepper cultivation practices in Micronesia, a project was developed to support local farmers and enhance agricultural productivity of black pepper in the region.

Based on the inputs of the stakeholders, farmers, agricultural professionals, and direct observations during black pepper farm visits, the project team identified the following issues that have caused serious decline in black pepper production in the Micronesian region:

1. Nonavailability of elite and disease-free seedlings.
2. Limited traditional tree-fern supports (standards).
3. Poor soil fertility management and fertilizer applications.
4. Occurrence of pests, diseases, and nutrient deficiencies.
5. Shortage of trained agricultural professionals.
6. Inadequate knowledge for fast propagation of cash crops.
7. Limited skills for commercial production and basic crop management.
8. Insufficient storage and processing facilities.
9. Stiff competition between local and imported products.

The nonavailability of disease-free and elite seedlings is a major bottleneck in quality black pepper production in the region. Micronesia is a small island state;

therefore, quarantine measures are very strict and the entry of any planting material is strictly prohibited. Considering the difficulty in maintaining disease-free parental stocks in the tropics, meristem culture is increasingly being appreciated as a potential means of germplasm preservation, and for the production of elite and disease-free planting materials on a mass scale.

Plant biotechnology is a powerful tool. Appropriate and skillful use of biotechnological approaches such as in vitro multiplication could help in successful multiplication of the elite and disease-free seedlings of cash crops and provide a means for germplasm conservation in an inexpensive way. In vitro multiplication is the best way to multiply the planting material of vegetatively propagated crops in bulk and produce disease-free, elite seedlings throughout the year.

The project aims to address some of the prime agricultural issues affecting the commercial black pepper production in Micronesia. It is specifically designed to develop micropropagation and nursery management systems in the region. The objectives of the project include: to produce elite black pepper seedlings in bulk quantities to ensure year-round availability of identical, disease-free, and high-quality planting material; to find alternatives for tree-fern supports; to determine appropriate fertilizer type and doses; to develop and publish a commercial black pepper cultivation guide appropriate for Micronesia. Adoption of new practices such as micropropagation of black pepper for improvement and enhanced productivity will ultimately help in reviving the local pepper industry. The following sections describe the health benefits, in vitro multiplication, and commercial cultivation of black pepper.

5.2 Black Pepper: Health Benefits

Black pepper is a spice widely consumed worldwide and is commonly used in traditional medicine. It is a rich source of minerals such as manganese, copper, magnesium, calcium, phosphorus, iron, potassium, and vitamins such as pyridoxine, riboflavin, thiamine, niacin, folic acid, and vitamins A, C, E, and K. Black pepper also has a high content of dietary fiber along with a moderate amount of protein and carbohydrates.

Piperine, a major alkaloid found in black pepper, has numerous beneficial properties and can improve human health in many ways. Piperine protects against oxidative damage by inhibiting free radicals and reactive oxygen species, as well as positively influencing antioxidant enzymes (Srinivasan 2014). Antioxidants in black pepper can prevent and repair the damage caused by free radicals and thus help prevent cancer, cardiovascular diseases, diabetes, and liver problems. Black pepper is also known to improve digestive health and enhance brain health. Recently, piperine has been shown to have fundamental effects on p-glycoprotein and many enzyme systems, leading to bio-transformative effects including chemoprevention, detoxification, and enhanced absorption and bioavailability of herbal and conventional drugs. Based on modern cell, animal, and human studies, piperine has been

found to have immunomodulatory, antioxidant, anti-asthmatic, anticarcinogenic, anti-inflammatory, anti-ulcer, and anti-amoebic properties (Meghwal and Goswami 2013). Specific health benefits of black pepper on various metabolic processes and diseases are described below.

5.2.1 Digestion

The consumption of black pepper facilitates proper digestion via piperine that stimulates the stomach to release more hydrochloric acid which aids in the digestion of food protein. Piperine also stimulates digestive enzymes in the pancreas and benefits the overall digestive process (Srinivasan 2007).

5.2.2 Respiratory Conditions

Black pepper is a good treatment for respiratory conditions due to its properties as an expectorant, as well as due to its strong anti-inflammatory properties. Black pepper with honey is a traditional approach to respiratory congestion, helping to expectorate and dry up mucus membranes (Frawley and Lad 1992). The pepper can also ease asthmatic symptoms (Clement et al. 2005).

5.2.3 Skincare

Black pepper is known to help in the cure of vitiligo, a condition where the skin loses pigmentation, and creates white patches. Piperine can stimulate the skin to produce melanocytes (Faas et al. 2008).

5.2.4 Cancer

Black pepper prevents cancer and also helps in transporting the benefits of other herbs to different parts of the body, thus maximizing the efficiency of other foods consumed. Piperine exerts protective activity against numerous forms of cancer such as breast, prostate, and colorectal cancers (Zheng et al. 2016). Piperine also increases the absorption of other nutrients like selenium, curcumin, beta-carotene, and B vitamins in the intestines, which are vital for cancer prevention. Piperine inhibits the in vitro growth of triple-negative breast cancer cells, as well as hormone-dependent breast cancer cells, without affecting normal mammary epithelial cell

growth (Greenshields et al. 2015). It reduces the stress on the rectum and helps prevent colon cancer (Yaffe et al. 2015). Piperine was also found to enhance the effectiveness of docetaxel, a chemotherapy medication used in prostate cancer (Samykutty et al. 2013).

5.2.5 Diabetes

The beneficial antioxidants in black pepper help in stabilizing blood sugar levels. They regulate hyperglycemia, thereby aiding in diabetes treatment (Sarfraz et al. 2017). A 2013 study has proved that black pepper oil can inhibit the two enzymes that break down starch into glucose and make diabetic symptoms worse. Ingesting black pepper can delay glucose absorption. Piperine can also be used as a bio-enhancing agent alongside metformin (a diabetes medication), which can help reduce the dose of metformin and even its side effects, all the while helping ease the symptoms of diabetes (Atal et al. 2016).

5.2.6 Alzheimer's Disease

Black pepper has great effects on brain health. Black pepper can delay brain aging and prevent Alzheimer's disease (Subedee et al. 2015). It can also enhance the nerve activity in the brain, thereby curing seizures. It also protects nerve cells and prevents early cell death.

5.2.7 Blood Pressure

The administration of piperine in combination with curcumin is able to partially prevent the increase of blood pressure (Hlavackova et al. 2011).

5.2.8 Obesity

The outer layer of peppercorn assists in the breakdown of fat cells and they are easily processed by the body and applied to other processes and enzymatic reactions, rather than settling in the body and making it overweight. Studies by Park et al. (2012) suggest that piperine could be a lead natural compound for the treatment of fat-related disorders.

5.3 Black Pepper: In Vitro Multiplication

5.3.1 Plant Material

To establish cultures for in vitro multiplication of black pepper (*Piper nigrum* cv. Sri Lanka), 10–15-cm-long vines with shoot apex were collected in the morning from visually healthy, 1-year-old plants that were growing in the farmers' fields. The collected vines were kept in a greenhouse where 100% relative humidity was maintained. Explants from these vines were obtained within 2–4 h of their collection.

To obtain explants, vines were further trimmed to 4-cm-size tips containing shoot apex. These trimmed vine tips were thoroughly washed with running tap water and were surface sterilized by immersion in 50% (v/v) ethanol for 10 min, which was followed by a treatment with 2% (v/v) sodium hypochlorite solution with five drops of Tween 20 for 5 min. Sterilized vine tips were then rinsed five times with sterile distilled water and were kept immersed in it until shoot apical meristem explants (including surrounding base tissue of 0.5 × 0.5 cm size) were excised for in vitro culture establishment.

5.3.2 Culture Medium

The Murashige and Skoog (1962) medium (MS) was used in this study as a basal medium. All media contained 0.8% agar and 3% sucrose. The pH was adjusted to 5.8 prior to autoclaving. Different concentrations and combinations of cytokinins and/or auxins were used to augment the media for culture establishment, multiplication, and rooting.

5.3.3 Micropropagation

Shoot apical meristem explants were inoculated on MS medium without any growth regulator and supplemented with 50 μM cupric sulfate pentahydrate (CSP). Excellent black pepper culture establishment was observed in 6 weeks. Adding CSP in the media reduced the rate of fungal and bacterial contamination rate to less than 1%. Micropropagation of black pepper occurred in three distinct phases: (1) shoot initiation phase (SIP), (2) shoot multiplication phase (SMP), and (3) shoot elongation phase (SEP) (Fig. 5.1a–c). To induce shoot initiation, the established cultures were given a transfer on MS medium augmented with 5 μM 6-benzylaminopurine (BA) on which they were kept for 6 weeks and a passage was given for another 6 weeks. After 12 weeks of incubation on SIP media, 100% shoot initiation was observed in all cultures. SIP cultures were then transferred on MS medium aug-

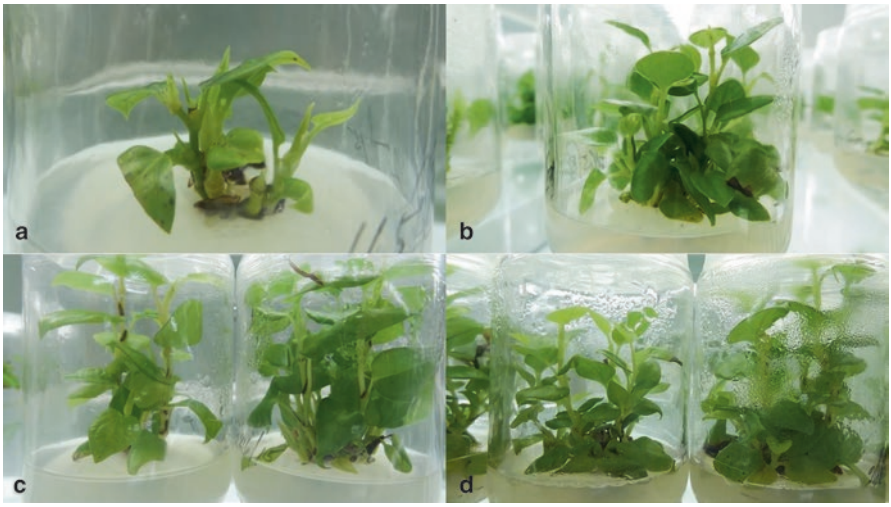

Fig. 5.1 Black pepper culture establishment (**a**); black pepper multiplication (**b**); rooting in black pepper multiple shoots (**c**); and complete black pepper plantlets (**d**)

mented with 7.5 μM BA and 5 μM indole-3-acetic acid (IAA) for 6 weeks to induce shoot multiplication, and two passages at 6 weeks each were given for subsequent multiplication during SMP. Multiple shoots induced during SMP were then transferred on MS medium without any growth regulator for 6 weeks, and two passages were given at 6 weeks each for further growth and shoot elongation during SEP. The total incubation time for culture establishment and all three phases was 54 weeks, with the duration of culture establishment being 6 weeks long, SIP being 12 weeks long, SMP being 18 weeks long, and SEP being 18 weeks long. The number of elongated shoots of 7–9 cm size produced from each explant after two subcultures varied from 8 to 20. Every 6 weeks, data were recorded and each experiment was replicated three times with 25 explants per replication. A photoperiod of 16 h with 40 μmol m^{-2} s^{-1} light intensity along with 24 °C day and 22 °C night temperature was maintained during all phases of micropropagation.

5.3.4 Rooting

MS medium augmented with 2 μM indole-3-butyric acid (IBA) proved best to induce rooting in multiple shoots obtained through SEP (Fig. 5.1b). Fully elongated shoots of 7–9 cm in height were transferred onto the rooting medium in groups of 20–25 shoots per culture (Fig. 5.1c). After 4 weeks on rooting medium (Fig. 5.1d), the percentage of rooting, number of roots per shoot, and root length were recorded and each experiment was replicated three times with 100 cultures per replication.

5.3.5 Acclimatization

Complete plantlets with 8–10-cm-long roots were transferred into 10 cm pots containing sterilized soil:vermiculite (1:1, v/v) mixture. These potted plantlets were kept in the greenhouse for first 6 weeks and then transferred into the screenhouse for next 12–18 weeks. The screenhouse was covered with 60% green-color knitted shade cloth and the temperature was maintained between 26 and 28 °C with 50–55% humidity. During these phases of acclimatization, the plants were irrigated once in 2 days with tap water and once per week with one-fourth strength of MS basal salts. The survival rate of the plants was recorded after 10 weeks, and a 68% survival rate was achieved. Each experiment was replicated three times with 100 plantlets per replication. After 12–18 weeks of ex vitro growth in the screenhouse, completely acclimatized plants were transferred to the nursery, where they were kept until field transfer (Fig. 5.2).

5.3.6 Statistical Analysis

Each experiment was replicated three times. A one-way analysis of variance was used to determine the level of significance between experimental treatments. Statistical significance of the results was determined using the least significant difference (LSD) test by Tukey (1953) at a 5% level of significance.

Fig. 5.2 Acclimatization of black pepper plantlets (**a**); disease-free acclimatized black pepper seedlings in nursery (**b**); raised beds for proper water drainage and durable reinforced cement-concrete standards to support black pepper vines (**c**); and planting pits 1.5 ft. radius from the standard and 1.5 ft. deep (**d**)

5.4 Black Pepper: Commercial Cultivation

Black pepper (*Piper nigrum* L.), a flowering vine of Piperaceae family, is valued for its dried berries called peppercorns, which are used as a spice and for medicinal purposes. Native to the humid jungles of the Malabar Coast of Southwestern India, the plant is cultivated in the tropics worldwide. In Micronesia, it is gaining commercial importance as an important cash crop because of premium price. Traditionally, the trunks of two cultivars of large native tree fern (*Cyathea nigricans*) are used as supports for black pepper cultivation. However, short life span of these tree ferns along with the rapid decline in their population due to increasing use of tree trunks for construction, is becoming a limitation for commercial black pepper cultivation in the region.

An in vitro multiplication protocol for locally preferred and commercially important black pepper cultivar *Piper nigrum* cv. Sri Lanka was developed and utilized for the multiplication and production of elite, uniform, and diseases-free black pepper plantlets in Micronesia. An efficient nursery management system was also standardized for the acclimatization of hundreds of plantlets into uniform and disease-free seedlings for sustainable commercial cultivation.

In Micronesia, traditionally, the trunks of tree fern (*Cyathea nigricans*) are used as living supports for commercial black pepper vines. These large native tree ferns are important sources of wood that are used for traditional house construction, and as supports for commercial black pepper cultivation. Out of the two cultivars of the tree ferns that are traditionally recognized, the one, which produces a red staining juice is preferred over the other cultivar, which produces a greyish juice. The increasing construction in Pohnpei, along with the short life span of the desired tree ferns, has resulted in drastic reduction in their lowland population. With newer roads now providing access to several inland locations, the upland populations of tree fern are also threatened (People and Plants of Micronesia 2014).

Considering the increasing demand for commercial black pepper cultivation and the extremely limited availability of traditional tree fern supports, nonliving supports such as reinforced cement-concrete standards have been specifically designed and constructed at the pilot site to support the vines of fully acclimatized black pepper plants in the field. Standards of reinforced cement-concrete (6 in. diameter in octagonal shape, and 13–15 ft. height) were constructed and used as a support for each plant. In addition, raised beds, which ensure perfect water drainage, were used for the establishment of black pepper commercial plantations. To provide perfect nutrition and maintain these plantations, organic fertilizers along with organic mulching and automatic fertilizer injectors, were used for soil amendment.

Thus, this project is integrating and employing multiple latest tools and technologies such as plant biotechnology, horticulture, microbiology, plant physiology, and plant pathology for the sustainable, climate-smart, and organic commercial cultivation of black pepper in Micronesia. The project team is utilizing plant biotechnological techniques such as in vitro multiplication for uniform black pepper plantlet production, greenhouse acclimatization of multiplied black pepper plantlets for elite disease-free seedling production, automatic fertilizer injectors for uniform

fertilizer application, and organic fertilizers to provide essential nutrients and maintain beneficial soil microorganisms along with appropriate site-specific and climate-smart horticultural, plant physiological, and integrated pest and disease management practices.

5.4.1 Climatic Conditions

Black pepper (*Piper nigrum* L.) originates from tropical, warm, humid latitudes, where temperatures of 77 °F and 80–120 in. annual rainfall predominate. Evenly distributed rainfall is ideal. Supplemental irrigation is necessary in dry, low-rainfall areas. Due to its tropical climate and adequate rainfall, pepper can be grown throughout the year in Micronesia.

5.4.2 Soil Characteristics

Black pepper can be grown on a wide range of soil types, but best results are obtained on deep, well-drained soils with good water-holding capacity. The best soil characteristics are sandy loam clay-to-clay loam with adequate essential plant nutrients and high organic content. Suitable soil pH is between 5.0 and 6.5. A slope not exceeding 10–15° is recommended for better soil conservation, easier harvesting, and farm management.

5.4.3 Field Preparation

Soil preparation for black pepper is similar to that for most dryland crops such as corn. Existing vegetation is turned under with a moldboard or disc plow, or by spading. Most soils benefit from adding compost at this stage. During cultivation, phosphate fertilizer can also be added if required. After turning, leave the soil for a few days to allow for decomposition, and then break soil clods by harrowing or rotovating, or with a hoe or rake in small gardens. After the soil has been pulverized, the surface should be smoothed in preparation for black pepper planting. Black pepper can be planted on ridges, in furrows, or on flat ground.

5.4.4 Preparation of Planting Materials

Traditionally black pepper has been propagated through cuttings that are prepared from main plants. The cuttings consist of the upper 5–7-node segments. Selected planting materials should come from varieties that are disease and pest resistant,

vigorous, and high yielding, with good productivity with respect to the final product. In recent years, owing to the advantages of disease-free planting material along with uniformity in growth and higher yields, the use of tissue-cultured plantlets as the planting material for black pepper has become increasingly popular among the farmers.

5.4.5 Standards and Planting

Traditionally in Micronesia, the trunks of the tree fern (*Cyathea nigricans*) are used as living supports for commercial black pepper vines. Considering the extremely limited availability of traditional tree fern supports and their very short life span, nonliving supports such as reinforced cement-concrete standards are a good alternative. Standards should be planted well before planting black pepper at a depth of 2.0–3.0 ft. The planting pits should have a depth of 1.5 ft. and a radius of at least 1.5 ft. from the standard. Prior to planting, the soil should be amended adequately with organic fertilizers such as compost. Disease-free seedlings should be planted in prepared pits at the onset of a rainy day or in the evening. Young vines should be tied loosely to the support and shaded with suitable plant material.

Considering the frequent and heavy rains, and poor drainage in the Micronesian region, the black pepper seedlings are recommended to be planted in rows on raised beds. The plants should be spaced in the rows at 8.0 ft. apart and a 10-ft.-wide alley is to be maintained between rows (Fig. 5.3).

5.4.6 Pruning

A couple of rounds of pruning should be carried out during the vegetative phase of vine growth. Initial pruning of terminal shoots is done 4–6 months after planting. The next pruning is done when the vines are about a year old, and the last pruning when the terminal shoots have reached the top of the standards (Fig. 5.4).

5.4.7 Irrigation

Often grown in areas with high rainfall, black pepper is generally a rain-fed crop. Black pepper plantations do not require irrigation under normal conditions, except perhaps during the initial establishment period or in drought-prone areas. The plantations should not be allowed to become waterlogged for any extended length of time. For best results, maintain soil moisture at or near field capacity (moist but fully drained) throughout the growing period.

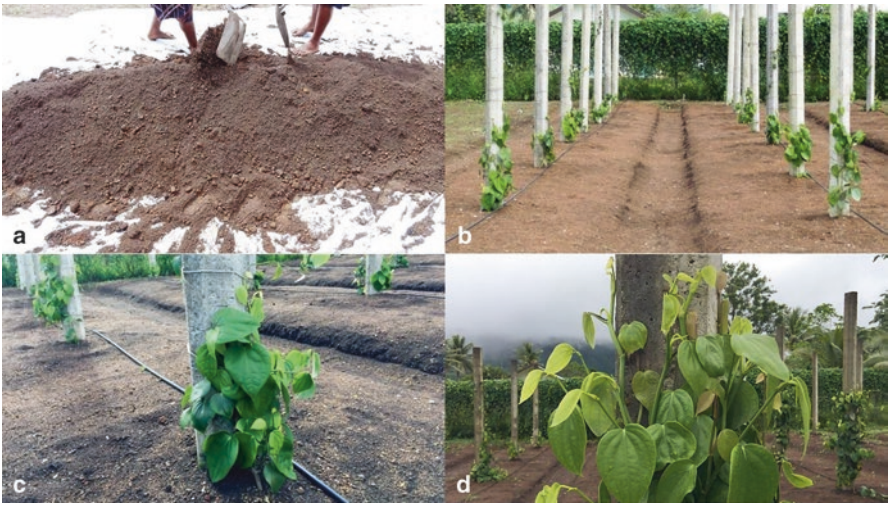

Fig. 5.3 Soil amended with organic fertilizers for black pepper planting (**a**); liquid fertilizer application to each plant at regular intervals by automatic fertilizer injector (**b**, **c**); and healthy and vigorous black pepper vine vegetative growth **(d)**

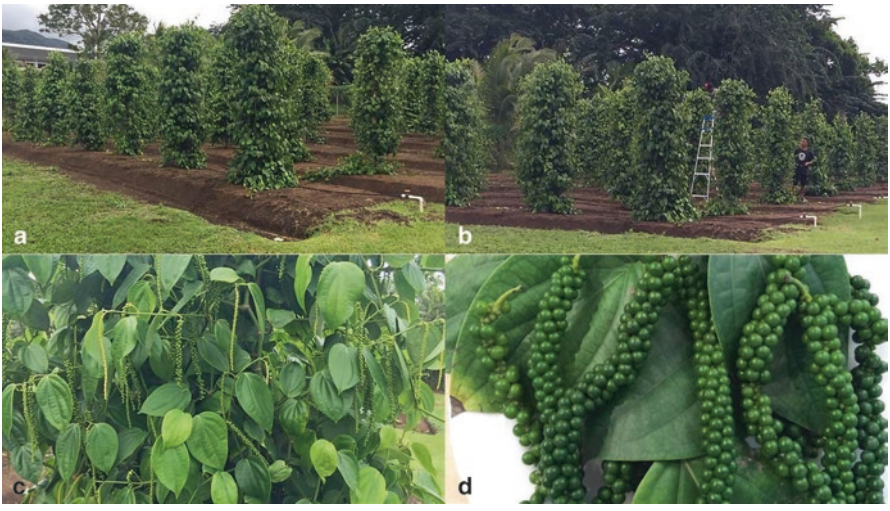

Fig. 5.4 Healthy and vigorous black pepper vines—1 year old after planting (**a**); black pepper vine pruning (**b**); black pepper flowering (**c**); and ready-to-harvest drupes of black pepper (**d**)

5.4.8 Fertilizer Application

Soils should be analyzed for nutrition status to determine nutrient requirements for growth and productivity of black pepper vines. In a tropical climate, it is better to apply small quantities of fertilizer often, rather than to add a large quantity in one

treatment. This makes the fertilizer application more profitable and prevents too rapid growth. Black pepper requires good soil fertility. In the first year, organic fertilizers such as compost may be applied at the rate of 4–6 lbs. along with 0.25 lbs. of inorganic fertilizer such as 12:2:14 nitrogen:phosphorus:potassium (NPK), plus microelements per planting pit at the interval of 3 months. In the second year, organic fertilizers may be applied at a rate of 8–10 lbs. along with 0.50 lbs. of inorganic fertilizer such as 16:16:16 NPK, plus microelements per planting pit at the interval of 3 months. In the third year and onwards, organic fertilizers may be applied at a rate of 10–12 lbs. along with 1.0–1.5 lbs. of inorganic fertilizer such as 12:12:17 NPK, plus microelements per planting pit at the interval of 3 months.

To apply compost or organic fertilizers, scrape the soil surface around the circumference of the canopy. Apply the fertilizer along with the organic fertilizers with the recommended dosage and then cover it with soil taken from the interspaces. Ensure sufficient moisture availability during fertilizer application.

5.4.9 Weed Control

Black pepper is susceptible to weed competition, especially during the first 8–12 months after planting, when the leaf canopy is being formed. During this time, control weeds by hand pulling or cultivating with a hoe. After the crop has attained the maximum vegetative stage, the lush foliage will shade out weed growth, and cultivation for weed control should be minimized to avoid injuring the roots. When necessary, limited weeding by hand may be carried out in the interspaces and around the base of the vine.

5.4.10 Insect Pests and Diseases

Nematode infestation by *Meloidogyne* spp. causes the main problem on conventional pepper cultivations. Soil-borne fungi are the most significant cause of disease to black pepper. They possess a wide spectrum of hosts and can affect practically all the crop types. Therefore, constant and frequent scrutiny is necessary to identify any incidence of disease or pest at an early stage, and to take immediate action to control them. Integrated pest and disease management principles need to be applied at all stages to maximize productivity and minimize crop loss. Phytosanitary measures, such as physical removal of pests, affected plant parts, and infected plants (virus-infected plants, severely disease-infected or pest-infested plants, including plants affected by *Phytophthora* spp. or slow decline or yellow wilt), are important to control the incidents.

Organic plant products and biocontrol agents such as neem oil, neem cake, hot chili solutions, and recommended predators for insect pest control may be used. Agrochemicals for control of pests and diseases may be used only when all other measures have been exhausted. Chemicals used should comply with the state regulations. Application of chemicals should follow recommended practices and these should be applied only under the supervision of qualified professionals.

5.4.11 Harvesting

Drupes that are almost mature with all green berries can be picked to process as green pepper. Drupes with one or two berries beginning to turn yellow can be picked to process into black pepper. To process into white pepper, drupes should be fully mature, with one or two ripe yellow-orange berries on each drupe. Drupes should be picked selectively and harvesting rounds should be carried out frequently throughout the year. Harvested drupes of pepper should be handled hygienically, collected, and transported in clean and closed baskets for the processing in peppercorns (International Pepper Community 2008).

5.4.12 Processing, Drying, and Storage

To ensure high quality, threshing of green pepper berries from the drupes is done manually in Micronesia. Separated green pepper should be washed in clean water to remove field dirt, insects, or other contaminants that may be present. Washed cleaned pepper should be soaked for 1–2 min in water at 194 °F temperature to eliminate contaminants. Soaking in hot water would also facilitate drying and improve the appearance of the dried peppercorns. In Micronesia, solar dryers and electric dehydrators are used because of frequent rain and extremely high relative humidity. Black peppercorns should be dried to a moisture level of 10% for long storage. To avoid the loss of volatiles in peppercorns, drying must not be done at temperatures above 131 °F.

5.4.13 Texture and Color

Different harvesting times and processing techniques could result in various colors and textures of peppercorns (Naturland 2001).

5.4.13.1 Green Pepper

Green peppercorns are produced when almost mature green berries are harvested, processed, and conserved in brine (salt water, vinegar, citric acid).

5.4.13.2 Black Pepper

Black peppercorns are produced when mature green berries are harvested, processed, and dried (Fig. 5.5).

Fig. 5.5 Harvested drupes of black pepper (**a**); processing of black pepper (**b**); processed white peppercorns (**c**); and processed black peppercorns (**d**)

5.4.13.3 White Pepper

White peppercorns are produced when ripe yellow-orange berries are harvested, processed, and dried.

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Chapter 6
Prospects for Goji Berry (*Lycium barbarum* L.) Production in North America

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6.1 Introduction

Lycium barbarum (goji berry) shows promise as a perennial, early-maturing, specialty crop capable of commanding high market prices and withstanding the subfreezing winter temperatures that are prevalent in the mountain west regions of the United States. In recent years increased popularity in Europe and the United States of so-called super foods such as goji berry, which are characterized by high nutritional content and antioxidant compounds, has generated a surge in importation of foreign commodities resulting in a disparity between local production and total consumption. Goji berry has long been utilized in traditional Chinese medicine, and is rich in flavonoids, phenols, carotenoids, and tocopherols (Potterat 2010). As US consumers become increasingly aware of the nutraceutical benefits of goji berry, market demand has followed in lockstep.

Goji berry is commercially cultivated in the Asian subcontinent with major production occurring in China followed by Japan, Korea, and Taiwan (Kulczynski and Grazma-Michalowska 2016). The American market has to date been supplied by goji products originating from China and market prices for organic product can easily reach $20 per pound. Concerns of pesticide contamination in imported shipments (U.S. FDA 2017) have led to an interest in the possibility of developing goji berry for production in the United States where quality can be better controlled and compliance with organic production standards can be easily assessed. Goji berry is currently being evaluated for its potential as a specialty crop in the Eastern

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(Virginia) and Southern (Louisiana) regions of the United States. Efforts are also ongoing to develop production practices for commercialization in California. Goji berry is stated to grow in cold-hardiness zones 2–7 (Demchak and Heidenreich 2014) but there is a lack of supporting freeze test research to validate these claims. The reported cold tolerance of goji berry indicates that mountain west regions of the United States and the Canadian prairies may be ideal regions for goji berry production in North America.

6.2 Origin and Uses

Goji berry has been used for regular consumption and in medicines in China for more than 2500 years (Amagase and Farnsworth 2011). The species was introduced in the European continent in the sixteenth century (Carnes et al. 2013). Goji berry is valued for its use in traditional Asian cooking and finished products like cosmetics, medicinal preparations, and nutraceuticals. Berries were eaten fresh or used for as a condiment in food and soup, and for herbal tea. Leaves also exhibit medicinal properties and were used for consumption as a vegetable. Ripe berries are characterized by a mild licorice flavor and are frequently sold as additives in confections, dry fruit mixes, candy, and breakfast cereals (Small and Catling 2007). Berries, leaves, and bark were historically used in traditional Chinese and Japanese medicine formulations with some of these formulations still being used for treatment of various ailments. A majority of the goji berry that is produced is consumed as dry fruit. Techniques such as osmotic dehydration in combination with air-drying can result in retention of berry color, higher antioxidant capacity, and improved shelf life compared to existing drying techniques (Dermesonlouoglou et al. 2018). Other commercial goji berry products include juice, teas, wine additives, and dietary supplements. Various plant parts are also processed into tinctures, powders, and tablets for medicinal purposes (Potterat 2010).

6.3 Botany and Distribution

The genus *Lycium* belongs to the family *Solanaceae* and includes 70 species which are distributed across five continents (Fukuda et al. 2001). Of primary economic interest in the *Lycium* genus are *L. barbarum* (syn. *Lycium halimifolium*) and *L. chinense* which have been traditionally utilized for their medicinal properties throughout China and other countries in East Asia (Potterat 2010). These two closely related species are both commonly referred to as goji berry, but additional names include boxthorn, wolfberry, and matrimony vine (Hummer et al. 2012; Potterat 2010). *L. barbarum* exhibits a vining growth habit (Hummer et al. 2012) capable of reaching 12 ft. in height if left unpruned while *L. chinense* exhibits comparatively lower vigor and can be identified by its lanceolate-shaped leaves.

Berries from *L. barbarum* are predominantly used for fresh consumption and development of commercial formulations. Molecular markers such as random amplified polymorphic DNA (RAPD) and sequence characterized amplified regions (SCAR) have been frequently used to distinguish *L. barbarum*-derived products from other *Lycium* species (Zhang et al. 2001). Two SCAR markers with 650 and 700 bp were developed to effectively screen potential adulterants in *L. barbarum* berries and their products (Sze et al. 2008).

Cultivated goji berries are deciduous shrubs (Fig. 6.1a) that exhibit rapid growth (Fig. 6.1b) under warm weather conditions (Rudolph and Busing 2008). Shrubs exhibit profuse flowering (Fig. 6.1c) and fruiting (Fig. 6.1d) throughout the growing season. Goji berry is stated to perform well on a wide variety of soils (Maughan and Black 2015). Flowering is observed in the axil of leaves with the production of perfect flowers (Fig. 6.2a). Flowers are funnel shaped with dark purple petals (Fig. 6.2b). Flowers are perfect/bisexual with five stamens (Fig. 6.2c) consisting of cream-colored, bilobed anthers and long filaments that are fused to the petals and pubescent at the fused end (Fig. 6.2d, e). The pistil (Fig. 6.1f) consists of a green stigma (Fig. 6.2g), a long style, and a globular ovary at the base of the flower that carried the ovules (Fig. 6.2h). Self-pollination is frequently observed in goji berry (Fig. 6.2i).

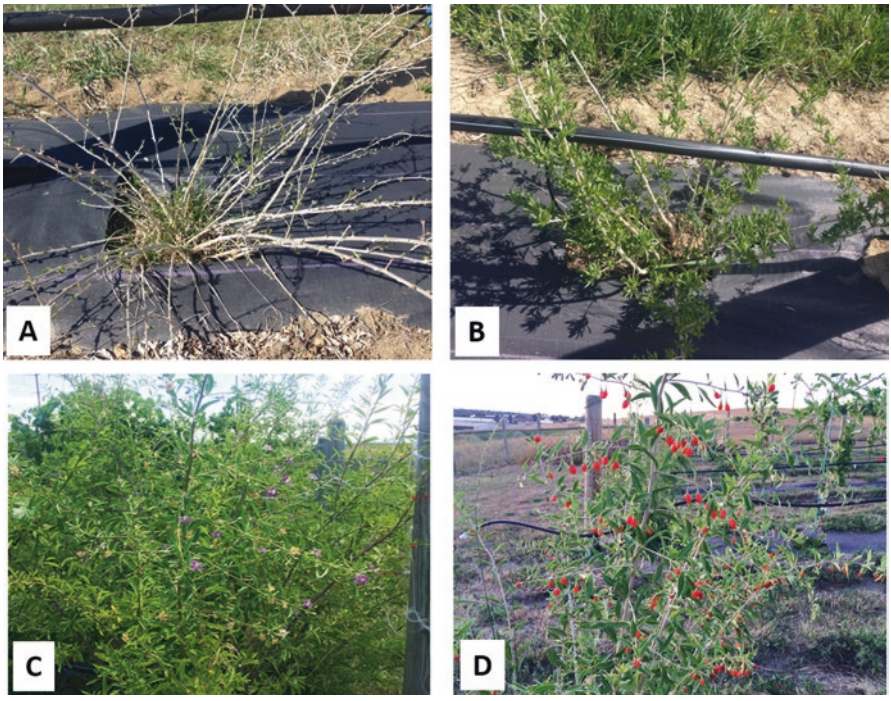

Fig. 6.1 Phenology and growth stages in *Lycium barbarum*. Bud break (**a**) and rapid growth are observed following warm weather conditions (**b**). Profuse flowering (**c**) and fruiting (**d**) are observed throughout the growing season

Fig. 6.2 Reproductive biology of *Lycium barbarum*. Unopened floral buds (**a**) develop into funnel shaped flowers with purple colored petals (**b**). Flowers are bisexual consisting of five stamens that are hirsute and fused at the base of the petals (**c**, **d**, **e**). The pistil (**f**) consists of a green colored stigma and slender style (**g**) with a basal ovary (**h**) containing the ovules. Self- pollination (**i**) is frequently observed in goji berry

Following fertilization the ovary continues to grown in size (Fig. 6.3a) to produce the berries, which gradually enlarge in size (Fig. 6.3b, c) prior to initiation of veraison (Fig. 6.3d). Fully ripe berries are bright orange to red in color (Fig. 6.3e) and bear several seeds in two rows (Fig. 6.2f). Individual berries may contain between

Fig. 6.3 Fruit development in *Lycium barbarum*. Ovary enlargement (**a**) is observed following pollination and fertilization leading to fruit expansion (**b**) and elongation (**c**). Following initiation of veraison (**d**), berries ripen to exhibit bright orange color (**e**) which contain 2–20 seeds (**f**)

3–20 seeds (Rudolph and Busing 2008), which are small averaging 2 mm in size (Small and Catling 2007). Goji berry is reportedly tolerant of alkaline soils, hardy to USDA zone 2 (Demchak and Heidenreich 2014), and has a blooming and fruiting period lasting from midsummer to fall which allows for multiple harvests throughout the growing season (Maughan and Black 2015).

Invasive potential of introduced crop species like goji berry must be evaluated on a regional scale as local climate influences growth and productivity. Over time, a number of introduced ornamental and agricultural species have been realized as weeds which can directly and negatively impact native organisms, habitats, and

human activities (Pimentel et al. 1989). *Lycium barbarum* has shown invasive capacity in Eastern Europe (Beniak et al. 2015), and a relative, *L. ferocissimum*, is listed on the federal noxious weed list (USDA 2010) making it imperative to study seed viability and germination rates in areas where *Lycium* spp. are grown.

6.4 Commercial Production

In China, more than 205,000 acres have been devoted to goji berry cultivation, mainly focused in the north central and western regions, with an annual production of 95,000 tons. Primary production of goji berry takes place on approximately 82,000 ha in China, with production yielding $120 million worth of product exports in 2010 (Donno et al. 2015). Most commercially produced goji berry comes from plantations in the Ningxia Hui region in north-central China and the Xinjiang Uyghur region in western China with some production occurring in inner Mongolia and Hebei (Potterat 2010; Yao et al. 2018). A majority of the studies on environmental condition requirements for goji berry production have been conducted in China (Lin et al. 2013; Liu et al. 2004). Significant differences in fruit morphology and quality have been observed in goji berry fruit grown in arid/semiarid and humid climates of China (Yao et al. 2018). Metabolic profiles of berries grown in varying climates indicated higher antioxidant and sugar levels in fruit obtained from humid regions than that grown in semiarid or plateau regions (Yao et al. 2018). The quality of goji berry fruits produced in various regions of China showed significant differences when analyzed using near-infrared spectroscopy (NIR) in combination with consumer sensory evaluation and chemometrics (Tingting et al. 2016). Additional studies have been conducted in recent years to study the feasibility of goji berry production in European regions (Dzhugalov et al. 2015; Mencinicopschi et al. 2012). Goji berry species and accessions grown in Southern Europe exhibited significant differences in fruit weight and quality including mineral composition, sugar and flavonoid levels, and antioxidant capacities (Kafkaletou et al. 2018). To date, large-scale cultivation of goji berry has been attempted in North America with production primarily occurring in Ontario and California (Demchak and Heidenreich 2014). A quick Internet search will yield a diversity of websites where small-scale growers and home gardeners across the United States both express interest in goji cultivation and boast successful production, but there is a lack of controlled research which lends any validity to claims of production in cold climates or high elevations, and no discernable yield data exists. Goji berry production trials in Wyoming indicate that *Lycium barbarum* is cold-hardy in USDA zone 3b, survives wide fluctuations in daily temperatures, and has a longer production season compared to other berry crops such as grape. Bud break in goji berry was observed to occur from March to May with profuse flowering and fruiting occurring from July until November.

6.5 Propagation

The US nursery industry has also kept up with the recent growing trend of goji production through the release of multiple goji cultivars such as Sweet Lifeberry® and Big Lifeberry® by Proven Winners (Sycamore, IL), "Phoenix Tears" by Phoenix Tears Nursery (Logan, UT), and continued production of the Chinese Ningxia #1 which is marketed as Crimson Star™. Most likely, successful vegetative propagation protocols have been established within the companies where the previously mentioned cultivars originate from, but this kind of knowledge is typically considered to be proprietary information and is not available to the general public.

Goji berry can be propagated using seed or asexually using soft- and hardwood cuttings (Baldwin 2018). Vegetative propagation of woody plant species is commonly achieved through collecting and rooting stem cuttings. To stimulate adventitious root production in cuttings, plant propagators primarily utilize synthetic auxins like indole-3-butyric acid or α-naphthaleneacetic acid to treat the wounds made by taking a cutting. Cuttings of different species may produce roots to varying degrees in response to the type of auxin and the concentration utilized for propagation (Al-Salem and Karam 2001). When cuttings are dipped in an auxin compound, hormonally regulated morphogenic reactions take place where callus cells proliferate at the site of wounding, and cells near the vascular cambium are dedifferentiated into meristematic cells and then re-differentiated into root primordia (Hartmann et al. 2002). It is the production of roots in this manner which allows for the mass multiplication of desirable genotypes.

The optimal media to utilize for vegetative propagation can vary by plant species and the type of cutting used. Factors to consider when selecting a propagation media include bulk density, porosity, water-holding capacity, and chemical properties. Commonly, plant propagators utilize Rockwool, Oasis foam, or sand, vermiculite, and perlite alone or in combination with organic materials like peat and coconut coir (Hartmann et al. 2002). The physical and chemical properties of a propagation medium in combination with temperature, light, and water availability may have a substantial influence on the quality and number of roots formed by a cutting (Gislerod 1983), and specific propagation protocol is often required for many species.

Vegetative propagation of a species related to *L. barbarum* and *L. andersonii* has been accomplished through the utilization of synthetic auxins and vermiculite as a propagation substrate (Wieland et al. 1971), but the researchers failed to report quantitative data resulting from their methodologies. A farmer/rancher project was previously funded by Southern SARE, which attempted to develop a commercial propagation system for goji berry (Wilson 2009). This study tested goji propagation using an aeroponic chamber for root induction, and the use of native soil with amendments for a subsequent growth substrate. Data resulting from the methods used are largely irrelevant to commercial horticultural systems elsewhere in the world as mineral soils are rarely used for commercial propagation (Hartmann et al. 2002),

and they may harbor pests and pathogens, and cannot be easily standardized or replicated. An additional farmer/rancher project which received funding from North Central SARE focused on goji berry establishment in Missouri (Nabelek 2008) which appears to have been successful, but the only data reported was a summary of approximate plant height.

Investigations into micropropagation of goji berry in vitro have resulted in the establishment of an efficient plant regeneration protocol. Early studies on goji berry androgenesis yielded mixed results in the production of haploid plants (Zenkteler 1972) where only low numbers of plantlets were obtained, and abnormal pigmentation was observed in a portion of the regenerated plants. In vitro regeneration of goji berry from leaf explants via direct organogenesis was shown to be an efficient means of micropropagation (Hu et al. 2001), where plant growth regulator combinations resulted in shoot proliferation and subsequent rooting of excised shoots. Further investigation by Hu et al. (2008) found that plant regeneration could additionally be achieved from root explants with some efficiency via somatic embryogenesis. The peak of goji berry micropropagation technology was reached in 2016 where following testing of shoot proliferation medium and rooting medium substrates an optimized in vitro regeneration system was found for large-scale plant production (Fira et al. 2016). In addition, ex vitro acclimatization methods for goji berry resulting from earlier research (Clapa et al. 2013) were tested in conjunction with the optimized micropropagation system (Fira et al. 2016) with results indicating that floatation hydroculture can be efficiently utilized for commercial-scale goji berry micropropagation.

6.6 Medicinal Uses

The health-beneficial properties of goji berry can be attributed to biochemical compounds found in the fruit, leaves, and roots including polysaccharides, lipids, flavonoids, organic acids, and amino acids. *L. barbarum* polysaccharides (LBP) consist of branched sugars and proteoglycans (Potterat 2010). Berries obtain their bright orange color from carotenoids, the predominant one being zeaxanthin followed by lower concentrations of beta-carotene, neoxanthin, and cryptoxanthin (Peng et al. 2005; Wang et al. 2010). Goji berries are rich in ascorbic acid while containing trace concentrations of other organic acids (Mikulic-Petkovsek et al. 2012). Berries also contain other vitamin precursors such as thiamin and riboflavin (Potterat 2010). A number of mineral elements including calcium, iron, magnesium, phosphorus, potassium, and sodium occur at varying levels in berries (Endes et al. 2015). Lipids such as palmitic, myristic, and linoleic acids are common fatty acids occurring in fruits (Endes et al. 2015). Goji berry leaves have been analyzed for identifying potential health-beneficial compounds (Potterat 2010). Several terpenoids including acyclic diterpene glycosides and lyciumosides have been identified. Additionally steroids with properties similar to those obtained from *Withania somnifera* have also been isolated from leaves (Potterat 2010).

The complex biochemical nature of goji berry fruit and leaves greatly contributes to their health-beneficial properties. Carotenoids, flavonoids, and polysaccharides exhibit high antioxidant activities and effectively scavenge free radicals; berries thus have high ORAC values similar to those observed with other berry fruits with high antioxidant levels (Kulczynski and Grazma-Michalowska 2016). Antioxidant activity and level of bioactive compounds in goji berry herbal teas are directly correlated with increasing temperature (Sun et al. 2017). The use of techniques such as pressurized liquid extraction could potentially improve the levels of phenolic compounds in extracts obtained from berries (Tripodo et al. 2018). Other postharvest treatments such as treatment of berries with lecithin significantly reduced fruit rot while retaining high quality for up to 8 days after storage (Jatoi et al. 2017). Flavonoids from organic goji berry fruit exhibited a high protection factor (PF) value for preventing oxidative degradation of soybean oil compared with synthetic antioxidants and could be used stabilizing additives for preventing oxidation of vegetable oils (Pedro et al. 2018). Goji berry fruit grown in Greece exhibited high antioxidant and radical scavenging activities through decreased lipid peroxidation, thereby indicating strong antimutagenic properties in fruit extracts (Benchennouf et al. 2017; Skenderidis et al. 2018). In clinical studies, human subjects consuming goji berry juice for a period of 30 days recorded a significant improvement in the serum levels of antioxidant markers including superoxide dismutase, and glutathione peroxidase with a concomitant decrease in lipid peroxidation-associated marker malondialdehyde (Amagase et al. 2009). The study suggested that consumption of goji berries on a routine basis could lead to a significant reduction in the production of free radicals in the body through increased antioxidant activities. In other studies, the antioxidative properties provided protection against dry eye disease in rats fed with goji berry extracts (Chien et al. 2018).

L. barbarum extracts administered at various concentrations significantly reduced levels of triglycerides, total cholesterol, and low-density lipoproteins (LDL) while simultaneously improving high-density lipoprotein (HDL) levels in rats, thereby indicating that fruit consumption may contribute to improved cardiovascular health (Luo et al. 2004). Goji berry leaf and fruit extract exhibit anti-apoptotic and antiproliferative activities, which may be correlated to the differential expression of genes involved in damage control and cryoprotection (Tang et al. 2011). Polysaccharides present in goji berry extract may provide protection against cancer and diabetes (Kulczynski and Grazma-Michalowska 2016). Polysaccharides present in goji berry extracts inhibited the proliferation of liver cancer cells possibly due to a reduction in lipid peroxidation (Zhang et al. 2005). Diabetic mice fed with goji berry extract exhibited decreased levels of blood sugar followed by a concomitant decrease in cholesterol and triglycerides compared to the control group (Luo et al. 2004; Zhang et al. 2005). The hypoglycemic effects and decreased lipid concentrations were attributed to the presence of polysaccharide fractions in the fruit extracts, which caused an increase in the activity of superoxide dismutase and catalase enzymes, and a subsequent decrease in oxidative stress.

The introduction of new foods with health-beneficial properties also raises the concern of potential allergenic reactions to such foods in the population. Allergic

reactions such as anaphylaxis following consumption of goji berry products and interaction of biochemical constituents from goji berry with existing medication use resulting in conditions such as epistaxis, bruising, and rectal bleeding have been reported in patients previously (Carnes et al. 2013; Rivera et al. 2012). General recommendations on the safe use of goji berry and its products include a safety analysis for patients who suffer from allergenic reactions from other foods in the Solanaceae family (Kocyigit and Sanlier 2017).

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Chapter 7
Skullcaps (*Scutellaria* spp.): Ethnobotany and Current Research

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7.1 Introduction

It is a testimony to the power of plants as healers that traditional medicine (TM) either acts as a pivot of the healthcare delivery system or serves as an addendum in many parts of the modern world. Medicinal plants have been an integral part of some of the ancient cultures (Arabian, Chinese, Indian) and their use dates back to the Neanderthal period (Solecki and Shanidor 1975). Ethnobotany seeks to investigate the relationship between plants and the way various cultures have used them in their everyday lives. In the past decades the ethnobotanical research has assumed even greater status as scientists explore new medicines to treat drug-resistant infections, cancers, viruses, and other ailments. To illustrate, globally, indigenous communities were the first to discover the use of plants to treat diseases through direct, first-hand observation of sick animals feeding on plants. Those observations led to the development of a plethora of medications we use today to treat an array of diseases (Tomimori et al. 1985; Moerman 1998, 2009; van Loon 1998; Higuchi and

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Terabayashi 2003; Shepard Jr 2004; Bodeker et al. 2005; Heo et al. 2009; Kunwar et al. 2010; Amiri et al. 2012; Zhao et al. 2016; Kosakowska 2017). For centuries, people have used plants for the prevention and treatment of diseases, nourishment, housing, ceremonial rights, dyes, clothing, oils, soaps, waxes, tannins, and ornamentals (Moerman 1998; Marshall and Chandrasekharan 2009).

The prevalence of plant-based medicines appears universal. In fact, an estimated 80% of the world's population incorporates plant-based medicine to treat and prevent diseases (Cole et al. 2007). Incredibly, there are over 1300 medicinal plants endorsed for use in Europe and 118 of the top 150 prescription drugs in the United States have natural sources as their basis (Chen et al. 2016). Traditional Chinese medicine (TCM) is practiced by 45% of all countries, clearly indicative of its role and acceptance. According to recent accounts, the annual global trade of medicinal plant material has reached USD 40 billion and it continues to grow at a rate of 10% per year (Li et al. 2015b). As these plants possess chemicals that have proven to have the potential as agrochemicals, flavors, fragrance ingredients, food additives, and pharmaceuticals, the popularity of medicinal plants has surged (Vanisree et al. 2004; Karuppusamy 2009). Examples of plant-derived drugs central to human health care are morphine and aspirin to treat pain and paclitaxol to treat ovarian, breast, lung, cervical, and pancreatic cancers. Plants will continue to exist as a significant source to modern medicine due to their ability to produce chemicals capable of treating human and animal ailments (Lahlou 2013).

Scutellaria species have been adopted in traditional and local medicine systems for many centuries (Shang et al. 2010). Comprised of over 360 species (Paton et al. 2016), the *Scutellaria* genus ranks as the second largest member of the mint family (Lamiaceae). Species from this genus have been targeted for their curative and therapeutic properties and have been employed during ceremonial rites by indigenous people from all around the world (Tomimori et al. 1985; Moerman 1998; van Loon 1998; Higuchi and Terabayashi 2003; Shepard Jr 2004; Bodeker et al. 2005; Moerman 2009; Heo et al. 2009; Kunwar et al. 2010; Amiri et al. 2012; Zhao et al. 2016; Kosakowska 2017). The English name, "skullcap" (also spelled "scullcap") was coined since the calyx forms a structure that resembles an ancient, miniature medieval helmet. The protuberance on top of the calyx, visible only in the early-flowering stage, provides the easiest and most reliable method of identifying the species (Paton 1990). At Fort Valley State University (FVSU), we have a collection of 21 *Scutellaria* species to conduct research on various aspects (Fig. 7.1).

Habitat and botany: *Scutellaria* species are herbaceous perennials and shrubs found worldwide, with the exception of the Amazon basin, lowland tropical Africa, and the Pacific Islands (Paton 1990; Paton et al. 2016). Some species grow in dry to damp woody areas, rocky or sandy soils, river banks, sunny grassy slopes, prairies, plains, meadows, pastures, or marginal land and in cultivated areas with full to little sunlight (Paton 1990; eFloras 2015; USDA 2016; Lady bird Johnson Wildflower Center 2017). The quadrangular stems feature opposite leaves that are linear, ovate, rhomboid, elliptic, or oblong with plant reaching a height of 5 cm to 1 m. A few species that are perennial develop woody stems and acquire the shape and size of a shrub (*S. scandens*, *S. suffrutescense*). Yellow, fibrous, and creeping roots originate

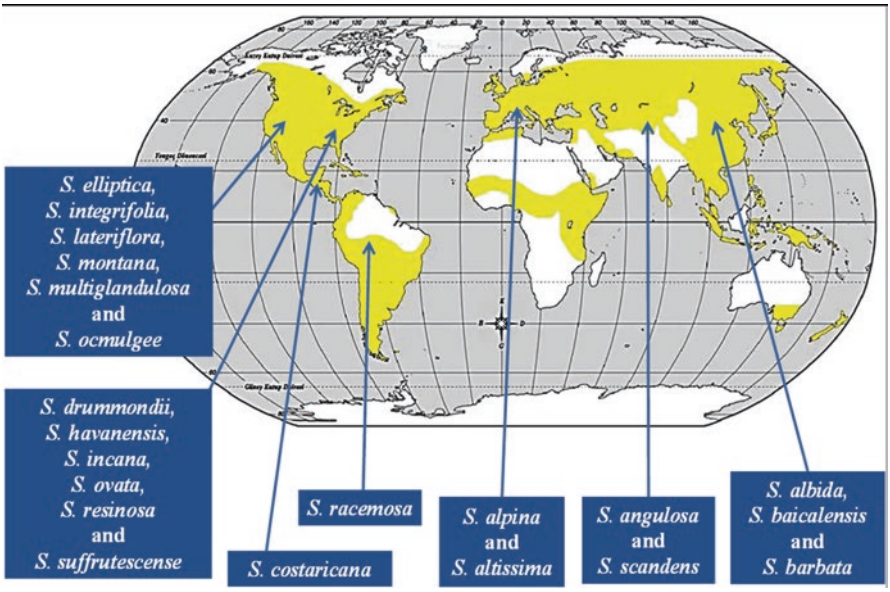

Fig. 7.1 Geographical distribution of *Scutellaria* germplasm maintained at Fort Valley State University

at the base of quadrangular stems. The blue to lavender flowers borne on the axis of the leaves bloom in late spring and continue throughout summer (Joshee et al. 2002). The shielded look that makes the genus recognizable actually originates from the bilabiate calyx sealed from the pressed-together lips of the fruit. The corolla is white, blue, pink, violet, yellow, or red (Paton 1990). A snapshot of a few medicinal Scutellaria species used in different parts of the world is provided in Table 7.1. A rich, traditional adoption of the genus in China, Japan, Korea, India, Nepal, and among South and North American countries has persisted for treatment of inflammation, infections, jaundice, high blood pressure, and tumors (Shang et al. 2010; Guo et al. 2011; Joshee et al. 2013). A compilation of reports enumerates over 295 phytochemicals isolated from 35 *Scutellaria* species (Shang et al. 2010).

7.2 *Scutellaria* in Various Traditional Medical Systems

The Baikal skullcap (*S. baicalensis*) is listed in the Chinese, Japanese, Korean, and European Pharmacopoeia (Kosakowska 2017). Though *Scutellaria* has been incorporated in Eastern medicine for thousands of years (Cole et al. 2008; Yuan et al. 2010; Kosakowska 2017) it has recently sparked the interest of Western medicine (Zhao et al. 2016). A brief account of the use of *Scutellaria* species in prominent native medical systems of the world is presented.

Table 7.1 Scientific and common names of *Scutellaria* species used as medicine in different parts of the world

Scientific name	Common name	Geography	References
Scutellaria spp.	Boshghabi (dish-like)	Persian	Ghannadi and Mehregan (2003)
S. angustifolia	Narrowleaf skullcap	The United States	Moerman (1998)
S. baicalensis	Huang-Chi, Huang-Qin	China	Guo et al. (2011)
S. barbata D. Don	Ban-Zhi-Lian	China	Joshee et al. (2010)
	Banjiryun	Korea	Shim (2014)
S. californica	California skullcap	The United States	Moerman (1998)
S. discolor Colebr.	Nilo Butte Ghans	Nepal	Tomimori et al. (1985)
S. discolor Colebr.	Wa Er Cao	China	Tomimori et al. (1985)
S. discolor Colebr.	Yenakha	India	Das et al. (2008)
S. elliptica	Hairy skullcap	The United States	Moerman (1998)
S. galericulata	Marsh skullcap	The United States	Moerman (1998)
S. incana	Hoary skullcap	The United States	Moerman (1998)
S. lateriflora	Blue skullcap, Hoodwort, Virginian skullcap, American skullcap, blue pimpernel, and mad dog	Canada and the United States	Moerman (1998); Joshee et al. (2002)
S. oblonga Benth.	Kaattutulsi	India	Jikku et al. (2015)
S. parvula	Small skullcap	The United States	Moerman (1998)
S. ramosissima	Sershoh ko'kamaron	Republic of Uzbekistan	Mamadalieva et al. (2017)
S. scandens	Charpate	Nepal	Miyaichi et al. (1988)
S. scordiifolia Fisch. Ex Schrank	Hong Len Ser Po	Siberia	Olennikov and Chirikova (2013)
S. violacea Heyne	Malainangai	India	Karuppusamy (2007)
S. violacea Heyne	Novu Pacchilai	India	Ayyanar and Ignacimuthu (2005)

7.2.1 Native Americans

Native Americans have appropriated herbal plants to heal their bodies, purify their spirit, and bring balance into their lives and surroundings for thousands of years. Native Americans learned of the cultural application of the medicinal properties of plants by observing natural instincts of sick animals ingesting specific plants and

vegetation for relief. They emulated acquired knowledge to identify potential benefits of plants to treat their own sicknesses (Legends of America 2003). In fact, some Native American tribes endorsed the use of skullcaps to treat gynecological conditions (menstrual cramps, breast pain, and to expel afterbirth), fevers, chills, diarrhea, sore throats, eye pain, kidney disorders, nerves, and heart conditions, as well as to prevent smallpox and insomnia (Moerman 1998, 2009; Legends of America 2003). Other tribes employed skullcap in purification ceremonies to induct young girls into womanhood. Miwok Indians used a decoction of *S. angustifolia* (narrowleaf skullcap) to wash sore eyes (Moerman 1998; Legends of America 2003). Documented medicinal uses of *Scutellaria* by the Cherokee, Delaware, Iroquois, Mendocino, Meskwaki, Mowok, Ojibwa, and Oklahoma Indian Tribes depict the impressive range of medicinal applications (Moerman 1998; Table 7.2). Native Americans generally favored seven different species prepared as decoctions or infusions through their roots or aerial parts, *S. angustifolia*, *S. californica*, *S. elliptica*, *S. galericulata*, *S. incana*, *S. lateriflora*, and *S. parvula* (Moerman 1998, 2009), though over 90 species have been reported from North America.

The Mendocino Indians used S. *californica* to treat chills and fevers. The Cherokee Indians, who called skullcaps ganigwiliski utanu, adopted an infusion of roots from *S. elliptica*, *S. incana*, and *S. lateriflora* (Upton 2009). In addition, Cherokee Indians preferred the infusion of roots from *S. elliptica*, *S. incana*, and *S. lateriflora* as an abortifacient for monthly periods and to expel afterbirth and a decoction of *S. lateriflora* roots to expel afterbirth. Compounds present in the roots of *S. elliptica*, *S. incana*, and *S. lateriflora* were used by the Cherokee Indians as a kidney medicine. The Meskwaki depended on the plant parts of *S. parvula* to treat diarrhea, while the Delaware and Oklahoma Indians appropriated the plant tips of *S. galericulata* as a laxative and stomach stimulant. To treat heart trouble the Ojibwa Indians relied upon *S. galericulata*. Further, the infusion of powdered roots of *S. lateriflora* was a smallpox preventative and a way to clean the throat for the Iroquois Indians (Moerman 1998, 2009). A skullcap decoction was sought by the Miwok for cough and colds (Moerman 2009). Interestingly, the Gosiutes, a tribe of Western Shoshone Native Americans, gathered the seeds of *Scutellaria* species as a source of food (Chamberlin 1911).

7.2.2 Traditional Chinese Medicine (TCM)

Fundamentally important to the TCM are about 50 herbs, and *Scutellaria* genus figures prominently in that list (Chen et al. 2014). Application of *Scutellaria* in TCM practice is primarily associated with heat cleansing, dry moisture, and toxin remover (van Loon 1998). Medicinal application of *S. baicalensis* dates back more than 2000 years in TCM and is known as Huang-Chi and Huang-Qin (Guo et al. 2011). Since the extract isolated from the root is yellow due to high flavonoid content (35%), *Scutellaria baicalensis* is often referred to as the “golden root” (van Loon 1998). First recordings (200–250 AD) in the oldest existing pharmacopoeia,

Table 7.2 *Scutellaria* species used by indigenous communities in various parts of the world

Country	Species	Users	Human ailment	References
Brazil	*Scutellaria agrestis*	Amazon, Solimões River floodplain community	Earache, fever, diarrhea, and high blood pressure	de Olivera et al. (2013)
Colombia	*S. agrestis*	Tikunas	Stomach disorders and diarrhea	de Olivera et al. (2013)
China	*S. baicalensis* Georgi	Chinese	Hepatitis, jaundice, diarrhea, inflammation, bronchitis, bacterial infections, high blood pressure, thrombosis, antioxidant, cholesterol, and tumors	Guo et al. (2011); Joshee et al. (2013)
	S. barbata D. Don	Chinese	Swelling of throat, edema, inflammation, cough, tumors, and hemorrhoids	Świader et al. (2003); Qu et al. (2010); Joshee et al. (2013)
	S. discolor Colebr.	Chinese	Fever, poisoning, inflammation due to cold, gastroenteritis, tympanitis, and other diseases	Tomimori et al. (1985)
Japan	*S. baicalensis* Georgi	Japanese Kampo	Inflammation, hepatitis, diarrhea, and tumors	Joshee et al. (2002); Jang et al. (2003); Cole et al. (2007)
Korea	*S. barbata* D. Don	Korean	Inflammation, tumors, high blood pressure, and edema	Qu et al. (2010); Joshee et al. (2013)
Native Americans	*S. angustifolia* Pursh	Miwok	Eye wash for sore eyes	Moerman (1998, 2009)
	S. californica Gray	Mendocino	Fever	Moerman (1998, 2009)
	S. elliptica Muhl. Ex Spreng.	Cherokee	Induces abortion and afterbirth, regulates menstruation, diarrhea, breast pain, nerves, and kidney disorders	Moerman (1998, 2009)
	S. galericulata L.	Delaware	Gastrointestinal	Moerman (1998, 2009)
		Ojibwe	Heart trouble	Moerman (1998, 2009)
	S. incana Biehler	Cherokee	Induces abortion and afterbirth, relieves symptoms of menstruation, diarrhea, breast pain, nerves, and kidney disorders	Moerman (1998, 2009)

(continued)

Table 7.2 (continued)

Country	Species	Users	Human ailment	References
	S. lateriflora L.	Cherokee	Induces abortion and afterbirth, regulation of menstruation, diarrhea, breast pain, nerves, and kidneys	Moerman (1998, 2009)
	S. lateriflora L.	Iroquois	Prevention of smallpox and to clean throat	Moerman (1998, 2009)
	S. parvula Michx.	Meskwaki	Diarrhea and flux	Moerman (1998, 2009)
	S. Spp	Miwok	Cough and colds	Moerman (1998)
Nepal	*S. discolor* Colebr.	Nepali	Colds, cuts, insect stings, headache, rheumatism fever, and indigestion	Tomimori et al. (1985); Kunwar et al. (2010)
	S. scandens	Nepali	Cuts and insect stings	Miyaichi et al. (1988)
Republic of Uzbekistan	*S. ramosissima*	Uzbekistan	Epilepsy, allergies, inflammation, St. Vitus's dance, nervous disorders, and hypertension	Yuldasheva et al. (2014)
Siberia	*S. Scordiifolia*	Buryatiyan lamas	Fever and infections	Olennikov and Chirikova (2013)
Turkey	*S. orientalis* subsp. *pinnatifida*	Anatolian	Diarrhea, stops bleeding and wound healing	Ersöz et al. (2002)

Materia Medica of Shen Nong of Huang-Qin, appeared in the *Shennong Bencaojing* in relation to the treatment of sores, fever, colds, and lung and liver issues (Shouzhong 1998; Higuchi and Terabayashi 2003). Huang-Qin was officially listed in the Chinese Pharmacopoeia (Zhao et al. 2016). In 1593, the *Bencao Gangmu* (*Compendium of Materia Medica*) reported *S. baicalensis* as a treatment for diarrhea, dysentery, hypertension, hemorrhaging, insomnia, inflammation, and respiratory infections (Zhao et al. 2016). *Scutellaria baicalensis* has been used to treat jaundice, infections, thrombosis, cholesterol, and tumors (Guo et al. 2011; Chen et al. 2014; Zhao et al. 2016). Two other *Scutellaria* species (*S. barbata* and *S. discolor*) have acknowledgement within TCM, but to a lesser extent. The Chinese Pharmacopoeia lists *Scutellaria barbata*, known as Bah-Zhi-Lian, Banzhilian, and Herba Scutellariae berbatae, to treat inflammation, edema, cough, tumors, urinary diseases, and hemorrhoids (Świader et al. 2003; Qu et al. 2010; Bardakci and Skaltsa 2015). Last, the Chinese people adopted *S. discolor* (Wa Er Cao) to treat fever, inflammation, food poisoning, and an array of other diseases (Tomimori et al. 1985).

7.2.3 Japanese Kampo

Traditional Chinese medicine serves as the basis of traditional Japanese Kampo medicine though it evolved into a personalized form of medicine based on extensive Japanese experience and knowledge (Eshima et al. 2015). The Japanese people have adopted these roots to prepare crude formulations to treat inflammatory diseases, bacterial infections, hypertension, renal disease, nervous system disorders, diarrhea anemia, fatigue, and edema. Higuchi and Terabayashi (2003) reported the import of *Scutellaria* seeds to Japan from the Korean peninsula to cultivate them in the Koishikawa Herb Garden around year 1726. Kampo contains over 100 formulations of crude drugs, herbs, and chemical substances. Groupings emerge from categorizations of desired effect, taste, nature, and other characteristics; some attributes include heat-clearing, purgative, and warming-interior, dampness-drying herbs, or toxin-resolving herbs. Japanese Kampo medicine employs the root of *Scutellaria* for heat-clearing and dampness-drying, with roots classified according to their shape (Eshima et al. 2015; Higuchi and Terabayashi 2003). When the plants are removed from the wild with the root tips or the periderm of the young roots removed, they are called "Sengon," "Shigon," or "Jogon." The term "Kogon" defines a rotted xylem with a black to brown appearance, or a hollow middle. Roots broken during the processing phase into semi-tubular or flat shapes are called "Hengon" (Higuchi and Terabayashi 2003). Age- or part-based classification of the plant material may have bearing on the chemical composition of the tissue related to specific bioactivity. The Japanese people have appropriated these roots to prepare crude formulations to treat inflammatory diseases, bacterial infections, hypertension, renal disease, nervous system disorders, diarrhea anemia, fatigue, and edema.

To illustrate, the crude formulation of Hangeshashinto embraces seven herbs, including *S. baicalensis* (Table 7.3). Currently the focus on Hangeshashinto research targets its potential in treating irinotecan hydrochloride-induced diarrhea resulting from chemotherapy. Clinical research has proven the use of skullcap to be effective when prescribed 2–3 days prior to the administration of irinotecan hydrochloride. Effectiveness of *S. baicalensis*-based formulation for the treatment of diarrhea has been observed by many physicians (Iwase et al. 2012; Yamakawa et al. 2013). Treatment of gastrointestinal inflammation with a combination of crude drugs, *Pinellia* Tuber, *Coptis* rhizome, and *Scutellaria* root, effectively targets anti-inflammatory properties (Yamakawa et al. 2013). Due to their potential in alleviating the adverse side effects of cancer treatment, Kampo medicines have generated a renewed global interest in the area of palliative care.

7.2.4 Traditional Korean Medicine (TKM)

Known as Hangul/Hanja and traced as far back as 3000 B.C., the TKM records *Scutellaria barbata,* or Banjiryun, in the treatment for inflammatory diseases and cancers. In TKM, *S. baicalensis* is used to treat cerebral ischemia, bacterial

Table 7.3 Japanese Kampo formulations that use *Scutellaria* species to treat various diseases

Formulations	Species	Uses	References
Hangeshashinto	*Scutellaria* spp.	Diarrhea, oral mucositis	Suzuki et al. (2009); Yamakawa et al. (2013); Fukamachi et al. (2015)
Keigai-rengyo-to	*S. baicalensis*	Acne vulgaris	Uchi et al. (2011)
Oren-gedoku-to	S. baicalensis	Fever, acute and chronic phases of various diseases, in vitro inhibitory activity of Th17-type immune responses, gastrointestinal disorders	Gao et al. (2005); Suzuki et al. (2009); Takagi et al. (2014)
Saiboku-to	*S. baicalensis*	Gastrointestinal disorders	Suzuki et al. (2009)
Saikokaryukotsu-boreito	*S. baicalensis*	Hypertension, arteriosclerosis, chronic renal disease, neurasthenia, neurotic palpitations, epilepsy, hysteria, and erectile dysfunction	Takashi et al. (2014)
Saikokeishikankyoto	*S. baicalensis*	Anemia, fatigue, GI disorders	Takano et al. (2009)
Saireito	*Scutellaria* spp.	Nephritic syndrome, cirrhosis, pregnancy, swelling, lymphedema after surgery and macular edema, gastrointestinal disorders	Suzuki et al. (2009); Nagai et al. (2013)
Shin'iseihaito	*S. baicalensis*	Sinusitis and acute *Streptococcus pneumoniae* murine sinusitis	Konishi et al. (2016); Minami et al. (2017)

infections, and inflammatory diseases (Heo et al. 2009). A herbal formulation called *Chunghyul-dan* (CHD), which translates as "purification of blood," combines plant parts from *Scutellariae radix*, *Coptidis rhizome*, *Phellodendri cortex*, *Gardeniae fructus*, and Rhizoma rhei (*Rheum officinale*). Chunghyul-dan has traditionally been recommended to treat cardiovascular and cerebrovascular diseases and still remains a treatment in Korean medical clinics. Hwang-Ryung-Haedok-Tand (HRHT) ranks as the most famous herbal formulation which contains everything in CHD with the exception of the Rhizoma rhei (Jung et al. 2016). Current research has focused on the use of CHD to treat unhealthy levels of fat in the blood, high blood pressure, arterial stiffness, and stroke prevention (Jung et al. 2016). The documented uses of *S. baicalensis* in Korean folk medicine have inspired researchers to investigate an understanding of the complex molecular mechanisms at work when treating human gastric cancer cells. An exciting conclusion has illustrated that *Scutellaria baicalensis* flavonoids significantly inhibited cell viability and induced apoptosis in human gastric cancer cells (Saralamma et al. 2017).

7.2.5 Nepali Traditional Medicine

Though small in area, the Himalayan country of Nepal boasts a large floristic wealth with 6653 flowering plant species (Kunwar et al. 2010). Among these, 25–50% species are considered ethnomedicinals (Manandhar 2002). Nepali Folk medicine

reports application of *Scutellaria discolor* Colebr. to treat cuts and wounds with the whole plant or with leaf paste to soothe insect stings (Sinha et al. 1999). Ayurveda traditions employ the plant juice to treat headaches, fever, wounds, viruses, and rheumatism (Kunwar et al. 2010). *Scutellaria scandens,* known as Nalsal and Chaarpaate in Nepali (Quattrocchi 2012), is used to treat wounds and swelling caused by insects (Sripathi and Ravi 2017). Currently, root juice of *S. discolor* is utilized to treat indigestion, anxiety, and rheumatism (Kunwar et al. 2010). *Scutellaria scandens* is used by the Tamang community in Kabhrepalanchok district, Nepal, for treating backaches (by applying root juice on the back) and is also fed to animals to prevent miscarriage (Manandhar 1991). Tibetan people residing in high-altitude areas in Dolpo, Nepal, use flowers of *S. prostata* classified as *jip-tsi* for the sweet nectar (Ghimire and Aumeeruddy-Thomas 2009).

7.2.6 Indian Traditional Medicine (ITM)

The ITM represents a valued way of healthy living steeped within a long and unique cultural history. Further, ITM assimilates the best foreign influences developed from contact with other civilizations, Greece (resulting in Unani medicine), Germany (Homeopathy), or the ancient scriptures which aided the creations of Ayurveda, Siddha, and naturopathy. The richness of formulations and their plethora of applications in daily life have necessitated a separate Department of Indian Systems of Medicine and Homoeopathy (ISM&H) developed in 1995. Since November 2003, it is currently known as the Department of AYUSH (an acronym for Ayurveda, Yoga and Naturopathy, Unani, Siddha, Homoeopathy). India has ascribed to plant-based medicine for over 3000 years as a part of Ayurveda practice (Karnick 1996; Dubey et al. 2004; Karuppusamy 2007). The tribal people of the Paliyan tribes in Sirumalai hills and the Kani tribes of southern and Western Ghats of India traditionally worked with the leaves of *S. violacea* Heyne to prepare a paste to treat cuts, wounds, and skin diseases (Ayyanar and Ignacimuthu 2005; Karuppusamy 2007). Different tribes in the state of Assam prepare the fresh leaf juice of *S. discolor* for wound healing (Das et al. 2008). Also, the local people of Urumbikkara preferred the juice of fresh leaves of *S. oblonga* to treat ear pain (Jikku et al. 2015).

7.2.7 Traditional Iranian and Central Asia Medicine (TICAM) Systems

Medicine has historically played a significant role in Iranian culture and the civilization has considered TICAM as the basic tenet in the maintenance of health and treatment of disease. Hence, a relationship exists between the factors affecting temperament and metabolism. Thousands of years of history and hundreds of books

have placed TICAM among the oldest and richest alternative medicine systems (Bodeker et al. 2005; Emtiazi et al. 2012). In Flora Iranica (Rechinger 1982), 40 species represent the genus, with 22 species growing in Iran while another 10 of these species being endemic. Some *Scutellaria* species used in the TICAM are *S. lindbergii*, *S. litwinowii*, *S. luteo-caerulea, S. platystegia,* and *S. pinnatifida.* Common distribution of *S. lindbergii* is limited to Iran and Afghanistan (Attar and Joharchi 2002; Jamzad 2012). However, distribution of *Scutellaria luteo-caerulea* in the neighboring regions of Iranian plateaus, such as Afghanistan, Turkmenistan, and Iran, is common (Rechinger 1982). It provides immune system stimulation and antibacterial effects in traditional medicine in Iran (Nikbin et al. 2014). The indigenous people of the Zangelanlo district in Northeast Iran traditionally relied upon the aerial parts of *S. luteo-coerulea* to treat indigestion, gas, and skin problems (Amiri et al. 2012). Additional regional areas of Iran consider *Scutellaria pinnatifida* ssp. *alpina* as a customary gastrointestinal remedy (Ghannadi and Mehregan 2003).

Of the 38 species of *Scutellaria* that grow in Uzbekistan one species in particular, *S. ramosissima,* acts as a foundation of Uzbek folk medicine to treat epilepsy, inflammation, allergies, chorea, nervous tension, and high blood pressure. Aerial parts of *S. ramosissima* are collected to prepare as a tea or infusion (Mamadalieva et al. 2017). In Anatolian folk medicine *Scutellaria*-based preparation is used to stop bleeding and heal wounds (Minareci and Pekönür 2017).

7.2.8 South American Medicine (SAM) Systems

Though the Central and South American countries maintained ancient medical systems, use of *Scutellaria* did not figure prominently into their formulations. A genus predominantly restricted to the Northern hemisphere, its lack of use can be probably attributed to the few *Scutellaria* species growing in that region. According to long-held tradition, the Azuay, Cañar, and Napo regions of Ecuador have traditionally used *S. volubilis* to treat ailments of the nervous system, heart, and kidneys (Valarezo et al. 2012). Further, in the Loja province of Southern Ecuador native markets sell a native species of *Scutellaria* to aid the nervous system, offered as standard fare for hundreds of years (Tinitana et al. 2016). The Yora tribe of the Peruvian Amazon Rainforest inhaled *Scutellaria* at the Shaman's behest which was offered during ceremonies and passed in a gourd. The tribe expected the wild herb to dispel *yõshi* spirits (Shepard Jr 2004).

7.3 Physical Nature of Traditional Medicine Formulations

Depending on the traditional medical system, the entire plant or a specific plant part can be used to prepare a medicine (Table 7.4). Physical manifestations of *Scutellaria* range from a singular crude drug to concoctions, decoctions, essential oils, infusions,

Table 7.4 *Scutellaria* species are used as an entire plant or specific plant parts

SN	Species	Plant parts	Uses	References
1	*Scutellaria pinnatifida* ssp. *alpine*	Aerial	Gastrointestinal	Ghannadi and Mehregan (2003)
2	*S. colebrookiana* and *S. violacea*	Root	Antioxidant and cytotoxic effect on Dalton's lymphoma ascites cells and Ehrlich's ascites carcinoma cells	Salini et al. (2013)
3	*S. sibthorpii, S. cypria* var. cypria, *and S. cypria* var. elatior	Aerial	Antibacterial and antifungal	Dereboylu et al. (2012)
4	*S. baicalensis*	Extract	Antibacterial and antioxidant	Li et al. (2015a)
5	*S. baicalensis*	Root	Induced apoptosis in the human hepatocellular carcinoma cell line	Li et al. (2015c)
6	*S. baicalensis* radix	Root	Induced apoptosis in human fibrosarcoma HT1080 cells	Zhang et al. (2014)
7	*S. baicalensis*	Root	Anti-inflammatory effect without cytotoxicity	Lee et al. (2014)
8	*S. baicalensis*	Root, baicalein	Improved hyperglycemia, glucose tolerance, and blood insulin levels in middle-aged obese diabetic male mice	Fu et al. (2014)
9	*S. baicalensis*	Root	Neuroprotective effect on rat cortical neuronal cultures at embryonic stage	Yang et al. (2014)
10	*S. baicalensis*	Extract, Wogonin	Downregulated OVA-induced Th2 immune responses (IgE and IL-5) in 6-week-old, female BALB/c mice	Shin et al. (2014)
11	*S. baicalensis*	Root	Downregulated the expression of ABCG2 protein which in turn decreased side population cells in human myeloma cell line RPMI-8226	Lin et al. (2013)
12	*S. baicalensis*	Root	Reduced cantharidin-induced (COX-2) hemorrhagic cystitis female Wistar rats	Huan et al. (2012)
13	*S. baicalensis*	Root, Wogonin	Induced cell death in human U251 and U87 glioma cells by generation of reactive oxygen species, resulting in apoptosis	Tsai et al. (2012)
14	*S. baicalensis*	Root, baicalin	Anti-inflammatory effects on male CD-1 mice (7–9 weeks old and 25–30 g) and Wistar rats (145–160 g)	Yimam et al. (2012)
15	*S. baicalensis*	Root	Wogonin inhibited the contraction of smooth muscle in the uterus of nonpregnant female Wistar rats (250–350 g)	Shih and Yang (2012)
16	*S. baicalensis*	Root, Oroxylin A	Inhibited spontaneous contractions in the uterus of nonpregnant female Wistar rats (250–350 g)	Shih et al. (2009)

tinctures, tonics, and teas (Zhao et al. 2016). A drug assigned "crude" must originate from dried, powdered plant material; must occur naturally; and must not undergo alteration for medicinal treatment. The normal application of *Scutellaria lateriflora* is commonly prescribed in tea form (Bardakci and Skaltsa 2015). A mixture of different herbs produces a "concoction," whereas a "decoction" relies on boiling water to extract chemical contents such as those producing medicinal teas. Infusions resemble decoctions with the extraction of chemical contents, but extraction proceeds only through soaking and with no heat application.

For example, Cherokee Native Americans used an infusion from leaves and flowers of *S. lateriflora* to regulate menstruation. In addition, roots and stem parts gathered to prepare decoctions to treat nerves and breast pains, as a postdelivery medication, or as a kidney medication (Wills and Stuart 2004). Chinese medical herbalists selected *S. baicalensis* for preparing a decoction to treat different types of cancer (Gao et al. 2008). Additionally, the crude plant material of *Scutellaria barbata* is used in TCM to make functional foods and drugs (Bardakci and Skaltsa 2015). Tinctures are solutions that use alcohol as the solvent and represent another physical manifestation of *Scutellaria*-based medicine. In Western herbal medicine, tinctures prepared using *S. lateriflora* and *S. baicalensis* plant parts have treated anxiety and inflammatory diseases, respectively (Gao et al. 2008). *Scutellaria lateriflora* tinctures have been primarily seen to be effective against symptoms of anxiety including insomnia, anorexia nervosa, and tension headache, as well as in curing symptoms of muscle spasms including fibromyalgia, seizure disorders, and mild forms of Tourette's syndrome (Gao et al. 2008). *Scutellaria baicalensis* tinctures have been prescribed to treat inflammation, bacterial conditions, and allergies (Gao et al. 2008). A documented history of the use of *Scutellaria* in various forms by Chinese medical herbalists has been compiled (Boyle et al. 2011).

Volatile components, generally referenced as essential oils, that impart natural odor and characteristics of a plant are obtained through distillation. The Uzbek *Scutellaria* species (*S. immaculata*, *S. ramosissima*, and *S. schachristanica*) produce essential oils by distilling the aerial plant parts that provide antioxidant effects (Mamadalieva et al. 2016). Essential oils from the aerial parts of *S. brevibracteata*, *S. hastifolia*, and *S. orientalis* spp. *alpina* were tested for antifeedant activity on *Spodoptera littoralis*, and the *S. brevibracteata* and *S. hastifolia* oils produced positive results (Formisano et al. 2013). Other species from which essential oils have been extracted include *S. barbata*, *S. utriculata*, *S. grossa*, *S. albida*, *S. baicalensis*, *S. diffusa*, *S. heterophylla*, *S. salviifolia*, *S. rupestris* ssp. *adenotricha*, *S. pinnatifida*, *S. laete vioacea*, *S. respens*, *S. volubilis*, *S. havanensis*, *S. lateriflora*, *S. porvula*, and *S. wightiana* (Sripathi and Ravi 2017).

Tonics represent another physical manifestation and owe their curative and invigorating properties to their preparation from herbs. The *Scutellaria lateriflora* tonic, either individually or in combination with tablet forms of other medicinal plants, has been proposed by traditionalists in combatting sleeping disorders in Canada (Bardakci and Skaltsa 2015). *Scutellaria lateriflora*, typically recommended for its sedative qualities, populates herb shops in the form of herbal tea,

tablets, tonics, capsules, and oral liquid preparations (Wills and Stuart 2004; Bardakci and Skaltsa 2015). The *S. lateriflora* leaves in combination with bear grease are used to dress and treat swelling, inflammation, sores, and wounds (Wills and Stuart 2004).

7.4 Bioactive Compounds

Secondary metabolites produced by plants that contain therapeutic properties are known as bioactive compounds. These compounds assist plants to defend against predator and pest attacks, and offer the additional benefits of pollination stimulation and of guiding insects to their food source (Cook and Samman 1996). These secondary metabolites have infiltrated pharmaceutical, agrochemical, aromatics, and food additive industries through multiple roles. Falling under a number of different chemical groups (Sidhu 2010), nearly 300 phytochemicals have been isolated from various *Scutellaria* species (Shang et al. 2010).

Phytochemical research of *Scutellaria* genus has continued since 1889, with phenolics and terpenes emerging as the two main groups of compounds reported from various species (Shang et al. 2010). The majority of the chemicals isolated from this genus fall under the chemical group flavonoids. Yellow when extracted, the diverse flavonoid group represents polyphenolic compounds that naturally occur in plants, fruit, vegetables, nuts, seeds, flowers, and bark (Cook and Samman 1996; Zhu et al. 2004; Procházková et al. 2011). Despite their yellow appearance, flavonoids comprise the anthocyanin pigments that give fruits and vegetables their pink, red, mauve, blue, and purple colors (Cook and Samman 1996; Procházková et al. 2011). As plants and vegetables constitute a regular part of many diets, humans and animals routinely consume flavonoids at a rate of 50–800 mg/day depending on the food and beverages ingested (Zhu et al. 2004). Dietary benefits of consuming flavonoids include decreased cardiovascular diseases, increased antioxidant activity, and increased longevity (Procházková et al. 2011). A brief description of the distribution of bioactive compounds in Scutellaria species and their potential activities is presented in Table 7.5.

In addition to dietary benefits, flavonoids prove biologically advantageous due to antibacterial, anticancer, anti-inflammatory, anti-ischemic, antiviral, and vasodilatory attributes. Further, through their antioxidant properties, flavonoids assist in the inhibition of reduced capillarity permeability and fragility, antagonistic enzyme systems (i.e., cyclooxygenase and lipoxygenase), lipid peroxidation, and platelet aggregation (Cook and Samman 1996; Zhu et al. 2004; Procházková et al. 2011). The following is a description of a few bioactive compounds isolated from various *Scutellaria* species and research conducted through different experimental platforms.

Table 7.5 Prominent bioactive compounds isolated from *Scutellaria* species and their activities

SN	Bioactive compound	Activities detected	*Scutellaria* spp.	References
1	Baicalein	Anti-allergic, antibacterial, anti-carcinogenic, anticonvulsant, anti-HIV, anti-inflammatory, anti-mutagenesis, antioxidant, antitumor, antiviral, anxiolytic properties, positive effects on dementia and memory loss, hepatoprotective and neuroprotective	*S. hypericifolia, S. amoena, S. viscidula, S. barbata, S. lateriflora, S. lateriflora*	Zhang et al. (2008); Zhu et al. (2004); Jelić et al. (2016); Shang et al. (2010)
2	Baicalin	Anti-allergic, antibacterial, anti-HIV-1, anti-inflammatory, anti-mutagenic, antioxidant, antipyretic, anti-RSV, antithrombotic, antitoxic, antitumor, antiviral, and anxiolytic properties, in addition to hepatoprotective and neuroprotective effects. Has the capability to inhibit prostaglandin E2 production, reduce high blood pressure, and relax arterial smooth muscle cells and is effective against several human prostatic cancer cell lines.	*S. baicalensis, S. amoena, S. viscidula, S. barbata, S. lateriflora*	Yang et al. (2013); Jelić et al. (2016); Shang et al. (2010); Marsh et al. (2014); Gao et al. (2008); Shang et al. (2010); Chen et al. (2014)
3	Chrysin	Anticancer property, anti-amyloidogenic, anti-atherogenic, antidepressant, anti-epileptic, anti-HIV, anti-inflammatory, antioxidant properties, chemopreventive, neuroinflammation, neurotrophic properties, anti-asthmatic anti-hypercholesterolemic properties, shown positive effects on Parkinson's disease and protection against spinal cord injury, a suppressive effect on vascular endothelial growth factor (VEGF)-induced angiogenesis, prevention of metastatic progression in breast cancer cells, inhibition of TNF-α and interleukin (IL)-1β activity, cardioprotective activity, prevention of osteoporosis, and renoprotective activity, effective against several different cancer types in vitro and in vivo by inhibiting proliferation and inducing apoptosis, and it can reduce proliferation and survival of cancer cells, especially in cervical cancer cell lines	*S. amoena, S. baicalensis, S. immaculata, S. linearis, S. ramosissima, S. strigillosa, S. viscidula, S. discolor*	Shang et al. (2010); Nabavi et al. (2015); Khoo et al. (2010); Laishram et al. (2015); Chrysin (2017); Chrysin C80105 -Sigma-Aldrich (2017)
4	Scutellarein	Antidiabetic, anti-inflammatory, anxiolytic, antioxidant, anti-mutagenic, anti-thrombotic, antitumor, hepatoprotective, and neuroprotective	*S. barbata, S. baicalensis, S. viscidula, S. rehderiana, S. lateriflora*	Shang et al. (2010); Marsh et al. (2014); Dong et al. (2016)

(continued)

Table 7.5 (continued)

SN	Bioactive compound	Activities detected	*Scutellaria* spp.	References
5	Scutellarin	Anticoagulant, antidiabetic, anti-HIV, anti-hypertrophic, anti-inflammatory, antioxidant, anti-RSV, and anti-thrombotic properties, as well as fibrinolysis effects shown to protect the brain and heart and suppress hypercholesterolemia	*S. barbata, S. racemosa, S. lateriflora, S. hastifolia*	Shang et al. (2010); Islam et al. (2011); Bardakci and Skaltsa (2015); Xing et al. (2011); Shi et al. (2015); Gu et al. (2017); Wang et al. (2017)
6	Wogonin	Anti-allergic, anti-HBV, anti-inflammatory, antioxidant, antitumor, antiviral properties, in addition to radical scavenging, neuroprotective, and anxiolytic effects, anti-fibrotic effects in renal tubular epithelial cells	*S. baicalensis*, *S. amoena*, *S. barbata*, *S. viscidula*, *S. rehderiana*, *S. linearis*, *S. racemosa, S. lateriflora, S. hypericifolia, S. likiangensis*	Lai et al. (2002); Islam et al. (2011); Shang et al. (2010); Marsh et al. (2014); Tai et al. (2005); Gao et al. (2008); Lin et al. (2013); Meng et al. (2016)
7	Wogonoside	Anti-allergic, anticoagulant, antiviral, anti-inflammatory, anti-inflammation-induced angiogenic, antioxidant, antitumor, and hepatoprotective properties, may have the potential to treat imatinib-resistant chronic myelogenous leukemia (CML) in vitro and in vivo by inducing growth inhibition and cell cycle arrest	*S. baicalensis*, *S. amoena*, *S. linearis*, *S. rehderiana*, *S. tenax*, *S. hypericifolia*, *S. likiangensis*, *S. viscidula, S. lateriflora*	Hattori (1930); Lui et al. (2002); Shang et al. (2010); Marsh et al. (2014); Lim (2003); Shang et al. (2010); Yang et al. (2013); Ku and Bae (2014); Li et al. (2015c, d)

7.5 Trichomes in *Scutellaria*

Trichomes are fine, nonglandular, or glandular outgrowths or appendages on plants (Glas et al. 2012; Huchelmann et al. 2017) that serve as the crucial primary sites where synthesis and storage of bioactive compounds take place (Bisio et al. 1998). Various types of lipophilic compounds accumulate in glandular trichomes. Since the gland cells display several ultrastructural features indicating active lipid metabolism and secretion, researchers believe that the terpenes are the major group of compounds found and synthesized within the glandular trichome (Gershenzon et al. 1989; Schilmiller et al. 2008). Either glandular or nonglandular, the size of trichomes can range from a few microns to several centimeters and they tend to exhibit tremendous species-specific shape diversity (Glas et al. 2012; Fig. 7.2). The characteristics of trichomes and the composition of the secretory substances show great variability among species and could act as a plant classification tool between related genera or species (Xiang et al. 2009). As single-celled or multicellular, the criterion mostly employed to classify trichomes is whether they are glandular or not (Glas et al. 2012). Depending on the plant organ and its location, the possibility exists of recording multiple types of trichomes on the same plant of a given species (Payne et al. 1999). Trichomes have been suggested as a signature organ (biomarker) to track adulteration in herbal biomass (Hill 2014).

7.6 Nonhuman Application of Medicinal *Scutellaria* Species

*7.6.1 Far Eastern Catfish (*Silurus asotus*)*

Incorporating natural herbs is an age-old practice that is advantageous as a dietary additive. *Scutellaria baicalensis* extract has been known to have antimicrobial, antibacterial, antiviral, anti-inflammatory, and/or antioxidant activities due to the presence of the active components baicalin and baicalein. The aquaculture industry has benefitted with the use of *S. baicalensis* extract in improving the survival of Far Eastern Catfish (*S. asotus)* infected with *Vibrio anguillarum* and *Streptococcus iniae* (Kim et al. 2013). To determine the effects on growth, body composition, and serum chemistry and in challenge tests of Far Eastern Catfish, various concentrations of *S. baicalensis* extract (100 ppm baicalin) used as a dietary additive were compared to a commercially available immune enhancer. Though no significant difference in weight gain or performance resulted, observations of increased survival after infection of bacteria occurred (Kim et al. 2013). Thus, the use of *S. baicalensis* in fish feed could provide beneficial results in other fish species raised for commercial purchase.

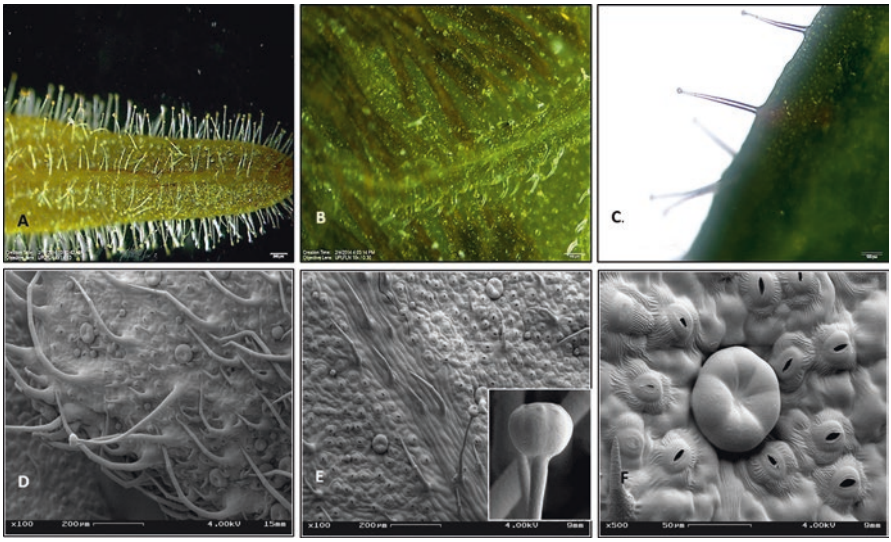

Fig. 7.2 General view of HistoChoice™ fixed leaves showing trichomes of *Scutellaria* species using light and scanning electron micrographs. (**a**) *S. baicalensis*. (**b**) *S. lateriflora*. (**c**) *S. ocmulgee*. (**d–f**) Nonglandular, glandular, and glandular-peltate type of trichomes in *Scutellaria* spp. (**e**) Shows magnified view of a glandular trichome (detailed view of the head cell in the inset)

*7.6.2 Olive Flounder (*Paralichthys olivaceus*)*

Olive flounder remains a popular fish in Eastern Asia because of its fast growth and disease resistance (Cho et al. 2013). In order to prevent economic losses, the development of new additives to fish feed was studied to determine whether disease resistance could be improved. Because of the antibacterial properties of *S. baicalensis* along with its safe consumption for humans and history of successful studies, researchers chose the *S. baicalensis* extract as a dietary supplement to the fish feed to access growth, feed utilization, and survival from infection of a pathogenic strain of *Edwardsiella tarda*. The study revealed that by adding 2% *S. baicalensis* extract to the feed, the specific growth rate of olive flounder improved and mortality of the fish decreased from pathogenic infection of *E. tarda* (Cho et al. 2013).

7.6.3 Poultry

Ongoing research seeks natural remedies to fight against foodborne pathogens, especially in the poultry industry. A study conducted using the root extract of *S. baicalensis* sought to determine the immune response in broiler chickens. Findings concluded that the root extract could act as a beneficial additive to feed, with the suggestion that future research could decide the dosage (Króliczewska et al. 2017). Another study conducted on inflammation and *Salmonella enteritidis* infection showed that a mixture of *Curcuma* and *S. baicalensis* extract added to feed

reduced such an infection in poultry (Varmuzova et al. 2015). Finally, research conducted by An et al. in 2010 mentioned that the egg weight of an experimental group of chickens given *S. baicalensis* extract significantly increased when compared to the control group's egg weight.

7.6.4 Pork

Veterinary medicine has historically utilized herbs and their extracts not only because of their antimicrobial, antiviral, anti-inflammatory, and antioxidant properties, but also for their improvement of digestion, nutrient absorption, increased immune response, and regulation of the intestinal microbiota (Liu et al. 2016). In a study incorporating a *S. baicalensis* and *Lonicera japonica* mixture as a herbal supplement for finishing pigs, growth, nutrient digestion, cortisol levels, and meat quality were analyzed. The results proved the positive role of the supplement in growth, digestion, decreased cortisol levels, and meat quality (Liu et al. 2016).

7.6.5 Cattle

Due to the high heat, relative humidity, and solar radiation during summer months, beef cattle in South China experience heat exhaustion, discomfort, and even death (Song et al. 2014). In an attempt to determine ways to alleviate these ailments researchers focused on two dietary supplements. One supplement contained *S. baicalensis* in the formulation. The study found at the conclusion a decrease in rectal temperature, an improved heat stress response, increased growth performance, and an increase in digestion. This study recognized the heat-clearing nature of *Scutellaria* and the other medicinal plants in the formulation (Song et al. 2014).

7.6.6 Kimchi

Considered a traditional food in Korean culture and consumed widely in northeastern Asian countries, preparation of the popular food kimchi involves fermenting baechu cabbage with other vegetables and lactic acid bacteria. Kimchi's fermentation process eliminates pathogenic bacteria and increases nutritional benefits. A problem with the preservation of kimchi, over-fermentation, leads to a sour taste and a major economic concern for the kimchi industry (Patra et al. 2016). Research has targeted the use of natural preservatives to stop the fermentation process and thereby increase the shelf life of kimchi (Moon et al. 1995). In one study conducted by incorporating medicinal plants as an antimicrobial agent in the preparation of kimchi, it was observed that the use of Baikal skullcap and Assam indigo effectively maintained the freshness of kimchi (Moon et al. 1995).

7.7 Threats to *Scutellaria* Populations

Unfortunately, many *Scutellaria* species possess no immunity from the threat of declining populations. In fact, there are many species that fall into the rare, threatened, or endangered categories (Cole et al. 2007; Joshee et al. 2010; Guo et al. 2011; Vaidya et al. 2013). The growing popularity of *Scutellaria* has resulted in declining populations through indiscriminate collection, adulteration, decreased seed set, invasive species, habitat loss, and diseases. In an effort to counteract the natural population loss, micropropagation, DNA barcoding, revitalization efforts, and conservation awareness programs have been set up (Joshee et al. 2010; Foster 2012; Gafner 2015). In addition, research groups have employed micropropagation to study the medicinal properties, reproductive biology, and its possible connection to decline in populations.

7.7.1 Seed Set

A few species suffer from an observed decrease of seed set. For example, a species endemic to Georgia and South Carolina, USA, *S. ocmulgee* (Ocmulgee skullcap), now exists as a threatened species in Georgia (Federal Register 2002). *S. montana* (large-flowered skullcap; mountain skullcap), found only in Georgia and Tennessee, shares prominence on the federally threatened and endangered state lists of Tennessee and Georgia (USDA 2012). Both species have poor seed set in the wild as well as declining populations (Vaidya 2013). At Fort Valley State University, we currently conduct research to analyze the reproductive biology aspects of these species, including the pollen behavior, so that assistance in establishing in situ conservation of these species can follow.

7.7.2 Adulteration

The 'Botanical Adulterants Program' addresses various techniques and analytical methods to differentiate *S. lateriflora* from *Teucrium chamaedrys* and some closely related species. However, when differentiating between closely related species the task becomes a little more difficult (Gafner 2015). A global push has emerged to encourage finding alternative ways to identify medicinal plants to ensure that consumers do not receive inferior products. One such approach involves DNA barcoding. DNA barcoding resembles DNA fingerprinting and would quickly allow inspectors to determine whether the consumer receives the correct product or not (CBOL Plant Working Group 2009).

Plants used for medicinal purpose cover a broad range of taxa that are morphologically similar. Oftentimes the similarities lead to misidentification and inappro-

priate use since phylogenetically they are unrelated (Chen et al. 2010). *Scutellaria lateriflora* has been adulterated with *Teucrium chamaedrys*, a germander that is toxic to the liver (Foster 2012). To highlight the taxa confusion, researchers designed a study to determine if supplements did in fact contain the raw material delineated on the product labels. Thirteen different supplements claiming to contain *Scutellaria* in them underwent testing. The study found that only five of the thirteen did in fact have *Scutellaria* in them while four of the thirteen tested contained the adulterant *Teucrium canadense* (Sun and Chen 2011; Foster 2012). *Scutellaria* is not only adulterated by other genera, but also by other species from their own genus (Guo et al. 2011).

At Fort Valley State University, we have *Scutellaria* germplasm consisting of 21 species found from all around the world (Fig. 7.1). Our ongoing research, to create a DNA barcode system to arrange *Scutellaria* species on a phylogenetic tree, will provide information to identify their adulterants for future scientific research and use. Thus, our goal to create a unique DNA barcode will differentiate the different species not only from each other, but from other medicinal plants as well.

7.8 Conclusion

Globally, people in this millennium have increasingly rejected pharmaceuticals when provided with effective and plant-based alternatives to manufactured drugs. It is a well-established fact that access to modern medicine does not eliminate the use of local medicinal practices. But modern medicine may trigger the disappearance of some age-old proven traditional practices. Efforts are on to record the practices native healers perform before the plant resources are depleted. A persistent issue central to the field of ethnobotany is the lack of mechanistic understanding, describing generalized mechanisms like "showing activity" in the experimental system. A closer link between ethnobotanical field researchers and laboratory pharmacologists will be very helpful to advance this field that has immense potential to serve humanity.

Scutellaria has existed as a traditional herbal remedy for thousands of years and has earned a positive reputation through the treatment and prevention of a range of simple and complex diseases. Most of the traditional uses of *Scutellaria* have already been scientifically proven through pharmacological and phytochemical analysis aimed at approximately 15% of the species. In addition, the most studied of the species are *Scutellaria baicalensis*, *S. barbata,* and *S. lateriflora*, leaving a wealth of germplasm to investigate further. That leaves over 300 *Scutellaria* species for future research to determine additional medicinal benefits. As people increasingly continue to turn back toward utilizing and embracing those natural plant-based remedies respected by their ancestors, the demand for *Scutellaria* plant-based therapies will increase exponentially. Since flavonoids are not produced in quantities high enough for commercial use, efforts to produce pure and higher quantities of these beneficial chemicals should intensify.

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Chapter 8
Cultivating Research Grade Cannabis for the Development of Phytopharmaceuticals

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8.1 Introduction

Cannabis sativa L. (Cannabaceae) is a widely spread plant which has been found wildly in nature and also cultivated by humans since millennia. It is one of the oldest plant sources for seed oil, intoxicant resin, medicine, and textile fiber (Ranalli et al. 1999; Kriese et al. 2004). In nature, cannabis grows in all kind of habitats ranging from sea level to temperate and alpine foothills around the world. Cannabis has a long history of being used as medicine to treat a variety of ailments such as asthma, epilepsy, fatigue, glaucoma, insomnia, nausea, pain, and rheumatism (Doyle and Spence 1995; Zuardi 2006). The benefits of cannabis derivatives have also been reported against HIV/AIDS (Abrams et al. 2007) and multiple sclerosis (Pryce and

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Baker 2005). Cannabis produces cannabinoids in its glandular trichomes. Among the cannabinoids, Δ^9-tetrahydrocannabinol (Δ^9-THC) is the main psychoactive compound which is naturally present in the form of an acid (Δ^9-tetrahydrocannabinolic acid, Δ^9-THCA) and undergoes decarboxylation with age or heating to form Δ^9-tetrahydrocannabinol (Turner et al. 1980; Pertwee 2006). The other important cannabinoid in cannabis of current interest is cannabidiol (CBD) which is reported to be active as an antiepileptic agent, particularly its promise for the treatment of intractable pediatric epilepsy (Mechoulam and Carlini 1978; Cunha et al. 1980).

Other than THC and CBD, tetrahydrocannabivarin (THCV), cannabinol (CBN), cannabigerol (CBG), and cannabichromene (CBC) are major cannabinoids isolated from *Cannabis sativa* (Fig. 8.1). Modern studies report that the pharmacological effects of phytocannabinoids are due to their ability to interact with cannabinoid receptors and/or with other kinds of pharmacological targets, including non-cannabinoid receptors which makes the pharmacology of the phytocannabinoids rather complex and interesting (Cascio et al. 2017).

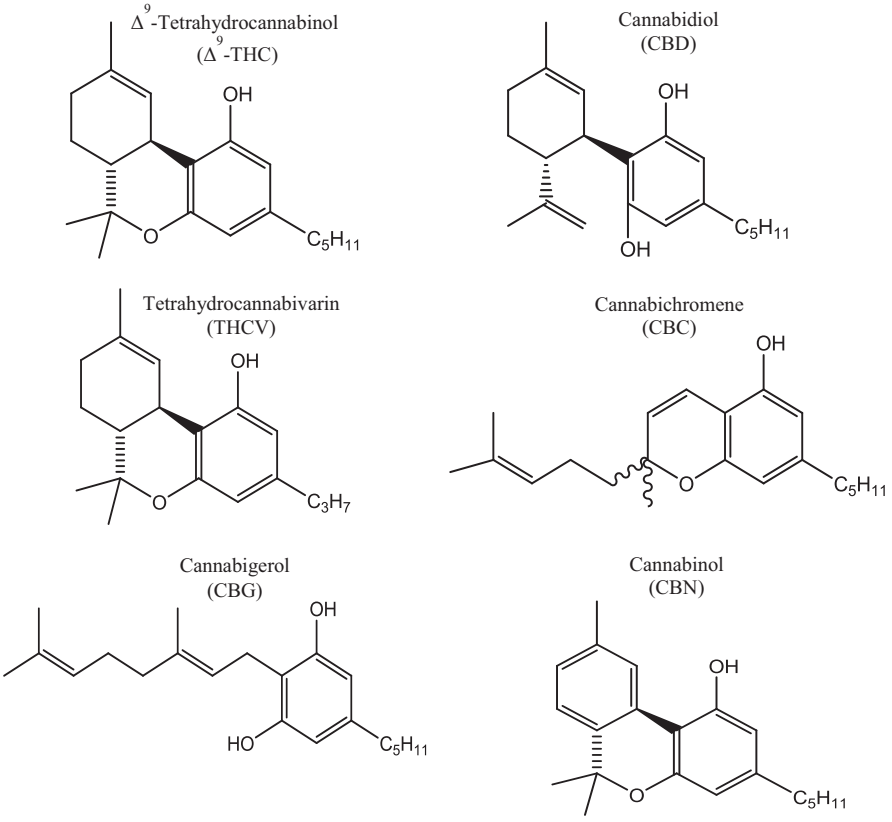

Fig. 8.1 Chemical structures of major cannabinoids commonly found in *Cannabis sativa*

8.2 The Plant Cannabis

Botanical nomenclature of *Cannabis sativa* L. is described in Table 8.1. Cannabis is highly variable and complex in its morphology and genetics and therefore the number of species in the Cannabis genus is a matter of debate for a long time. Modern hybrid varieties and/or cultivars with manipulation in genetics and chemical constituents make the nomenclature of cannabis more difficult. The pioneer modern taxonomist Swede Carl Linnaeus L. (1737) treated cannabis as a single species, whereas Lamarck (1785) described "Indian cannabis strain" taxonomically different than "European hemp" and gave it a specific name "*Cannabis indica*."

Due to the morphological and phenotypic variations, a number of reports proposed cannabis as a polytypic genus, whereas others suggest as monotypic but highly polymorphic species, *Cannabis sativa* L. (Emboden 1974; Hillig 2004, 2005; Hillig and Mahlberg 2004; Small 1975a, b, 2017; Small and Cronquist 1976; Gilmore et al. 2003). At the present day, cannabis is considered as a single but highly diverse species, *C. sativa* L., with different varieties such as *C. sativa* var. *sativa*, *C. sativa* var. *indica*, and *C. sativa* var. *ruderalis*. Common cannabis names are shown in (Table 8.2).

Cannabis is a dioecious and occasionally monoecious plant. It flowers under the shorter photoperiod (below 12-h light) and continues growing vegetatively under the longer days. At the vegetative stage, it is difficult to differentiate male and female plants due to their morphological similarities. However, separation of male from female is possible at early flowering stage based on their different morphological appearances. Cannabis is a wind-pollinated plant. For the production of cannabinoids (or phytocannabinoids) sinsemilla female plants are preferred over male

Table 8.1 Botanical nomenclature of *Cannabis sativa* L.

Kingdom: Plants

Subkingdom: Vascular plants

Family: Cannabaceae

Genus: Cannabis

Species: *Cannabis sativa* L.

Table 8.2 Popular **c**annabis names in different languages

Arabic: Bhang, hasheesh kenneb, tîl

Chinese: Ye ma, xian ma

English: Cannabis, hemp, marihuana

French: Chanvre, chanvre indien, chanvrier

German: Hanf, haschisch, indischer hanf

Hindi: Bhang, charas, ganja

Japanese: Mashinin

Spanish: Cáñamo, grifa, hachís, mariguana

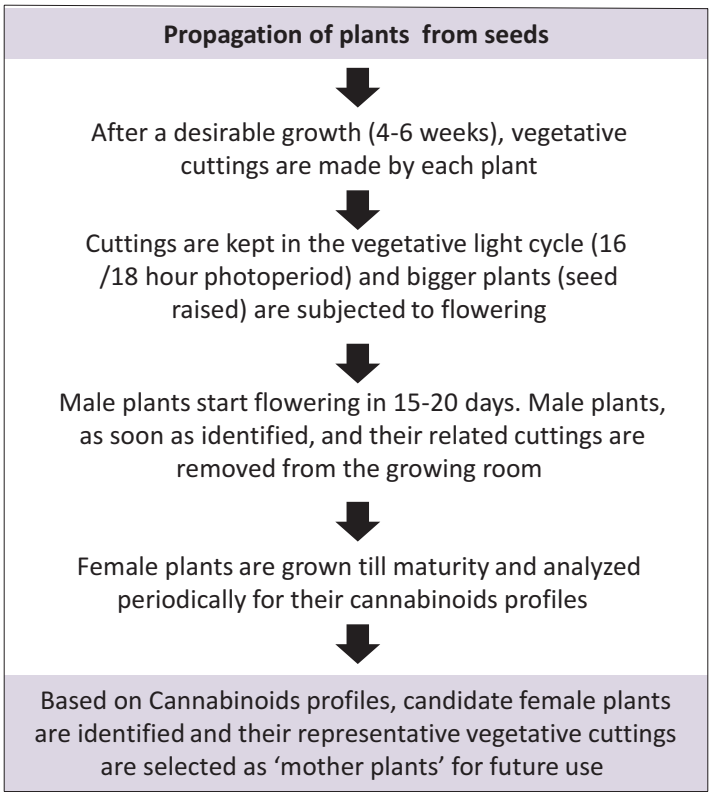

Fig. 8.2 Schematic diagram of screening and selection of high-yielding mother plant in cannabis varieties

plants. In the presence of males, female plants produce seeds at maturity and if several cannabis varieties are being grown together cross-pollination among the varieties would affect the cannabinoid profile of the final product. Therefore, male plants are normally removed as they appear. Among females, high-yielding elite clones are chosen based on their chemical profile and multiplied using conventional (vegetative cutting) and biotechnological tools (tissue culture) to maintain a batch-to-batch consistency in the final product (Chandra et al. 2010). A schematic diagram of screening and selection process of elite mother plants is shown in Fig. 8.2.

8.3 Chemical Constituents of *Cannabis sativa*

The first cannabinoid to be isolated (Wood et al. 1899) and identified (Adams et al. 1940a; Cahn 1932; Ghosh et al. 1940) from *Cannabis sativa* was cannabinol (CBN). Cannabidiol (CBD) was subsequently isolated from Mexican marijuana

Table 8.3 Constituents of *C. sativa* L.

Chemical class	Number of constituents
Δ^8-THC type	5
Δ^9-THC type	23
CBG type	16
CBC type	9
CBD type	7
CBND type	2
CBE type	5
CBL type	3
CBN type	11
CBT type	9
Cannabinoids (see Table 8.4 for details)	120
Non-cannabinoids	445
Miscellaneous type	30
Total	**565**

(Adams et al. 1940b) and the structure was determined (Mechoulam and Shvo 1963). The most psychoactive compound Δ^9-THC was isolated and the structure was determined by Gaoni and Mechoulam in 1964 (Gaoni and Mechoulam 1964). Since then, the number of cannabinoids and other compounds isolated from cannabis has been continually increasing.

Till today, 565 constituents are reported from cannabis (Table 8.3). Among them 120 are phytocannabinoids (Table 8.4) (ElSohly and Slade 2005; ElSohly and Gul 2014; Radwan et al. 2017). Out of 50 new cannabinoids in the last 10 years, 43 are isolated by the ElSohly group at the National Center for Natural Product Research (NCNPR), School of Pharmacy at the University of Mississippi. Based on the chemical constituents, their complexity, and individual and combined biological effects, cannabis is considered as a complex plant (ElSohly and Slade 2005).

Cannabis is classified into three distinct groups based on their chemical profiles, drug type (THC/CBD ratio > 1), intermediate type (THC/CBD ratio close to 1.0), and fiber type (THC/CBD ratio < 1) (Small and Beckstead 1973a, b).

8.4 Cannabis Biosynthesis

A schematic diagram of cannabinoid biosynthesis is shown in Fig. 8.3. In Cannabis plant, Δ^9-THC, CBD, CBC, and CBG are produced as their acid forms (Shoyama et al. 1975; Kajima and Piraux 1982; Fellermeier and Zenk 1998), and so are all other cannabinoids.

Two independent pathways, the cytosolic mevalonate and the plastidial methylerythritol phosphate (MEP), are responsible for plant terpenoid biosynthesis. The plastidial methylerythritol phosphate pathway is reported to be responsible for

Table 8.4 An update on phytocannabinoids isolated from *Cannabis sativa* L.

No.	Name of phytocannabinoids
1	(−)-Δ^9-*Trans*-tetrahydrocannabinolic acid A (Δ^9-THCA-C_5 A)
2	(−)-Δ^9-*Trans*-tetrahydrocannabinolic acid B (Δ^9-THCA-C_5 B)
3	(−)-Δ^9-*Trans*-tetrahydrocannabinol (Δ^9-THC-C_5)
4	(−)-Δ^9-*Trans*-tetrahydrocannabinol-C4 (Δ^9-THC-C_4)
5	(−)-Δ^9-*Trans*-tetrahydrocannabinolic acid A-C4 (Δ^9-THCA-C_4)
6	(−)-Δ^9-*Trans*-tetrahydrocannabivarin (Δ^9-THCV-C_3)
7	(−)-Δ^9-*Trans*-tetrahydrocannabivarinic acid (Δ^9-THCVA-C_3)
8	(−)-Δ^9-*Trans*-tetrahydrocannabiorcolic acid (Δ^9-THCOA-C1 A)
9	(−)-Δ^9-*Trans*-tetrahydrocannabiorcol (Δ^9-THCO-C_1)
10	β-Fenchyl-Δ^9-*trans*-tetrahydrocannabinolate
11	α-Fenchyl-Δ^9-*trans*-tetrahydrocannabinolate
12	Epi-bornyl-Δ^9-*trans*-tetrahydrocannabinolate
13	Bornyl-Δ^9-*trans*-tetrahydrocannabinolate
14	α-Terpenyl-Δ^9-*trans*-tetrahydrocannabinolate
15	4-Terpenyl-Δ^9-*trans*-tetrahydrocannabinolate
16	α-Cadinyl-Δ^9-*trans*-tetrahydrocannabinolate
17	γ-Eudesmyl-Δ^9-*trans*-tetrahydrocannabinolate
18	Cannabisol
19	8α-Hydroxy-Δ^9-*trans*-tetrahydrocannabinolate
20	8β-Hydroxy-Δ^9-*trans*-tetrahydrocannabinolate
21	11-Acetoxy-Δ^9-*trans*-tetrahydrocannabinolic acid A
22	Δ^9-THC aldehyde
23	8-Oxo-Δ^9-*trans*-tetrahydrocannabinol
24	Δ^8-Tetrahydrocannabinolic acid A (Δ^8-THCA-C_5 A)
25	Δ^8-tetrahydrocannabinol (Δ^8-THC-C_5)
26	10*α*-Hydroxy-Δ^8-tetra-hydrocannabinol
27	10*β*-Hydroxy-Δ^8-tetrahydrocannabinol
28	10a*α*-Hydroxy-10-oxo-Δ^8-tetrahydrocannabinol
29	Cannabigerol {(*E*)-CBG-C_5}
30	Cannabigerolic acid {(*E*)-CBGA-C_5}
31	Cannabigerol monomethyl ether {(*E*)-CBG-C_5}
32	Cannabigerolic acid monomethyl ether {(*E*)-CBGAM-C_5}
33	Cannabigerovarinic acid A {(*E*)-CBGVA-C_3}
34	Cannabigerovarin {(*E*)-CBGV-C_3}
35	Cannabinerolic acid A {(*Z*)-CBGVA-C_5}
36	Camagerol
37	γ-Eudesmyl-cannabigerolate
38	α-Cadinyl-cannabigerolate
39	Sesquicannabigerol
40	5-Acetyl-4-hydroxy-cannabigerol
41	(±)-6,7-*Trans*-epoxycannabigerolic acid
42	(±)-6,7-*Cis*-epoxycannabigerolic acid

(continued)

Table 8.4 (continued)

No.	Name of phytocannabinoids
43	(±)-6,7-*Trans*-epxoycannabigerol
44	(±)-6,7-*Cis*-epoxycannabigerol
45	(±)-Cannabichromenic acid (CBCA-C_5)
46	(±)-Cannabichromene (CBC-C_5)
47	(±)-Cannabivarichromevarinic acid (CBCVA-C_3)
48	(±)-Cannabivarichromene (CBCV-C_3)
49	(+)-Cannabichromevarin (CBCV-C_3)
50	2-Methyl-2-(4-methyl-2-pentyl)-7-propyl-2H-1-benzopyran-5-ol
51	(±)-4-Acetoxycannabichromene
52	(±)-3″-Hydroxy-Δ4″-cannabichromene
53	(−)-7-Hydroxycannabichromane
54	Cannabidiol (CBD-C_5)
55	Cannabidiolic acid (CBDA-C_5)
56	Cannabidiol monomethyl ether (CBDM-C_5)
57	Cannabidiol-C_4 (CBD-C_4)
58	Cannabidivarin (CBDV-C_3)
59	Cannabidivarinic acid (CBDVA-C_3)
60	Cannabidiorcol (CBD-C_1)
61	Cannabinodiol (CBND-C_5)
62	Cannabinovarin (CBND-C_3)
63	Cannabielsoic acid A (CBEA-C_5-A)
64	Cannabielsoin (CBE-C_5)
65	Cannabielsoic acid B (CBEA-C_5-B)
66	C_3-Cannabielsoic acid B (CBEA-C_3-B)
67	C_3-Cannabielsoin (CBE-C_3)
68	Cannabicyclol (CBL-C_5)
69	Cannabicyclolic acid (CBLA-C_5)
70	Cannabicyclovarin (CBLV-C_3)
71	Cannabinol (CBN-C_5)
72	Cannabinolic acid (CBNA-C_5)
73	Cannabinol methyl ether (CBNM-C_5)
74	Cannabinol-C_4 (CBN-C_4)
75	Cannabivarin (CBN-C_3)
76	Cannabinol-C_2 (CBN-C_2)
77	Cannabiorcol-C_1 (CBN-C_1)
78	4-Terpenyl cannabinolate
79	8-Hydroxy cannabinolic acid A
80	8-Hydroxycannabinol
81	1'S-hydroxy-cannabinol
82	(−)-*Trans*-cannabitriol {(−)-*trans*-CBT-C_5}
83	(+)-*Trans*-cannabitriol {(+)-*trans*-CBT-C_5}
84	(±)-*Cis*-cannabitriol {(±)-*cis*-CBT-C_5}

(continued)

Table 8.4 (continued)

No.	Name of phytocannabinoids
85	(−)-*Trans*-10-ethoxy-9-hydroxy-$\Delta^{6a(10a)}$-tetrahydrocannabinol {(−)-*trans*-CBT-OEt-C_5}
86	(±)-*Trans*-cannabitriol-C_3 {(±)-*trans*-CBT-C_3}
87	Cannabitriol-C_3-homologue (CBT-C_3-homologue)
88	(−)-*Trans*-10-ethoxy-9-hydroxy-$\Delta^{6a(10a)}$-tetrahydrocannabivarin-C_3 {(−)-*trans*-CBT-OEt-C_3}
89	8,9-Dihydroxy-$\Delta^{6a(10a)}$-tetrahydrocannabinol (8,9-di-OH-CBT-C_5)
90	Cannabidiolic acid tetrahydrocannabitriol ester (CBDA-C_5–9-OH-CBT-C5-ester)
91	Dehydrocannabifuran (DCBF-C_5)
92	Cannabifuran (CBF-C_5)
93	10-Oxo-Δ6a(10a)-tetrahydrocannabinol (OTHC)
94	8-Hydroxy-isohexahydrocannabivirin (OH-iso-HHCV-C_3)
95	Cannabichromanone-C5 (CBCN-C_5)
96	Cannabichromanone-C3 (CBCN-C_3)
97	Cannabicitran
98	(−)-Δ^9-*Cis*-(6aS,10aR)-tetrahydro-cannabinol (*cis*-Δ^9-THC)
99	Cannabicoumaronone-C5 (CBCON-C_5)
100	Cannabiripsol (CBR)
101	Cannabitetrol (CBTT)
102	(±)-Δ^7-*Cis*-isotetrahydrocannabivarin-C3 (*cis*-iso-Δ^7-THCV)
103	(−)-Δ^7-*Trans*-(1R,3R,6R)-isotetrahydrocanna-bivarin-C3 (*trans*-iso-Δ^7-THCV)
104	(−)-Δ^7-*Trans*-(1R,3R,6R)-isotetrahydrocannabinol-C5 (*trans*-iso-Δ^7-THC)
105	Cannabichromanone B
106	Cannabichromanone C
107	Cannabichromanone D
108	(−)-(7R)-cannabicoumarononic acid
109	4-Actoxy-2-geranyl-5-hydroxy-3-n-pentylphenol
110	2-Geranyl-5-hydroxy-3-n-pentyl-1,4-benzoquinone
111	5-Acetoxy-6-geranyl-3-n-pentyl-1,4-benzoquinone
112	Cannabimovone (CBM)
113	Cannabioxepane (CBX)
114	10α-Hydroxy-Δ^9,11-hexahydrocannabinol
115	9β,10β-Epoxyhexahydrocannabinol
116	9α-Hydroxyhexahydrocannabinol
117	7-Oxo-9α-hydroxyhexahydrocannabinol
118	9α-Hydroxy-10-oxo-$\Delta^{6a,10a}$-THC
119	10α-Hydroxyhexahydrocannabinol
120	10aα-Hydroxyhexahydrocannabinol

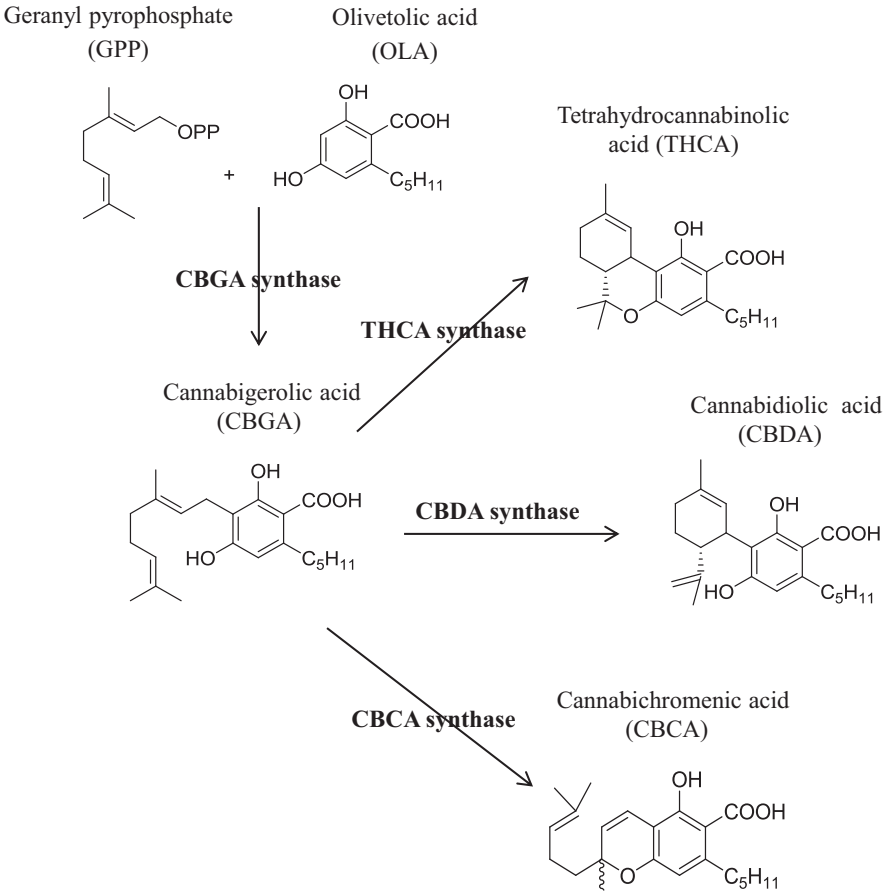

Fig. 8.3 Biosynthetic pathway of cannabinoids

the biosynthesis of the cannabinoid terpenoid moiety (Sirikantaramas et al. 2007). Olivetolic acid (OLA) and geranyl diphosphate (GPP) are derived from the polyketide and the deoxyxylulose phosphate (DOXP)/MEP pathways, respectively, followed by condensation under the influence of the prenylase, olivetolate geranyltransferase, yielding cannabigerolic acid (CBGA). CBGA, in turn, is oxidocyclized by flavin adenine dinucleotide-dependent oxidases, namely, cannabichromenic acid (CBCA) synthase, cannabidiolic acid (CBDA) synthase, and Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) synthase, producing CBCA, CBDA, and Δ^9-THCA, respectively (Flores-Sanchez and Verpoorte 2008a, b).

8.5 Biomass Production

Since cannabis is a dioecious and cross-pollinated plant, screening of high-yielding female plant is the first step towards the production of biomass which is consistent in chemical profile. Once screened, cuttings of mother plants can be cultivated at 18-h photoperiod for vegetative growth.

Cannabis can be grown under indoor and outdoor conditions from seed or vegetative cuttings. Under the climatic controlled indoor environmental conditions, 3–4 crops per year (depending upon required per plant biomass yield) can be cultivated whereas outdoor cultivation limits to one crop per year.

Cultivation through seeds is a traditional method to raise cannabis crop. Being a dioecious species, seed-raised plants normally turn 50% male and 50% female subject to the variety used. Furthermore, seed-raised plants exhibit considerable difference in the chemical profile of their biomass content, even when seeds are acquired from a signal mother plant (Potter 2015). Therefore, to maintain consistency in the biomass product, it is always better to select high-yielding mother plants and multiply them through vegetative propagation.

8.6 Indoor Cultivation

Once a high-yielding female clone with specific chemical profile is screened and selected, a fresh nodal segment containing two or three leaves can be used for vegetative propagation either in soil or in hydroponics system. A cut at a 45° angle below a node is made and dipped in the rooting hormone. The cutting is then planted in biodegradable jiffy pots with node covered by soil for efficient rooting. Plants are regularly irrigated and kept under controlled environmental conditions. Rooting initiates in 2–3 weeks, followed by transplantation to bigger pots after 5–6 weeks. The cuttings can be maintained at a constant vegetative state under 18-h photoperiod (Fig. 8.4a). For hydroponics cultivation instead of soil, rockwool cubes or a hydroton clay ball is used as a supporting medium. Plants are supplied with vegetative fertilizer formula and exposed to fluorescent light under 18-h photoperiod for vegetative growth. Rooting initiates in 2–3 weeks.

Tissue culture is an alternative method for conservation and mass multiplication of selected mother clones. Several different routes of propagation using different plant parts from apical segments to roots have been reported. In our laboratory at the University of Mississippi we have developed several protocols to micropropagate cannabis germplasm using apical segment (direct organogenesis, Lata et al. 2009a, 2016, Fig. 8.5), leaf disc (indirect organogenesis, Lata et al. 2010a), nodal segment in slow-growth medium (Lata et al. 2012), and nodal segment in sodium alginate gels (synthetic seeds, Lata et al. 2009b) for the conservation and further cultivation (Lata et al. 2010b, 2013). Tissue-cultured plants were successfully rooted and hardened in climatic controlled indoor growing room. For hardening, plants were first kept under 18-h fluorescent light for few weeks and then transferred under regular grow lights with mother plants for vegetative growth.

Fig. 8.4 Indoor cultivation of *Cannabis sativa*. (**a**) Crop at vegetative stage, (**b**) crop at flowering stage

Growing environment is an important factor for cannabis cultivation. Light quality, light intensity, photoperiod, temperature, carbon dioxide level, air circulation, and relative humidity are the main factors affecting cannabis growth. For vegetative growth, plants are grown under photosynthetically active radiation (PAR) with 18-h photoperiod whereas flowering activates under 12-h or less photoperiod in 2 weeks. Depending on the variety, plants normally mature in 6–9 weeks (Fig. 8.4b). Length of vegetative growth period can be increased or decreased based on the plant growth and biomass yield/plant needed. Our studies show that cannabis plants can utilize high photosynthetic photon flux density (PPFD, ~1500 $\mu molm^2s^{-1}$) for the efficient photosynthesis and transpiration (Chandra et al. 2008, 2015).

Fig. 8.5 Micropropagation of *Cannabis sativa*. (**a**) Fully rooted micropropagated plants, (**b**) fully grown micropropagated plants under controlled climatic condition

Depending on genetics, the temperature response of photosynthesis varies with the cannabis variety. Twenty-five to 30 °C growth temperature is however found to be ideal for the most varieties of cannabis (Chandra et al. 2008, 2009, 2011a). Cannabis grows better in high CO_2 environment. Our results show a 50% increase in the rate of photosynthesis under doubling of CO_2 concentration as compared to ambient CO_2 concentration (Chandra et al. 2008, 2011b). Humidity around 75% is recommended during the juvenile stage and in the range of 50–60% during the active vegetative and flowering stages.

8.7 Outdoor Cultivation

A representative cultivation of cannabis sativa is shown in Fig. 8.6. Cannabis is an annual plant. It grows vegetatively during summer time due to long days and flowers during the fall with days turning shorter. If not harvested, plants go to senescence and die. Outdoor planting normally starts during late March or early April depending upon the weather conditions. Cannabis can be grown by one of the following ways: (a) It can be grown by planting seeds directly in the ground, (b) by planting seeds in biodegradable jiffy pots for germination and then planting the seedlings in ground,

Fig. 8.6 Outdoor cultivation of *Cannabis sativa*. (**a**) Crop at vegetative stage, (**b**) crop at flowering stage

and (c) by planting through rooted vegetative cuttings. As explained earlier, rooted cuttings of screened and selected high-yielding female plants are preferred for the production of biomass, consistent in its cannabinoid profile.

8.8 Harvesting

Cannabis plants start flowering during late summer or early fall irrespective of plant age and harvest could last till November or early December depending on the variety. Harvesting time is determined by visual observation, color change of buds, odor of flowers, cannabinoid analysis, or a combination of all these methods. In cannabis, Δ^9-THC increases with plant age, reaching the highest level at the budding stage, and achieves a plateau before the onset of senescence. This gives a window of 7–10 days for harvesting the plants. Since the entire plant does not mature at one time, mature buds can be harvested first and lower branches are given more time to achieve their maturity.

8.9 Postharvest Handling

After harvesting, dead leaves are removed, and branches are cut into small pieces before they are dried. Harvested plants can be dried by hanging manicured branches or whole plant in dry area with circulating air or under forced warm air-drying. It is important to keep the temperature within a limit so that it won't affect the level of cannabinoids and terpenes. Air temperature of drying barn should be kept at 125 ± 5 °F. Depending upon the weather on the day of harvest drying can take 10–15 h in a drying barn. The dried plant material can be hand manicured, by removing any remaining dead leaves from the buds. The buds are carefully rubbed through different-sized screens to separate small stems and seeds. An automated plant-processing machine can also be used to separate big stems and seeds from the useable biomass. Adequately dried biomass is stored in FDA-approved polyethylene bags placed in sealable fiber drums at 18–20 °C for short-term and at ≤10 °C for long-term storage in the dark to avoid oxidation.

8.10 Extraction of Cannabinoids

Extraction of plant material is accomplished by one of the two different methods: (1) by solvent extraction or (2) by supercritical fluid extraction. Decarboxylation of the acid cannabinoids to the neutral cannabinoids can be done either before extraction by heating the biomass or after extraction by heating the extract. At the University of Mississippi, decarboxylation is accomplished after extraction whereas at GW Pharmaceuticals the plant material itself is subjected to the decarboxylation step before extraction.

8.11 Cannabis: A Natural Candidate for Botanical Drug Development

Medicinal plants have been used for the prevention and treatment of a variety of illnesses in all cultures and still play an important role in primary healthcare systems in many countries. The development of natural products in modern medicine has its own place but it is costly and time consuming. Therefore, scientific focus is being directed to the development of botanical drugs whereby the total plant extract (fully acclimatized) is being used for the treatment of a specific disease condition. In this regard the US Food and Drug Administration (FDA) has developed guidance for the development of "Botanical Drugs" products (FDA 2006). The first botanical drug approved by FDA was green tea extract (Veregen®) for the topical treatment of genital and perianal warts in 2006.

In case of cannabis, Sativex®, an oromucosal spray (GW Pharmaceuticals), is the only one pharmaceutical product available on the market (mainly in Europe and Canada) that contains phytocannabinoids. Sativex is a formulated mixture of two extracts of two varieties of the *Cannabis sativa* (high THC variety and high CBD variety) and the final product contains Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) in a 1:1 ratio. This product was approved in England in 2010 as a "botanical drug" in the form of a mouth spray for people with multiple sclerosis who can use it to alleviate neuropathic pain, spasticity, overactive bladder, and other symptoms of multiple sclerosis. It is also marketed with the name of nabiximol (Chandra et al. 2017).

The advantage in producing a "botanical drug" is the traditional knowledge, which is associated with plants and the way they are used in practice. On the other hand, either collected from nature or cultivated, one of the major challenges in making a botanical drug is to obtain a standardized plant material with consistency in secondary metabolites. With cannabis, it can be achieved by screening a variety and mother plant/s depending upon their chemical profiles and multiplying them using vegetative propagation and/or micropropagation.

In the United States, cannabis is illegal to grow without permission from the Federal Government and its cultivation is highly regulated. However, there is a growing pressure for legalization of cannabis for medical use all over the country. Many states have passed their own "medical marijuana" laws for legalization of cannabis cultivation for medical and/or recreational purpose. The biggest dilemma for scientific institutions and/or companies to develop cannabis-based drug is a conflict between state and federal laws. According to the federal laws, cannabis (drug type or fiber type/hemp) is categorized as a schedule I drug. By definition, that means with no currently accepted medical use and a high potential for abuse. Furthermore, any kind of drug development (botanical or purified) is regulated by FDA. Therefore, in spite of legalization of cannabis cultivation and production in any given state, any kind of drug development activity has to be approved by FDA and Drug Enforcement Administration (DEA). Due to these regulatory steps, there are only few controlled trials that have been undertaken.

At the University of Mississippi, a variety of research activities dealing with cannabis are underway through a contract with the National Institute on Drug Abuse (NIDA). This includes growing, harvesting, and processing the cannabis biomass for research purposes and making it available for licensed researchers across the country. This program is federally funded and approved by DEA and FDA and serves as an ideal platform for future cannabis-based drug development. Under a state legislation, the National Center for Development of Natural Products and the University of Mississippi Medical Center are currently working on making a CBD-rich extract formulation available for clinical trial in children with severe, intractable epilepsy. The research is underway and is in full compliance with the federal laws.

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Chapter 9
Natural Products as Possible Vaccine Adjuvants for Infectious Diseases and Cancer

Anna-Mari Reid and Namrita Lall

9.1 A Short History of Vaccines and their Mechanism of Action

Vaccines, despite all the negative publicity, have a significant impact on human and animal health. According to the World Health Organization (WHO), vaccines have prevented the deaths of more than 2.5 million individuals on a yearly basis, especially in preventing the deaths of children (WHO 2018). The vaccine repertoire has now also been expanded to include treatment for the elderly, infants and pregnant women. A whole new range of newly licenced vaccines are ready for use as well as many that have successfully passed Phase III clinical trials (Rappuoli and De Gregorio 2016).

The history of vaccines started with Edward Jenner in the late eighteenth century who showed that it was possible to protect yourself against a disease, without transmitting the disease itself. During his experimentation, he looked into the protective properties of mild cowpox in preventing infection with smallpox (Di Pasquale et al. 2015). After the initial investigations made by Edward Jenner, Louis Pasteur, the famed French chemist, reportedly developed the first vaccine for rabies disease in 1885. The term 'vaccine' was also formally coined and included all inoculating agents used in this process (Stern and Markel 2005). After this initial period, the twentieth century started off with the identification of toxins from bacteria. These bacterial components were turned non-toxic but still possessed highly immunogenic properties (Di Pasquale et al. 2015).

The goal of vaccination has always been to induce protective immunity against diseases by initiating innate immunity and activating antigen-presenting cells

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(APCs) which then induce an adaptive immunity against antigens originating from pathogens or an infectious agent (Fig. 9.1) (Di Pasquale et al. 2015). The whole immunisation process is dependent on two phases. The first phase occurs when the foreign infectious agent is introduced into the body of an individual, usually through the mucosal route. The first line of defence after introduction is the recognition of

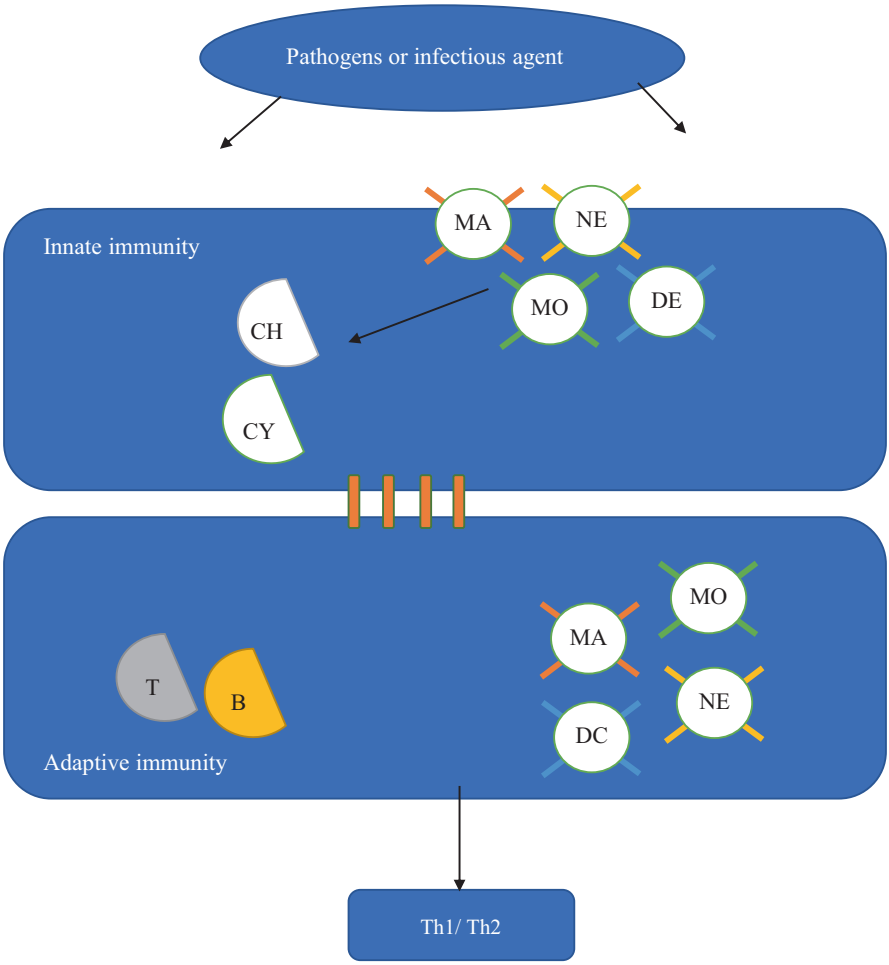

Fig. 9.1 The process of protective immunity. Protective immunity starts with the initiation of the innate immunity by the recognition of the PAMPS by PRRs of macrophages (MA), monocytes (MO), neutrophils (NE) and dendritic cells (DC). The migration of the PRRs to the draining lymph nodes (in orange) links and activates the adaptive immune response (B cells [B] and T cells [T]) which will direct the resulting immune response towards either a Th1 or a Th2 response. *CY* cytokines, *CH* chemokines

surface patterns or pathogen-associated molecular patterns (PAMPS) by pattern recognition receptors (PRRs) of macrophages, monocytes, neutrophils and dendritic cells. This process is followed by phagocytosis and release of inflammatory mediators which include chemokines and cytokines. The APCs are then responsible for migrating to the draining lymph nodes where they will cross-link with the adaptive immune response as the innate immune response has no capacity for memory (El-Ashmawy et al. 2015; Di Pasquale et al. 2015). The APCs therefore play a very important role in linking the innate and adaptive immune response and the direction into which the adaptive immune response will be steered, either a Th1 or a Th2 response (El-Ashmawy et al. 2015; Di Pasquale et al. 2015). The adaptive immune response is comprised of both T and B cells. The type of immune response to the infection is determined by these specific cells. After recognition of the specific antigen, B cells will differentiate into plasma cells and release antibodies. B cells develop memory and a rapid response after re-exposure of the same antigen only after a T-cell response. The $CD4^+$ T-helper cells on the other hand have an inability to recognise antigens without them being processed by the APCs that originate after activation during the innate immune response. Activated T-helper cells will then release inflammatory mediators which are specific to a T-helper cell subpopulation (Th1, Th2, Th17 and Thf) all with their own downstream responses on how the infection is removed or contained (Ohnmacht et al. 2009; Garlapati 2012; Luckheeram et al. 2012; El-Ashmawy et al. 2015; Di Pasquale et al. 2015). Th1 cells are normally needed for removal of intracellular pathogens, Th2 cells for removal of extracellular parasites, Th17 for removal of bacteria and fungi and Thf cells for activating the specific T-cell-dependent B-cell response needed for immune memory (Luckheeram et al. 2012; Di Pasquale et al. 2015).

9.2 Vaccines Currently in Use

Three different types of vaccines are currently in use: live-attenuated vaccines that are composed of either a virus or a bacterium which can replicate within an individual; inactivated vaccines which are parts of a pathogen which are inactivated either chemically or by heat; and subunit vaccines which are derived from nonliving vaccine antigens (Riese et al. 2013; Lee and Nguyen 2015). Live-attenuated vaccines are able to induce a mild response within patients and therefore an immune response that is similar to the infection of the specific disease (Di Pasquale et al. 2015).

Some inactivated whole-pathogen vaccines are also effective due to their heterogeneous nature of antigens and other pathogenic components which have the ability to act as natural, innate adjuvants. These adjuvants are however not suitable when the natural infection itself does not give long-standing immunity and if the pathogen is unable to grow in culture (Di Pasquale et al. 2015). Highly purified

vaccines lack PAMPs, which means that the initial reaction to induce the innate immunity is lacking and therefore no downstream responses, i.e. the adaptive immune responses, are activated (Di Pasquale et al. 2015).

9.3 Vaccine Adjuvants: A History and their Mechanism of Action

Normally vaccines are of low immunogenicity and do not induce a sufficient response against invading pathogens. This was circumvented by the use of adjuvants. The word adjuvant was derived from the Latin word a*djuvare* which means 'to help or aid'. Adjuvants are therefore a perfect way to create a longer lasting immunity in infected individuals. Particularly, subunit vaccines which are normally poorly immunogenic require the presence of an adjuvant to stimulate the needed immune response. The inability of highly purified vaccines to initiate an immune response has been said to be due to their failure to induce maturation of the APCs (Moser and Leo 2010; Di Pasquale et al. 2015). Vaccine adjuvants were first discovered by a French veterinarian named Gaston Ramon, who observed that the anti-sera yield was much higher when abscesses occur at the site of injection. He introduced sterile abscesses with breadcrumbs, tapioca and starch with an inactivated toxin. This confirmed that inducing local inflammation at the site of injection could increase the yield of anti-sera produced (Vogel and Hem 2004; Di Pasquale et al. 2015). Alexander Glenny and colleagues at around the same time as Gaston Ramon discovered aluminium salts and their ability to enhance the immune system (Marrack et al. 2009). In 1932, aluminium salt was used for the first time in human vaccines and was also the only licenced adjuvant used in humans for more than 70 years (Di Pasquale et al. 2015). The mechanism of action of aluminium salts is not completely understood but is said to be based on the fact that they increase antibody production and are best suited to target pathogens which are eliminated by antibodies (De Gregorio et al. 2013).

9.4 Vaccine Adjuvants: Delivery Systems Vs. Immunostimulators

Adjuvants work on the basis that signals originating from pathogen recognition receptors (PRRs) would activate dendritic cells, which would then prime and activate T cells, leading to an adaptive immune response (Rey-Ladino et al. 2011). Based on their proposed mechanism of actions, adjuvants have been divided into two broad categories, namely delivery systems and immunostimulatory adjuvants (Singh and O'Hagan 2003; Awate et al. 2013). The adjuvants that optimise the delivery of antigens to immune cells are also known as delivery systems. Examples

of vehicle adjuvants are mineral salts, emulsions such as water-in-oil emulsions, liposomes, virosomes as well as polymer microspheres (Mohan et al. 2013). Quil-A (saponin-based vaccine adjuvant) and QS-21 (mixture of soluble triterpene glycosides) are known as complexes that stimulate the immune system. Other examples include ISCOM and ISCO-MATRIX (Massa and Franconi 2012). The immunopotentiator class of adjuvants are a class of adjuvants that enhance the immune response of the innate immunity by the activation of various PRRs such as the toll-like receptors (TLRs) (Massa and Franconi 2012). Immunostimulatory adjuvants mainly work on a cytokine level through the activation of major histocompatibility complex molecules, through the signals of costimulatory molecules or through intracellular signal pathways (Singh and O'Hagan 1999). Saponins derived from *Quillaja saponaria* (Fig. 9.2) are also known as immunostimulatory adjuvants (Massa and Franconi 2012).

Other classes of adjuvants are polymeric microsphere adjuvants. These adjuvants which are biodegradable and biocompatible microspheres incorporate antigens as well as carbohydrate-based adjuvants. They are naturally derived which gives them the ability to induce both the humoral and cellular immunity. They have the ability to enhance cellular immunity and bacterial products that consist of cell wall components such as lipopolysaccharides (LPS) that target the TLR pathway (Banday et al. 2015). Available evidence suggests that adjuvants employ one or more of the following mechanisms to elicit an immune response (Cox and Coulter 1997; Hoebe et al. 2004; Fraser et al. 2007; Awate et al. 2013):

- Sustained release of an antigen at the site of injection (depot effect).
- Upregulation of cytokines and chemokines.
- Cellular recruitment at the site of injection.
- Increased antigen uptake and delivery to antigen-presenting cells (APCs).

Fig. 9.2 Pods and husks of the *Quillaja saponaria* tree (Culbert 2013)

- Activation and maturation of APCs [increased major histocompatibility complex (MHC) class II and costimulatory molecule expression] and migration to the draining lymph node.
- Activation of inflammasomes.

These mechanisms are described in detail below:

9.4.1 Formation of Depot at the Site of Infection (Depot Effect)

Antigen trapping and slow release at the site of injection ensure that constant stimulation of the immune system occurs for the production of high antibody titres (Siskind and Benacerraf 1969; Awate et al. 2013). This was considered the classic mechanism of action of many adjuvants. This is also the proposed mechanism of adjuvant activity for alum, water and oil emulsions and biodegradable micro- and nanoparticles that generate a prolonged and sustained high antibody titre (Herbert 1968; Kreuter 1988; Awate et al. 2013).

9.4.2 Upregulation of Cytokines and Chemokines

Particulate adjuvants have been shown to create a local pro-inflammatory environment to recruit immune cells (Goto and Akama 1982; Awate et al. 2013). Normally it follows with the recruitment of neutrophils, monocytes, eosinophils and macrophages followed by dendritic cells after injection. The recruited cells, especially the neutrophils, monocytes and B cells, take up both antigen and adjuvant and traffic them to the draining lymph nodes. Neutrophils are the first cells to be recruited at the site of adjuvant injection and also the highest in numbers (Calabro et al. 2011; Awate et al. 2013). They attract other immune cells by producing an increased number of chemokines (Calabro et al. 2011; Morel et al. 2011; Awate et al. 2013).

9.4.3 Antigen Presentation and Activation/Maturation of Dendritic Cells

Efficient antigen presentation by APCs like dendritic cells is important for the induction of an adaptive immune response (Summerfield and McCullough 2009).

Activation of dendritic cells is essential for induction of an adaptive immune response. Increased expression of MHC class II, activation marker CD86 and maturation marker CD83 leads to the enhanced ability of APCs to induce T-lymphocyte activation and differentiation (Coyle and Gutierrez-Ramos 2001; Awate et al. 2013).

9.4.4 Activation of Inflammasomes

Innate immune cells express various pathogen recognition receptors (PRRs) to recognise infectious agents. In recent years, various new families of PRRs have been identified including TLRs, C-type lectin-like receptors (CLRs), nucleotide oligomerisation domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs). Many immunological adjuvants signal via PRRs or act as ligands for innate immune receptors. The inflammasome belongs to the NLR family, which also includes various other receptors, such as NODs (NOD1-5), (NLRPs (NLRP1-14), NLRP1 (NAIP), NLRC4 (IPAF) and major histocompatibility complex II transactivator (CIITA) (Martinon et al. 2009; Awate et al. 2013). Apart from damage-associated molecular patterns (DAMPS), inflammasomes can be activated by PAMPS such as bacterial flagellin through NLRC4 activation (Zhao et al. 2011; Awate et al. 2013).

Adjuvants have the ability to reduce the amount of antigen to be used in a single dose. It may also reduce the amount of vaccine doses to be given to a patient to achieve a complete immune response, thereby reducing the cost of manufacturing a vaccine. Adjuvants may also increase the responses of the immune system to a vaccine in populations of immunocompromised individuals, the elderly and infants (Rey-Ladino et al. 2011; Di Pasquale et al. 2015). Overall, adjuvants improve the immune response and do so through a variety of ways such as the increase of the immunogenicity of an antigen too weak to elicit a response. They can modify the speed and duration of this response and they can also induce mucosal immunity. Individuals that have low immunogenicity either by being immunologically immature or senescent can be enhanced. They have also proven to overcome competition between antigens in combined vaccines (Singh and O'Hagan 1999). To further improve the immune response elicited by an adjuvant, adjuvant systems, which are adjuvants that contain more than one immunostimulatory component, were created. An example of this is a combination of the RTS, S/AS01 for use against malaria. This combination initiated a higher cell-mediated immune response and provided greater clinical protection during studies (Kester et al. 2009; Leroux-Roels et al. 2014; Di Pasquale et al. 2015). It is currently a candidate malaria vaccine in Phase III of clinical trials with efficacy shown in vaccinated children (Bejon et al. 2008; Di Pasquale et al. 2015). Another vaccine that did not use aluminium salt as an adjuvant was a vaccine against hepatitis A, licensed in the 1990s, which uses a virosome adjuvant system (Bovier 2008; Di Pasquale et al. 2015). Virosomes have spherical layers of phospholipids that carry influenza antigen either bound on the surface or encapsulated within the lumen (Moser et al. 2013). After aluminium, the second most commonly administered adjuvant is oil-in-water emulsions using oils which have increased reactogenicity as compared to Freud's original adjuvant. Freud's incomplete adjuvant consisted of a mineral oil-in-water emulsion but caused too much adverse reactions in humans. Squalene, which is a naturally occurring oil, is mostly used in oil-in-water emulsions. These emulsions have the ability to induce both a humoral and cellular immune response (Fox and Haensler 2013).

9.5 An Ideal Vaccine Adjuvant

An ideal adjuvant would have the ability to initiate an immune response as well as the ability to direct the adaptive immune response towards effective inactivation and removal of the specific pathogen after which it will then develop an immune memory for a rapid response next time infection occurs with the same pathogen (Di Pasquale et al. 2015). Components used as adjuvants may aggregate in water and interact with surrounding proteins and also may reorganise themselves according to their surrounding environment which may lead to products that are not as effective. Coating these adjuvants with bilayers made up of synthetic liposomes or multilamellar vesicles may increase their stability and potency. GlaxoSmithKline™ has an adjuvant formulation known as AS01B/E, which consists of monophosphoryl lipid A and QS-21 encapsulation within a liposome. Encapsulation is also possible with nanoparticles made of polymer (Garçon et al. 2007; Bejon et al. 2008; Kasturi et al. 2011; Ilyinskii et al. 2014; Hanson et al. 2015; Moyer et al. 2016). These encapsulation methods have been shown to increase accumulation and stimulation of draining lymph nodes, decreasing the systemic exposure which lowers the toxicity levels of the compounds (Moyer et al. 2016).

Several adjuvants with effective potency have advanced through to clinical trials but have been shown to be too toxic for safe use in humans (Nicholls et al. 2010). Only in certain aspects such as treating diseases that are life threatening such as cancer will toxicity levels that are slightly higher be accepted. Other aspects to be taken into consideration when developing an effective adjuvant are the fact that they need to be biodegradable, stable, and easy to manufacture; should have low cost; and need to be able to be effective over a variety of different vaccines (Singh and O'Hagan 1999). For compliance, the administration route of vaccines is also very important, and the mucosal route is the most preferred route of administration (Singh and O'Hagan 1999). Research into the chemical and biological characteristics should be well documented and presented so that minimal variations are created during administration. The adjuvant also needs to be stable and have a minimum of 2-year shelf life (Banday et al. 2015).

According to Halmuthur and Irfan (2012), the following structural requirements are needed of a molecule to act as a perfect adjuvant. There has to be a hydrophilic-hydrophobic balance and for depot formation the presence of micellar structures. There should also be a lipophilic structure that has the ability to envelope the antigen which will preserve the antigen structure and is required for its immunogenicity potential. These lipophilic structures should also have the ability of cytosol trafficking. For an effective Th1 response, several functional groups are also needed to stimulate co-stimulatory signals. The overall design should be able to stimulate both Th1 and Th2 responses and also act as a ligand for T-cell or dendritic cell surface receptors.

9.6 Adjuvants in Clinical Trials, Licenced Out and in the Pipeline

Adjuvants that are currently approved and safe for human use are alum, mineral salts and MF59, an oil-in-water emulsion, generally used in a variety of vaccines, including influenza and pandemic flu as shown in Table 9.1 (Atmar et al. 2006). AS03, an oil-in-water emulsion, and tocopherol are also used for the pandemic flu (Lambrecht et al. 2009). AS04 which is monophosphoryl lipid A and alum are used in combination for the hepatitis B virus and human papilloma virus (HPV) (Garçon et al. 2007). Virosomes in liposomes are used for hepatitis A virus and flu (Banday et al. 2015) with Pam_3Cys, and a lipopeptide and TLR 2 agonist used for Lyme disease, cancer and HPV (Poland and Jacobson 2001). Alum adjuvants' mechanism of action is said to be due to the formation of an antigen depot at the site of inoculation that traps the antigens in the gel. This trapping causes the slow and sustainable release of the antigen as well as an extension of the interaction with the immune system. This aggregate of the antigen that forms promotes phagocytosis of the antigen by dendritic cells, macrophages and B cells which presents the antigen with the major histocompatibility complex II molecules. Alum is also said to activate eosinophils, macrophages and inflammasomes (Banday et al. 2015). AS03 is a squalene-based oil-in-water emulsion adjuvant. MF59 is also a squalene-based oil-in-water-type nano-emulsion which has shown balanced Th1 and Th2 responses. The mechanism of action of MF59 is through the stimulation of macrophages, monocytes and granulocytes that produces cytokines and chemokines after an initial depot formation (Banday et al. 2015). AS04 is a successor for AS03 and is a combination of aluminium phosphate and MPL. The MPL constituents are known agonists of TLR4 which stimulates a T- and B-cell response. It also has the ability to downregulate Th2 responses, known to be enhanced in allergies (Banday et al. 2015).

9.7 Hurdles Facing the Development of Vaccine Adjuvants

The most prominent adjuvants currently being used in human vaccines are alum salts, MF59 from Novartis™ and AS03 from GlaxoSmithKline™. Alum salts have been effective for use in most vaccines but are not very effective against diseases such as cancer, which require a cell-mediated response (Massa and Franconi 2012). Vaccines and adjuvants used for cancer therapy need to overcome suppressant factors that are used by cancer to evade T- and B-cell-mediated immune response. These vaccines need to be T-cell specific to tumours that are ineffectively primed as well and be effective within the tumour environment. These include factors such

Table 9.1 Summary of the current available adjuvants, their stage of clinical development or approved for use in humans

Name of the adjuvant	Class of compounds	Mechanism of action	Progress/stage of clinical development	Indications	References
Advax	Delta inulin-based synthetic polysaccharide adjuvant	Stimulating the immune system through the complement pathway through antigen-presenting upregulation and antigen-specific T-and B-cell activation	Phase II	Broad use, hepatitis B (HBV) and influenza	Honda-Okubo et al. (2012); Massa and Franconi (2012); Petrovsky (2013); Banday et al. (2015)
Alum	Mineral salts	Depot formation	Approved for human use	Used in several vaccines for various diseases	De Veer et al. (2010); Banday et al. (2015); Moyer et al. (2016)
AS01	MPL, liposomes and QS21	Induces a great number of neutrophils into the site of injection	Phase II	Malaria and tuberculosis	Didierlaurent et al. (2014); Schleiss et al. (2014); Banday et al. (2015); Reed et al. (2016)
AS02	MPL, oil-in-water emulsion and QS21	Enhances cellular and humoral immune response	Phase II	Malaria	Garcon and Van Mechelen (2011); Schleiss et al. (2014); Banday et al. (2015)
AS03	Oil-in-water emulsion and tocopherol	Promotion of the recruitment of monocytes and granulocytes, also upregulates cytokine secretion	Approved for human use	Pandemic flu	Lambrecht et al. (2009), Morel et al. (2011); Banday et al. (2015)
AS04	Monophosphoryl lipid A and alum	TLR 4 agonist and stimulates cytokine production at injection site	Approved for human use	Hepatitis B virus and human papilloma virus	Garçon et al. (2007); Garçon et al. (2011); Banday et al. (2015)
CpG	Oligonucleotide, alum and MF59	Upregulation of MHC class II and co-stimulatory molecules	Phase I	HBV, malaria and cancer	Jakob et al. (1998); Singh and O'Hagan (1999); Klinman (2006); Bode et al. (2011); Banday et al. (2015)

Flagellin	Flagellin linked to antigen	TLR 5 agonist through recognition of the antigen attached	Phase I	Flu	Turley et al. (2011); De Gregorio et al. (2013); Ben-Yadidia and Arnon (2005); Banday et al. (2015)
IC31	Peptide and oligonucleotide	Ligation of endosomal TLRs	Phase 1	Tuberculosis	Kaufmann (2010); Szabo et al. (2013); Banday et al. (2015)
Imidazoquinolines	Small-molecule immunomodulators	TLR7 and TLR 8 agonists	Phase II	Broad use	Shi et al. (2012); Banday et al. (2015)
ISCOMs	Saponins, cholesterol and phospholipids	Stimulates all classes of immunoglobulins and induces specific CD8+ cytotoxic responses	Phase I	Broad use	Sjolander et al. (1998); Mohan et al. (2013); Banday et al. (2015)
MF59	Oil-in-water emulsion	Depot formation	Approved for use in humans	Influenza and pandemic flu	Atmar et al. (2006); De Veer et al. (2010); Banday et al. (2015); Moyer et al. (2016)
MF59 and MTP-PE	Lipidated MDP and oil-in-water emulsion	Immunostimulant	Phase I	HIV and flu	Podda et al. (2006); Durando et al. (2008); Banday et al. (2015)
Montanide	Oil-in-water emulsion	Depot formation	Phase III	Malaria and cancer	Aucouturier et al. (2002); Levast et al. (2014); Banday et al. (2015)
Pam_3Cys	Lipopeptide	TLR 2 agonist	Approved for human use	Lyme disease, cancer and HPV	Poland and Jacobson (2001); Steinhagen et al. (2011); Banday et al. (2015)
PLG	Polymeric microparticles	Increases the secretion of interleukin 1/3	Phase I	DNA vaccine (HIV)	Vajdy et al. (2004); Sharp et al. (2009); Banday et al. (2015)

(continued)

Table 9.1 (continued)

Name of the adjuvant	Class of compounds	Mechanism of action	Progress/stage of clinical development	Indications	References
QS21	Saponin	Still yet to be discovered but membrane lysis may play a role	Phase II	Broad use	Garcon and Van Mechelen (2011); Banday et al. (2015)
RC-529	Synthetic MPL and alum	TLR 4 agonist	Phase II	Broad use	Thompson et al. (2005); De Gregorio et al. (2013); Banday et al. (2015)
Virosomes	Liposomes	Has both delivery and immunostimulatory properties	Approved for human use	Hepatitis A virus (HAV) and flu	Moser et al. (2013); Banday et al. (2015)

as Treg cells and IL-10 cytokines, which have the ability to reduce the response of adaptive immunity. Other factors include the lack of appropriate animal models and single-directional treatment (Massa and Franconi 2012). MPL, a known immunostimulant vaccine adjuvant, is currently the only licenced adjuvant that is categorised as a TLR ligand (Didierlaurent et al. 2009; Massa and Franconi 2012). Current hurdles for the development of novel adjuvants are the costs involved, the fact that they only receive approval in combination with an already registered vaccine as well as several uncertainties pertaining to the regulatory approval process (Massa and Franconi 2012). In the year 2011, three cancer vaccines were ready for use in human patients, namely Provenge, Yervoy-Ipilimumab and CimaVax. Provenge is patient specific and used for patients with prostate cancer. Yervoy-Ipilimumab is a human monoclonal antibody used for the treatment of melanoma and CimaVax, a vaccine used for lung cancer, released in September of 2011 (Massa and Franconi 2012). Bacille Calmette-Guerin (BCG) vaccine, which is derived from *Mycobacterium bovis*, uses both TLR2 and TLR4 signalling pathways and also interacts with NLR2. BCG is also being used as an adjuvant in vaccines for the treatment of melanoma (Massa and Franconi 2012). Side effects such as toxicity, local reactions such as inflammation and pain at the injection site, and pyrogenicity and reactogenicity that are associated with synthetic and bacterial products have limited the ability of the development of several adjuvants (Petrovsky 2015).

Plants have the potential of providing potent, safer and more natural alternative adjuvants. Crude extracts and compounds isolated from plants have been used as immunostimulators since the dawn of time and therefore have the potential of being used as adjuvants for their immunostimulatory properties (Harikrishnan et al. 2011). Various classes of compounds from plant sources have been identified over the years as immunostimulators. These classes are alkaloids, saponins, polysaccharide, triterpenoids, iridoids and organic acids to name a few (Alamgir and Uddin 2010).

9.8 Natural Vaccine Adjuvants

Many diverse classes of compounds have been investigated for their potential as adjuvants such as mineral salts, microbial products, emulsions, saponins, cytokines, polymers, microparticles and liposomes (Guy 2007; Awate et al. 2013). In general, delivery systems were previously thought to act by providing a depot while immunostimulatory adjuvants activate cells of the innate immune system (Pashine et al. 2005; Awate et al. 2013). It is however no longer appropriate, since now there is evidence that some delivery systems can also activate innate immunity (Awate et al. 2013).

9.9 Current Herbals and Compounds Used for Vaccine Adjuvants

QS-21, isolated from the bark of *Quillaja saponaria* Molina., has the ability to stimulate both Th1 and Th2 responses including the production of cytotoxic T lymphocytes, which are antigen specific (Ng et al. 2016). QS-21 has been used in its pure form or as a component of adjuvant mixtures (Quil A, ISCOMs, ISCOMATRIX, AS01 and AS02). QS-21 has been studied as an adjuvant for many diseases such as cancers, infectious diseases and Alzheimer's disease (Fernandez-Tejada et al. 2016). This form of adjuvant has severe limitations such as limited access due to its low yield; it is also responsible for systemic reactions such as swelling, erythema at the injection site and flulike symptoms (Ragupathi et al. 2011). QS-21 also suffers from spontaneous hydrolysis which produces an inactive adjuvant and haemolytic by-products which complicates the manufacture and storage process (Cleland et al. 1996; Fernandez-Tejada et al. 2016). Fractions that are rich in iridoid glycosides have been identified from *Picrorhiza kurroa* Royle ex. Benth, a medicinal plant that is known to possess immunostimulatory potential (Puri et al. 1992). RLJ-NE-299A is a mixture of picroside-I and picroside-II that was isolated from *P. kurroa* and has been investigated for its adjuvant potential (Khajuria et al. 2007). During these investigations, it was found to have only limited sustained immune memory and depot formation properties and therefore this restricted its use as an adjuvant alternative (Khajuria et al. 2007). Further investigations however led to the discovery of the potential of the acylated analogues of picroside II. Two of these analogues, namely PK-II-3 and PK-II-4, stimulated anti-OVA IgG titre of which the antigen activity of ovalbumin is known to be very weak. The titres of the neutralising antibody (IgG1 and IgG2a) as well as a Th1 response (IL-2 and IFN-γ) were also

Fig. 9.3 *Taxus brevifolia* (**a**) (Brewbooks 2015) and *Papaver somniferum* (**b**) (Lestat 2008)

reported during the investigations. IL-4 along with their T-lymphocyte subsets consisting of CD4 and CD8 cells was also present. All these constituents clearly show the potential of acylated analogues as enhancers of Th1 and Th2 responses that are antigen specific, all indicating the potential of a plant-based adjuvant alternative (Kumar et al. 2010).

9.10 Natural Products as Adjuvants

Natural products are known for their ability to harness novel compounds that can be used against cancer and infectious diseases such as tuberculosis. Many pharmaceuticals have their origins traced back to their novel counterparts originating from natural products. Examples of currently used drugs that originate from natural products are taxol, the anticancer therapy that was isolated from *Taxus brevifolia* Nutt. and morphine and codeine isolated from *Papaver somniferum* L. (Fig. 9.3.). Other examples include salbutamol, aspirin and digoxin (De Smet 1997; Chen and Yu 2016). A neem tree (*Azadirachta indica*) leaf preparation and specifically the Rb1 fraction of ginseng (*Panax ginseng*) have previously been studied for their potential as adjuvants (Wang et al. 2005; Rivera et al. 2005). Plant saponins are the best known components derived from plants that are used as adjuvants. As previously mentioned, QS-21 which is plant based, is a 3,28-*o*-bisdesmonic triterpene saponin that is extracted from the bark of the tree *Quillaja saponaria* Molina. It is highly soluble in water and stable in combination with an antigen (Massa and Franconi 2012). With regard to its adjuvanticity properties, QS-21 has the potential to enhance both Th1 and Th2 responses within the body (Moore et al. 1999).

Several drawbacks have however caused restrictions to its use as it was seen to be very toxic and unstable and had haemolytic effects (Waite et al. 2001). Several investigations also look into the development of mucosal vaccines of plant origin that are incorporated into edible crops. These saponin-based components will then act as endogenous adjuvants with immunomodulating capabilities (Massa and Franconi 2012).

9.10.1 Immunostimulating Herbals as Vaccine Adjuvants

There is the potential of novel immunomodulating compounds found within plant-derived resources. Novel immunomodulating compounds from natural products may lead to the discovery of several naturally derived adjuvants to aid current or new vaccines. Due to the importance of dendritic cells as the initiator of an innate immune response as well as being the link between the innate and adaptive immune response, they have been thoroughly investigated as potential targets for novel adjuvants to help poor immunogenicity of vaccines. The state of maturation of dendritic cells determines what type of immune response will develop. The process of

maturation results into mature antigen-presenting dendritic cells, which are morphologically and phenotypically different from their immature counterparts. Several natural products have been linked to the differentiation and maturation of dendritic cells. *Polypodium leucotomos* (L.) J. Sm. increases the production of interleukin (IL)-1α, IL-1β and tumour necrosis factor (TNF)-α (Fig. 9.4) (Bernd et al. 1995; Aldahlawi 2016). *Astragalus mongholicus* (Bunge) increased the co-expression of integrins and major histocompatibility complex (MHC) molecules on the surfaces of murine bone marrow-derived dendritic cells (Shao et al. 2006; Aldahlawi 2016).

Astragalus mongholicus also reduces the uptake of fluorescein isothiocyanate-dextran and increases the production of IL-12 (Shao et al. 2006, Aldahlawi 2016). A Chinese traditional medicine, *Achyranthes bidentata* (Blume), causes phenotypic and functional maturation of dendritic cells (Zou et al. 2011; Aldahlawi 2016). Seeds from *Plantago asiatica* L. showed the ability to increase the levels of MHC class II molecules as well as the levels of major co-stimulatory molecules found on the surface of the dendritic cells. Therefore, the plant has the potential to increase the antigen-presenting capabilities to T lymphocytes, which indicates maturation of dendritic cells (Huang et al. 2009; Aldahlawi 2016). Investigation into the modulating properties of *Abelmoschus esculentus* L. showed that the extract had the capability of stimulating dendritic cells by upregulating the expression of MHC class II and co-stimulatory CD80/86 molecules. The extract also increased the expression of IL-12 and interferon (IFN)-γ (Aldahlawi 2016; Sheu and Lai 2012). Triterpene esters isolated from the hooks from *Uncaria rhynchophylla* (Miq.) Jacks. (uncarinic acid C and uncarinic acid D) showed activation of cytokines that were responsible for a Th1-steered response. Modulating dendritic cells is at the forefront of research into novel adjuvants. Many functions of dendritic cells seem to be altered when cancer is present and therefore modulating the immune system plays a very important role in preventing and treating this disease (Okamoto et al. 2004; Aldahlawi 2016).

Mucuna pruriens var. *utilis* (L.) DC. also had the ability to induce the maturation and differentiation of dendritic cells as well as induce apoptosis in vitro in human cancer cells (Kurokawa et al. 2011). Similarly, *Vigna angularis* heat-stable extract also induced apoptosis in controlling the growth of U937 cells (Nakaya et al. 2012; Aldahlawi 2016). Several plants also showed potential in vivo in reducing tumour metastasis within a mice model such as the injection of *Astragalus* spp. against metastatic lung cancer (Jing et al. 2014; Aldahlawi 2016).

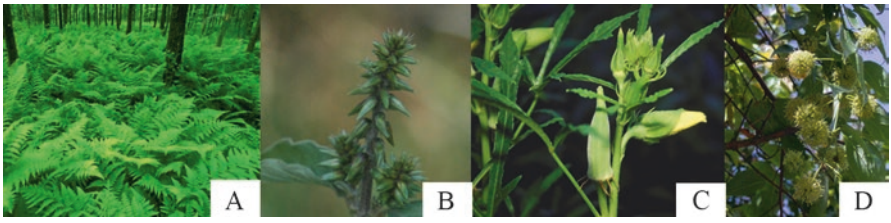

Fig. 9.4 *Polypodium leucotomos* (**a**) (Leucotomos 2014), *Achyranthes bidentata* (**b**) (Doronenko 2011), *Plantago asiatica* (**c**) (Dalgial 2009), *Uncaria rhynchophylla* (**d**) (Keisotyo 2003)

The root of an *Astragalus* spp. and an elderberry (*Sambucus nigra*) extract showed upon investigation by Frokiaer et al. (2012) to induce the production of IFN-β, reducing the inflammatory response posed by *Escherichia coli*, and also improve the endocytosis capabilities of immature dendritic cells (Frokiaer et al. 2012; Aldahlawi 2016).

9.10.2 TLR Agonists and Ligands

Several researchers have investigated the potential of natural products as TLR agonists, specifically TLR4 agonists, which are known for recognising lipopolysaccharides (LPS) that are found in bacteria as well as other pathogens (Chen and Yu 2016). An edible mushroom, *Pleurotus ferulae* (Lanzi.) Sacc., increased the function as well as the maturation of dendritic cells derived from murine bone marrow through the TLR4 signalling pathway (Li et al. 2015) (Fig. 9.5). Polysaccharides from the sclerotium of *Polyporus umbellatus* (Pers.) (Fr.) also activated and matured the dendritic cells of murine bone marrow through TLR4 (Aldahlawi 2016). TLR4 agonists and their abilities to use this signalling pathway have been shown to inhibit cancer growth (Ghochikyan et al. 2014).

Currently a gap exists in finding an active TLR agonist that is both effective in activating dendritic cells and safe for use in humans, as many TLR4 agonists have been declared too toxic for use in humans, such as LPS (Ghochikyan et al. 2014). Many polysaccharides have also undergone intensive research as adjuvants. Polysaccharide purified from potato sprouts and suspended in saline, or more commonly known as Immunomax, is an injectable that has previously been used as a treatment against many infectious diseases but has also been investigated for its potential as an adjuvant in the treatment of breast cancer. Immunomax has been shown to act as a specific agonist for TLR4 and works by activating dendritic cells and enhances anti-tumour properties of natural killer cells (Ghochikyan et al. 2014). Other polysaccharide-based adjuvants include Advax, which is a modified version of inulin, a compound extracted from the tubers of dahlias (Petrovsky 2008). It has the ability to stimulate T-cell and antibody immunity and has proven its adjuvant properties against hepatitis B during animal testing as well as malaria and influenza (Cooper et al. 1991; Saul et al. 1992; Amorij et al. 2007; Petrovsky 2008). It is also

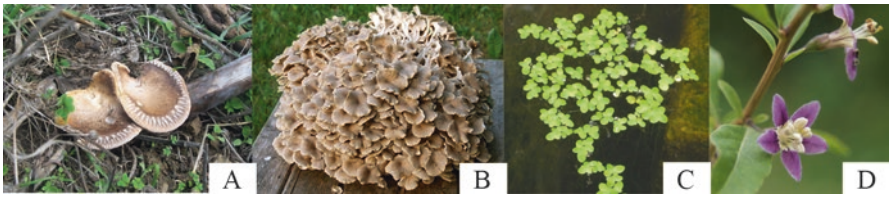

Fig. 9.5 *Pleurotus ferulae* (**a**) (Kkarageorgos 2010), *Polyporus umbellatus* (**b**) (NI74 2007), *Lemna minor* (**c**) (Rasbak 2005), *Lycium barbarum* (**d**) (Danny 2011)

currently in clinical trials for an HPV vaccine for use against cervical cancer (Massa and Franconi 2012). Other polysaccharides under investigation include apiogalacturonic peptin from *Lemna minor* L., a polysaccharide from *Actinidia eriantha* Benth and a polysaccharide-protein complex originating from *Lycium barbarum* L. which has shown the potential as an adjuvant in dendritic cell-based vaccines (Chen et al. 2009; Massa and Franconi 2012).

9.10.3 Plant Proteins, Polysaccharides and Fungi as Vaccine Adjuvants

Plant proteins that defend plants against invading pathogens have also had its fair share of investigation for providing protection against pathogens infecting humans. Proteins from a fungus *Ganoderma lucidum* (Curtis.) P. Karst. have the ability to inhibit bacterial growth, inhibit HIV-1 reverse transcriptase and cause proliferation of Hepg2 and L1210 cells. Glycoproteins extracted from the neem tree have also been shown to restrict the growth of tumours in mice by increasing the expression of IFN-γ (Wang et al., Massa and Franconi 2012). Various natural polysaccharides such as mannan and inulin are naturally found in bacteria and plants. The adjuvanticity of inulin (Fig. 9.6) has been proven and various derivatives of this polysaccharide have been found to be potent adjuvants that activate the immune system. δ inulin has been used in conjunction with Japanese encephalitis and HIV vaccine antigens and proven to be effective (Bauer et al. 2010).

Mannan binds to mannose receptors and then enhances the phagocytotic activity of the antigens (Thiel and Gadjeva 2009). Mannan can be derivatised according to which immune response is needed, as the reduced form of mannan combined with an antigen activates the Th2 pathway, while the oxidised form of mannan activates the Th1 pathway (McKenziea et al. 1998). Chitosan, derived from the shells of shrimp, in combination with an antigen has also been shown to activate the immune response through activation of macrophages, production of cytokines and increased

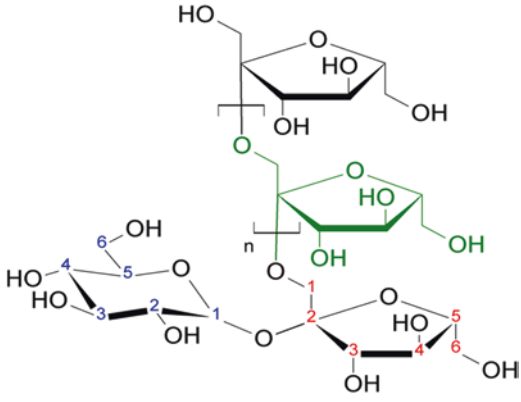

Fig. 9.6 The structural formula of inulin (Fisch 2006)

synthesis of antibodies (Shakya and Nandakumar 2012). Chitosan has mainly been used for effective delivery of antigens, and the influenza virus vaccine entrapped in chitosan has shown a significant increase in the production of antibodies in comparison with controls (Ghendon et al. 2009). KRN700 is an extremely immunopotentiating compound isolated from *Agelas mauritianus*, an ocean sponge found in southern Japan. This glycosylceramide has the potential to stimulate the immune system through activation of dendritic cells, natural killer cells, T cells and macrophages (Santini and Belardelli 2003). Investigations on this compound have shown that KRN7000 has the ability to reduce experimental tumours in murine models (Nakagawa et al. 1998, 2000) and is also safe to administer to cancer patients and those individuals that have lowered levels of natural killer cells (Santini and Belardelli 2003).

9.11 Adjuvants Specific for Cancer Therapy (The Desired Properties)

The desired response for the treatment of cancer includes the activation of IFN type 1 T-helper cells (Th1) as well as induction of cytotoxic T lymphocytes (Lim 2015). The immunotherapeutic and anti-tumour activity of TLRs used for the treatment of cancer rest on the principles that the anti-tumour activity is due to the activation of natural killer cells, as well as macrophages, monocytes and several cytokines that are directly or indirectly involved.

9.12 Adjuvants Specific for Infectious Diseases (The Desired Properties)

Several TLR agonists especially those for TLR 3, TLR7, TLR8 and TRL9 have shown the potential for treatment against infectious diseases (Lim 2015). Several agonists have been researched for their potential in treating viral infections. The main mechanism of action for antiviral activity is based on the induction of type I IFNs and IFN-γ-dependent antiviral effects, the upregulation of natural killer cells and their cytotoxicity and T-cell responses which are specific to certain viruses (Lim 2015).

9.13 Future Prospects for Natural Products as Adjuvants for Vaccines

Technologies exist today that have the ability to recognise the type of immune response needed to eliminate a specific pathogen (Di Pasquale et al. 2015). Effective vaccines have been found for a myriad of infections such as diphtheria, smallpox

and measles but diseases such as malaria, tuberculosis and human immunodeficiency virus (HIV) infection are capable of evading the immune system and are therefore not effectively prevented. Several challenges face the development of future vaccines such as pathogens that are intracellular in nature, those that consist of complex life cycles, those that compromise the host immunity and those that possess latent phases (Di Pasquale et al. 2015).

Synthetic polymer matrices were engineered to ensure the prolonged release of vaccines over a period of time. Robert Langer and his research group used non-biodegradable poly(ethylene-co-vinyl acetate) implants that had the ability of releasing protein antigens for a period of more than a month, which resulted in antibody responses that were equivalent to multiple injections made by Freund's adjuvant (Moyer et al. 2016). Biodegradable poly-lactic-co-glycolic acid (PLGA) microspheres that encapsulated and caused slow release of antigens were the pursuit of many research groups but had several problems such as antigen degradation due to acidic conditions within the PLGA environment; scale-up processes during manufacturing; and the dominant use of alum (Moyer et al. 2016).

PLGA formulations that made use of PRRs have been tested in clinical trials (Moyer et al. 2016). This gave rise to the new research field of vaccine adjuvant kinetics based on PRR signals. One of these, a microneedle skin patch, is made up of an array of pyramidal or cylindrical shaped projections (Moyer et al. 2016). They are a fraction of a millimetre in height and only a few hundred microns in size at their base. They were made to only mechanically perforate the upper surface of the skin (stratum corneum) so as to enter the epidermis and/or the upper dermis of the skin. The basic principles include coating microneedles with dried vaccine or the needles itself are dissolved within the layers of the skin with the subsequent release of the vaccine (Moyer et al. 2016). The advantages of this novel method of vaccine delivery are that the vaccine antigen targets a wide variety of immune cells (maximum exposure), it has minimum pain and discomfort as compared to traditional intramuscular injections and it provides encapsulation of the vaccine before use (Moyer et al. 2016). Several designs have been engineered and investigated in mice and in phase I clinical trials. These include microneedles of polymers that have the ability to controllably swell or dissolve at certain rates, those that are made up of polymer micro- and nanoparticles which consist of an encapsulated vaccine in a matrix which is also dissolvable, and microneedles that are made up of a solid tip or a polymer coating with a dissolving base (Moyer et al. 2016).

The wide variety of plants and herbal extracts that have not been researched yet for their immunomodulating capabilities gives us an untapped reservoir of potential to discover and enhance vaccines for more effective treatment of infectious diseases and cancer. This will also lead the way for dispensing of inexpensive vaccines in many developing countries who cannot afford a rollout of a new vaccine for infectious diseases and cancer.

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Chapter 10
In Vitro Plant Cell Cultures: A Route to Production of Natural Molecules and Systematic In Vitro Assays for their Biological Properties

Peeyushi Verma and Rakhi Chaturvedi

10.1 Introduction

The connection between humans and their search of drugs from nature is boundless since ancient times. Written documents, preserved plant materials, and original plant medicines are some of the evidences that elucidate the use of plants as medicines by humans (Petrovska 2012a). After many experiences, enormous sufferings, and diseases, people attained the knowledge of using leaves, barks, fruits, and other parts of the plants for the treatment of ailments (Petrovska 2012b). The primeval document describing the use of medicinal plants for drug preparation was found on a Sumerian clay slab from Nagpur. It is about 5000 years old and encloses 12 drug preparation recipes using more than 250 plants (Petrovska 2012b). The Indian books like "Vedas" emphasize to utilize abundant plants, like clove and pepper for the benefit of mankind. Theophrastus (371–287 BC), "The Father of Botany," established botanical sciences and contributed books, namely, "De Causis Plantarum" and "Historia Plantarum," to the society. In his book, he classified more than 500 medicinal plants. Dioscorides, "The Father of Pharmcognosy," one of the most prominent writers on plant drugs in ancient history, described the utilization of parsley and oak bark for diuretic and gynecological problems (Petrovska 2012b). Galen (131 AD–200) compiled the whole list of medicinal plants and introduced some novel plant drugs, which were not described by Dioscorides, such as *Uvae ursi folium* which is used for uroantiseptic and mild diuretic ailments even today. In the seventh century AD Slavic people started using *Rosmarinus officinalis, Ocimum basilicum,* and *Mentha viridis* in cosmetics and *Cucumis sativus, Urtica dioica, Artemisia maritime* L., and *Lavandula officinalis* were used against various injurious insects, like moths, mosquitos, and spiders. In Middle Ages, Charles the Great

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(742 AD–814) used some medicinal plants for planting. Old people used plants in simple forms, like decoctions, maceration, and infusion, while sixteenth- and eighteenth-century people used compound drugs, which comprised medicinal plants with drugs of plant and animal origin. In the eighteenth century, alkaloids from poppy, quinine, pomegranate, and glycosides were isolated from various plants and in the nineteenth and twentieth centuries, regular use of plants for making medicines led to depletion of plant species. Also, many authors suggested that the healing property of the plant depends upon its mode of drying. The condition or environment while drying decides the efficacy of plant. Till today, medicinal plants are being used by people themselves or due to recommendation by doctor (Petrovska 2012a).

Even today, most of the pharmacologists prefer to use only plant resources as medicine due to its less toxic side effects. About 80% of world population depends upon herbal medicine to treat various kinds of ailments. Today it is known that the medicinal properties in plants lie in specific category of compounds, called secondary metabolites, produced by them (Wink 2015). Plants possess mainly two kinds of metabolites: primary and secondary. Primary metabolites are responsible for growth and reproduction of plants, for example nucleic acids, fats, and carbohydrates. On contrary to this, secondary metabolites have no role in any of the primary functions of the plant, like growth and reproduction, but are mostly produced by plants for their defense against herbivores, plants, and pathogens. Several research articles have proved that secondary metabolites possess a wide range of bioactivities; for example camptothecin and paclitaxel, isolated from *Camptotheca acuminate* and *Taxus brevifolia*, respectively, are widely used in tumor therapy (Wink 2015).

Different parts of the plants and in vitro callus cultures of *Baliospermum montanum* were tested for their phytochemical as well as antibacterial potential. Leaves and roots of naturally growing plant are used for in vivo studies while callus obtained from these explants is used for in vitro studies. During phytochemical studies the amount of steroids, terpenes, saponins, glycosides, alkaloids, flavonoids, phenols, tannins, and sugars was found to be higher in in vitro-derived callus cultures than their respective plant parts, i.e., leaves and roots. Also, the ethanol extract of in vitro leaf callus showed the maximum antimicrobial activity (Johnson et al. 2010). *Commiphora wightii* (guggul), a critically endangered plant, is well known for its oleo-gum-resin, which reduces cholesterol. The boon to save these plants was "plant tissue culture technique." It was observed that in vitro micropropagation using nodal cuttings was more effective than conventional propagation system using long cuttings as well as seedlings from mother plants which has limitations, like seasonal dependency, low success rate, and damage to mother plants.

This chapter focuses on the importance of in vitro plant cultures, ways for establishing in vitro callus cultures, and impact of in vitro cultures on metabolite production and bioactivity followed by in vitro assays to investigate the efficacy of in vitro cultures. In vitro assays help in understanding the mechanism of action of plant-based drugs (Sect. 10.2); it is either in crude form or in purified form. Moreover, in vitro assays also aid in assessing antioxidant, antifungal, antiulcer, antimalarial, and anticancer properties of the plants.

10.2 Significance of In Vitro Cultures

Plant-based drugs or plant extracts are drugs obtained from plants. Basically, these drugs are available in two forms: crude form and isolated or purified form. Crude form of drug is prepared by drying the plant or its parts, followed by extraction of its metabolites in specific solvent while purified form of drug is prepared in the same way as crude form but with an additional step of separation of active compounds using various analytical techniques. However, the usage of crude extracts and isolated metabolites from naturally growing plants has some limitations, like varying amounts of metabolite production associated with the environmental changes and regional variations (Sampaio et al. 2016). It causes difficulty in scale-up and downstream processing of metabolites (Hussain et al. 2012). Furthermore, consistent accessing of the medicinal plants from natural environment can lead the plants to their extinction. Thus, plant tissue culture technique is the finest solution to solve these problems. Haberlandt, "Father of Plant Tissue Culture," devised the concept of cell culture in 1902. In plant tissue culture technique, plant cells, tissues, and organs are grown in a controlled environment using appropriate media composition. In vitro cultures obtained via plant tissue culture technique provide consistent and optimum metabolite production throughout the year, irrespective of seasonal and regional variations. Furthermore, in vitro cultures can accumulate a wide variety of secondary metabolites ranging from simpler to complex, like terpenes, saponins, alkaloids, polyphenols, anthraquinones, tannin, and flavonoids (Anulika et al. 2016). Further, in vitro cultures also possess some additional metabolites as compared to their wild donor plants. For example, in vitro cultures of *Lantana camara* L. produce betulinic acid, which is usually absent in wild plants (Srivastava et al. 2010). The presence of additional metabolites along with the provision for their scale-up makes in vitro cultures more valuable.

10.3 Establishment of In Vitro Plant Cell Cultures

10.3.1 Micropropagation

The method of producing multiple copies of donor plants from any somatic tissues under in vitro conditions, using plant tissue culture technique, is known as micropropagation or more specifically clonal propagation to generate true-to-type plantations. Micropropagation involves two methods: meristem culture using apical or axillary buds and adventitiously from any somatic/vegetative tissues other than pre-existing meristems. This is an alternative to conventional method of vegetative propagation by long stem cuttings (>30 cm) where success rate varies from season to season and region to region and is implemented in a few species only. On the other hand, micropropagation requires a small explant (<1 cm), such as nodal segments, shoot tips, and leaf discs, and hence favors large-scale propagation of

uniform plantations within a short duration, irrespective of the environmental fluctuations. Furthermore, it does not promote variations as happens with conventional methods by seed propagation where the seeds are the products of fertilization and resulting into variability. Thus, micropropagation perpetuates the parental characters to the progeny. Micropropagation by nodal segment cultures and adventitious shoot proliferation are two widely used methods for large-scale production.

10.3.2 Callus Cultures

The undifferentiated mass of cells obtained after inoculating an explant on a particular media in sterile conditions is known as callus culture. The explant could be any part of the plant showing totipotency. Some of the important benefits of callus cultures are their fast growth, availability of single or small cluster of cells, easy scale-up of cell biomass, and high metabolite content. The initiation and multiplication of callus from leaf disc cultures of *Lantana camara* (Fig. 10.1). Fresh and young leaves were collected, and washed with Tween-20, followed by surface sterilization with $HgCl_2$. Under aseptic conditions, leaf discs were prepared from

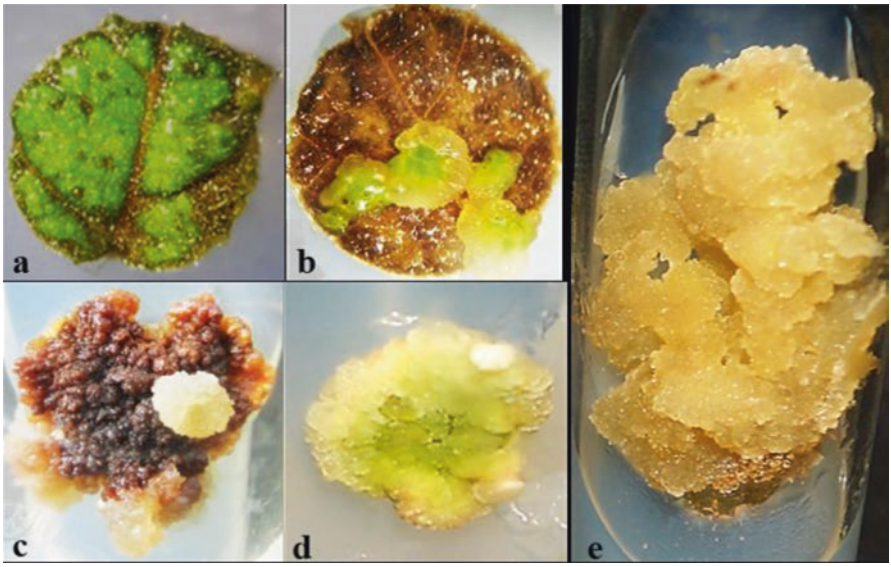

Fig. 10.1 Establishment of in vitro callus cultures of *Lantana camara* L. using leaf disc as an explant: (**a**) expansion and browning of the leaf disc began after 1 day of inoculation in media; (**b**) induction of callus from the incised ends of leaf disc occurred after 15 days; (**c**) same, after 28 days, develops compact brown callus with initiation of cream calli; (**d**) proliferation of fresh, friable, and green-color callus from compact brown callus occurred after another 30 days; (**e**) massively grown fresh, friable, cream-color callus proliferated after subsequent subcultures, every 2 weeks

sterilized leaves using cork borer and explants were inoculated on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP; 5 μM), 1-naphthaleneacetic acid (NAA; 1 μM), and 2,4-dichlorophenoxyacetic acid (2,4-D; 1 μM) (Srivastava et al. 2010).

10.3.3 Cell Suspension Culture

The method to culture cells and tissues in liquid nutrient media is known as cell suspension cultures. In suspension cultures, each individual cell is in direct contact with the medium nutrients, resulting in faster cell growth compared to that in semi-solid media. Cell suspension cultures are basically of two types: batch and continuous culture. In batch culture, also called as closed system, the cell inoculum is added to the media in the beginning and is allowed to grow without subsequent addition of fresh media. With the time, the nutrient is used up by the growing cells and, hence, the cells pass through the four distinct stages, lag phase, log phase or exponential phase, stationary phase, and death phase. At the end of the process, the cells are harvested for product isolation. On the other hand, in continuous culture system, media is added after certain intervals and spent media is replaced with the fresh media at the same rate while keeping the rest of the conditions at optimum. Due to continual exchange of nutrient medium, it is considered as an open system and aims to maintain the cells at exponential phase. The remarkable benefit of cell suspension cultures is that they can serve as inoculum for large-scale production of biomass and metabolites in batch or continuous systems.

10.4 Plant Secondary Metabolites

10.4.1 Chemical Classes of Secondary Metabolites

10.4.1.1 Terpenes

Terpenes or terpenoids are the largest class of secondary metabolites. Majority of the compounds of this class are commonly water insoluble. They are made up of five carbon atoms with branched carbon skeleton of isopentane. Saponins and carotenoids are two important derivatives of triterpenes. These are mainly known for their defensive role in plants. Some of the plants, like basil, lemon, sage, and tobacco, are known to have terpenes. Terpenoids perform several functions in plants (Table 10.1), primarily plant pollination and defense against herbivores. For example, the eugenol, isolated from *Ocimum gratissimum*, serves as fragrance to attract insects for pollination and the *Nicotiana attenuata* plant secretes trans-β-ocimene, cis-α-bergamotene, and trans-β-farnesene terpenoids after herbivore attack (Anulika et al. 2016; Kessler and Baldwin 2001)

10.4.1.2 Phenolic Compounds

Phenolic compounds from the plant sources are the secondary metabolites containing hydroxyl group on aromatic ring. Plant phenols are highly heterogeneous as some dissolves only in organic solvents and a few in water and others remain as insoluble polymers. Anthocyanin, tannins, lignin, flavonoids, and isoflavones are few derivatives of phenols (Table 10.1). Flavonoids are one of the largest classes of phenols and their basic function is pigmentation and defense (Anulika et al. 2016). Apple, blackberries, tea, and woody plants possess tannins in them (Lazar 2003). A broad range of phenolic compounds from plants impart organoleptic properties (i.e., taste and odor) to the food products (O'Connell and Fox 2001).

10.4.1.3 Nitrogenous Compounds

Nitrogenous compounds include secondary metabolites containing nitrogen in their structures like alkaloids and glucosides (Lazar 2003) (Table 10.1). Morphine is the first medically applicable alkaloid, isolated from *Papaver somniferum* (Thummel 1979).

Table 10.1 Types of secondary metabolites occur in plants and their uses (Karban and Baldwin 1997, Bidlack 2000, Rosenthal and Berenbaum 1991)

Class	Compounds	Sources	Effects and uses
Nitrogen containing			
Alkaloids	Nicotine	*Nicotiana tabacum*	Interfere with neurotransmission
Nitrogen and sulfur containing			
Glucosinolates	Sinigrin	Cabbage relatives	–
Terpenoids			
Monoterpenes	Menthol	Mint and relatives	Interfere with neurotransmission, anesthetic
Sesquiterpenes	Parthenolide	Parthenium and relatives	Contact dermatitis
Diterpenes	Gossypol	Cotton	Block phosphorylation; toxic
Triterpenes	Betulinic acid, oleanolic and ursolic acid	*Lantana camara*	Cytotoxic effect on HeLa cancer cells
Tetraterpenoids	Carotene	Several plants	Antioxidant; orange coloring
Sterols	Spinasterol	Spinach	Interfere with animal hormone action
Phenolics			
Phenolic acids	Caffeic, chlorogenic	All plants	Oxidative damage, browning in fruits and wine
Coumarins	Umbelliferone	Carrots, parsnip	Cross-link DNA, block cell division
Flavonoids	Anthocyanin, catechin	Almost all plants	Flower, leaf color; inhibit enzymes, antioxidants
Phenols	Hydroxytectoquinone	*Rubia cordifolia*	Anticancer
Lignin	Lignin	All land plants	Toughness, structure, fiber

Many alkaloids provide defense mechanism, especially against mammals due to their toxicity (Hartmann 1999).

10.4.2 Quantification of Secondary Metabolites

Continuous monitoring of secondary metabolites is necessary for the successful establishment of production technology. Analytical techniques, like high-performance liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography-mass spectrometry (LC-MS), and spectrophotometry, are used for quantification of metabolites from in vitro plant cultures (Matkowski 2008). To prepare plant sample for quantification, the plant materials are dried and then soaked in a suitable solvent to extract metabolites. Of the various extraction methods, the most commonly used ones are Soxhlet extraction, microwave-assisted extraction, sonication, etc. (Kim and Verpoorte 2010).

10.5 Strategies to Enhance Secondary Metabolite Production

The metabolite content from in vitro-grown cultures can further be enhanced by several ways such as optimizing media and culture conditions, selecting high metabolite-producing cell lines, precursor feeding, elicitation, and biotransformation.

10.5.1 Optimization of Metabolite Synthesis by Culture Conditions

Nutrients and environment highly influence the metabolic pathway of secondary metabolites for example light-induced anthocyanin pigments. The anthocyanidin profile is different in both leaves and fruits; for example, cyanidin-3-rutoside occurs dominantly in leaves. Media optimization is needed for higher biomass as well as metabolite production. Auxins and cytokinins are two important plant growth regulators, responsible for the stimulation of metabolic pathways. In anthocyanin-producing *Glehnia littoralis* callus cultures, NAA (1 mg/L) was preferred as auxin over IAA (indole-3-acetic acid; 1 mg/L) and 2,4-D (1 mg/L) and further addition of kinetin (0.01 mg/L) enhanced the cell growth and pigment biosynthesis (Miura et al. 1998). In some reports two-stage system is used to optimize the production of secondary metabolites. The first stage is optimized to achieve maximum cell proliferation and faster biomass growth while second stage aims to accumulate more products. For example, when two-stage system was applied on *Crocus sativus*, crocin accumulation increased to 430 mg/L, by using IAA (2 mg/L) and BAP (0.5 mg/L) instead of NAA (2 mg/L) and BAP (1 mg/L) (Chen et al. 2003).

10.5.2 Selection of High Metabolite-Yielding Tissues

The production of metabolites depends on the type of tissues they are present. For example, in *Salvia officinalis* (Grzegorczyk et al. 2007) and *Rosmarinus officinalis* (Caruso et al. 2000) in vitro cultures, carnosic acid (a diterpene) is found only in shoot cultures but not in callus, suspension, or hairy roots, while higher phenols are accumulated by undifferentiated cell suspensions. The active cell lines chosen for metabolic production should produce metabolites higher than the other cultures and the normal producing cell lines. The efficient cell lines can be selected on the basis of the amount of their compounds or by selecting the agents supporting the process of production of those compounds. The cell line selected for the study should exhibit both the levels of production obtained in unselected cultures and the natural biosynthetic productivity of an intact organism. When a phenylalanine analogue is added to the culture medium, it will damage majority of the cells except those ones expressing high PAL (phenylalanine ammonia lysate) activity. The selected cells exhibiting overexpression of PAL produce more phenylpropanoid compounds (Matkowski 2008).

10.5.3 Precursor Feeding and Biotransformation

In a few in vitro-grown medicinal plants, the intact plant forms various valuable metabolites through its biosynthetic pathway whereas the dedifferentiated form is unable to complete the biosynthesis. This could be due to lack of environmental stimuli, insufficient expression of related genes, or improper enzymatic machinery. If the enzymes responsible for production of a particular valuable metabolite are expressed in one portion of the metabolic pathway, then precursor applications are recommended to overcome such type of problem. For example, strawberry cultures were producing negligible amount of anthocyanin but feeding phenylalanine (precursor) increased its production (Edahiro et al. 2005). Likewise curcumin is a yellow-colored potent antioxidant compound obtained from the roots of *Cucuma longa* commonly known as turmeric but due to its insolubility in water it is not absorbed by intestine and, thus, exhibits no pharmaceutical importance (Sharma et al. 2007). Later, in *Catharanthus roseus* cell suspension cultures, the supplied curcumins were modified into peculiar glycosides that are much more soluble in water. The water solubility of these curcumin-modified glycosides (curcumin-4′,4″-*O*-β-D-digentiobioside) was 20 million folds higher than that of curcumin (Kaminaga et al. 2003).

10.5.4 Elicitation and Stress-Induced Production

Many medicinal plants when exposed to stress exhibit increased production of secondary metabolites (Verpoorte et al. 2002). In vitro cultures can be elicited by using stress-related growth regulators, such as jasmonic acid or its esters, biotic elicitors

(for example, bacteria and fungus), and abiotic factors (for example, metals and radiation). Methyl jasmonate (MeJa), a stress-related growth regulator, is widely used in inducing the metabolite biosynthesis including anticancer alkaloids (Verpoorte et al. 2002; Mulabagal Vanisree et al. 2004). Yeast extract, chitin, or chitosan (biotic elicitors) was independently tested for production of silymarin where only yeast extract was found to be effective and its efficacy was increased when supplemented along with MeJa (Sanchez-Sampedro et al. 2005). Chitin intensified the production of flavonoids in *Cephalocereus senilis* (cactus) cell cultures (Qin et al. 1993). Vanadium salts and rare elements like La^{3+} and Ce^{3+} have shown to be increasing the accumulation of rosmarinic acid (Georgiev et al. 2007) and crocin in in vitro cell cultures (Chen et al. 2003). In certain plant cells, UV irradiation was found to increase the production of antioxidants.

10.5.5 Agrobacterium-Mediated Transformation

Agrobacterium-mediated transformation is one of the highly preferred methods to increase the secondary metabolite production. The plant species to be transformed are co-cultivated with *Agrobacterium rhizogenes* to induce hairy-root development. Using the method, rosmarinic acid (RA) production and its antioxidant activity were increased to much higher level in *Salvia officinalis* hairy roots as compared to that in untransformed organs (Grzegorczyk et al. 2006, 2007).

10.5.6 Scale-Up in Bioreactor

The ultimate aim of raising cell biomass and cell suspension cultures from medicinal plants, using plant tissue culture techniques, is to increase the availability of cell biomass as a raw material and also to increase the production of valuable metabolites at industrial scale. This could be possible when the callus and cell suspension cultures grown successfully in small culture vials and shake flasks, respectively, are to be grown at the same rate in large-size bioreactors. As the volume of the cultures increases in bioreactors, several parameters, like rotational speed, medium pH, cell concentration, temperature, and illumination, are to be optimized. However, the plant cell cultures are not as commercially utilized as microbial cell cultures due to the increased size of plant cells making it more sensitive to shear stress. The other major limitations are lack of availability of data; mostly the published work are dealt with plant cells at lab scale due to their comparatively large size and rigid cell wall than animal cells (Griffits 1985). Till 2001, the best examples of success were shikonin, paclitaxel, and ginseng production, where the industries employ plant cell culture at pilot scale (Zhong 2001). Bioprocess parameters like engineering considerations, optimization of process parameters, and process strategies can be applied to increase the production of secondary metabolites from plant cell suspension

cultures (Chattopadhyay et al. 2002). Stirred tank reactor with setric impeller was used to optimize podophyllotoxin production in suspension cultures of *Podophyllum hexandrum*. Stirred tank reactor with setric and turbine impeller was used to optimize azadirachtin production in cell cultures of *Azadirachta indica*. In this case, less shear and better mass transfer were observed. Biomass and azadirachtin yield with setric and turbine impeller was obtained as 18.7 and 15.5 and 0.071 and 0.05 g/L, respectively, at 125 agitation and 0.2 vvm aeration rates (Prakash and Srivastava 2006). Cell cultures of *Scrophularia striata* Boiss were grown in shake flasks and in bioreactor and analyzed for increase in biomass and phenylethanoid glycoside (PeG) production (Falahi et al. 2017). Cell biomass and PeG content were found higher in bioreactor than shake flasks, i.e., 15.64 g/L DW and 1404.20 µg/g and 14.16 g/L DW and 459.71 µg/g (Pavlov et al. 2007).

10.6 In Vitro Methods for Assessment of Biological Properties

10.6.1 Antibacterial Activity

Today, most of the antibiotics like tetracycline, cephalosporin, and aminoglycosides are synthesized chemically. The existence of these essential compounds is at risk due to developing resistance in microbes, against these antibiotics. As a result, drugs become inactive and infections persist and spread. Hence, these multidrug-resistant microbes are a threat to human health (Balouiri et al. 2016; Mayers et al. 2009). Thus, the discovery of new antibiotics is a crucial issue among many researchers. The production of antibiotics via in vitro plant tissue culture technique is a biological route of synthesis as well as sustainability of cultures. In order to measure the antibacterial potential of plant-based drugs, following assays are generally performed (Guschin et al. 2015).

10.6.1.1 Diffusion Methods

Diffusion methods are based on the principle of mass transfer due to diffusion. In diffusion, the "movement of molecules takes place from higher concentration to lower concentration." Most of the lifesaving molecules, including water, are transported by diffusion. Using the similar phenomena, the antibacterial activity of plant-based drugs is measured using the assays described in the following lines. The efficacy of the active compound is quantified in terms of zone of inhibition (ZOI), followed by minimum inhibitory concentration (MIC). The clear zone around the filter disc containing antibiotic defines the ZOI, where there is no visible growth of bacteria. Higher ZOI represents higher potency of the drugs. The activity of the compounds is compared with that of positive and negative controls for all the experiments. Positive control is any antibiotic known to kill the test bacteria while the

negative control is usually the solvent in which the test organism is suspended without any antibiotic. As the name suggests, positive control surely produces ZOI while negative control gives no ZOI (Balouiri et al. 2016). The minimum concentration of the antibacterial test compound (i.e., compound to be tested for antibacterial activity) is called minimum inhibitory concentration (MIC), which is the minimum concentration required to kill the visible population of test bacteria. It is generally expressed as μg/mL or mg/mL.

Agar disc diffusion method is one of the most commonly used methods in many clinical laboratories for testing antibacterial susceptibility of various drug candidates. It offers many advantages over other methods like simplicity of procedure, low cost, facility to test multiple test bacteria (i.e., bacteria used for the study), and antibacterial compounds with the ease to interpret results. The procedure to test the antibacterial potency of in vitro plant extract (IPE) through disc diffusion method is represented in Fig. 10.2. Nutrient agar (NA) plates are prepared by pouring 20 mL of NA media in 90 mm petri plates inside the laminar hood and once the media is solidified the plates are ready for testing the antimicrobial activity. Thereafter, the bacterial cultures ($\approx 1 \times 10^6$ cells) are spread over NA plates with the help of spreader. Afterwards, known concentrations of IPE are added to filter paper discs (6 mm dia). After drying these filter discs, these are placed over the petri plates inoculated with test bacteria (Choma and Grzelak 2011). Antibacterial compounds of IPE diffuse into the agar and form a ZOI, which is measured in millimeter (mm). This method is not suitable for MIC determination as the quantification of antibacterial compounds diffused into the agar is very difficult (Balouiri et al. 2016).

Agar well diffusion method is similar to the disc diffusion method except that the wells are made in the agar plate, instead of using the filter discs. After spreading the test bacteria on agar plates, wells (of dia 6–8mm) are made aseptically.

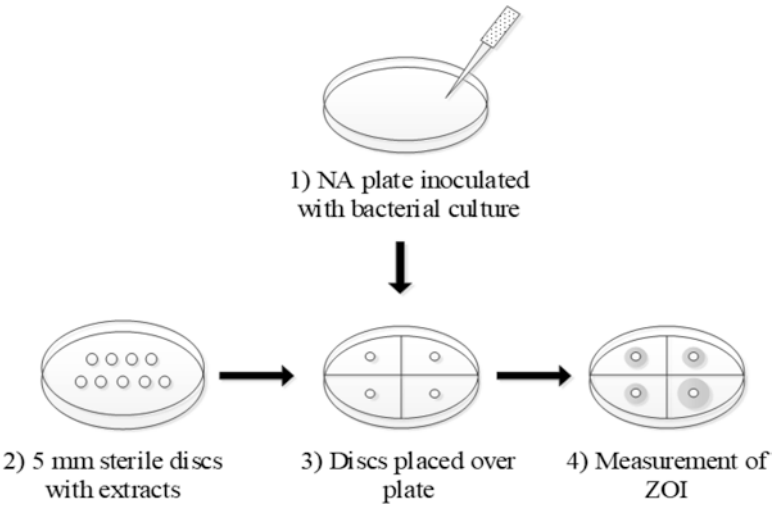

Fig. 10.2 Protocol for agar disc diffusion method

Subsequently, 20–100 μL of IPE is added and plates are incubated at optimum growth conditions of test bacteria. The diffusion of IPE occurs through agar and produces ZOI, which in turn gives the information about antimicrobial potential of the IPE. The more the ZOI (in mm), the more the antimicrobial potential of the IPE (Balouiri et al. 2016).

10.6.1.2 Dilution Methods

Dilution method is the most applicable technique for MIC determination as it gives the quantified amount of antimicrobial drug (Choma and Grzelak 2011). The broth and agar dilution methods are widely used methods for quantitative measurement of the antimicrobial activity against bacteria and fungus.

Broth dilution method is a basic method to test antibacterial activity. It is of two types: broth macro- or micro-dilution methods. If the twofold dilution (i.e., 1, 2, 4, 8, and 32 μg/mL) of test antimicrobial compound is performed in 2 mL tube it is called as macro-dilution and if it is performed in lower volumes in 96-well plate it is known as micro-dilution (Balouiri et al. 2016). Bacterial inoculum preparation is one of the most important factors influencing the MIC value. The EUCAST guidelines, standardized for broth dilution for testing antimicrobial activity by broth dilution method, are described in the following lines. As per the EUCAST guidelines if the inoculum is fungus (conidium and spores), the inoculum to be used for activity test will be adjusted to 0.4×10^4–5.0×10^4 CFU/mL by adding phosphate-buffered saline (PBS) to the inoculum. The dilution of inoculum, in order to get the required number of cells, can be achieved in two ways: by preparing 0.5 McFarland solution and comparing the inoculum with 0.5 McFarland scale (reference) or by measuring absorbance by spectrophotometer (Fig. 10.3). After inoculum preparation, the test antimicrobial compounds (twofold diluted, i.e., 1, 2, 4, 8, and 32 μg/mL) were added to inoculum (in tubes or in 96-well plates) and incubated (mostly without agitation) at optimum growth conditions of the test bacteria (Balouiri et al. 2016; Choma and Grzelak 2011) and their absorbance is measured using spectrophotometer at 625 nm (Fig. 10.4).

Agar dilution method is used particularly when IPE color inhibits the detection of bacterial growth. It involves the addition of antimicrobial agent to molten agar medium, using twofold serial dilution method, followed by addition of bacterial inoculum, measurement of absorbance, and MIC determination (Balouiri et al. 2016, Choma and Grzelak 2011).

10.6.1.3 Antimicrobial Gradient Method (E-test)

This method combines the principle of dilution and diffusion methods. An inert and nonporous plastic reagent stripe comprising increasing concentration of IPE is introduced to the agar medium (Choma and Grzelak 2011). To detect the synergy between two drugs, stripe containing the first drug is placed on pre-inoculated agar

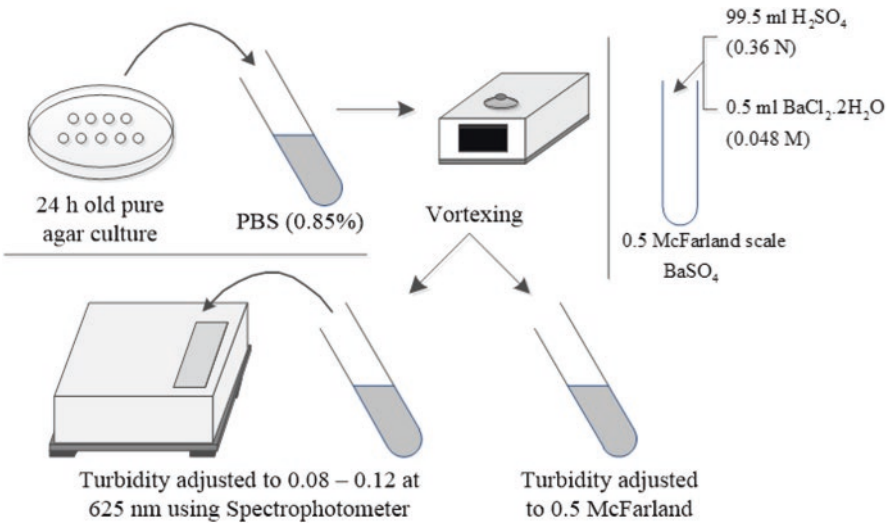

Fig. 10.3 Protocol for inoculum preparation for antimicrobial activity

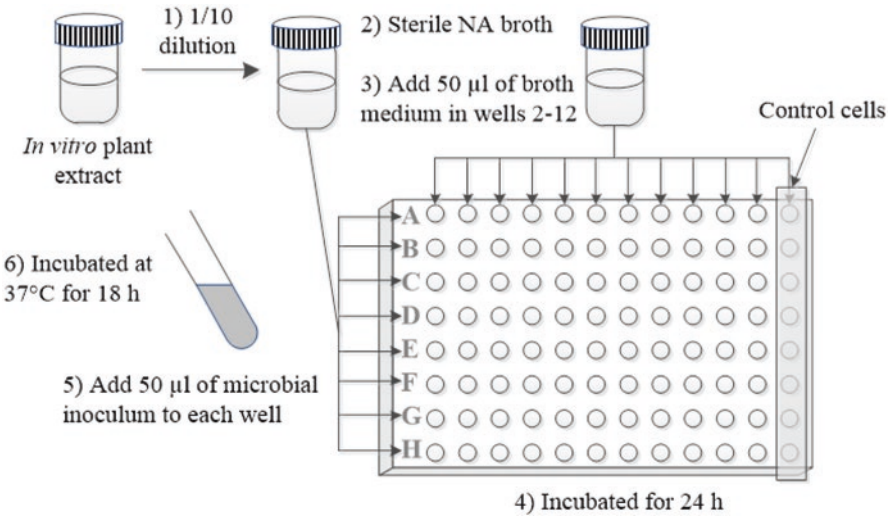

Fig. 10.4 Broth microdilution method for determination of MIC of test compound

media followed by another stripe containing the second drug and the MIC is calculated. The drugs were considered to be synergistic in action, when the MIC value of both the drugs in combination is at least more than two dilutions as compared to the most active drug tested alone (Balouiri et al. 2016). Two drugs are considered to be synergistic if their combined effect is more than the effect of individual drugs individually (Fig. 10.5).

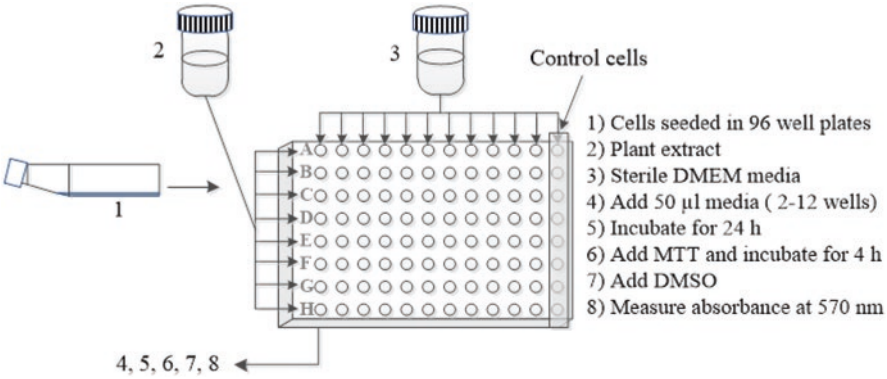

Fig. 10.5 MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay for determination of cytotoxicity of test compounds

10.6.1.4 TLC Bioautography

Bioautography is a microbe detection technique grouped with planar chromatography. Bioautography technique coupled with thin-layer chromatography (TLC) is known as TLC bioautography. The protocol for this assay is identical to agar diffusion methods except that the diffusion of test compound occurs from chromatographic layer (i.e., adsorbent or paper) to the agar plate. This method is of three types: agar diffusion or agar contact method, direct bioautography, and agar overlay bioassay (Balouiri et al. 2016, Choma and Grzelak 2011).

10.6.1.5 Time Kill Test

Time kill test or time kill curve is one of the important techniques to determine bactericidal or fungicidal activity. To perform this, 1×10^5 CFU/mL bacterial cultures were taken in three tubes, followed by the addition of test compound at concentrations of 0.25 × MIC and 1 × MIC in two tubes and control (without test compound) in third tube. All the tubes were incubated for a period of 0, 4, 8, 12, and 24 h. Thereafter, percentage of dead cell is calculated by comparing with the number of living cells in control at every stage of respective time interval (Balouiri et al. 2016).

10.6.1.6 ATP Bioluminescence Assay

ATP is a chemical form of energy universally present in all the living cells. This assay calculates the ATP generated by bacteria or fungi, which in turn gives the amount of microbe in a sample. However, the sample is treated with a potent antimicrobial agent. In the presence of ATP, D-luciferin gets converted into oxyluciferin which gives illumination. It is measured by a luminometer and is expressed as a relative light unit (RLU). Based on the RLU values, the linear relationship between

cell viability and luminescence is established for measuring the bioactivity of a particular antimicrobial agent (Balouiri et al. 2016).

10.6.1.7 Flow Cytofluorometric Method

Flow cytometry is used to quantify the damaged cells by using appropriate dyes that stain specific organelles of the cell. Many studies reported the use of flow cytometric method in antibacterial assays. For example, propidium iodide (PI) is used for membrane disruption study and carboxyfluorescein diacetate (cFDA) is used to detect esterase activity. Apart from lysed cells, these methods also differentiate live, dead, and injured cells (Balouiri et al. 2016).

10.6.2 Antidiabetic Activity

10.6.2.1 α-Amylase Inhibition Assay

α-Amylase inhibition assay is performed by 3,5-dinitrosalicylic acid (DNSA) method. Leaf extract of *Adenanthera pavonina* was initially dissolved in a minimum amount of 10% dimethyl sulfoxide (DMSO), followed by its further dissolution in *reagent* 1 to prepare concentrations ranging from 10 to 1000 μg/mL. Thereafter, 200 μL of *reagent* 2 is added to 200 μL of extract, followed by incubation at 30 °C for 10 min. The measured volume of 1 mL of *reagent* 3 is added. After 3 min of incubation, *reagent* 4 is added to stop the reaction and this reaction mixture is boiled in water bath for 10 min at 85–90 °C. Thereafter, the reaction mixture is cooled to room temperature, followed by its dilution with 5 mL of distilled water. The absorbance is taken at 540 nm via spectrophotometer with the help of Eq. (10.1). Plant extract and acarbose (2–100 μg/mL) were used as blank and positive controls, respectively (Wickramaratne et al. 2016). The detailed list of reagents required is provided in Table 10.2:

$$\%\text{Inhibition} = \frac{A_{540\text{CONTROL}} - A_{540\text{EXTRACT}}}{A_{540\text{CONTROL}}} \times 100 \tag{10.1}$$

10.6.2.2 Glucose Diffusion Inhibitory Assay

This assay describes the protocol of with some minor modifications. *Reagent* 5 (2 mL) was laden into a dialysis tube containing IPE (50 mg/mL) and then the dialysis tube was tied from both its ends. It is then placed in a centrifuge tube containing 45 mL solution of 0.15 M NaCl. Thereafter, the centrifuge tube was placed in an orbital shaker at room temperature. Diffusion of glucose into the external solution was monitored after every 1 h by measuring the concentration of glucose in the

external solution (Gray and Flatt 1997). The control consists of 1 mL of 0.15 M NaCl containing *reagent* 5 and 1 mL distilled water (Vijayalakshmi et al. 2014).

10.6.3 Antioxidant Activity

Antioxidants are compounds that delay or inhibit the oxidation of a substrate even if it is in lower concentration than substrate (Halliwell and Gutteridge 1995). It investigates the potential of natural antioxidants in both forms, as pure compounds and plant extracts. Due to high sensitivity, these methods have become popular (Salazar et al. 2008). Some of the methods are described below.

10.6.3.1 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Assay

One of the widely used methods to measure the radical scavenging activity (RSA) of antioxidant compounds is DPPH assay (Blois 1958; Mensor et al. 2001; Singh et al. 2014). It involves the reduction of DPPH to form DPPH-H in methanol when a hydrogen-donating antioxidant is present. The assay is performed in author's laboratory where 2 mL of extract (stock solutions of 10 mg/mL in methanol) is mixed with 500 mL of *reagent* 6. After a duration of 30 min, absorbance is recorded at 517 nm by using control having same amount of DPPH. Decrease in absorbance shows higher RSA. It is calculated using Eq. (10.2)

$$\%\text{Inhibition} = \frac{A_0 - A_1}{A_0} \times 100 \tag{10.2}$$

where A_0 and A_1 are absorbance of control and test sample, respectively. Positive controls can be gallic acid, ascorbic acid (Blois 1958), and α-tocopherol (Shimada et al. 1992).

10.6.3.2 Superoxide Anion Radical Scavenging (SO) Assay

Superoxide anion is known to be a weak oxidant but it generates hazardous hydroxyl radicals and singlet oxygen (Meyer and Isaksen 1995). Many other biological reactions also generate highly toxic superoxide radicals. This method is performed using the protocol of Robak and Gryglewski (1988). To generate superoxide anion radicals, 0.5 mL of *reagent* 8, 0.5 mL of *reagent* 9, 1.0 mL of extract, and 0.5 mL of *reagent* 7 are mixed. Thereafter, 0.5 mL of r*eagent* 10 is added to start the reaction. After addition of antioxidants, depletion of superoxide anion in the reaction mixture causes decrease in absorbance which is measured at 560 nm using spectrophotometer. Positive controls such as gallic acid (Robak and Gryglewski 1988), curcumin, BHA, ascorbic acid, and α-tocopherol can be used (Nishikimi et al. 1972).

Table 10.2 Reagents required to test particular bioactivity

Activity	In vitro assays	Reagents required
Antidiabetic activity	DNSA activity	1. Sodium phosphate buffer (0.02 M) with NaCl (0.006 M) pH 6.9 2. α-amylase solution (0.5 mg/mL) 3. Starch solution (1% in water (w/v)) 4. DNSA color reagent
	Glucose diffusion inhibitory assay	5. Glucose solution (0.22 mM in 0.15 M sodium chloride)
Antioxidant activity	DPPH assay	6. DPPH in methanol (0.5 mg/mL)
	SO assay	7. Tris–HCl buffer (16 mM, pH 8.0) 8. Nitro-blue tetrazolium dye, (NBT; 0.3 mM) 9. β-Nicotinamide adenine dinucleotide reduced sodium salt, (NADH solution; 0.936 mM) 10. PMS solution (0.12 mM)
	XO method	11. Phosphate buffer (0.05 M, pH 7.5) 12. Xanthine oxidase solution (0.2 units/mL) 13. Xanthine substrate solution (0.15 M)
	H_2O_2 assay	14. H_2O_2 (40 mM) 15. Phosphate buffer (50 mM; pH 7.4)
	NO assay	16. Sodium nitroprusside in phosphate buffer (10 mM) 17. Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H3PO3)
	HO assay	18. 2-Deoxy-D-ribose (28 mM) in KH_2PO_4-KOH buffer (20 mM); pH 7.4 19. Ethylenediaminetetraacetic acid (EDTA; 1.04 mM) 20. $FeCl_3$ (1:1 v/v) 21. H_2O_2 (1.0 mM) 22. Ascorbic acid (1.0 mM) 23. Thiobarbituric acid (1%) 24. Trichloroacetic acid (2.8%)
	ORAC assay	25. 2, 2′- Azo-bis, 2-amidinopropane dihydrochloride (AAPH)
	FRAP assay	26. 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) 27. FRAP reagent (10 parts of 300 mM sodium acetate buffer; pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20.0 mM FeCl3. $6H_2O$ solution)
Anticancer activity	MTT assay	28. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
	XTT assay	29. 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H* tetrazolium-5-carboxyanilide inner salt (XTT)
	MTS assay	30. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS)
	Trypan blue dye exclusion assay	31. Hanks' buffered salt solution (HBSS) 32. Trypan blue (0.4%)
	Microscopic observations of cell morphology	33. Acridine orange or ethidium bromide (AO/EB)
	Resazurin cell growth inhibition assay	34. Trypsin (0.025%) 35. EDTA (0.25 mM) 36. Resazurin (0.01% w/v)

10.6.3.3 Xanthine Oxidase (XO) Method

This method is performed using the protocol of Noro et al. (1983). Allopurinol of 100 µg/mL in methanol and 500 µL (0.1 mg/mL) of extract were mixed with 1.3 mL and 0.2 mL of *reagent*s 11 and 12, respectively. This mixture is incubated for 30 min at 25°C, followed by addition of 1.5 mL of *reagent* 13 reaction mixture. It is again incubated for 30 min at 25°C and absorbance is recorded at 293 nm by spectrophotometer. The solution of 1.3 mL of *reagent* 11, 0.2 mL of *reagent* 12, and 0.5 mL of methanol is used as blank. The mixture of 1.3 mL of *reagent* 11, 0.2 mL of *reagent* 12, 1.5 mL of *reagent* 13, and 0.5 mL of methanol is used as control. It is calculated using Eq. (10.3)

$$\%\text{Inhibition} = \left(1 - \frac{A_s}{A_c}\right) \times 100 \tag{10.3}$$

where A_s and A_c are the absorbance values of test sample and control, respectively. Catechin can be used as positive control (Schmeda-Hirschmann et al. 1996).

10.6.3.4 Hydrogen Peroxide Radical Scavenging (H_2O_2) Assay

Hydrogen peroxide is present at low levels of concentration in plants, human body, microorganisms, air, and water. In human body, it can enter while breathing or by contact with skin and eye. After entering inside, H_2O_2 decomposes into water and oxygen. It can also form hydroxyl radicals ($OH^{\cdot}$), which can even damage DNA. The H_2O_2 scavenging potential of plant extracts is analyzed by method of Ruch et al. (1989). *Reagent* 14 is mixed with *reagent* 15. Extract (20–60 µg/mL) is prepared in distilled water and added to H_2O_2. Absorbance is measured at 230 nm by spectrophotometer. *Reagent* 15 alone is used as blank. It is measured by Eq. (10.4):

$$\%\text{Scavenged}\left(H_2O_2\right) = \left(A_0 - \frac{A_1}{A_0}\right) \times 100 \tag{10.4}$$

where A_0 and A_1 are absorbance of control and test sample. Positive controls can be α-tocopherol (Gülçın et al. 2003), ascorbic acid, and BHA (Jayaprakasha et al. 2004).

10.6.3.5 Nitric Oxide (NO) Assay

Nitric oxide is produced in solution when *reagent* 16 reacts with oxygen to produce nitrite ions at physiological pH which is observed by *reagent* 17 (Green et al. 1982). A measured volume of 3 mL of *reagent* 16 is added to 2 mL of extract and reference compound, in various concentrations ranging from 20 to 100 µg/mL, followed by

incubation at 25 °C for 60 min. Methanol is used as control. An amount of 5 mL each, from incubated sample and *reagent* 17, is taken and mixed. Absorbance is measured at 540 nm. The percentage inhibition is calculated by comparing the absorbance values of test and control samples. Positive controls used are α-tocopherol (Garrat 1964), ascorbic acid, BHA (Jayaprakasha et al. 2004), or caffeic acid, sodium nitrite, and curcumin (Sreejayan and Rao 1997).

10.6.3.6 Hydroxyl Radical Scavenging (HO) Assay

Hydroxyl radicals are one of the most effective reactive oxygen species (ROS) in the biological system. The method measures the scavenging activity (Kunchandy and Rao 1990). An amount of 1 mL of reaction mixture is prepared by adding 100 μL of *reagent* 18, 500 μL of extract, 200 μL of *reagent* 19, 200 μM of *reagent*-20, 100 μL of *reagent* 21, and 100 μL of *reagent* 22. Then, it is kept for incubation at 37 °C for 1 h. The mixture is heated for 15 min at 95 °C and 1.0 mL of both *reagents* 23 and 24 is added to it. After 20 min of incubation at 100 °C, it is cooled and centrifuged at 5000 rpm for 15 min. Absorbance of supernatant is recorded at 532 nm. Positive controls are taken as vitamin E (Halliwell et al. 1987), ascorbic acid, or rutin (Jayaprakasha et al. 2004).

10.6.3.7 Oxygen Radical Absorbance Capacity (ORAC) Assay

Investigation of the peroxyl radical scavenging potential of the compounds, produced by spontaneous decomposition of *reagent* 25, is done by ORAC assay (Prior et al. 2005). For the estimation, reaction mixture is prepared by adding 0.5 mL of extract in phosphate buffer (75 mM, pH 7.2) with 3.0 mL of fluorescein solution, followed by preincubation for 10 min at 37 °C. After the addition of 0.5 mL of *reagent* 25, decrease in fluorescence (FL) is observed and difference in area under the FL decay curves is calculated for both sample and control. O.

XYGEN-RADICAL ABSORBANCE CAP

10.6.3.8 Ferric Reducing Antioxidant Power (FRAP) Assay

The potential of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of reagent 26, where a blue complex Fe^{2+} TPTZ is formed with an absorption maximum at 593 nm, is measured by FRAP assay. The antioxidant potential is proportional to a decrease in absorbance (Benzie and Strain 1996). For reaction mixture, 0.2 mL of extract and 3.8 mL of *reagent* 27 are mixed, followed by incubation at 37 °C for 30 min. Absorbance is taken at 593 nm. Positive controls used are BHA, BHT, ascorbic acid, quercetin, or catechin (Benzie and Strain 1996).

10.6.4 Anticancer Activity

A number of methods are available to investigate the anticancer potential of plants such as 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H tetrazolium-5-carboxyanilide inner salt (XTT) assay, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay, lactic acid dehydrogenase (LDH) assay, sulforhodamine B (SRB) assay, Resazurin cell growth inhibition assay, and trypan blue dye exclusion assay. The description of reagents is provided in Table 10.2.

10.6.4.1 MTT (3-[4,5-Dimethylthiazole-2-yl]-2,5-Diphenyltetrazolium Bromide) Assay

One of the preliminary assays to test toxic effects of a compound or drug on various cell lines is MTT assay. It is an extremely fast, reliable, convenient, and economical method (McCauley et al. 2013) to determine the number of viable cells. Anticancer potential of callus extract of *Lantana camara* was tested on cervical cancer cell line (HeLa) cells using normal fibroblast cell lines (BHK-21) as control in the author's laboratory. The HeLa and BHK-21 cells were grown on T-25 culture flasks in 10% Dulbecco's modified Eagle medium (DMEM). The cells were harvested by trypsinization, followed by seeding in 96-well plates (density $\approx 10^4$ cells/mL) and incubated for 24 h. Thereafter, cells seeded in 96-well plate were washed with phosphate-buffered saline (PBS) followed by addition of different concentrations of callus extract (2.5–200 μg/well) and curcumin (positive control; 1–10 μg/well). Plates were incubated for various time intervals like 24, 36, 48, 60, and 70 h, followed by addition of *reagent* 28 and 4-h incubation. Thereafter, addition of dimethyl sulfoxide (DMSO) dissolves the formazan granules formed inside the living cells and absorbance is measured at 570 nm via multi-plate reader (Srivastava et al. 2010).

10.6.4.2 XTT (2,3-Bis[2-Methoxy-4-Nitro-5-Sulfophenyl]-2*H* Tetrazolium-5-Carboxyanilide Inner Salt) Assay

This assay depends on the splitting of yellow tetrazolium salt (XTT) for the formation of an orange formazan dye by mitochondrial enzyme, formazan dehydrogenase, in living cells. It measures the amount of viable cells by spectrophotometry. Cells were grown in medium supplemented with 10% fetal bovine serum (FBS) in 96-well plates until 70–80% confluency followed by drug treatment and 24-h incubation. Each well is supplied with 50 mL of XTT followed by 4-h incubation at 37 °C. Thereafter, formazan dye is solubilized using aqueous solutions and optical density is measured at 450 nm and is compared with control wells by a multi-well spectrophotometer enzyme-linked immunosorbent assay (ELISA) reader.

10.6.4.3 MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2*H*-Tetrazolium) Assay

Like MTT, it is also a reliable, convenient, and economical method for cytotoxicity determination. This method is performed in similar way like MTT but *reagent* 30 is used to perform this assay (McCauley et al. 2013). Some of the routine in vitro assays which are performed to investigate the bioactivity of in vitro cultures are provided in Table 10.3.

10.6.4.4 Trypan Blue Dye Exclusion Assay

It is the most commonly performed test to assess the viability of cells. For this assay, cells are washed with *reagent* 31 and centrifuged for 10–15 min at 10,000 rpm and this entire step is repeated thrice. Cells are added to *reagent* 31 and cell count is adjusted to 1×10^6 cells/mL. These cells were transferred into Eppendorf tubes (2×10^5 cells in 0.1 mL), followed by addition of plant extracts and incubation for 3 h at 37 °C. Thereafter, these treated cells were mixed with *reagent* 32, incubated for 1 min, and assessed for their viability using hemocytometer. The growth inhibition percentage is calculated by Eq. (10.5):

$$\%\text{Growth inhibition} = \left(\frac{\text{Total cells} - \text{Dead cells}}{\text{Total cells}} \right) \times 100 \tag{10.5}$$

10.6.4.5 Resazurin Cell Growth Inhibition Assay

Resazurin cell growth inhibition assay or Alamar blue assay measure the cellular viability as well as function of mitochondria. Cells harvested from tissue culture flasks were treated with *reagents* 34 and 35 for 5 min. Subsequently, cells were washed with PBS, counted, seeded in 96-well plate containing 5×10^3 cells/well, and incubated for overnight growth. Then, cells were treated with samples and kept for 48-h incubation, followed by addition of 20 μL of *reagent* 36 and further incubation for 1–2 h at 37 °C. Fluorescence of 96-well plate is measured by multi-plate reader at excitation and emission wavelength of 540 and 590 nm, respectively, and inhibitory concentration (IC_{50}) values are calculated. The IC_{50} value is the amount of anticancer drug (here, plant extract) needed to inhibit 50% of cell proliferation (Kuete et al. 2011).

10.6.5 Anthelmintic Activity

This assay is performed in author's laboratory using the protocol described by Singh et al. (2014). Live fluke worms, commonly parasitizing inside the rumen of cattle livestock, were collected in 0.9% phosphate-buffered saline (PBS; 8 g NaCl, 1.21 g

Table 10.3 In vitro assays to investigate the bioactive potential of in vitro cell cultures

S. No.	In vitro plant/ part/callus	In vitro assay	Inference	References
1	In vitro leaf callus extract of *Spilanthes acmella* Murr.	Anthelmintic (time of paralysis)	Aqueous extract of in vitro callus showed stronger anthelmintic activity than extract of field-grown plant	Singh et al. (2014)
2	In vitro leaf callus extract of *Spilanthes acmella* Murr.	Antioxidant (1,1-diphenyl-2-picrylhydrazyl: DPPH)	Methanol extract of *Spilanthes acmella* possesses antioxidant activity with IC_{50} = 1342.9 µg/mL	Singh et al. (2014)
3	In vitro flower callus extract of *Spilanthes acmella* Murr.	Schizonticidal	In vitro flower callus extract showed significant antimalarial activity	Rajendran et al. (2017)
4	In vitro leaf callus extract of *Lantana camara* L.	Anticancer (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide: MTT)	Aqueous fraction showed anticancer activity at 36 h (100 µg/mL) to 72 h (25 µg/mL) whereas ethyl acetate fraction at 1500–3000 µg/mL	Srivastava et al. (2009, 2010)
5	In vitro leaf extracts of 14 plants	Anticancer (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide: MTT)	Extract showed activity against NCI-H292 (lung cancer) cell line	Gomes de Melo et al. (2010)
6	In vitro callus of *Decalepis*	Antioxidant ferric reducing antioxidant power (FRAP)	In vitro callus of *Decalepis* showed significant antioxidant activity with IC_{50} = 20 ± 1.54 µg/mL	Umesh (2014)
7	In vitro leaf, stem and root callus extract with wild leaf, stem and root of *Tephrosia tinctoria*	Antioxidant (1,1-diphenyl-2-picrylhydrazyl: DPPH) and anticancer (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide: MTT)	More amount of anticancer compound (phenol, 2,4-bis (1,1-dimethylethyl)) is obtained in stem callus extract and root callus extract showed significant inhibition of cell growth of HepG2 cells (IC_{50} = 20 µg/mL) after 72-h treatment	Rajaram et al. (2013)
8	In vivo and in vitro leaf callus cultures of *Baliospermum montanum*	Antibacterial (well-diffusion method)	Ethanol extract of in vitro leaf callus showed the maximum antimicrobial activity than in vivo leaf	Johnson et al. (2010)

K_2HPO_4, and 0.34 g KH_2PO_4, pH 7 ± 0.3). Thereafter, the worms were incubated at 37 ± 1 °C in PBS supplemented with 1% dimethyl sulfoxide (DMSO) with extract concentrations of 10, 20, and 30 mg/mL or without extract (control) and time taken for paralysis and death of worm is recorded. Distocide (drug) is used as reference.

10.6.6 Schizonticidal Activity

Plasmodium falciparum is a protozoan parasite which causes malaria in humans. It is the most fatal strain of genus plasmodium (Rich et al. 2009). Malaria is transferred by biting of female anopheles mosquito. Wild natural flowers and in vitro flower callus were utilized for extract preparation followed by isolation of two important metabolites, i.e., spilanthol and UDA from these extracts. *P. falciparum* 3D7 parasites were initially maintained in red blood corpuscles (RBCs) and later transferred to RPMI 1640 media (25 mM HEPES, 0.4% glucose, 0.2% sodium bicarbonate, 0.5% albumax, 50 mg/L hypoxanthine, 40 μg/mL gentamycin, and 25 μg/mL amphotericin B, at 37 °C). The synchronous development of erythrocytic stages of these parasites is observed on transferring to 5% D-sorbitol. These parasitic cultures were then incubated with different concentrations (0–50 μg/mL) of extract as well as isolated metabolites followed by 36-h incubation (Parveen et al. 2013; Rajendran et al. 2017). A thin blood smear is prepared and stained with Giemsa and number of schizont-containing RBCs was counted under microscope (Lambros and Vanderberg 1979; Zhang et al. 2015).

10.7 Conclusion and Future Prospects

Plants are an outstanding source of medicine. As mentioned in Sect. 10.2, the major benefit of using plants as drugs is that they rarely show any lethal or toxic effects to cells which is a major issue with most of the synthetic drugs. Therefore, establishment of in vitro cultures and investigating their potential by using various in vitro assays is a route to conquer many life-threatening diseases. To conclude, if explored suitably, they can even replace the allopathic drugs.

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Chapter 11
Antioxidant, Antimicrobial, Analgesic, Anti-inflammatory and Antipyretic Effects of Bioactive Compounds from Passiflora Species

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11.1 Introduction

More than 500 species are known to exist in genus Passiflora which is the largest in the family of Passifloraceae. Passion fruit possesses strong aroma and is consumed widely as fresh fruit or in the production of industrialized juice and other fruit products including jam and jellies. It has also been utilized as an ingredient for dessert, ice cream, candy and gourmet preparations. Worldwide, this plant species have been used extensively in the traditional medicine system as well as in the therapeutic system. The species of this genus are distributed in the warm temperate and tropical regions. Despite some records of species in India, China, Australia and the Pacific islands, the majority of Passiflora species are found in tropical regions, and among them, the most prominent species is *Passiflora edulis* Sims, the edible passion fruit (Cerqueira-Silva et al. 2014a; Cutri et al. 2013). This tropical fruit with intense, delightful, peculiar aroma and flavour owes its name to the shape of its beautiful flowers, of which the early Christian missionaries to South America described it as the symbol of the Passion of Christ (Miroddi et al. 2013).

The most popular edible passion fruit species are the *Passiflora edulis* Sims (purple passion fruit), *Passiflora ligularis* Juss (granadilla), *Passiflora alata* Curtis (sweet passion fruit), *Passiflora mollisima* (Kunth) Spreng, (Banana passion fruit) and the *Passiflora edulis* var. *flavicarpa* Degenerer (yellow passion fruit) (Fig. 11.1). The *P. edulis* forma Flavicarpa, known as "sour passion fruit", is also widely commercialized (Carr 2013). Passion fruit is rich in minerals (calcium and phosphorus) and vitamins, especially A, C, thiamine, riboflavin and niacin. It is also a good

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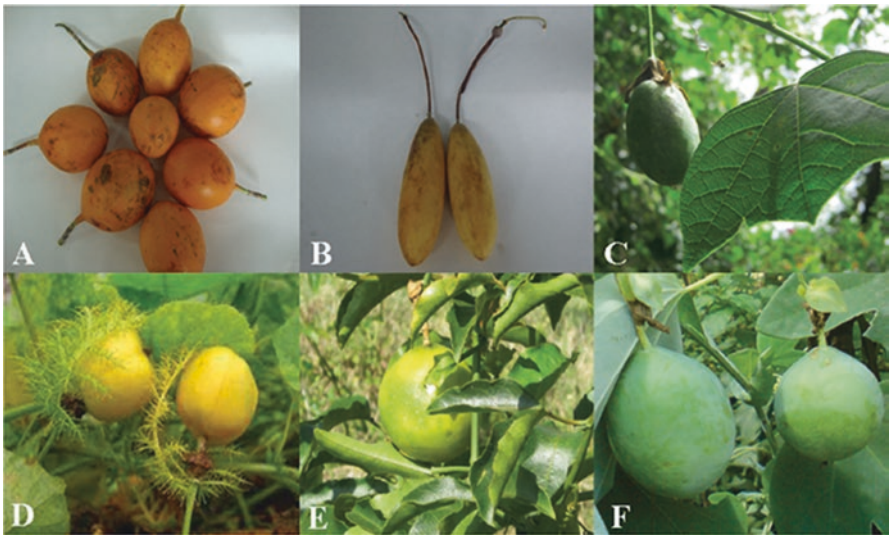

Fig. 11.1 Images of various Passiflora species. (**a**) *Passiflora ligularis* (Granadilla); (**b**) *P. mollisima* (Banana fassion fruit); (**c**) *P. leschenaultii*; (**d**) *P. foetida*; (**e**) *P. edulis* (yellow passion fruit); (**f**) *P. subpeltata* (white passion flower)

source of carotenoids, anthocyanins and alkaloids (Jiménez et al. 2011; Porto-Figueira et al. 2015). The fruits also contain significant amounts of β-carotene, calcium, phosphorus, and ascorbic acid per 100 g of edible part, as much as low fat contents and low caloric contributions. In addition to the expected composition variations among the different Passiflora species, the nutritional value of these fruits as well as their phenolic content and antioxidant capacities may vary according to cultivars, the fruit ripeness and edaphoclimatic factors (Casierra-Posada and Jarma-Orozco 2016; Devi Ramaiya et al. 2013).

Passiflora species are known to possess a large number of functional properties which include: antioxidant (Dhawan et al. 2004; Zeraik and Yariwake 2010), anti-diabetic (Colomeu et al. 2014; Saravanan and Parimelazhagan 2014), anti-inflammatory, antipyretic and analgesic (Saravanan et al. 2014), sedative and hypotensive properties (Cazarin et al. 2014). Recent reports documented also that fruits of Passiflora have neuroactive functions, demonstrating antianxiety and anti-convulsant effects (Dembitsky et al. 2011; Porto-Figueira et al. 2015; Xu et al. 2016). Furthermore, peel flour of *Passiflora edulis* fruit is used for antiretroviral therapy for the HIV patients (do Socorro Fernandes Marques et al. 2016). Similarly, several biotechnological consumptions of the Passiflora genus and correspondent industrial co-products have been contemplated to enhance anti-diabetic and enzyme regulation reported previously (Almeida et al. 2015; Machado et al. 2008; Ueatrongchit et al. 2010; Zilly et al. 2011). Of late, the scientific papers regarding Passiflora spp. have dealt with unravelling taxonomic and conservation issues (Pereira et al. 2015; Porto-Figueira et al. 2015; Wosch et al. 2015).

In this perspective, the present chapter aims to summarize and evaluate the recent findings related to antioxidant, antimicrobial, analgesic, anti-inflammatory, antipyretic effects and biotechnological applications of Passiflora spp., with special attention to the potentialities of their bioactive compounds.

11.2 Biodiversity and Taxonomy of Genus Passiflora

The Passiflora genus plants are shrubs and herbs, mostly climbers with auxiliary tendrils. According to Cerqueira-Silva et al. (2014b), the number of estimated species in the Passifloraceae family varies between 520 and 700. Taxonomical inconsistencies aside, Passiflora, the principal genus, is indubitably diverse. Being tropical, this genus includes more than 500 species distributed in five continents, the Galapagos Islands, and the islands of the Pacific Ocean, among which at least 120 species are native to Brazil and about 50 of them present potential commercial value (Cerqueira-Silva et al. 2014a, b; Pereira et al. 2015). The universal morphology of Passiflora genus has been extensively reviewed. The stem is herbaceous or woody, generally climbing, very rarely arborescent. Alternate leaves, sometimes simple, entire, lobed or palmate, sometimes compound, imparipinnate; stipules germinate at the base of petioles, rarely absent; tendril axillary, arising from sterile pedicels; Flowers bisexual or unisexual, regular; Calyx of 3–5 free or basely connate, imbricate sepals; Corolla of 3–5 free or basely connate petals, rarely absent. Stamens 3–5 (10) inserted either at the bottom of the perianth; ovary superior, more or less stipitate, very rarely sessile, unilocular, of 3–5 united carpels containing several or many anatropous ovules on parietal placentas. Styles equal in number to the placentas. The size of the flowers and the degree of complexity in the corona vary widely throughout the genus. The innermost row of the corona, the operculum, interacts with a membrane (limen) at the base of the androgynophore to form a lip or cup over the nectary preventing access by ineffective pollinators. These three characters, corona, operculum, and limen, have historically been heavily relied upon as taxonomic characters for delimiting relationships within Passiflora fruit 1-celled, an indehiscent berry or a capsule with 3–5 semi-placentiferous valves with numerous seeds (Dhawan et al. 2004).

The leaves of most of the members of the genus Passiflora are dorsiventral in structure. However, *P. mooreana* Hook. and *P. reticulate* Engl. have centric or almost centric structure. The mesophyll shows only a few special features. The middle layers of spongy tissues contain thickened and pitted cells; in *P. arborea* Spreng. and *P.citrifolia* Mast., numerous spicular cells with an irregular course occur in the mesophyll. *P. leschenaultii* also have the morphological variation in their leaves which looks like a half moon shape. The wood of Passiflora species is characterized by variable breadth of medullary rays. Broad medullary rays occur both in climbing species and in those which do not climb (*P. arborea* Spruce). In the genus Passiflora, many of the species which climb by means of tendrils, especially in *P. spicata* Mart (Dhawan et al. 2004; Zeraik and Yariwake 2010).

11.3 Ethnopharmacology

The use of Passiflora as a medicine was discovered for the first time by a Spanish researcher Monardus in Peru in 1569 as the beautiful flowers of Passiflora appeared to him to be symbolic of the passion of Christ (Taylor 1996). Various species of Passiflora have been used extensively in the traditional system of therapeutics in many countries. In Brazil, the said species, known as "Maracujá" has been put to use as an anxiolytic, sedative, diuretic and an analgesic (Oga et al. 1984). *Passiflora caerulea* (blue passion flower), native of Brazil and introduced into Britain in seventeenth century, is the most vigorous and tender species having traditional use of its fruit as a sedative and anxiolytic (Hickey and King 1988; Kirtikar and Basu 1975; Rendle 1959). *Passiflora caerulea* was used medicinally in Uruguay, but no details are available (Watt and Breyer-Brandwijk 1962).

Passiflora species are commonly used for their fruits and their products, and as ornamental and medicinal plants. The most widely grown are the yellow and purple passion fruit (*P. edulis* Sims). In Brazil, *Passiflora alata* Curtis. is the second most cultivated species of the genus. Consumption of some wild species, such as *P. cincinnata, P. nitida, P. quadrangularis,* and *P. setacea* have already been reported (Cerqueira-Silva et al. 2014a, b). The use of passion fruit as an ornamental plant is due to the diversity and exoticness of its leaves, flowers, and fruits. Although its ornamental value is still little explored in Brazil, it is highly valued in the United States and certain European countries (Abreu et al. 2009). The use of Passiflora species as medicinal plants has been well documented. The leaves, flowers, roots and fruits of wild and commercial species are used in folk medicine for the treatment of insomnia, anxiety, helminthic infestations and stress (Dhawan et al. 2004; Doyama et al. 2005; Ferreres et al. 2007; Miroddi et al. 2013; Zeraik et al. 2011). In addition, passion fruit is a rich source of alkaloids, flavonoids, saponins, essential oils and carotenoids, as well as minerals, fibres and vitamins A, C and D (Ulmer and MacDougal 2004), beneficial to human health. Besides their pharmaceutical significance, the Passiflora also have potential for exploitation by the cosmetic industries.

There have been many reports about studies on the neuropharmacological activities of the species of Passiflora. *P. incarnate* was confirmed by all the related reports to be anxiolytic and sedative (Dhawan et al. 2001; Soulimani et al. 1997; Speroni and Minghetti 1988). The extracts of *P. edulis* are known to be active in rodent model of anxiety (Coleta et al. 2006; Petry et al. 2001). However, Dhawan et al. (2001) concluded that *P. edulis* was devoid of anxiolytic activity. *P. foetida* L. (Stinking passion flower) is South American in origin, which has been spread to many tropical areas. It is found in riverbeds, dry forest floors, and wayside thickets, covering the top thorny shrubs and also growing near hamlets. The ethnobotanical views of *P. foetida*, suggest that decoction of leaves, fruits are used for the treatment of asthma and biliousness, leaves, root decoction is emmenagogue, used for hysteria, and leaf paste is applied on the head for giddiness and headache (Nwosu 1999). The plant is said to be used for curing itches. The major phytoconstituents of this plant are alkaloids, phenols, glycosides, flavonoids and cyanogenic compounds (Dhawan et al. 2004) and passifloricins, polypeptides and alpha-pyrones in *P. foetida.* Several years

ago a flavonoid compound in the resin of leaves of *P. foetida* was screened which exhibited anti-feedant activity in in vitro assays against the phytophagous larvae of *Dione juno* (Echeverri et al. 2001).

P. leschenaultii DC belongs to the family Passifloraceae which possesses rich polyphenolic compounds with antioxidant, analgesic, anti-inflammatory and antipyretic properties (Shanmugam et al. 2016a). Traditionally, the plant leaves, fruit juices and seeds have been used to treat dysentery, urinary stone disease, wound healing and other ailments (Farook et al. 2009; Ignacimuthu et al. 2008; Salave 2012). In an earlier study that identified and quantified the functional compounds from acetone extract of leaves of this plant through HPLC-DAD-MS analysis, it was reported that the leaves extract possessed higher amounts of polyphenolic compounds such as hyperin (quercetin-3-*O*-galactoside), chlorogenic acid, caffeic acid and rutin (Shanmugam et al. 2016a). In addition, the pretreatment with acetone extract of *P. leschenaultii* leaves could attenuate acute paracetamol-induced hepatic injury in rats. Probably this action is due to multiple mechanisms involving the elimination of the free radicals and thus inhibiting the elevation of serum biochemicals due to the presence of major phytochemical compounds. Furthermore, the modulation of PXR and FXR might play an important role in deteriorating the disease level.

Among the various Passiflora species, *P. subpeltata* (Synonym *P. calcarata*) commonly known as white passion flower; is found to grow in the higher altitudes. The plant bears attractive flowers and their fruits and leaves are edible. In Western Ghats of India, *P. subpeltata* leaves are widely consumed as a leafy vegetable. They are combined with *Centella asiatica*, *Trianthema portulacastrum* or with crabs and fish as a healthy diet by the tribal peoples of Kurumba, Paniya and Kattunaikka living across the Western Ghats of India. Traditionally, leaves of this plant are also used to treat pain and inflammation related health disorders (Ratheesh Narayanan and Anil Kumar 2007). A coumarin derivative (cyanogenic glycoside barterin) has been isolated from the leaves of *P. subpeltata* earlier (Olafsdottir et al. 1997). This compound is reported to possess sedative and anti-plasmodic properties. The prior report stated that the seeds of this plant species possess antioxidant and antimicrobial properties (Saravanan and Parimelazhagan 2013). Moreover, the phenolic and flavonoid compounds like gallic acid, apigenin, catechin and coumarin derivatives cyanogenic glycosides have been identified and described earlier (Olafsdottir et al. 1997; Saravanan et al. 2014). Previous report also revealed that the acetone extract of leaves of *P. subpeltata* exhibit antioxidant, analgesic, antipyretic and anti-inflammatory properties (Saravanan et al. 2014). Moreover, a recent study reveals that the acetone extract of *P. subpeltata* leaves possesses strong hepatoprotective activity. The changes resulting from the paracetamol induction in the rat serum biochemical and enzymatic antioxidant levels were retained to normal level in the plant extract treated animals. It might be due to the presence of luteolin and quercetin 3-β-D-glucoside compounds. The most important mechanisms underlying the defensive effects of *P. subpeltata* might be due to its alleviation of cellular antioxidant enzymes like catalase, superoxide dismutase (SOD), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione *S*-transferase (GST) and inhibition of lipid peroxidation (Shanmugam et al. 2016b).

P. mollissima (Kunth) L. H. Bailey, commonly known as "curuba de Castilla" or "banana passion fruit", is a native species of the southern Andes. It grows between 2000 and 3000 m above sea level as a vigorous climber, in climates with average temperatures varying from 13 and 16 C (Lobo Mario 2009). The fruit has an oval form of about 8–15 cm length, its skin is thin but consistent, and pale yellow when ripe. The pulp is gelatinous and surrounds small black seeds. Its flavour is smooth and pleasant, with an acid taste. *P. mollissima* is a source of vitamins A, B and C (93 mg/100 g fresh fruit), calcium, iron, phosphorus, and potassium, and also contains fibre (Leterme et al. 2006). The edible part of the fruit shows a high antioxidant activity in FRAP (114 ± 3.28 μmol TEs/g of FW), ABTS (131 ± 0.64 μmol TEs/g of FW) and a high phenolic compounds content (635 ± 2.71 P mg of GAEs/100 g of FW) (Contreras-Calderón et al. 2011). In Colombia, this fruit has a year-round production and is exported to Europe, because it is versatile and not as perishable as fruits of other species. Usually, the fruit of this species is eaten either fresh or in the form of juices and sorbets. Banana passion fruit may be a particularly good source of bioactive agents because of its relatively high levels of phenolic compounds, carotenoids, and dietary fibres (Gil et al. 2014), which are known to be beneficial to human health and wellbeing (Wootton-Beard and Ryan 2011). Previous studies have shown that dietary fibres from fruits have a positive effect on the treatment of diseases such as hyperlipidaemia, coronary heart disease and certain types of cancer (Kumar et al. 2012). The major source of noncellulosic dietary fibre in fruits is pectins (Voragen et al. 1983), which are acidic heteropolysaccharides, composed mainly of alpha-(1,4)-linked D-galacturonic acid (GalA) residues (Ridley et al. 2001).

11.4 Pharmacological Activities

Since the genus Passiflora contains a large amount of phytoconstituents, a few reports regarding the pharmacological investigations on the plants of this genus are available (Corrêa et al. 2016; Saravanan et al. 2014; Shanmugam et al. 2016a, b). Most of the pharmacological work has been carried out on the CNS depressant effects, antioxidant and radical scavenging activities of various species. A group of Brazilian researchers have performed thorough pharmacological studies on *P. alata* leaves using mice as the experimental animals. On intraperitoneal administration to mice at a dose of 150 mg/kg, the *P. alata* extract reduced amphetamine-induced spontaneous motor activity (Oga et al. 1984).

11.4.1 Antioxidant

Oxygen and its importance in living beings are well known as it plays vital role in several biological processes. However, during oxidative stress, oxygen can lead to formation of free radicals (electrically charged molecules having unpaired electron)

including superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and lipid peroxide radicals, on interacting with certain molecules of body during natural processes. Free radicals so formed are also essential at low to moderate concentrations for production of energy to fuel biological processes in most living organisms. When they are generated in higher concentrations they are very harmful to body as they damage or modify the major components of a cell, such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, cell membranes and enzymes and form grounds of various neurodegenerative (Alzheimer and Parkinson) diseases. Moreover, ageing and degenerative diseases of ageing such as cancer, diabetes, rheumatoid arthritis, cardiovascular diseases (atherosclerosis), inflammation, ocular and pulmonary diseases also occur.

Antioxidants are the radical scavengers which help in delaying or prevention of oxidation by trapping the free radicals. The normal vital concentration of the free radicals or reactive oxygen species (ROS) in living organisms is maintained by the enzymatic antioxidants such as glutathione peroxidase, superoxide dismutase, glutathione reductase, catalase and non-enzymic antioxidants like alpha-tocopherol, glutathione and ascorbic acid, which provides protection to the organism against free radical induced oxidative damage (Peuchant et al. 2004). Sometimes these enzymes present in our body are not able to maintain the proper balance of actual required concentration of free radicals due to its unlimited or uncontrolled production and hence leads to several health problems (Steer et al. 2002; Uchida 2000).

In such cases additional antioxidants from outside are required in order to supply to maintain the proper balance between free radicals and enzymatic antioxidants in the body. Many natural and synthetic compounds such as polyphenols, flavonoids, vitamins C and E, and terpenoids have been reported to function as antioxidants due to their ability to donate a hydrogen atom or an electron to chelate redox active metals and inhibit lipoxygenases (Fritz et al. 2003; Oteiza et al. 2005).

Despite the consumption of whole fruits and vegetables intake, typically non-edible parts could also be a good alternative to add dietary bioactive compounds (Leite-Legatti et al. 2012). The literature data indicates that the *P. edulis* leaf extracts possess in vitro and ex vivo antioxidant activity against protein oxidative damage, being considered as new sources of natural antioxidants (Rudnicki et al. 2007). da Silva et al. (2013) reported in their study that aqueous extract of passion fruit (*P. edulis*) leaves has the antioxidant status which was analyzed by FRAP, ORAC in serum and by SOD, GR and GPx activities, GSH contents and TBARS in liver, brain and kidneys.

In our recent studies on the plant species of *P. ligularis* fruit, *P. subpeltata* leaves and seeds, *P. leschenaultii* leaves and *P. foetida* fruits and leaves possess higher amount of antioxidant activity which is analysed by the various in vitro antioxidant assays (Tables 11.1 and 11.2). The obtained results from the *P. leschenaultii* revealed that the acetone extract of leaves exhibited higher phenolic (440.24 mg GAE/g extract) and flavonoid (253.33 mg RE/g extract) contents and scavenged the $DPPH^{\bullet}$ (IC_{50} 29.14 μg/mL), $ABTS^{+\bullet}$ (10509.69 μM TEAC/g extract) effectively (Shanmugam et al. 2016a) while the acetone extract of *P. subpeltata* seed recorded highest total phenolic (340.70 mg GAE/g extract) and tannin (214.30 mg GAE/g extract) contents

Table 11.1 Total phenolics, tannin and flavonoid contents of Passiflora species

Content	Solvents	*P. subpeltata*		*P. leschenaultia*	*P. ligularis*	*P. foetida*	
		Leaves	Seeds	Leaves	Pulp	Leaves	Seeds
Total phenolics (mg GAE/g extract)	Petroleum ether	115.13 ± 8.42^{c}	122.90 ± 1.89^{c}	125.24 ± 1.80^{e}	102.40 ± 1.89^{c}	7.8 ± 0.1	5.0 ± 0.0
	Chloroform	113.45 ± 6.19^{c}	137.90 ± 3.52^{b}	141.67 ± 1.49^{d}	125.70 ± 2.60^{d}	–	–
	Acetone	417.65 ± 7.33^{a}	340.70 ± 4.95^{a}	440.24 ± 0.82^{a}	640.70 ± 2.95^{a}	–	–
	Methanol	227.17 ± 10.97^{b}	115.70 ± 3.60^{d}	290.00 ± 0.71^{b}	137.90 ± 1.52^{b}	–	–
	Ethanol	–	–	–	–	4.8 ± 0.2	4.5 ± 0.1
Tannin content (mg GAE/g extract)	Petroleum ether	48.18 ± 10.19^{b}	31.90 ± 1.32^{c}	20.24 ± 2.97^{d}	38.60 ± 1.89^{c}	3.1 ± 0.2	0.6 ± 0.1
	Chloroform	25.77 ± 4.20^{c}	67.90 ± 5.02^{b}	22.38 ± 0.82^{d}	65.23 ± 2.93^{b}	–	–
	Acetone	182.91 ± 11.44^{a}	214.30 ± 2.95^{a}	229.29 ± 1.24^{a}	234.30 ± 4.50^{a}	–	–
	Methanol	40.90 ± 5.47^{b}	60.23 ± 2.93^{b}	128.81 ± 0.41^{b}	67.90 ± 3.02^{b}	–	–
	Ethanol	–	–	–	–	0.004 ± 0.3	0.7 ± 0.1
Total flavonoids (mg RE/g extract)	Petroleum ether	166.22 ± 10.78^{b}	228.33 ± 2.31^{b}	141.67 ± 11.55^{c}	218.33 ± 1.31^{b}	–	–
	Chloroform	72.22 ± 4.44^{d}	128.00 ± 1.00^{c}	90.00 ± 5.00^{e}	178.00 ± 2.00^{c}	–	–
	Acetone	241.33 ± 6.11^{a}	287.33 ± 1.08^{a}	253.33 ± 7.64^{a}	387.33 ± 1.08^{a}	–	–
	Methanol	121.11 ± 15.79^{c}	231.31 ± 1.50^{b}	210.00 ± 5.00^{b}	233.33 ± 0.53^{b}	–	–

Values expressed as mean ± standard deviation ($n = 3$)
Mean values followed by different superscript letters (a, b, c, d, e) in a column indicate significant statistical difference ($p < 0.05$)
GAE gallic acid equivalent, *RE* rutin equivalent

Table 11.2 $ABTS^{\bullet+}$ scavenging, FRAP, metal chelating and phosphomolybdenum activities of various Passiflora species

Content	Solvent	*P. subpeltata*		*P. leschenaultia*	*P. ligularis*
		Leaves	Seeds	Leaves	Pulp
$ABTS^{\bullet+}$ (μM TEAC/g extract)	Petroleum ether	1430.82 ± 14.58^{b}	1887.69 ± 12.59^{d}	3054.36 ± 4.59^{c}	2020.24 ± 4.48^{d}
	Chloroform	2620.84 ± 6.80^{b}	1942.24 ± 8.21^{c}	2973.36 ± 5.27^{d}	2452.74 ± 5.86^{c}
	Acetone	10108.91 ± 3.28^{a}	3276.98 ± 3.55^{a}	10509.69 ± 3.80^{a}	9800.94 ± 3.80^{a}
	Methanol	3357.60 ± 6.98^{b}	3074.23 ± 5.81^{b}	6349.46 ± 8.23^{b}	5228.17 ± 17.44^{b}
FRAP (mM Fe (II) E/g extract)	Petroleum ether	425.56 ± 26.94^{c}	30.11 ± 0.78^{d}	528.77 ± 15.47^{c}	20.51 ± 0.18^{d}
	Chloroform	3698.89 ± 25.05^{a}	28.12 ± 0.28^{c}	587.17 ± 5.85^{c}	22.82 ± 0.18^{c}
	Acetone	3863.33 ± 20.24^{a}	63.03 ± 0.10^{a}	1511.74 ± 20.25^{a}	43.06 ± 0.15^{a}
	Methanol	1841.11 ± 18.25^{b}	45.20 ± 1.15^{b}	1107.24 ± 20.62^{b}	35.30 ± 0.15^{b}
Metal chelating activity (mg EDTAE/g extract)	Petroleum ether	155.37 ± 16.18^{a}	45.22 ± 1.55^{d}	156.31 ± 2.07^{c}	61.32 ± 0.55^{d}
	Chloroform	124.61 ± 13.19^{a}	39.15 ± 1.78^{c}	175.05 ± 0.72^{b}	79.25 ± 0.78^{c}
	Acetone	236.72 ± 11.59^{a}	144.23 ± 0.20^{a}	279.41 ± 0.60^{a}	134.53 ± 0.30^{a}
	Methanol	202.66 ± 17.89^{a}	123.29 ± 0.10^{b}	234.13 ± 0.20^{a}	113.79 ± 0.70^{b}
Phosphomolybdenum assay (mg AAE/g extract)	Petroleum ether	–	–	32.93 ± 0.61^{d}	–
	Chloroform	–	–	48.00 ± 1.44^{c}	–
	Acetone	–	–	250.27 ± 1.80^{a}	–
	Methanol	–	–	123.33 ± 5.00^{b}	–

Values expressed as mean ± standard deviation ($n = 3$)
Mean values followed by different superscript letters (a, b, c, d) in a column indicate significant statistical difference ($p < 0.05$)
TEAC trolox equivalent antioxidant capacity, *Fe (II) E* Fe (II) equivalent, *EDTAE* ethylene diamine tetra acetic acid equivalent, *AAE* ascorbic acid equivalent

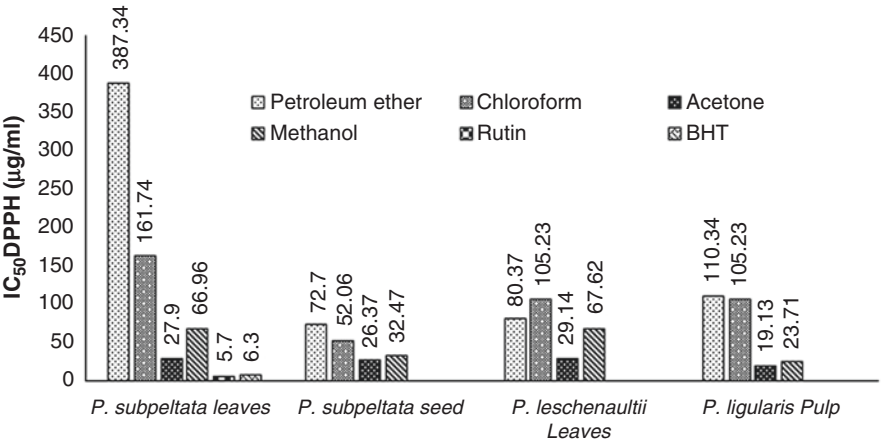

Fig. 11.2 DPPH radical scavenging activity of Passiflora species. Values expressed as mean ± standard deviation ($n = 3$). (Source: Adapted from the publications by Shanmugam et al., (2016a) and Saravanan et al. (2013, 2014))

and possess high antioxidant activity (IC_{50} value of DPPH$^{\bullet}$ being 26.37 µg/mL) while the superoxide radical scavenging activity was 75.18% (Saravanan and Parimelazhagan 2013). At the same time, the acetone extract of *P. subpeltata* leaves registered higher total phenolic (417.65 mg GAE/g extract) and tannin (182.91 mg GAE/g extract) contents and exhibited highest DPPH$^{\bullet}$ (IC50 of 27.9 µg/mL) and ABTS$^{\bullet+}$ (10108.91 µM trolox equivalent/g extract) scavenging activities (Saravanan et al. 2014).

Furthermore, the fruits of *P. ligularis* also revealed that among the various solvents, acetone extract displayed maximum total phenolic (640.70 mg GAE/g extract), tannin (214.30 mg GAE/g extract) and flavonoid contents (387.33 mg RE/g extract). The acetone extract has an efficient DPPH$^{\bullet}$ value of IC_{50} 19.13 µg/mL, ABTS$^{\bullet+}$ value of 9800.94 µmol/L Trolox equivalent/g extract, superoxide (78.27%), nitric oxide (79.95%) radical scavenging activities along with ferric reducing antioxidant power (43.06 mmol Fe (II)/mg extract) and metal chelating (134.53 mg EDTA/g extract) abilities (Saravanan and Parimelazhagan 2014). The in vitro antioxidant activities of petroleum ether, ethanol and hot water extracts of *P. foetida* root, leaves, flower, fruit peel and seeds showed better antioxidant properties analysed by various in vitro assays such as reducing power, metal chelating, hydroxyl radical scavenging, nitric oxide radical scavenging, ABTS$^{\bullet+}$, DPPH$^{\bullet}$, antihaemolytic and β-carotene assays (Sasikala et al. 2011a) (Fig. 11.2).

Alessandra et al. (2006) reported that Passiflora species, namely, *P. nitida, P. foetida*, *P. tenuifila*, *P. coriacea* and *P. palmeri* showed good antioxidant capacity with the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging property. Zeraik et al. (2011) studied that antioxidant activity of the plant species of *P. edulis* and *P. alata* by stimulated neutrophils and myeloperoxidase activity assays. Oliveira et al. (2016) studied different methodologies for recovering fatty acids and phenolic compounds from the seeds and seed cakes discharged by both passion fruit juice and seed oil industries. They reported promising antioxidant potentials of ethanolic extracts of these co-products of the *P. edulis* species.

11.4.2 Antimicrobial

Clinical microbiologists have two reasons for being interested in plant extracts with antimicrobial activity. In the first place, it is very likely that the phytochemicals found have a role in the arsenal of antimicrobial agents. Despite the development of numerous antimicrobial drugs in recent years, the incidence of multi drug resistance by pathogenic microorganisms has increased. The therapeutic properties of plant polyphenols have also demonstrated antimicrobial effects by causing structural or functional damage to the bacterial cell membrane. Several studies have also shown that fruits rich in polyphenols exert both antioxidant and antimicrobial effects (Puupponen-Pimia et al. 2001).

The *in vitro* antimicrobial activities of the different solvents extracts of *P. ligularis* fruit pulp against the tested microorganisms *Salmonella paratyphi*, *S. typhi* A, *S. typhi* B, *Klebsiella pneumonia*, *Candida albicans* and *Aspergillus niger* were qualitatively assessed by the inhibition zones. According to the results, the acetone extract of *P. ligularis* fruit pulp exhibited a potent inhibitory effect against all bacteria especially *S. paratyphi* (28 mm), *S. typhi* A (31 mm), *S. typhi* B (32 mm) and *K. pneumonia* (32 mm). The acetone extract showed more inhibition against *C. albicans* (14.85 mm) and *A. niger* (13.91 mm). The greater inhibition zone, indicate the greater antibacterial and antifungal activity of the extract. The results from the disc diffusion method indicated that the tested *P. ligularis* fruit pulp extracts have higher inhibition of antibacterial and antifungal effects against both Gram positive and Gram-negative bacteria and fungal strains. The results were comparable with the standard drug, ampicillin and fluconazole (Saravanan and Parimelazhagan 2014) (Fig. 11.3).

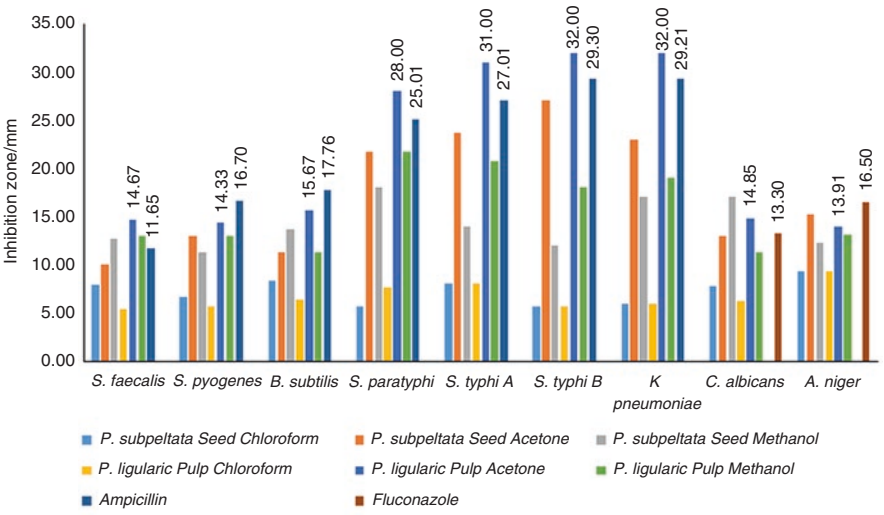

Fig. 11.3 Antimicrobial activity of *P. subpeltata* seed and *P. ligularis* fruit pulp. (Source: Adapted from the publications by Saravanan et al. (2014))

The disc diffusion method of evaluation of antimicrobial activity was tested for *P. subpeltata* seed extract, which showed comparable antibacterial effects against both Gram-positive and Gram negative bacteria. The acetone and methanol extracts were tested against *S. typhi B* and *K. pneumonia.* However, the acetone extract showed better antibacterial activity (27 and 23 mm, respectively) followed by methanol extract (14 and 17 mm, respectively). Moreover, both acetone and methanol extracts of *P. subpeltata* seed exhibited excellent antifungal activity against *A. niger* (13.23 and 15.21 mm). The results were comparable with the standard drugs, ampicillin and fluconazole. Results from the antimicrobial study suggest that phenolic compounds are responsible for the antibacterial and antifungal activities of different extracts of *P. subpeltata* seed (Saravanan and Parimelazhagan 2013).

Recently, Siebra et al. (2018) reported that hydroalcoholic extracts of leaves, stems, bark, pulp and seeds of *P. cincinnata* show the antimicrobial and antibiotic-modifying activity potential by inhibiting the microorganisms such as *Staphylococcus aureus* and *Escherichia coli.* Further, the extracts from both raw materials obtained by supercritical fluid extraction (SFE), low pressure extractions (LPE)—cold maceration (MAC) and ultrasonic assisted extraction (UE) using a mixture EtOH–H_2O were selected to perform the antimicrobial activity analysis of *P. edulis*. The minimum inhibitory concentration (MIC) results show that the extracts were mostly effective against the Gram-positive bacteria (*L. innocua*), compared to the results for the Gram-negative one (*E. coli*) (Oliveira et al. 2016).

The broth microdilution method was used to determine the antimicrobial activity of yellow passion fruit (*P. edulis* var. *flavicarpa*) seeds and pulp fibre (PFSP), passion fruit albedo (PFA) and nisin against gram-positive and gram-negative bacteria. PFSP and PFA showed antimicrobial activity against all the bacteria analyzed. The MIC values of PFSP varied between 6.25 mg/mL for *Aeromonas hydrophila* and 50 mg/mL for *Pseudomonas fragi* and *P. fluorescens*. The rest of the bacteria tested had a MIC value of 12.50 mg/mL. As regards PFA, the MIC values ranged between 3.125 mg/mL for *A. denitrificans* and 50 mg/mL for *P. fragi* and *P. fluorescens*. Other bacteria assayed had an MIC of 25 mg/mL. All the bacteria tested in this assay had higher resistance to PFSP and PFA than to nisin, which is the only bacteriocin widely accepted as a natural food preserver (López-Vargas et al. 2013). Antibacterial activities of the leaves and stems of *P. quadrangularis*, *P. maliformis* and *P. edulis* extracted by using three different solvents: petroleum ether, acetone and methanol were tested using the disc diffusion method against ten human pathogenic bacteria. The largest inhibition zone was observed for the methanol extract of *P. maliformis* against *B. subtilis*. Generally, extracts from the Passiflora species exhibit distinct inhibition against Gram-positive but not Gram-negative bacteria (Ramaiya et al. 2014).

11.4.3 Analgesic

Analgesics are agents which selectively relieve pain by acting in the CNS and peripheral pain mediators without changing consciousness. Analgesics may be narcotic or non-narcotic. Pain is a disabling accompaniment of many medical conditions and pain control is one of the most important therapeutic priorities. Pain has been officially defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. It is always a warning signal and primarily protective in nature but often causes a lot of discomfort and lead to many adverse effects. Analgesics are drugs used to treat or reduce pain and the classical analgesic drugs notably opiates and nonsteroidal anti-inflammatory drugs have their origin in natural products (Raquibul Hasan et al. 2010).

The acetic acid-induced writhing test methods have been postulated as useful technique for evaluating the peripherally acting analgesic drugs (Eddy and Leimbach 1953; Koster et al. 1959; Williamson et al. 1996). Injection of acetic acid into the control mice resulted in 17.25 ± 0.48 writhes. Pretreatment with acetone extract of *P. subpeltata* at doses of 200 and 400 mg/kg reduced the number of writhes to 8.5 ± 0.29 (50.65% inhibition) and 3 ± 0.82 (82.73% inhibition), respectively. Remarkably, the extract at a dose of 400 mg/kg registered higher levels of statistically significant ($p < 0.001$) analgesic activity. The abdominal constriction response induced by acetic acid is a sensitive procedure to establish peripherally acting antinociceptives. The results indicated that the acetone extract of *P. subpeltata* leaves significantly inhibited the number of writhes in comparison to the control group ($p < 0.001$) (Saravanan et al. 2014).

The oral administration of acetone extracts of *P. leschenaultii* showed a dose-dependent analgesic activity. Intraperitoneal administration of acetic acid to the control mice resulted in 17.25 writhings. Pretreatment with acetone extract of *P. leschenaultii* leaves at 200 and 400 mg/kg doses reduced the writhings to 9.50 and 3.25 (44.75% and 81.43% inhibition), respectively. The extracts at a dose of 400 mg/kg registered a significantly ($p < 0.001$) higher level (81.43%) of analgesic activity when compared to control. Regarding formalin test, the results obtained from the acute phase (10 min) showed that morphine and the acetone extracts possessed only mild inhibitory effects. During the delayed phase (30 min), morphine and acetone extracts exhibited significant ($p < 0.001$) analgesic effect. Morphine showed 72.19% of analgesic activity while the acetone extracts showed an analgesic effect of 57.26% and 70.80% at the doses of 200 mg/kg and 400 mg/kg p.o., respectively. In the case of hot plate test, results showed that in early phase period of 30 min, 400 mg/kg of leaves extract and 10 mg/kg of morphine showed a significant ($p < 0.05$) analgesic activity. However, in the late phase period of 120 min, 400 mg/kg of leaves extract (81.96%) and 10 mg/kg of morphine (85.92%), showed very high analgesic activity and increased the reaction time when compared with the control group at significant level ($p < 0.001$). In this study, oral pretreatment with acetone extract of *P. leschenaultii* leaves strongly inhibited both phases especially at the 400 mg/kg dose which reduced significantly ($p > 0.001$) the formalin-induced licking or biting

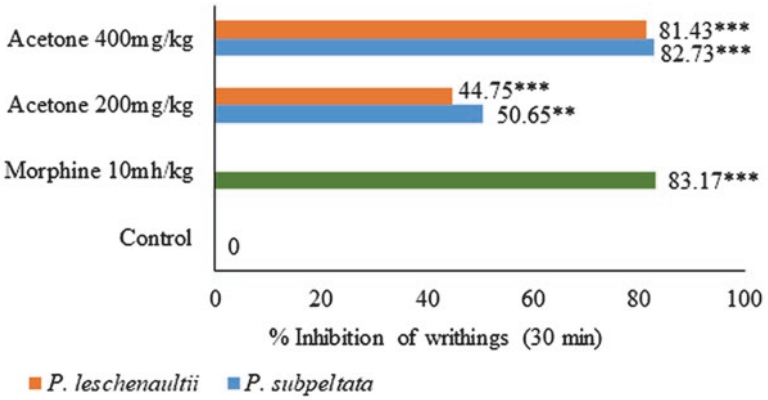

Fig. 11.4 Analgesic effect of acetone extract obtained from leaves of *P. leschenaultii* and *P. subpeltata*, inhibition (%) of writhings by acetic acid-induced writhing test. Values expressed as mean ± SEM ($n = 6$); ***Significantly different at $p < 0.001$ when compared to control. (Source: Adapted from the publications by Shanmugam et al., (2016a) and Saravanan et al. (2014))

responses in mice. Moreover, the plant extract also increases the latency time of the animals on the hot plate test by reducing the licking and jumping responses which prove that the plant extracts stimulate the thermal stability of the animals. The results also suggest that the plant extract counteracts both peripheral neurogenic pain and central inflammatory nociception responses (Shanmugam et al. 2016a).

The analgesic effect of ethanol extract of *P. foetida* leaves using hot plate test indicated that oral administration of the extract (200 mg/kg) significantly attenuated the hot-plate thermal stimulation. Analgesic activity of the extract was comparable to the standard drug, morphine sulphate (5 mg/kg). Among the two doses tested, 200 mg/kg showed highest analgesic activity (13.50 ± 0.43) min at a reaction time of 120 min. Furthermore, it was also observed that the ethanol extract of leaves significantly inhibited the acetic acid induced writhing response in a dose dependent manner. Even a 200 mg/kg dose of plant extract produced 37.50 ± 0.65 writhing as compared to standard dose of acetyl salicylic acid (41.3 ± 31.62) (Sasikala et al. 2011b) (Figs. 11.4 and 11.5).

11.4.4 Anti-inflammatory

Inflammation is a multifarious pathophysiological process intermediated by a variety of signaling molecules produced by leukocytes and macrophages which include phagocytic uptake and the production of inflammatory mediators such as nitric oxide (NO), prostaglandin enzyme (PGE2) and tumour necrosis factor alpha (TNF-α) (Sengar et al. 2015). In the meantime, the production of cytokines either from immune or from central nervous system cells might directly sensitize the peripheral

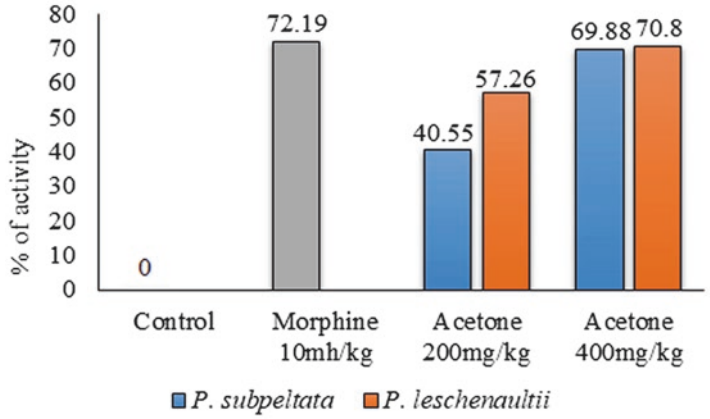

Fig. 11.5 Inhibition (%) of writhings by formalin-induced paw licking analgesic test of *P. subpeltata* and *P. leschenaultia* leaves. Values expressed as mean ± SEM ($n = 6$). (Source: Adapted from the publications by Shanmugam et al., (2016a) and Saravanan et al. (2014))

nociceptors; the prostaglandins (PGs) induce hyperalgesia by affecting the transducing property of free nerve endings, bradykinins, TNF-α, and interleukins (ILs) and induce pain (Obreja et al. 2002).

Ethanol extract of *P. foetida* leaves produced significant higher acute anti-inflammatory effect. Carrageenan induced paw oedema model was used for the evaluation of anti-inflammatory activity of the ethanol extract of *P. foetida* leaves. Carrageenan-induced rat paw oedema was markedly inhibited by plant extract (100 mg/kg) and indomethacin (10 mg/kg). The ethanol extract of leaves produced a significant acute anti-inflammatory effect (1.302 ± 0.079 mL) in mice. Moreover, the ethanol extract of leaves also demonstrated a significant anti-inflammatory effect against histamine-induced inflammation at a dose of 100 mg/kg. The anti-inflammatory effect of the extract was significantly (1.523 ± 0.052) mL less than that of indomethacin (1.576 ± 0.055) mL (Sasikala et al. 2011b).

The anti-inflammatory effect of the acetone extract of *P. subpeltata* leaves on carrageenan-induced oedema in hind paws of the experimental rats shows gradual increase in paw oedema volume of rats in the control (carrageenan treated group). However, in the test groups, the leaves extract showed a significant reduction in the paw oedema volume. The results revealed that the acetone extract of leaves of *P. subpeltata* at 200 and 400 mg/kg exhibited 47.08% and 81.54% inhibition, respectively while indomethacin showed 89.16% inhibition. The development of oedema induced by carrageenan is a biphasic event; the early phase (90–180 min) of the inflammation is due to the release of histamine, serotonin and similar substances and the later phase (270–360 min) is associated with the activation of kinin-like substances and the release of prostaglandins; proteases and lysosome (Olajide et al. 1999). Acetone extract of leaves of *P. subpeltata* inhibited hind paw oedema and showed a dose-dependent anti-inflammatory activity. Plant extract inhibited both

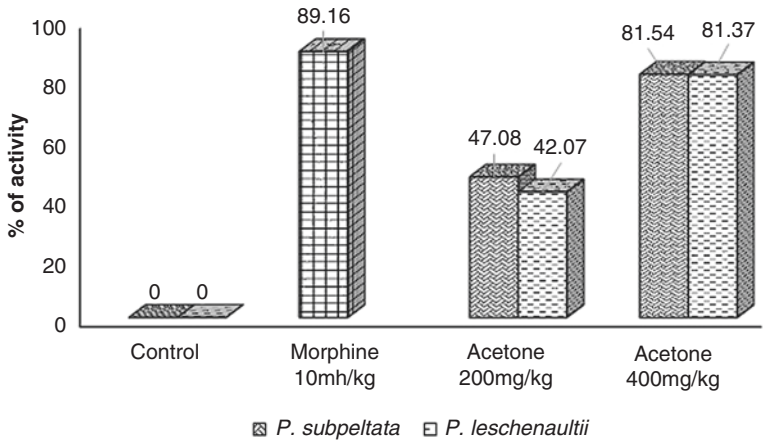

Fig. 11.6 Anti-inflammatory effect of *P. subpeltata* and *P. leschenaultii* leaves acetone extract on carrageenan-induced paw oedema in rats. Values expressed as mean ± SEM (n = 6). (Source: Adapted from the publications by Shanmugam et al. (2014, 2016))

the phases of carrageenan-induced oedema, which may be by reducing the release of histamine and serotonin and also the kinin-like substances and prostaglandins (Saravanan et al. 2014).

The anti-inflammatory effect of the acetone extract of *P. leschenaultii* leaves on carrageenan-induced oedema in hind paws and cotton pellet-implanted chronic inflammatory response of the experimental rats shows gradual increase in paw oedema volume of rats in the control (carrageenan treated group). However, at the same time, the standard drug and plant extracts treated groups showed a significant reduction in the paw oedema volume but these were not significantly different ($p < 0.001$). The results also revealed that the acetone extract of *P. leschenaultii* leaves expressed inhibitory activity at the doses of 200 mg/kg and 400 mg/kg p.o., being 42.07% and 81.37%, respectively. However, standard indomethacin showed highest activity (89.16%) (Fig. 11.6). Meanwhile, in the cotton pellet-implanted chronic inflammatory response the plant extract exhibited a significant ($p < 0.001$) anti-inflammatory effect in a dose dependent manner and these results were comparable to that of standard drug indomethacin. The doses of 200 mg/kg and 400 mg/kg of acetone extracts of *P. leschenaultii* leaves exhibited 56% and 79.04% granuloma inhibition, respectively which is equal to the standard indomethacin (80.31%) at a dose of 10 mg/kg (Fig. 11.7).

Naturally occurring phenolic and flavonoid compounds exert a wide range of pharmacological activities such as antioxidant, anti-inflammatory, analgesic and anti-carcinogenic actions with different mechanism (Liu and Lin 2012). Thus, the quantitative results of acetone extract of *P. leschenaultii* leaves encompass higher amount of phenolics and flavonoid compounds that may be correlated to the anti-inflammatory properties (Shanmugam et al. 2016a).

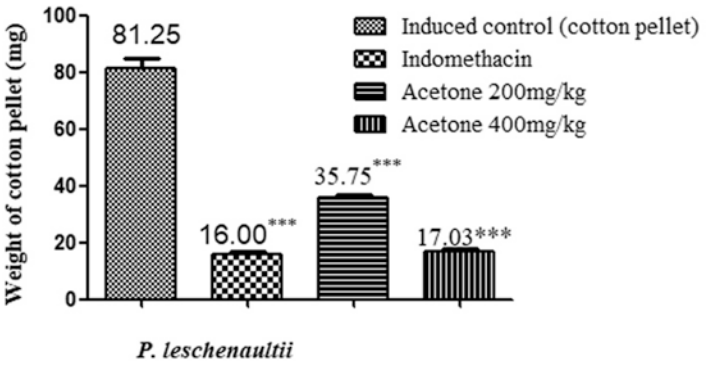

Fig. 11.7 Inhibition (%) of granuloma formation by cotton pellet implanted granuloma in rats treated with *P. subpeltata and P. leschenaultii* leaves. Values expressed as mean ± SEM ($n = 6$); ***Significantly different at $p < 0.001$ when compared to control. (Source: Adapted from the publications by Shanmugam et al. (2016))

11.4.5 Antipyretic

Fever or pyrexia is a result of secondary impact of inflammation (Shukla et al. 2010). During the course of fever, the generation of pyrogens including ILs, TNF-α and interferon induces PGE2 production in hypothalamus which subsequently increases the body temperature (Sengar et al. 2015). Subcutaneous injection of Brewer's yeast induced pyrexia is considered as a useful test for the screening of plants materials as well as synthetic drugs for their antipyretic effect. Yeast induced pyrexia is also called pathogenic fever and its aetiology could be the production of higher level of PGs synthesis. The inhibition of PGs synthesis could be the possible mechanism of antipyretic action, as it can be achieved by blocking the cyclo-oxygenase enzyme activity. There are several mediators for pyrexia and the inhibition of these mediators is responsible for the antipyretic effect (Muhammad et al. 2012).

The antipyretic activity of leaf extract of *P. subpeltata* and that of the acetone extract of leaves at doses of 200 and 400 mg/kg caused significant lowering of the body temperature up to 5 h following extract administration in a dose-dependent manner. The maximum lowering of body temperature was noticed at 400 mg/kg as the mean temperature of 38.03 °C was reduced to 35.48 °C within 5 h period of pyrexia induced by yeast. The animals treated with acetone extract at 200 and 400 mg/kg doses showed a decrease in the rectal temperature by 0.14 °C and 0.38 °C respectively within 1 h. Furthermore, 1.68 °C and 2.55 °C temperature was reduced after 5 h, in all animals, which received the test sample extract (200 and 400 mg/kg doses, respectively) (Saravanan et al. 2014). The results of antipyretic activity revealed that the acetone extract of *P. subpeltata* leaves possesses a significant antipyretic effect in maintaining normal body temperature and reducing yeast induced elevated body temperature in rats and its effect is comparable to that of the standard antipyretic drug paracetamol.

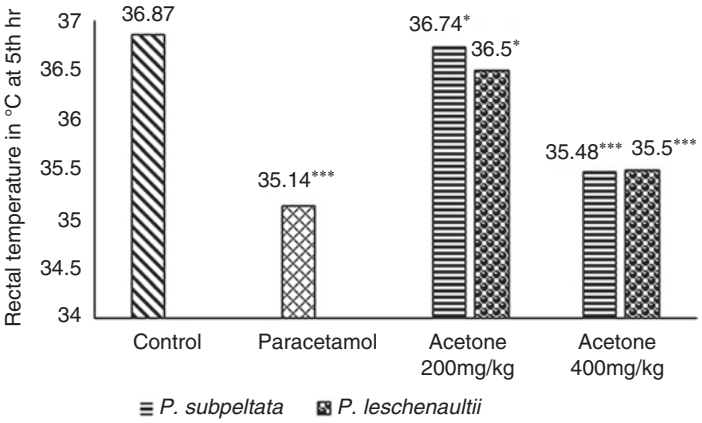

Fig. 11.8 Brewer's yeast-induced pyrexia in experimental rats at fifth hr time interval. Values expressed as mean ± SEM ($n = 6$); *Significantly different at $p < 0.05$ when compared to control; ***Significantly different at $p < 0.001$ when compared to control. (Source: Adapted from the publications by Shanmugam et al. (2014) and Saravanan et al. (2016))

The antipyretic activity of acetone extracts of *P. leschenaultii* leaves showed that acetone extracts of leaves at doses of 200 and 400 mg/kg p.o. cause significant ($p < 0.001$) lowering of the body temperature up to 5 h following the plant extract administration in a dose dependent manner. The maximum lowering of body temperature was noticed at dose of 400 mg/kg p.o. where the mean temperature of 37.92 °C was reduced to 35.50 °C within 5 h period of pyrexia induced by yeast. The animals treated with acetone extracts of *P. leschenaultii* leaves at 200 and 400 mg/kg doses showed a decrease in the rectal temperature by 0.14 °C and 0.34 °C, respectively within 1 h while after 5 h, the temperature was reduced by 1.05 °C and 2.42 °C, respectively. The results of antipyretic activity revealed that the acetone extract of *P. leschenaultii* leaves possesses a significant ($p < 0.001$) difference when compared with paracetamol antipyretic effect in maintaining normal body temperature and reducing yeast induced elevated body temperature in rats and their effects are comparable to those of the standard antipyretic drug paracetamol. The result obtained could be attributed to the fact that the plant extract may possess certain influence on the inhibition of PGs development because the prostaglandins are well-thought-out to stand a foremost controller of fever (Shanmugam et al. 2016a) (Fig. 11.8).

11.5 Bioactive Compounds

The ethnopharmacology associated with the chemical study has become an important tool in bioprospecting. Studies have associated information on the traditional use of medicinal plants with phytochemical and pharmacological studies, searching

for new drugs and herbal medicines (de Medeiros et al. 2013). It is widely accepted that oxidative stress is involved in the development and/or secondary pathology of various human diseases (Halliwell and Gutteridge 2007). Several studies show evidence that regular consumption of plant foods is associated with lowered risk of incidence of these (Alasalvar and Shahidi 2013). It is believed that health beneficial effect of plant foodstuffs can mainly be credited to a number of phenolic compounds and their ability to promote antioxidant effect (Brewer 2011).

Phenolic compounds are considered to be the most abundant source of natural antioxidants and are usually found in sources such as different plant organs (leaves, roots, etc.), fruits and vegetables (Farhadi et al. 2016; Fiorentino et al. 2008; Moo-Huchin et al. 2015; Velioglu et al. 1998). Studies have found a high correlation between the concentration of phenolic compounds and antioxidant activity (Einbond et al. 2004); therefore their ingestion either through natural sources or through dietary supplements can be associated with a reduced risk of cardiovascular diseases, stroke and certain forms of cancer (Klimczak et al. 2007).

The genus Passiflora, have been used extensively in the traditional medicine in many countries. Regarding their chemical composition, the compounds more frequently reported for the genus are flavonoids, especially C-glycosyl flavonoids, which are usually described as the main components (Zucolotto et al. 2012). Harman alkaloids are also frequently associated with Passiflora species, especially *P. incarnata* (Lutomski et al. 1975). Additionally, several saponins have been described for this genus, although their occurrence is restricted to certain species (Doyama et al. 2005). Xu et al. (2013) identified four novel 2,6-dideoxyhexose-C-glycosyl flavones from the stems and leaves *n*-butanolic fractions of P. edulis, namely, luteolin-8-C-*b*-digitoxopyranosy l-40-O-*b*-D-glucopyranoside; apigenin-8-C-*β*-digitoxopyranoside; apigenin-8-C-*β*-boivinopyranoside and luteolin-8-C-*β*-boivinopyranoside. A previously known compound, luteolin-8-C-*b*-digitoxopyranoside (50.0 mM), showed neuroprotective effects in culture cell assays. Recently, Costa et al. (2015) identified six C-glycosylflavonoids with in vitro α-glucosidase inhibitory activities in the ethanolic leaf extract of *P. bogotensis*, among which the novel compounds apigenin-6-C-*α*-L-rhamnopyranosyl-(1–2)-(600-Oacetyl)-*β*-D-glucopyranoside and luteolin-6-C-*α*-L-rhamnopyranosyl-(1–2)-(600-O-acetyl)-*β*-D-glucopyranoside were present.

A previous study of *P. mollissima* volatile composition showed the presence of aliphatic and aromatic hydrocarbons, terpenes, aldehydes, ketones, esters (acetates as well as hexyl and hexenyl esters), alcohols and lactones as main constituents (Froehlich et al. 1989). Recently, volatile composition of three fruits belonging to Passiflora genus was analysed by HS-SPME (headspace solid-phase microextraction) coupled to mass spectrometry (MS). The fruits of *P. mollissima* contained 22 volatile compounds and among these (*Z*)-β-ocimene, hexyl butanoate, hexyl hexanoate and 1-hexanol were the major constituents (Pontes et al. 2009). Phenolic compounds are present in many Passiflora species, such as *P. edulis* and *P. alata*, mainly belonging to the flavones C-glucoside class (Dhawan et al. 2004). Isoorientin, C-glucoside flavone found in *P. edulis* (Dhawan et al. 2004), was also found to be the major flavonoid in pulp extracts of this species. In fact, the total flavonoid content in *P. edulis* pulp was reported to be quite significant in comparison with other

beverages that are sources of flavonoids, such as orange juice and sugarcane juice (Zeraik and Yariwake 2010). The major phytochemical constituents of *P. foetida* have several active constituents like hydrocyanic acid, groups of flavonoids, harman alkaloids, passifloricins, polyketides, α-pyrones and vitexin (Pongpan et al. 2007).

The HPLC-DAD/MS analysis of the acetone extract of *P. leschenaultii* leaves revealed the presence of four major peaks. The separated peaks were identified according to retention time in comparison with standard compounds and were quantified as hyperin (quercetin-3-*O*-galactoside) (P1–2.7 mg/g extract), chlorogenic acid (P2–1.9 mg/g extract), caffeic acid (P3–4.6 mg/g extract) and rutin (P4–1.8 mg/g extract). The acetone extract of *P. leschenaultii* leaves contained 1.9 mg/g extract of chlorogenic acid. Thus this research finding revealed that the leaves of *P. leschenaultii* are good source of phenolic compounds, which possess strong antioxidants, antinociceptive, anti-inflammatory and antipyretic properties (Shanmugam et al. 2016a).

The quantification of polyphenolic compounds showed that *P. ligularis* fruit contained gallic acid (21.22 mg/g extract), caffeic acid (26.22 mg/g extract), rutin (33.89 mg/g extract), ellagic acid (62.44 mg/g extract) and kaempferol (3.05 mg/g extract). From the results, the presence of ellagic acid in higher concentration could attribute to the excellent antioxidant, anti-proliferative, chemopreventive and anti-atherogenic properties of the plant extracts. The presence of these compounds may be the reason for the excellent antioxidant, antidiabetic, antibacterial and antifungal activity seen in the fruit pulp of *P. ligularis* (Saravanan and Parimelazhagan 2014) (Table 11.3). Figure 11.9 shows the principal bioactive compounds present in various Passiflora species along with their pharmaceutical applications.

The quantification of phenolic compounds shows that *P. subpeltata* contained quercetin (22.36 mg/g extract), gallic acid (20.56 mg/g extract) apigenin (21.26 mg/g extract) and catechin (18.03 mg/g extract) (Saravanan et al. 2014). However, during analysis, variations in the retention time from the corresponding reference standard, even though the experiments were performed under uniform conditions, were observed in the cases of apigenin and catechin. This could be attributed to the fact that the observed peaks at the relative retention time may correspond to compounds that are derivatives of apigenin and catechin (Saravanan et al. 2014). The extensive HPLC-DAD analysis of the same plant species showed two major compounds, namely, luteolin and quercetin 3-β-D-glucoside, which were identified. The presence of luteolin and quercetin 3-β-D-glucoside in the leaves of *P. subpeltata* was reported for the first time in this species. The pharmacological activities of these compounds have been studied and it has been reported that both these compounds are used to treat different human ailments such as inflammation, pain, cancer, and diabetic and hepatic disorders (Lee et al. 2010; Shanmugam et al. 2016a).

11.6 Conclusion

In the recent years, there has been an extensive volume of research work undertaken to identify and quantify bioactive compounds present in different parts (pulp, peel and seeds) of various Passiflora species. Another focus on research has been to optimize

Table 11.3 Antioxidant, antimicrobial, analgesic, anti-inflammatory and antipyretic effects of bioactive compounds from Passiflora species

Passiflora species	Plant part used	Compounds	Functional property	Activity based on in vitro and in vivo experiments	References
P. edulis	Seeds	Pe-AFP1 (peptide)	Antifungal activity	Pe-AFP1, a protein similar to 2S albumins obtained from *P. edulis* seeds, inhibited the mycelial growth of *Trichoderma harzianum*, *Fusarium oxysporum*, and *Aspergillus fumigatus*	Pelegrini et al. (2006)
	Seeds	Passiflin	Antifungal activity	Passiflin, a dimeric protein isolated from *P. edulis*, inhibited the mycelial growth of *Rhizoctonia solani*	Lam and Ng (2009)
	Peel flour	Thermogenic/ergogenic	Antioxidant activity	The intake of *P. edulis* peel flour modulated enzymatic antioxidant in tissue status of rats	da Silva et al. (2014)
	Leaves	Isoorientin, Orientin, Vitexin, isovitexin	2,4,6-trinitrobenzenesulphonic acid induced inflammatory colitis	The intake of the aqueous *P. edulis* extract improved the enzymatic antioxidant levels in the affected colon tissues and also reduced the inflammatory condition in the tissue	Cazarin et al. (2015)
			Anti-inflammatory activity	*P. edulis* aqueous extract and its derived fractions are effective in treating experimental inflammation, and this was accompanied by a decrease in IL-1b and MIP-2 levels	Benincá et al. (2007)
P. alata	Seeds	Pe-AFP1 (peptide)	Antifungal activity	*P. alata* seeds. Pa-AFP1 inhibited the mycelial growth of *Colletotrichum gloeosporioides*, a remarkable fruit trees pathogen	Ribeiro et al. (2011)
	Leaves	Vitexin, Isovitexin, Isoorientin	Antioxidant activity	The aqueous extract of *P. alata* was able to reduce the stable radical DPPH by 100%	Colomeu et al. (2014)

(continued)

Table 11.3 (continued)

Passiflora species	Plant part used	Compounds	Functional property	Activity based on in vitro and in vivo experiments	References
P. ligularis	Fruit pulp	Ellagic acid, gallic acid, rutin, kaempferol and caffeic acid	Antioxidant, antimicrobial activity	The fruit pulp and extracts demonstrated potent antioxidant, anti-diabetic properties and also restricted the growth of seven tested bacteria (*Streptococcus faecalis, S. pyogenes, Bacillus subtilis, Klebsiella pneumoniae, Salmonella paratyphi*, *S. typhi A*, *S. typhi B*.) and two tested fungi (*Candida albicans* and *Aspergillus niger*) in antimicrobial studies	Saravanan and Parimelazhagan (2014)
P. subpeltata	Seeds	Total flavonoids	Antioxidant and antimicrobial	The acetone extract of *P. subpeltata* showed the highest antioxidant potential, among five tested seed extracts. It also presented effective anti-microbial properties, inhibiting the growth of seven tested bacteria and two tested fungi	Saravanan and Parimelazhagan (2013)
	Leaves	Luteolin and quercetin 3-β-D glucoside	Antioxidant, analgesic, anti-inflammatory and antipyretic	*P. subpeltata* leaves, a potential resource of natural antioxidant revealed from various in vitro antioxidant assays followed by evaluation of remedy for the treatment of analgesic, inflammatory and pyretic diseases.	Saravanan et al. (2014)
P. leschenaultii	Leaves	Hyperin, chlorogenic acid, rutin and caffeic acids	Antioxidant, analgesic, anti-inflammatory and antipyretic	The acetone extracts obtained from *P. leschenaultii* leaves revealed significant antioxidant, anti-nociceptive, anti-inflammatory and antipyretic activities in all the animal model tested	Shanmugam et al. (2016a)

P. foetida	Leaves, root, fruit, seed and fruit peel	Total flavonoids	Antioxidant activity	All the plant parts proved that the effect of scavenging of the free radicals by donating the electron or hydrogen ions	Sasikala et al. (2011a)
	Leaves	Total flavonoids	Analgesic and anti-inflammatory activity	The ethanolic extract of *P. foetida* leaves proved that the analgesic activity by reducing the acetic acid induced writhing response and reduced the paw volume induced by the carrageenan	Sasikala et al. (2011a)
P. incarnata		Chlorogenic acid, hyperoside, isovetixin, caffeic acid, quercitin, luteolin, orentin, rutin, scutelarein, vitexin	Antioxidant activity	The aqueous extract of passion flower (APE) and ethanol passion flower extract (EPE) reliably neutralized the free radicals	Masteikova et al. (2008)
P. mollissima	Fruit	Glycosides of (epi)-afzelechin	Antioxidant activity	The antioxidant capacity of microencapsulated banana passion fruit and freeze-dried fresh fruit showed significant results in DPPH$^{\bullet}$ (48.34 ± 1.64 and 50.12 ± 1.78 mmol Trolox/100 g DW) and ORAC (103.59 ± 4.55 and 105.22 ± 3.05 mmol Trolox/100 g DW) assays	García-Ruiz et al. (2017)
		Pectin	Anti-inflammatory activity	The observed results clearly revealed that the pectin substance obtained from the fruits of *P. mollissima* could reduce the disease level	Espinal-Ruiz et al. (2016)
P. caerulea	Aerial parts	Vitexin, Isovitexin, Isoorientin and vicenin-2	Antioxidant activity	Since lipid peroxidation is a biomarker of oxidative stress, the reduction of TBARS levels in colon induced by *P. caerulea* extract indicated that the extract could possess an antioxidant effect in this experimental model	Anzoise et al. (2016)
			Anti-inflammatory activity	*P. caerulea* showed anti-inflammatory, anti-diarrhoeal and spasmolytic activities on preclinical models	Anzoise et al. (2016)

(continued)

Table 11.3 (continued)

Passiflora species	Plant part used	Compounds	Functional property	Activity based on in vitro and in vivo experiments	References
P. nitida	Leaves	Catechins, Flavonols, flavanones	Antioxidant, anti-inflammatory activity	*P. nitida* leaves might hold potential as an active agent in the treatment of disorders caused by oxidative stress, *P. nitida* extract presented an anti-inflammatory activity, in which Wistar rats that were used in the method of carrageenan-induced paw oedema and treated with oral doses of 50, 100, and 150 mg/kg showed volume decrease of paw oedema of 6, 6, and 72.2%, respectively	Montefusco-Pereira et al. (2013)
P. manicata	Leaves	Vitexin, Isovitexin, Isoorientin	In vitro and in vivo antioxidant activity	The potential of *P. manicata* to scavenge peroxyl radicals in vitro, by using TRAP/TAR assays, indicated a significant antioxidant capacity. Also *P. manicata* leaves possess protective properties against protein oxidation and lipid peroxidation	da Silva Morrone et al. (2013)

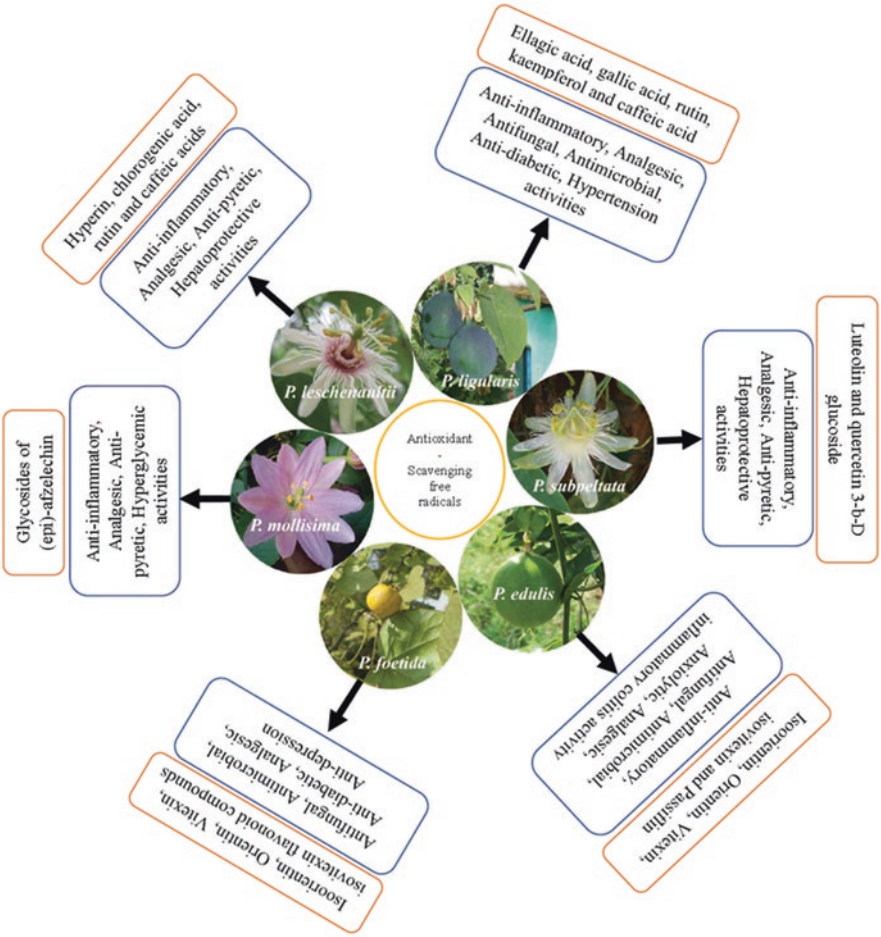

Fig. 11.9 Principal bioactive compounds present in various Passiflora species as related to their pharmaceutical applications

the extraction conditions so as to obtain higher yield of bioactive compounds from the fruit or its residues. In Passiflora specie, the major class of bioactive compounds belong to flavonoids. On the other hand research has also been concentrated in evaluating the therapeutic effects of the extracts obtained from different parts of the fruit and these profiles compared with the efficacy of standard medicines commonly used for curing various diseases. This chapter discusses the potential of different dosages of various extracts isolated from the different species of Passiflora fruit as applied in various pharmaceutical applications such as antioxidant, antimicrobial, analgesic, anti-nociceptive, anti-inflammatory and antipyretic activities caused by newly identified flavonoid compounds. The data assembled and discussed in this chapter reveal that the various species of Passiflora fruit present high potential in development of products which have wide applications in curing or controlling many diseases.

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Chapter 12
Modulation of Tumor Immunity by Medicinal Plant or Functional Food-Derived Compounds

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12.1 Introduction

Most conventional cancer treatments rely heavily on compounds that directly target cancerous cells. However, noncancerous stromal components, most of which are immune cells, constitute as much as 50% of the cancer "tissue" and play a critical role in cancer progression. This leaves an open window for possible atypical adjuvant therapy in the form of naturally derived compounds that target the mechanism for cancer/stromal (immune) interaction. Research into natural compounds may illuminate hidden potentials and offer possible breakthroughs in more effective cancer treatment.

By administering compounds which modulate a proper immune response, we also provide the powerful tool of an additional and alternate avenue to target cancer. Unilateral treatment, targeting only the proliferating cancer cells, has shown to result in chemoresistant forms of cancer (Chang 2011). Thus, by discovering and utilizing secondary or tertiary adjuvant treatments, successful elimination of cancer could be achieved without causing unnecessary harm to patients, could be achieved. Naturally derived compounds have one characteristic that should be emphasized, and that is the lack of injurious side effects when taken in therapeutic doses (Storka et al. 2015).

The importance of the immune system and the central role it plays in eradicating newly forming cancer from the body should be greatly emphasized. As most cancer therapeutics target the rapidly dividing property of cancer cells, they will also destroy rapidly dividing normal cells, such as the bone marrow cells and hair

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follicles. A proper antitumor immune response is capable of specifically recognizing and eliminating cancer cells, including ones that have infiltrated into normal tissue or metastasized into distant organs—tumor cells that are often missed by conventional therapeutics. However, continual expression of particular immune responses, i.e., inflammation, can actually aid in the progression of cancer via various mechanisms (Grivennikov et al. 2010).

Inflammation is a normal response to different forms of tissue assault or injury. It is necessary in certain pathological states to allow the body to mount defense and eventual repair, e.g., combatting extracellular pathogens (Singer and Clark 1999). The activation of the innate immune system and subsequent Th2 response aid in production of inflammatory molecules and eradication of the pathogen (Damsker et al. 2010; Kaiko et al. 2008). In case of cancer, neoplastic cells send out danger signals in the form of hypoxia-inducible factor (HIF), heat-shock proteins (HSPs), or high mobility group box 1 (HMGB1) protein, which may lead to a cytotoxic, antitumor immune response or a prolonged/chronic inflammation resulting in an advantageous environment for tumor progression (Tsan 2006). Studies have concluded that interfering with chronic inflammation and eliciting a cytotoxic response can reverse the pro-tumorigenic environment, and therefore provides an ideal target for adjuvant therapy. Research has shown that many food- and plant-derived molecules can greatly benefit cancer patients by directly inhibiting inflammation (Zubair et al. 2017). These compounds potentially work by altering the expression of certain signal transduction pathways within immune system, which would help prevent tumor-promoting inflammation.

Naturally derived compounds, such as soy isoflavones, curcumin, apigenin, and wogonin, can therefore be used to negate pro-tumor inflammation and help bring forth the necessary antitumor cytotoxic capabilities (Pavese et al. 2010; Lee 2013; Dandawate et al. 2012). In this chapter we cover the development and consequences of aberrant inflammation and the important pathways commonly found in inflammation and consider mechanisms by which plant-derived compounds would modulate such inflammatory immune responses. We also aim to bring forth evidence that supports naturally derived compounds as a possible addition to conventional treatment.

12.2 An Introduction to Natural Compounds

Natural compounds have shown promising results both in vitro and in vivo studies as anti-tumorigenic agents, especially regarding their capacity to modulate an anti-inflammatory response. Various cellular pathways are targeted by these compounds, ranging from induction of pro-apoptotic pathways to providing antioxidant support within the stromal cells. Plant extracts provide readily accessible phytochemicals which aid in cancer treatment and prevention at a relatively inexpensive cost. Plant phytochemicals such as polyphenolic flavonoids and terpenoids have shown to be quite effective as anticancer agents (Thoppil and Bishayee 2011). Although a large number of natural compounds have been discovered as having potential anticancer

activity, the extent of this chapter will be geared towards compounds which directly or indirectly modulate the tumor-immune interaction. Most of the discussed plant-derived compounds/polyphenols are "anti-inflammatory" in nature. Therefore, it is plausible that they modulate the pro-tumor inflammatory response by affecting the stromal immune components as much as, if not more than, its direct effects on the cancer cells.

Many naturally-derived compounds lie under the classification of phytochemicals, a group of compounds that exhibit a wide range of beneficial biological activity. Phytochemicals can be subdivided into groups, depending on their origin and chemical composition. Their activity can range from directly promoting apoptosis, to providing vital antioxidant capabilities, to inhibiting the production of pro-inflammatory molecules (Zubair et al. 2017; Surh et al. 2001). Examples of phytochemical classes include anthocyanins, flavonoids, glycosides, terpenoids, and tannins. Phytochemicals found in soy, turmeric, and Scutellaria are of particular interest because of their immunomodulating capabilities. More specifically, active phytochemicals found in these extracts help inhibit pro-tumor inflammatory states. The active phytochemicals found in the extract from Scutellaria include apigenin, baicalin, oroxylin A, scutellarin, and wogonin, among many others (Hussain et al. 2016). The main compound in soy pertinent to this chapter includes the isoflavone genistein (Banerjee et al. 2008). From turmeric, the active compound we will focus on is curcumin. Each of these compounds exhibits immunomodulatory forms of action, with some compounds having overlapping mechanisms of modulating the immune system. These phytochemicals work to prevent the induction or expansion of immune-suppressing mechanisms or provide additional support to move the immune system towards antitumor, cytotoxic immune response.

12.3 Innate Immune Components of Anti- or Pro-tumor Inflammation

The innate immune system is composed of cells which include monocytes/macrophages, dendritic cells, granulocytes (neutrophils and basophils/mast cells), innate lymphoid cells, and natural killer (NK) cells. The innate immune system responds systematically in an attempt to rectify perceived danger or distress due to pathogen attack or tissue damage, respectively. Danger signals are received via different receptors including toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like helicases (RLHs), or glycan receptors (Roach et al. 2005). TLR's primary role is to activate specific pathways by dimerization in response to the recognition of their respective ligands. These ligands are molecules called pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs) (Charles and Janeway 2002; Garg et al. 2013; Lotze et al. 2007; Tang et al. 2012). An example of PAMP is LPS, a lipopolysaccharide found in a large majority of gram-negative bacterial cell walls. The stimulation of TLR4 by LPS transduces a signal which can proceed to promote a pro-inflammatory response.

DAMPs are important host cellular molecules which are released during injury, such as a necrotic event. Because of cancer's virulent nature, the stress due to overactivation of metabolic and catabolic pathways can result in the accumulation of reactive oxygen species, ROS. This can cause excessive oxidative stress pushing cells into apoptosis or oxidative burst, which in turn leads to the release of distress signals such as hypoxia inducible factor (HIF), heat-shock proteins (HSP), and high mobility group box 1 (HMGB1) (Oberley 2002; Reuter et al. 2010). DAMPs act as signals to alert the body of distress, allowing for a proper immune and repair response. Via the DAMP–TLR interaction, the innate immune system responds by producing pro-inflammatory cytokines such as Il-6 and tumor necrosis factor alpha (TNF-α) (Zhang and An 2007). In a normal immune response, pro-inflammatory molecules are regulated in such a way that allows for either activation or inactivation of pro-survival or pro-death pathways, dependent on intracellular conditions (Hehlgans and Pfeffer 2005).

After the release of DAMPs into circulation from necrotic events, an immune response is elicited by activation of cellular receptors on resident macrophages and neutrophils (Newton and Dixit 2012). Detection can also occur by patrolling NK cells (Wu 2003). This promotes the production and release of cytokines and chemokines that aid in the chemotaxis of other immune cells. In response, specific intracellular signal transduction pathways are activated, and depending on the ligand present different signal transduction pathways can induce a cytotoxic or inflammatory response (Zhang and An 2007; Lu et al. 2008; Wong et al. 2001).

Neutrophils, NK cells, and monocytes/macrophages form a front-line defense system to stop and prevent further damage from perceived harm. Immune surveillance provided by these innate immune cells also provides a mechanism to recognize and provide a cytotoxic response to emerging hyperplastic cells. NK cells are one of the first in line during the development of an active antitumor immune response. NK cells monitor for cellular abnormalities throughout the body to prevent the proliferation of hyperplastic cells. Several TLRs are also expressed on the NK cell's surface. Activation of these receptors via DAMPs from necrotic events allows for the secretion of interferon-gamma (IFNγ), a potent cytokine necessary in the activation of macrophages (Vivier et al. 2008; Mosser and Edwards 2008).

Macrophages perform fundamental housekeeping throughout the body, mainly by phagocytizing cellular remnants and debris. The role of housekeeper is essential in order to deal with the constant cell turnover and need to recycle any leftover cellular material. During times without threat, macrophages remain in an inactivated state; their morphology and capabilities tend to be focused solely on their housekeeping duties and perform these tasks without provocation. In comparison, an activated macrophage takes a more protective stance, focusing on phagocytizing pathogens, with the combined ability of antigen presentation. Activation only occurs once molecular signals have been received via danger signal receptors along with cytokines, such as IFNγ, secreted from either NK cells or helper T cells. Activation of macrophages can result in a M1 or M2 phenotype, each with distinct capabilities, with M1 leaning towards a more cytotoxic oriented response and M2 more oriented to a regulatory, wound-healing-type response. M1 and M2 macrophages tend to

coincide with whichever helper T-cell response has been elicited (Wang et al. 2014a). Noteable is the debate surrounding the simplistic nomenclature, as macrophage capabilities vary and lie on a spectrum not solely dependent on a Th1 or Th2 response (Martinez and Gordon 2014). IFNγ or TNF-α is an important cytokine needed to activate macrophages and helps to provoke either an antitumor or pro-inflammatory response, respectively (Mosser and Edwards 2008).

As tumorigenesis progresses, the tumor stroma can trick macrophages into providing support for a pro-tumor environment. Tumor-associated macrophages, or TAMs, are a result of advanced cancer and aid in anti-cytotoxic, pro-metastatic activities preventing the immune system from mounting a correct response against the progressing cancer (Cortez-Retamozo et al. 2012). Production of inflammatory molecules, angiogenic factors, and metalloproteases all result from TAM transforming from M1- into M2-type macrophages and this creates a stromal environment rife for tumor progression and metastasis. Major cytokines produced by TAMs include TGF-β and EGF, which can cause immunosuppression and support tumor cell extrication.

Neutrophils are the most abundant cells found in the human innate immune system and one of the shortest-lived with an average life span of well under 24 h. They are important mediators in the progression of inflammation, purveyors of both cytokines and reactive oxygen species (Kolaczkowska and Kubes 2013). Neutrophils are in constant circulation throughout the body and utilize cell-specific adhesion molecules and cytokines, such as IL-8, during activation. Activation is a multistep process which includes the priming of neutrophils by typically an inflammatory molecule, such as TNF-α. After priming, neutrophils are activated by stimulation of TLRs. Once activated, the life span of neutrophils dramatically increases to 1–3 days. Activated neutrophils perform a multitude of important tasks, including trafficking dendritic cells, promoting inflammation and phagocytosis, or providing a wound repair response (Mayadas et al. 2014).

Interleukin 8, or IL-8, or CXCL8 is a member of the CXC chemokine family and regarded as a pro-inflammatory cytokine that is heavily involved in the chemotaxis and extravasation of neutrophils (Bickel 1993). Production involves TNF-α activation of the NF-kB and Akt pathways. IL-8 can also cause degranulation of histamines in target tissues (Lacy 2006). IL-8 is an autocrine growth factor in certain cancers and can promote angiogenesis in several others, including both colon cancer and lung cancer. As a cytokine, IL-8 is an important mediator of mitogenic factors which promote cell survival and exacerbate inflammation. IL-8 works through G protein-coupled receptors called CXCR1 and CXCR2. Activation of these receptors has the capability of stimulating multiple pathways including PI3k/Akt, NF-kB, STAT3, and AP-1. Its proliferative and immune-modulating nature makes it a key component to target in attempts to prevent inflammation (Waugh and Wilson 2008).

Medicinal plant- or food-derived phytochemicals have been shown to modulate various components of tumor-associated innate immune components via various mechanisms. Research done by Peng et al. indicated that administration of Scutellaria extract in a dose-dependent manner lowered serum levels of IL-8 in mice bearing U14 cervical cancer (Peng 2014). The study did show a contradiction as

levels of TNF-α were elevated in a dose-dependent manner as well. Even so, the study displayed suppressed immune responses in the control group, while an enhanced immune response was observed in mice who received Scutellaria extract. The elevated levels of TNF-α may indicate the extract played a role in recruiting NK cells to combat the cancer (Pilaro et al. 1994). More research is needed to confirm this theory (Fridlender et al. 2009; Fridlender and Albelda 2012).

There is a conspicuous correlation between higher levels of expressed cell adhesion molecules and monocyte migration. To initiate chemotaxis from TANs and TAMs, they require IL-1β, IL-6, and CXCL8 (IL-8). Production of these molecules is often regulated by the Akt pathway and in many cancer cells the Akt pathway is constitutively active (Testa and Tsichlis 2005). Gong et al. reported that genistein, a key soy isoflavone, inhibits the Akt pathway in MDA-MB-231 breast cancer cells (Gong et al. 2003). Inhibition of this pathway prevents the production of cell adhesion molecules preventing the migration of TANs and TAMs and their capability to modulate the immune system and prevent inflammation. Our group has previously shown that a leaf extract of Scutellaria inhibits glioma growth in mouse models via inhibition of the Akt pathway (Parajuli et al. 2011).

TGF-β exerts robust pro-tumorigenic activities and plays a tremendous role in controlling the immune system. In general TGF-β inhibits cytotoxic responses from the immune system and blocks antitumor activities. The culmination of these effects on macrophages then promotes their activities to become pro-inflammatory. It stimulates T cells to become regulatory, immune-suppressing cells, also known as T regulatory cells. Enhanced Treg activity has been attributed to more advanced stages of cancer (Takeuchi and Nishikawa 2016). Li et al. displayed that genistein prevents RNA expression of TGF-β, therefore inhibiting all of TGF-β downstream effects (Li 2002).

12.4 The Important Roles of $CD4^+$ T-Helper and Treg Cells

Different pathways are activated in the immune system to help dictate whether a continued aggressive cytotoxic response is needed, or if the body should be preparing for a wound-healing response. Coordination of these responses is an important factor when dealing with a pathogen or an injury. There are dichotomous differences between these two response pathways and result in largely divergent actions. There needs to be a logical system the body can take to prevent potentially dangerous consequences due to an incorrect response. The body does not want to promote a healing environment when pathogens are still present. Orchestration for a proper response comes in part by means of a subset of T cells named T-helper cells, or Th cells.

Naïve helper T cells are involved in a process of differentiation that aids in the progression towards an effective adaptive immune response (Janeway 1989). There are large differences between effector Th1, Th2, and Th17 $CD4^+$ cells and each promotes a specific agenda which, under normal circumstances, is supposed to

reflect the nature of the pathogen (Kaiko et al. 2008). Each T-helper cell response has a profoundly different outcome and is evoked by specific cytokines and chemokines secreted by surrounding stromal cells. Aberrant helper T-cell differentiation is also known to play a role in multitude of autoimmune diseases (Charlton and Lafferty 1995; Lafaille 1998; Yuan Zhang et al. 2014).

$CD4^+$ Th1 cells are geared towards intercellular assault, such as viral invasion and cancer proliferation. Excess inflammation is not required for a proper immune response within the tumor environment; therefore, a Th1-mediated response is more advantageous in preventing tumorigenesis. Extracellular pathogens on the other hand require a response geared towards removing and expelling them from the body (Anthony et al. 2007). The consequences of a prolonged inflammation mediated by Th2 cells are explained later in this chapter.

$FoxP3^+$ regulatory T cells, or Treg cells, are an important part of the immune system originally geared to prevent autoimmune responses towards self-antigens. Their capability to suppress the immune system is far reaching, with mechanisms that inhibit T cells, B cells, macrophages, dendritic cells, and NK cells. Treg cells work through a few different methods of actions, with the capacity to produce cytokines (TGF-β and IL-10) and cell-surface molecules (CTLA-4) involved in promoting anergy and apoptosis in surrounding immune cells. TGF-β also plays a critical role in the expansion of Treg cell population in the tumor microenvironment (Takeuchi and Nishikawa 2016). We have reported that Scutellaria extract and its active constituent wogonin reduce the accumulation of $FoxP3^+$ Treg cells in animal models of glioma via inhibition of TGF-β signaling (Dandawate et al. 2012). Scutellaria extracts prevented the secretion of TGF-β from glioma cells while also inhibiting the generation/expansion of Treg cells via modulation of intracellular, SMAD-mediated TGF-β signaling (Dandawate et al. 2012).

In a study by Xu et al. Treg cells were shown to be downregulated by curcumin via inhibition of the FoxP3 gene transcription and subsequently converted into Th1 cells. This prevented the immunosuppressive effects of Treg cells while also promoting a cytotoxic immune response in patients with advanced colon cancer (Xu et al. 2017). Kan et al. displayed that Scutellaria extract has additional Treg-modulating activities in H22 hematoma-bearing mice. This was observed via downregulation of the cytokines IL-17, TGF-β, and IL-10, while the levels of Th1 cytokines IL-2 and IL-12p70 were elevated. Furthermore, tumor-infiltrating Th17 and Treg cells were also inhibited after treatment with Scutellaria extract (Kan et al. 2017). As a noteworthy point, there is a lack of evidence that supports the definitive upregulation of Th1 cells. No tests were performed to show the levels of Th1 cells before or after administration, only measurements of relevant cytokines.

Clinical studies have also exhibited promising results in the ability to modulate $FoxP3^+$ Treg cells. A study by Lesinski et al. showed that consumption of soy isoflavone-fortified bread by 32 men with prostate cancer displayed a large decrease in Treg cells after 56 days (Lesinski et al. 2015). This provides key clinical evidence supporting claims that digestible soy isoflavone intake has immune-modulating capabilities.

12.5 TNF-α and NF-κB

Immune cells are greatly influenced by the cytokine TNF-α. TNF-α receptors, or TNFR, come in two types, I and II, with type II primarily found on immune cells. Activation of these receptors is a key step in the inflammatory process (Parameswaran and Patial 2010). After induction of the TNF-α-TNFR complex, a key downstream effector, nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), can be activated (Hayden 2004). Many of the inflammatory properties of immune and stromal cells are elicited through activation of NF-κB (Sabroe et al. 2008; Mantovani et al. 2008; Hoesel and Schmid 2013). Natural compounds have proven to be great inhibitors of NF-κB, therefore preventing the inflammatory effects of TNF-α.

TNF-α can act in paradoxical roles within the cell depending on the microenvironment. TNF-α is primarily secreted by either activated macrophages, or effector T cells, but can also be secreted by other surrounding stromal cells (Parameswaran and Patial 2010). Under normal homeostatic conditions, TNF-α works as a pro-survival molecule (MacEwan 2002). TNF-α can mediate apoptosis but only once both PI3K/Akt and NF-κB pathways have been inhibited (Rath 1999). Because TNF-α is such a potent cytokine and activator of many inflammatory responses, its downstream effector molecules provide possible targets to prevent inflammation while also inducing apoptosis in cancer. In both cancer and some autoimmune diseases, TNF-α is produced in excess, and has already been used as a target to inhibit the initiation and progression of inflammation (Bradley 2008).

NF-κB is an essential transcription factor needed for initiating inflammation in cancer (Hoesel and Schmid 2013; Fan et al. 2013). Its activation causes a snowball effect, with the activation of multiple cellular processes, mostly geared towards cell survival and production of pro-inflammatory molecules such as LOX-5 and COX-2. NF-κB can also cause the production and activation of inducible nitric oxide synthase (iNOS), a producer of powerful reactive nitrogen species. Tumor-related inflammation and immune suppression rely on the constitutive activity of NF-κB and its downstream effectors.

Curcumin has been shown to inhibit NF-κB and prevent the actions of its downstream effectors (Bharti et al. 2003). Curcumin directly binds to IκBα and inhibits the necessary phosphorylation and cleavage of said complex to activate NF-κB (Singh and Aggarwal 1995). Without these steps, NF-κB cannot translocate into the nucleus and start the initiation of transcription. Curcumin has also been shown to directly attach to COX-2, a molecule widely known for its role in promoting inflammation (Lee 2013). Furthermore, iNOS is also a target for curcumin, which further helps inhibit inflammation (Brouet and Ohshima 1995).

Genistein also works by regulating and inhibiting NF-κB (Zhou 2014). Genistein has been shown to prevent the translocation of NF-κB after exposure to TNF-α (Davis et al. 1999). The interpreted explanation was inhibition of phosphorylation of the IκBα complex.

Scutellaria extract has also been shown to inhibit NF-κB activation. NF-κB is activated through multiple mechanisms. One mode of activation occurs when the precursor complex IKK is exposed to excess reactive oxygen species. Scutellaria extract, which contains the active antioxidants wogonin, oroxylin A, baicalin, and baicalin, can scavenge reactive oxygen and nitrogen species and inhibit IKK and NF-κB activation (Hussain et al. 2016). In glutathione-depleted cells, curcumin has also shown signs of an inverse mechanism of inhibiting NF-κB. This occurs by excess production of ROS that then leads to degradation of IKK instead of ROS activation (Sandur et al. 2007).

As previously stated, activation of NF-κB results in a wide range of outcomes and its inhibition causes widespread intracellular and extracellular effects. Curcumin also prevents TNF-α-mediated activation of NF-κB by interaction with IκBα and prevents nuclear translocation of NF-κB (Bharti et al. 2003).

Inhibition of NF-κB can allow for apoptosis of cells responsible for perpetuating inflammation. The pro-apoptotic effects of curcumin have been attributed to the inhibition of PI3k/Akt pathway, preventing the necessary phosphorylation of Akt, which is needed to continue signal transduction. This causes inhibition of downstream effectors including two major anti-apoptotic proteins BCL-2 and BCL-XL. Inhibition of this pathway also helps promote activation of caspase-3, a key regulatory molecule in the apoptotic pathway (Lee 2013).

12.6 Natural Compounds, a Plausible Alternative

Naturally derived compounds have been used for centuries to combat inflammation. For instance, written history has documented the use of willow bark, which contains acetylsalicylic acid, the active ingredient in aspirin, as a form of treatment for various ailments dating back to the Roman empire (Norn 2009). It should then be noted that some of the most important anticancer drugs, such as vinca alkaloids, are naturally derived (Moudi et al. 2013). Anticancer and immune-modulatory activities of select phytochemicals are summarized in Table 12.1.

Soy isoflavones have exhibited great potential as immune-modulating compounds (Banerjee et al. 2008; Abernathy et al. 2015, 2017). Genistein has emerged as a multifaceted compound, showing signs as a potential form of adjuvant cancer therapy with possible applications outside of cancer treatment, including treatment for Alzheimer's and atherosclerosis, both via inhibition of inflammation (Zhou 2014; Wang et al. 2008). Although soy products have been under debate over the efficacy and safety of use in certain types of cancer, specifically breast and prostate cancer, research has leaned towards soy possessing beneficial effects rather than the commonly believed negative impact when consumed or used therapeutically (Davis et al. 1999). Soy products are known to have weak estrogenic effects. In certain breast cancer, specifically ER^+ breast cancer, growth is considered to be estrogen dependent. Logically there has been speculation as to whether soy and possible

Table 12.1 Naturally derived compounds: functions and molecular mechanism

Origin of compound	Compound	Function	Molecular mechanism	References
Turmeric	Curcumin	Inhibits production of pro-inflammatory molecules	Direct inhibition of NF-κB, COX-2, iNOS	Lee (2013); Brouet and Ohshima (1995)
		Pro-apoptotic	Inhibition of the PI3k/Akt pathway, inhibition of NF-κB	Lee (2013); Singh and Aggarwal (1995)
		Inhibits TNF-α stimulation	Attachment to IκBα	Bharti et al. (2003)
		Upregulation of Th1 cells	Prevention of FoxP3 expression	Xu et al. (2017)
Soy	Genistein	Inhibition of Treg cells	Downregulation of multiple pro-inflammatory cytokines	Lesinski et al. (2015)
		Inhibits production of pro-inflammatory molecules	Inhibition of NF-κB	Zhou (2014)
		Inhibits TNF-α stimulation	Prevention of translocation of NF-κB	Davis et al. (1999)
		Inhibits TGF-β production	Prevention of RNA transcription of TGF-β	Li (2002)
Scutellaria	Apigenin	Inhibits production of pro-inflammatory molecules	Inhibition of COX-2, iNOS and NF-κB (p65)	Choi et al. (2004); Wang et al. (2014b); Liang et al. (1999); Patil et al. (2016)
	Wogonin	Inhibition of Treg cells	Inhibition of TGF-β	Dandawate et al. (2012)
		Inhibition of pro-inflammatory pathways	Inhibition of IKK and NF-κB activation	Hussain et al. (2016)
	Oroxylin A	Inhibits release of IL-6, IL-1b	Inhibition of Jak/STAT pathway	Hussain et al. (2016)
	Baicalin	Inhibition of pro-inflammatory pathways	Inhibition of TLR2 formation, inhibition of Th17 cell	Hussain et al. (2016)
	Scutellarin	Inhibition of pro-inflammatory pathways	Inhibition of TLR4 formation	Hussain et al. (2016)
	Extract (nonspecific)	Inhibition of pro-inflammatory pathways	Inhibition of Akt, GSK-3α/β and NF-κB pathways	Parajuli et al. (2011)
		Inhibition of Treg cells	Inhibition of production of IL-17, TGF-β, and IL-10	Kan et al. (2017)

estrogenic effects could be deleterious in such cancers, the rationale being that it could promote further growth. Yet even after multiple clinical studies, a definitive decision on possible negative effects due to soy intake has resulted in inconclusive evidence. Rather, more current studies have shown evidence that soy products may have the capacity to be competitive inhibitors of estrogen in ER^+ breast cancers and therefore aid in the prevention of tumor growth and progression (Messina and Badger 2017; Kwon 2014).

Turmeric has an extensive and long history of being used as an anti-inflammatory agent (Prasad and Aggarwal 2011). Curcumin, the active compound in turmeric, has shown great versatility in controlling and preventing inflammation. One very positive aspect of curcumin is its low toxicity even at very high doses (Gota et al. 2010). This versatility of curcumin comes in part due to its structural diversity. The tautomerization between a keto-enol form is dependent on the surrounding pH and can perform different tasks depending on the current form curcumin is in (Lee 2013). Many of the benefits from curcumin stem from the inhibition of NF-κB and the Akt pathways, allowing for the prevention of pro-inflammatory cytokines and potential apoptosis of cells which produce them.

Most of the active flavonoids in Scutellaria extract display potent antioxidant capabilities. Some key targets for each of these include the following: Baicalin inhibits Th17 cell maturation, lowering pro-inflammatory cytokines and positive feedback loop that perpetuates inflammation. Baicalin also prevents the expression of TLR2s, a member of the TLR family known to promote the production of TNF-α once activated. Scutellarin inhibits TLR4 expression, a receptor associated with perpetuating low-grade inflammation. Oroxylin A functions through the JAK/STAT pathway, unlike other active compounds in Scutellaria. It does so by inhibiting the phosphorylation of STAT in the JAK/STAT pathway, because of this, cytokines IL-1β and IL-6 cannot be released, preventing the activation of other inflammatory genes in the surrounding environment (Hussain et al. 2016).

Another compound with characteristics as a potential immune modulator is the flavonoid apigenin. Found in a wide variety of fruits, vegetables, and other plant-derived products, e.g., chamomile tea, parsley, oranges, and Scutellaria, apigenin has been found to be nontoxic at levels which correlate to normal human consumption (Janssen et al. 1998; Fernandez de Simon et al. 1992; Lemberkovics 1998). Apigenin can be found in detectable levels in urine samples after administration of a 2-gram bolus of parsley, suggesting a relatively good bioavailability with a calculated half-life of 12 h in human subjects (Nielsen et al. 2007). Apigenin can inhibit both COX-2 and iNOS, as well as TNF-α-induced NF-κB activity by inhibiting p65-DNA complexes needed for transcription of important pro-inflammatory molecules (Choi et al. 2004; Wang et al. 2014b; Liang et al. 1999; Patil et al. 2016). Apigenin also directly represses the activation of the LPS-induced inflammatory response in macrophages. Zhang et al. have reported a sharp, dose-dependent, downregulation of NF-κB activity and production of the inflammatory cytokines IL-1β, IL-6, IL-12, and CCL5, as well as the cell adhesion molecules ICAM and VCAM (Zhang et al. 2014). This is done via downregulation of both the transcription of pro-inflammatory genes and inhibiting the production of cytokines central to promoting an inflammatory response.

There is promising evidence for naturally derived adjuvant therapy in the treatment of cancer. Because many cancers share similarities in the activation of onco genes and repression of tumor-suppressor genes, especially those involved in mediating inflammation, this creates a consistent target that naturally derived compound can inhibit. Thus, natural compounds can potentially provide a multifaceted nontoxic approach towards treating cancer without deleterious side effects.

12.7 Conclusion

Plants have proven time and again to have great versatility in producing multifaceted compounds for use as instruments for their own survival. This versatility allows for such compounds to be utilized elsewhere as a potential modulator of the human inflammatory immune responses. Many of these anti-inflammatory compounds contain chemical structures which allow for multiple modes of interaction with minimal side effects to the host at therapeutic dosage. Proper application of these compounds and their distinct properties should be considered as potential novel therapeutic agents.

The immune response to cancer varies throughout the progression of cancer. During the initial stages, it provides a cytotoxic response, aimed at targeting aberrant cells proliferating rapidly. The formation of a tumor provides a hypoxic environment which activates innate immune cells, including NK cells, starting the cascade of events which results in acute inflammation and activation of an adaptive cellular and humoral immune responses. However, as time progresses, and inflammation becomes a chronic situation, it can create an environment rife for tumorigenesis. This transition into a chronic, inflammatory, pro-tumor, Th2-mediated, TGF-β-producing environment results in a suppressed immune response and allows for production of pro-tumor cytokines and growth factors which help in the progression of tumorigenesis. In progressing cancer, the immune response can be polarized and eventually used against the body, perpetuated through continued immunosuppression via the development of Treg cells, TAMs, and TANs.

Immunotherapy can be a robust tool in the fight against cancer. It can provide the functionality needed to stop tumor progression without affecting surrounding normal cells. Immune-regulatory activities mediated by TGF-β, Treg, and TAM are great impediments to successful immunotherapy. By administering naturally derived compounds with immune-modulatory capabilities, we can not only inhibit pro-tumor inflammation but also potentially redeploy an antitumor, cytotoxic immune response. Phytochemicals found in turmeric, soy, and Scutellaria have shown profound effects on attenuating and modulating inflammation. Further research could pave ways toward combining these natural compounds with various forms of immunotherapeutic strategies against cancer.

To suitably implement these natural compounds as immune-modulating agents during tumorigenesis, further research should be done to fully understand the range of possibilities and capabilities each of these compounds has. Contemporary modes

of cancer therapeutics need nontoxic forms of adjuvant immunomodulatory agents. By properly applying these naturally derived compounds, a mediator that provides multiple modes of action can be used to significantly improve the clinical outcome of conventional therapies.

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Chapter 13
Dietary Brown Seaweed Extract Supplementation in Small Ruminants

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13.1 Introduction

A feed supplement containing an extract from the brown seaweed (BSW), *Ascophyllum nodosum*, has been reported to influence antioxidant activity (Fike et al. 2001), vitamin E status (Montgomery et al. 2001), immune cell response (Saker et al. 2001), and carcass characteristics (Allen et al. 2001a) in farm animals. The mode of action is not clear, but some specific vitamins or antioxidants may be involved in the antioxidant action of Tasco (Allen et al. 2001b).

Brown seaweed contains some natural antioxidants, such as substituted phenols, polyphenolic compounds, and vitamin precursors, like α-tocopherol (Le Tutour 1990). Saker et al. (1998) reported that the antioxidant activity of BSW lowers oxidative stress in cattle. Oxygen free radicals are produced during stress and disease, and the antioxidant nature of BSW could increase stress tolerance by reducing the effect of toxic oxygen radicals, which are responsible for cell damage (Allen et al. 2001a, b). Antioxidants are essential for reducing the formation of oxygen radicals, such as superoxide, in order to protect the immune system (Dubeski 1999).

Antioxidants like vitamin E act as scavengers of oxygen free radicals, protect cell membranes from oxidative destruction, and maintain cell integrity (Liu et al. 1995). This may in turn reduce protein degradation and muscle fiber disruption (Mitsumoto et al. 1998). Antioxidant effects are prominent in animals that graze BSW-treated forage or are fed with BSW-containing commercial products such as Tasco-Ex and Tasco-14 (Allen et al. 2001b).

Brown seaweed has been reported to reduce the number of bacteria, such as *Escherichia coli*, in the gastrointestinal tract of livestock (Braden et al. 2004; Behrends et al. 2000; Evans and Critchley 2014), probably due to the effect of

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phlorotannins present in *A. nodosum.* Dietary BSW supplementation is seen as a potential preharvest intervention strategy to improve food safety.

Stress in animals prior to processing for food not only induces immunosuppression and releases free radicals, but also affects meat quality by altering muscle glycogen content (Kannan et al. 2003). Preslaughter stress lowers muscle glycogen levels, which results in elevated ultimate pH of meat. Brown seaweed increases vitamin E levels in muscle, liver, and blood in steers (Saker et al. 2001; Allen et al. 2001b), and vitamin E has been reported to increase muscle glycogen content in pigs (Rosenvold et al. 2002). Supplementation with BSW may alter the undesirable pH that results from depletion of muscle glycogen during preslaughter stress.

Goat meat is prone to rapid lipid and pigment oxidation on refrigerated display (Kannan et al. 2001). Feeding BSW has been reported to increase the shelf life of beef by preventing or delaying muscle pigment and lipid oxidation (Montgomery et al. 2001). Brown seaweed can act as an antioxidant, either by increasing vitamin E levels or by using its natural antioxidants. The effects of BSW supplementation have not been adequately tested in small ruminants. The existing literature on the effects of BSW on stress tolerance, immune function, antioxidant activity, rumen metabolism, rumen microflora, body composition, and meat quality in ruminants, particularly in goats and sheep is reviewed in the following sections of this chapter.

13.2 Taxonomy of Brown Seaweed and History of Use in Livestock

Ascophyllum nodosum, commonly known as brown seaweed, knotted wrack, knotted kelp, egg wrack, rockweed, and Norwegian kelp, is an alga. Algae are generally classified based on size into macroalgae (seaweeds) and microalgae. While the large-sized seaweeds are found in the coastal and shorefront areas of certain ocean waters, small-sized microalgae are found throughout the ocean waters. Marine macroalgae are classified into red algae, green algae, and brown algae, and they vary in color, size, shape, and habitat.

A. nodosum belongs to the phylum Chromophycota, class Phaeophyceae, order Fucales, and family Fucaceae. This brown algae group is among the largest, growing more than 30 m in length. They are found in littoral zones, particularly seen attached to the moderately exposed to extremely sheltered shorefront rocks of the northern Atlantic, with distributions extending from the White Sea to Portugal in the east and from Baffin Island to New Jersey in the west. Exposure to wave movement is a major factor determining the extent of local distribution of BSW, with the number of plants and fronds decreasing with increasing exposure to waves. *A. nodosum* has flexible stems with long leathery fronds and air bladders (Fig. 13.1), and has a tendency to grow slowly. This seaweed also has the unique feature of discarding its outer skin periodically.

Fig. 13.1 Fronds of brown seaweed (*Ascophyllum nodosum*) from Nova Scotia, Canada. (Source: Adapted from Feedipedia; CC BY-SA 3.0; Wikimedia Commons)

Although there are thousands of species of seaweed, only a few are used in livestock feeding. The most frequently used species for livestock feeding is *A. nodosum*, probably due to its abundance and easy accessibility for harvesting. The attachment portion of plants is believed to be permanent and survive for decades, giving rise to new fronds. *A. nodosum* fronds may break from holdfasts after several years (~15 years), and the detached spherical masses can migrate in sheltered waters. When fragments of the seaweed settle on the shore, they can establish and propagate independently. According to Evans and Critchley (2014), the estimated global yearly harvest of *A. nodosum* for livestock feed is approximately 100,000 t wet biomass. Commercial production of this seaweed occurs mainly in Canada, Iceland, Ireland, France, and Norway. After storm harvesting in some regions is done on the shore or in the intertidal zones. In some regions, mechanical harvesters are used, such that the natural beds are allowed to regrow to enable periodic repeated harvesting (Evans and Critchley 2014).

Seaweed has been used for feeding livestock for thousands of years, based on Greek and Icelandic chronicles. There is evidence that during the beginning of the twentieth century, sheep grazed on seaweed on the shores or the seaweed was harvested and fed to livestock in Iceland due to scarcity of forages round the year (Evans and Critchley 2014; Makkar et al. 2016). There are also reports that seaweed was stored in barns in dried form or silaged for use as winter feed for sheep. Evidently, during approximately the same time period (nineteenth and early twentieth centuries), seaweed was also fed to ruminants in France, the Scottish Islands, and Scandinavian countries. Hansen et al. (2003) reported that even today Orkney sheep in Northern Scotland thrive on a predominantly seaweed diet. Seaweed as a potential livestock feed ingredient apparently received a renewed focus in the 1960s when Norway started producing kelp meal. With a good body of scientific research evidence available today on the benefits of BSW in livestock, *A. nodosum* is widely harvested commercially and marketed as a livestock feed supplement. However, some factions of the society resist commercial harvesting of seaweed due to the perceived negative impact on the ecosystem.

13.3 Nutritional Value of Brown Seaweed

Brown seaweeds have a number of bioactive compounds that could be beneficial to livestock and poultry. The brown color is due to the predominance of fucoxanthin pigments that conceal the chlorophylls, β-carotene, and other xanthophylls (Guiry 2014). The nutritional value of brown microalgae may be lower due to their lower protein and higher mineral contents compared to green or red macroalgae, although all fresh seaweeds contain 70–90% of water (Makkar et al. 2016).

The crude protein content of BSW can depend on the season in temperate regions. The crude protein values of *A. nodosum* range from 5% to 12% (Table 13.1). Seaweeds are generally considered to be deficient in essential amino acids, with the exception of sulfur-containing amino acids. For example, the methionine content of *A. nodosum* is comparable with soybean; however, lysine, phenylalanine, and tyrosine levels in *A. nodosum* are lower than those in soybean protein (Cruz-Suarez et al. 2008; Dawczynski et al. 2007; Makkar et al. 2016; Table 13.2). A polysaccharide of glucose, laminarin, is the primary carbohydrate in brown seaweeds and the cell walls are comprised of alginic acid and cellulose. Alginic acid is found in abundance in BSW, and *A. nodosum* contains about 15–30% of this long-chained heteropolysaccharide (Guiry 2014).

Table 13.1 Chemical composition of *Ascophyllum nodosum*

Analysis	*Ascophyllum nodosum*
Crude protein (%)	8 ± 2.7 (5)
Crude fiber (%)	5.5 (4.1–6.8)
NDF (%)	20.9 (19.8–22.0)
ADF (%)	13.1
Lignin (%)	13.8 (6.2–21.4)
Ether extract (%)	3.9 ± 1.6 (3)
Ash (%)	22.5 ± 2.1 (12)
Gross energy (MJ/kg)	14.6 (14.5–14.7)
Ca (g/kg)	20
P (g/kg)	1
K (g/kg)	24
Na (g/kg)	NA
Mg (g/kg)	8
Mn (mg/kg)	12 ± 3 (8)
Zn (mg/kg)	181 ± 114 (8)
Cu (mg/kg)	28 ± 16 (1)
Fe (mg/kg)	134 ± 36 (8)

All values are on DM basis
Values are mean ± SD (*n*); other values are either single or average of two values, each value given in parenthesis
NA not available
Source: Adapted from Makkar et al. (2016)

Table 13.2 Amino acid composition of *Ascophyllum nodosum* compared with soybean

Amino acids	*Ascophyllum nodosum*	Soybean
Essential		
Methionine	1.3	1.32
Cystine	NA	1.38
Valine	4.1	4.5
Isoleucine	3.1	4.16
Leucine	5.3	7.58
Phenylalanine	3.2	5.16
Tyrosine	0.9	3.35
Histidine	1.4	3.06
Lysine	4.6	6.18
Threonine	3.6	3.78
Tryptophan	NA	1.36
Nonessential		
Serine	3.5	5.18
Arginine	6.0	7.64
Glutamic acid	11.6	19.92
Aspartic acid	8.4	14.14
Proline	3.0	5.99
Glycine	4.8	4.52
Alanine	5.4	4.54

Values are g/16 g nitrogen
NA not available
Source: Cruz-Suarez et al. (2008) and Dawczynski et al. (2007); adapted from Makkar et al. (2016)

Brown seaweeds are rich in certain minerals and deficient in others (Table 13.1). The sodium and potassium contents of *A. nodosum* are 3–4% and 2–3%, respectively, and its iodine content can be up to 0.1% (Rodriguez-Montesinos and Hernandez-Carmonal 1991). Mineral content of seaweed can vary based on season and geographical region.

13.4 Effects of Brown Seaweed Extract in Livestock

13.4.1 Stress Responses

Physiological responses to common stressors that animals are typically exposed to in commercial production situations, with particular emphasis on small ruminants, and putative beneficial effects of brown seaweed supplementation are considered in this section. Unlike other livestock species, meat goats are frequently transported long distances to processing facilities before being slaughtered in the USA (Kannan et al. 2002). During transportation, animals are exposed to unfavorable environmental

conditions which bring about intense physiological changes indicative of stress. The major stress factors in livestock transportation may be classified as noise, vibration, lack of exercise, prolonged standing, and changes in environmental temperature and humidity. Stress can be indicated by plasma cortisol, glucose, nonesterified fatty acids (NEFA), urea nitrogen (PUN) concentrations, creatine kinase (CK) activity, differential leukocyte counts, and immune function. Physiological changes induced by stress primarily increase glucocorticoid activity, and can also negatively affect immune function in animals. Kannan et al. (2000) reported that Spanish goats subjected to a 2-h transportation and an 18-h feed deprivation showed elevated cortisol concentrations. Loading onto trailers also markedly elevates plasma cortisol concentrations in sheep (Broom et al. 1996) and goats (Kannan et al. 2000). In addition, fasting has been shown to elevate circulating levels of corticosteroids in sheep (Murayama et al. 1986). In different experiments conducted in Spanish, Spanish × Boer, and Boer breeds of goats, dietary BSW extract supplementation did not influence plasma cortisol concentrations measured following transportation stress (Galipalli et al. 2004a; Kannan et al. 2007a).

During initial hours of stress, small ruminants utilize glucose as their primary source of energy (Kannan et al. 2002). High blood glucose in an animal indicates metabolic responses to stress, which are related to sympathomedullary and corticoadrenal activities. Catecholamines secreted from the sympathetic nerve endings and adrenal medulla induce excessive glycogenolysis in liver and muscle (Nwe et al. 1996). In muscle, glycogenolysis forms lactate, and in liver it forms glucose, leading to an increase in blood glucose. Murray et al. (1990) reported that liver glycogenolysis is the fundamental process involved in increasing plasma glucose concentrations. Kannan et al. (2000) observed that plasma glucose concentrations remained elevated during the first 2 h after transportation and then began decreasing at 3 h. Nwe et al. (1996) reported that plasma glucose concentrations returned to baseline level at 3 h after a 6-h journey in male adult Japanese native goats. Sanhouri et al. (1992) observed that a 20-min journey increased only plasma cortisol concentrations, but a 2-h van journey increased both cortisol and glucose concentrations in goats. The authors also noted that as a response to stress, elevation of glucose concentration was preceded by an elevation of cortisol concentration. Nwe et al. (1996) showed that plasma cortisol and glucose concentrations changed in a similar fashion during and after transportation. Eiger et al. (1979) suggested that cortisol may be acting synergistically with other hormones to sustain marked hyperglycemia. Therefore, plasma glucose concentration may be a useful indicator of the intensity of stress (Sanhouri et al. 1991). Galipalli et al. (2004a) reported that BSW extract supplementation did not have any effect on plasma glucose concentration in Boer goats; however, Kannan et al. (2007a) found that BSW extract supplementation caused an increase in glucose concentration in Spanish goats but caused a decrease in blood glucose in Spanish × Boer goats when measured after 2 h of transportation. These results imply that BSW supplementation elicits differential glycogenolytic effects in different breeds, at least in goats.

Plasma CK activity indicates the extent of muscular activity or damage (Wilson et al. 1990) during transportation and handling of animals. Kenny and Tarrant (1987) observed that cattle tend to lose balance in a moving truck due to sudden application of brakes or cornering of animals. When animals try to maintain balance in a moving

vehicle, it is likely that they may place more burden on the muscles involved, resulting in elevation of CK activity. These stressors can lead to tissue perfusion, hypoxia, focal muscle necrosis (Spraker 1982), and an increase in CK activity in animals (Kannan et al. 2000). Dietary BSW supplementation does not influence plasma CK activities in goats exposed to transportation stress (Galipalli et al. 2004a; Kannan et al. 2007a). Acute stress may also lead to increased levels of catecholamines and subsequent lipolysis that result in increased circulating free fatty acids (Shaw and Tume 1992). Warriss et al. (1989) stated that fasting may further increase the free fatty acid response to catecholamines. During fasting, free fatty acids become the main source of energy in muscles after liver glycogen depletes, resulting in a decrease in blood glucose and an increase in nonesterified fatty acid (NEFA) concentrations. Fasting also results in increased urea nitrogen concentrations in circulation by increasing protein catabolism in the animal's body. Other workers also observed that nutritional stress increased PUN concentrations in livestock (Kannan et al. 2000). Corticosteroids may cause protein and nucleic acid catabolism in muscles. The effects of transportation and feed deprivation stresses on NEFA and PUN concentrations were not influenced by dietary BSW supplementation in goats (Galipalli et al. 2004a; Kannan et al. 2007a).

Brown seaweed extract supplementation has been reported to reduce rectal temperatures when beef cattle were exposed to elevated ambient temperatures (Williams et al. 2009). A similar effect was noticed by Pompeu et al. (2011) in dairy cattle fed a BSW extract supplement, indicating that seaweed supplementation helps animals cope with heat stress. No data could be found in the literature on the effects of BSW on heat stress in small ruminants.

13.4.2 Antioxidant Activity

The antioxidant effect of BSW in livestock is well documented. A feed supplement containing BSW extract has been reported to improve serum antioxidant status and alter monocyte cell function in ruminants (Fike et al. 2001). Oxygen radicals, such as superoxide, produced due to incomplete reduction of O_2 contribute to certain diseases and the natural aging process. By catalyzing the dismutation reaction, superoxide dismutase (SOD) converts superoxide anion into oxygen and hydrogen peroxide. Saker et al. (2004) reported that superoxide dismutase activity increased in lambs exposed to prolonged heat stress. However, Kannan et al. (2007b) observed that BSW extract supplementation decreased superoxide dismutase activities in goats exposed to transportation stress, and that the extent of decrease was influenced by breed (Fig. 13.2). In addition to antioxidants in the diet, the antioxidant status in animals is also influenced by selenium, copper, zinc, manganese, and iron availability (Dubeski 1999). The hydrogen peroxide converted from superoxide dismutase is destroyed via several mechanisms. Glutathione peroxidase (GSH-Px), a selenium-containing protein, reduces hydrogen peroxide when glutathione is available. Kannan et al. (2007b) noted that both RBC GSH-Px and WBC GSH-Px activities were higher in seaweed extract-supplemented goats subjected to transportation stress (Fig. 13.3).

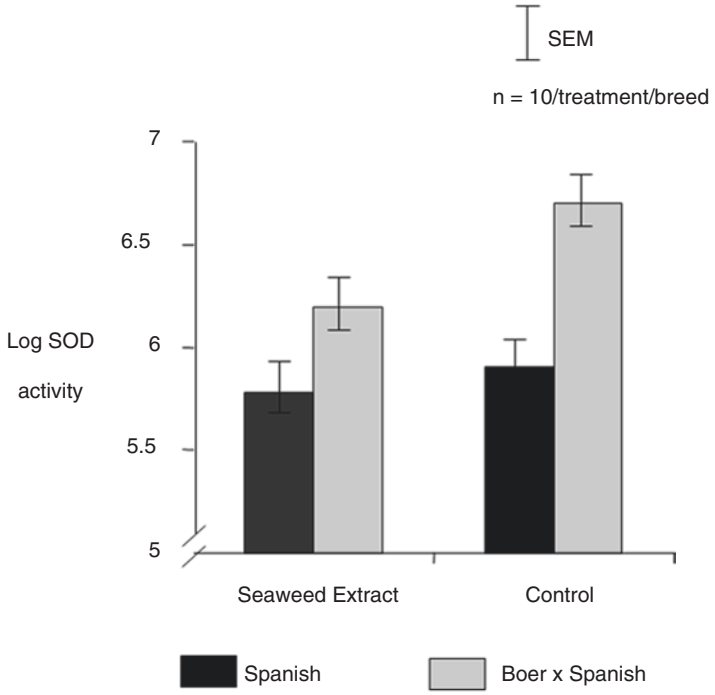

Fig. 13.2 Effect of dietary brown seaweed (*Ascophyllum nodosum*) extract supplementation on blood superoxide dismutase (SOD, mU/mg protein) activity. (Source: Kannan et al. (2007b))

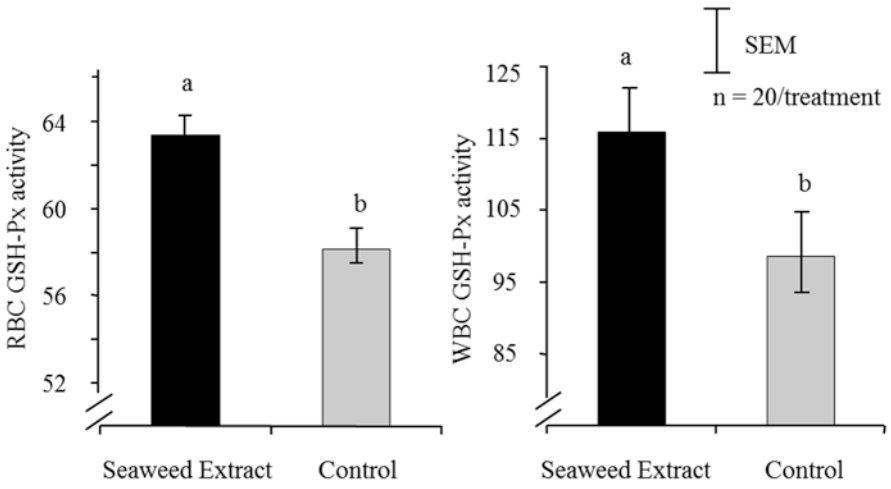

Fig. 13.3 Effect of dietary brown seaweed (*Ascophyllum nodosum*) extract supplementation on red blood cell glutathione peroxidase (RBC GSH-Px) and white blood cell glutathione peroxidase (WBC GSH-Px) activities in goats. Bars within a graph with different alphabets differ significantly ($P < 0.05$). (Source: Adapted from Kannan et al. (2007b))

The RBC GSH-Px activities increased in supplemented goats during the first 2 h of transportation and during the post-transport holding period. The WBC GSH-Px also increased during the first 2 h in supplemented goats, but decreased during the holding period. Saker et al. (2004) also observed increased GSH-Px activities in BSW extract-supplemented wether lambs exposed to extended periods of heat stress.

Lipid peroxidation was also lower during transportation in goats and remained at a lower level throughout post-transport holding in goats supplemented with BSW extract (Kannan et al. 2007b). Lipid peroxidation in control goats remained higher throughout the 6-h transportation and 18-h holding periods (Fig. 13.4). Seaweed extract supplementation could minimize oxidative stress and increase antioxidant activity in small ruminants and help them combat the negative effects of various stressors.

13.4.3 Immune Function

Stress affects the immune system of farm animals since both cortisol and catecholamines reduce immunity in animals. Cortisol is thought to be the primary agent mediating the negative effects of stress on the immune system. Prolonged stress and elevated corticosteroids in circulation alter the leukogram in animals. The percentage

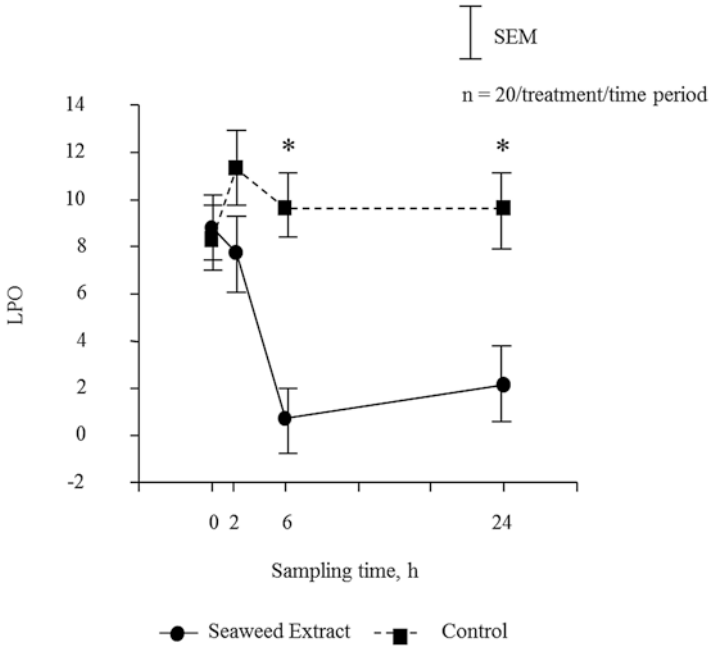

Fig. 13.4 Effect of dietary brown seaweed (*Ascophyllum nodosum*) extract supplementation on blood lipid peroxidase activity (LPO; lipid hydroxyperoxides per sample, μM) in goats. Asterisks indicate that mean values differ significantly ($P < 0.05$). (Source: Kannan et al. (2007b))

of neutrophils increases and that of lymphocytes decreases compared with baseline percentages in goats (Kannan et al. 2000) and cattle (Tarrant et al. 1992) due to transportation. Galipalli et al. (2004a) reported that differential leukocyte counts were not influenced by BSW extract supplementation in goats. Although the antioxidant properties of BSW have been reported to increase immune function in cattle (Saker et al. 2001) and lambs (Saker et al. 2004), the effect was not significant enough to cause changes in leukocyte counts in intact Boer goats. Similarly, Swanson and Morrow-Tesch (2001) observed that calves supplemented with 20 mg/kg of vitamin E (tocopherol-acetate) to boost the antioxidant effect before transport showed no significant improvement in immune response. Minton and Blecha (1990) suggested that both intensity and duration of stressors may be important in causing changes in immunological function in animals.

Kannan et al. (2007b) reported that BSW extract supplementation did not have any effect on phagocytosis despite having significant effects on GSH-Px and SOD activities, although transportation stress increased percent phagocytosis in goats. Phagocytic cell function is influenced by GSH-Px and SOD activities (Saker et al. 2001) and BSW extract has been reported earlier to alter both monocytic cell function and antioxidant status in ruminants (Fike et al. 2001). Major histocompatibility complex (MHC) class II expression and monocyte phagocytic activity were found to be higher in cattle due to BSW extract treatment (Allen et al. 2001a). The enhanced immune function in BSW-supplemented ruminants may be due to the antioxidant activity of phenolic compounds in BSW (Fike et al. 2001) or its ability to increase blood vitamin E (Montgomery et al. 2001). Vitamin E increases phagocytic activity and MHC class II expression in ruminants, while vitamin E deficiency has a reverse effect on phagocytosis.

The neutrophil, lymphocyte, and monocyte counts are not affected by BSW extract supplementation in goats (Kannan et al. 2007b), although a change in leukocyte counts does not necessarily indicate a change in immune function. Eosinophil counts, however, decrease due to BSW supplementation in transported goats (Kannan et al. 2007b).

13.4.4 Rumen Metabolism

Diet composition has been shown to have a significant effect on rumen volatile fatty acid (VFA) concentrations in small ruminants, and this is well documented. For example, rumen acetate and total VFA concentrations were higher in goats fed a diet consisting of 75% bermudagrass (*Cynodon dactylon*) hay than those fed a diet consisting of 75% sericea lespedeza (*Lespedeza cuneate*), a perennial legume high in condensed tannins (Lee et al. 2009a). The authors also found that butyrate, isobutyrate, isovalerate, and valerate concentrations were higher in sericea lespedeza hay-fed goats than bermudagrass hay-fed goats. The concomitant prevalence and activity of high fiber-digesting microbes with high roughage diets are responsible for producing higher acetate concentrations as a by-product of rumen fermentation. Feeding goats an alfalfa hay diet for 90 days resulted in higher rumen acetate

concentration compared with a regimen of 45 days of alfalfa hay diet followed by 45 days of concentrate diet containing 18% crude protein (Lee et al. 2009b). There appears to be a strong influence of dietary fiber and nutrient quality on rumen VFA and pH in goats. High-fiber low-nutrient feeds generally decrease rumen VFA and increase pH, while low-fiber high-nutrient feeds have the opposite effect. In addition, the type of roughage can also influence rumen VFA production. A legume roughage is digested at a higher rate than a grass-type roughage.

Galipalli (2004) found that rumen pH and total VFA concentrations were not influenced by BSW extract supplementation in goats (Table 13.3). This effect was probably due to the fact that the experimental feeds consisted of a base diet of alfalfa pellets supplemented with a commercial pellet feed either with or without seaweed extract, and the diets were not different in roughage-concentrate ratio or particle size. Rumen pH is influenced by roughage-concentrate ratio (Owens and Goetsch 1988), and decreasing forage particle size and ruminally fermentable carbohydrates decreases rumen pH in cows (Krause et al. 2002). Galipalli (2004) also found that propionate concentration decreased as a result of BSW supplementation in goats (Table 13.3).

Kannan et al. (2007a) reported that in Spanish and Boer × Spanish goats, the pH values of rumen fluid were lower in BSW-supplemented animals than the control animals. Generally, higher rumen ammonia and total VFA contents can cause a decrease in ruminal pH values. Dietary antioxidant (vitamin E) supplementation in sheep increases the total VFA and decreases the pH in the rumen, possibly by altering fermentation patterns (Narizoğlu et al. 1997). However, Kannan et al. (2007a) found that BSW supplementation did not significantly increase the total VFA in the rumen of goats, and BSW supplementation did not change the proportion of volatile fatty acids in the rumen. Han et al. (2002) found a decrease in the proportion of propionate and increase in the proportions of butyrate and isobutyrate in the rumen as a result of antioxidant supplementation in cattle. In contrast, butyrate concentrations decreased due to vitamin E supplementation in lambs (Hidiroglu and Lessard 1976). The effect of antioxidants on the proportions of rumen volatile fatty acids does not seem to be consistent in animals.

Table 13.3 Effects of dietary brown seaweed (*Ascophyllum nodosum*) extract supplementation on rumen pH and volatile fatty acid (VFA) concentration in goats

Item	Seaweed extract	Control	*P*-value
Rumen pH	6.3 ± 0.06	6.3 ± 0.06	0.87
Total VFA, mM	31.2 ± 3.58	26.1 ± 3.58	0.32
Acetate, %	56.2 ± 3.94	46.2 ± 3.81	0.08
Propionate, %	17.5 ± 1.84	22.5 ± 1.78	0.05
Butyrate, %	12.1 ± 1.01	13.7 ± 0.98	0.27
Isobutyrate, %	6.8 ± 0.85	8.4 ± 0.82	0.16
Valerate, %	5.3 ± 0.58	5.9 ± 0.56	0.39
Isovalerate, %	2.1 ± 0.33	2.9 ± 0.32	0.09
Acetate-propionate ratio	4.7 ± 0.45	4.4 ± 0.43	0.63

Source: Galipalli (2004)

Breed can have an effect on rumen metabolism. With identical diets, the rumen fluid pH recorded was lower and the ammonia levels were higher in the Spanish goats compared to the crossbreds (Kannan et al. 2007a). There is strong evidence that feeding ruminants with diets containing seaweed can change rumen microflora; however, Makkar et al. (2016) suggested that further investigations may be required to better understand the adaptation of microflora and the mechanisms involved in altering rumen metabolism.

13.4.5 Gut Microbial Population and Food Safety

The digestive tracts of sheep and goats are natural reservoirs of *Escherichia coli,* and their prevalence has been well documented (Shukla et al. 1995; Kudva et al. 1996). Controlling pathogens in live animals is regarded as a critical step in the production of wholesome and safe meat since fecal shedding of pathogens has been correlated with carcass contamination (Elder et al. 2000). Consumer exposure to pathogens could be reduced by employing effective dietary strategies in small ruminants that reduce food-borne microorganisms prior to slaughter. Diet can significantly influence fecal shedding of pathogens in animals (Kudva et al. 1997).

Studies on dietary strategies in small ruminant animals have included varying the proportions of hay and concentrate, switching to a hay diet 2 days prior to slaughter, varying the duration of preslaughter feed deprivation, and feeding a diet containing BSW extract supplement for 2 weeks prior to slaughter, to name a few. Gutta et al. (2009) observed that *E. coli* counts tended to be higher in concentrate-fed sheep and goats than in hay-fed animals. A similar trend was also reported by Kudva et al. (1997) in sheep and by Diez-Gonzalez et al. (1998) in cattle. High-grain diets result in lower rumen pH due to increased starch fermentation and the resultant increased production of VFAs. Lower pH environment is generally expected to result in lower *E. coli* and coliform counts. It is hypothesized that fermentation of easily digestible carbohydrate portion of concentrate diets results in the production of simple sugars such as glucose, carbon skeletons, nitrogenous precursors, and adenosine triphosphate molecules, in addition to VFA. All of these substances are likely to promote microbial growth in the rumen. The absence of such readily available nutrients in the rumen of hay-fed animals results in lowered *E. coli* counts despite higher pH. However, in vitro studies have shown higher *E. coli* O157:H7 multiplication in ruminal fluid from cattle fed a high-forage diet than in ruminal fluid from cattle fed a high-grain diet (Tkalcic et al. 2000). Laboratory strains of *E. coli* are found to be more sensitive to VFA-dependent pH decrease, while natural strains of *E. coli* are more pH and VFA resistant.

Dietary BSW seaweed extract supplementation in feedlot cattle has been reported to reduce *E. coli* proliferation in the digestive tract and reduce fecal shedding (Behrends et al. 2000; Braden et al. 2004). The increased *Salmonella* shedding normally seen after transportation in cattle was significantly reduced due to seaweed supplementation (Braden et al. 2004), although this effect could not be seen in

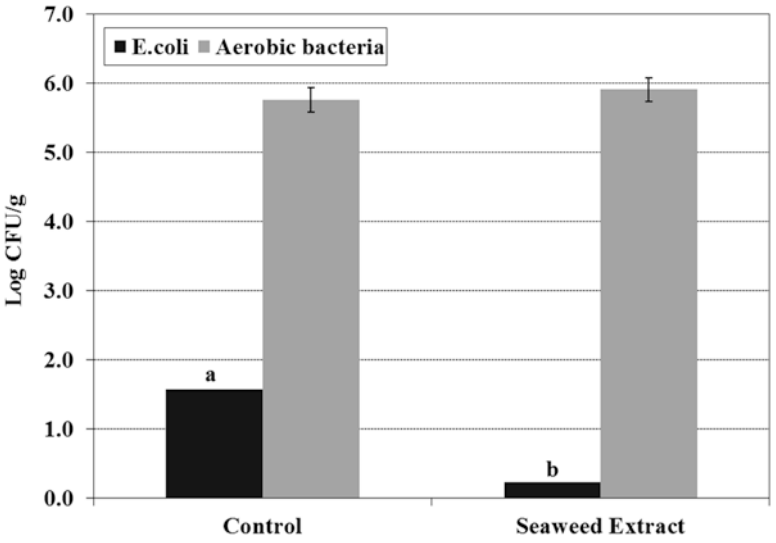

Fig. 13.5 Effect of dietary seaweed extract supplementation on bacterial counts in rumen liquor of goats. Bars with different alphabets differ significantly ($P < 0.05$). (Source: Kannan et al. (unpublished data))

in vitro trials (Behrends et al. 2000). Studies in small ruminants have also shown reduction of *E. coli* proliferation as a result of dietary BSW supplementation. Zhou et al. (2018) reported that BSW meal feeding in sheep reduced fecal shedding of *E. coli* with minimum effect on rumen fermentation, which was consistent with the findings in goats (Kannan et al. unpublished data). While the rumen contents of pH did not change due to seaweed extract supplementation in goats, *E. coli* counts were significantly reduced as a result of supplementation (Fig. 13.5). The results in small ruminants confirm those in cattle that the reduction in microbial proliferation is not pH dependent, but rather due to the direct effect of seaweed on microbes and/or due to the overall positive effect of seaweed extract on the heath of animals.

13.4.6 Body Composition

Although overnight holding of goats at slaughter facilities helps livestock recover from transportation stress prior to slaughter (Kannan et al. 2000), prolonged feed withdrawal in holding pens may contribute to live weight loss (shrinkage). Shrinkage due to transportation or holding after transportation or both is not affected by BSW supplementation in goats (Table 13.4; Galipalli 2004). Transportation stress causes perspiration, resulting in dehydration and higher blood packed cell volume (PCV). Packed cell volume is not influenced by BSW supplementation in goats, although there is an indication that BSW-treated goats can have lower PCV than control goats when measured

Table 13.4 Effect of dietary brown seaweed (*Ascophyllum nodosum*) extract supplementation on body composition and carcass yield characteristics in goats

Item	Seaweed extract	Control	*P*-value
Live weights			
Body weight, kg	49.9 ± 1.33	47.6 ± 1.30	0.22
BWAT,[a] kg	47.3 ± 1.23	45.2 ± 1.19	0.24
BWAH,[b] kg	44.7 ± 1.19	43.2 ± 1.16	0.38
T Shrink,[c] %	5.2 ± 0.70	4.9 ± 0.70	0.82
H Shrink,[d] %	5.5 ± 0.51	4.5 ± 0.49	0.15
TH Shrink,[e] %	10.5 ± 0.70	9.2 ± 0.68	0.24
ADG,[f] kg	0.25 ± 0.02	0.22 ± 0.02	0.17
Carcass yield characteristics			
HOTCW,[g] kg	22.2 ± 0.62	21.9 ± 0.60	0.70
COLDCW,[h] kg	21.5 ± 0.58	20.9 ± 0.55	0.49
C Shrink,[i] %	3.4 ± 0.49	4.3 ± 0.47	0.18
M Fat,[g] g	1179.0 ± 122.12	1025.7 ± 117.98	0.37
Carcass yield, %	50.1 ± 0.67	49.3 ± 0.65	0.38
Organ weights			
Heart, g	216.3 ± 8.30	217.6 ± 8.02	0.91
Liver, g	833.0 ± 26.34	754.4 ± 25.45	0.04
Lungs, g	801.6 ± 36.69	720.1 ± 35.44	0.12
Kidneys, g	146.5 ± 4.10	136.3 ± 3.96	0.08
Spleen, g	62.4 ± 3.26	61.3 ± 3.15	0.80
GIT,[h] g	7261.1 ± 270.30	6552.9 ± 261.14	0.07

Source: Galipalli (2004)
[a]Body weight after transport
[b]Body weight after holding
[c]Shrinkage due to transportation
[d]Shrinkage due to holding
[e]Shrinkage due to transportation plus holding
[f]Average daily gain
[g]Hot carcass weight
[h]Cold carcass weight
[i]Shrinkage due to cooling
[g]Mesenteric fat
[h]Gastrointestinal tract

immediately after a 6-h transportation (Galipalli 2004). There is evidence that BSW supplementation can help ruminants better regulate body temperatures and cope with heat stress (Williams et al. 2009; Pompeu et al. 2011); however, BSW does not seem to have a significant effect on reducing body dehydration and shrinkage.

Brown seaweed supplementation does not influence average daily gain (ADG) in goats (Galipalli 2004); it is used as a feed supplement for its antioxidant activity and mineral contents rather than for its protein or energy content. The antioxidant properties alone are unlikely to influence ADG, since Waylan et al. (2002) found that vitamin E did not influence ADG of growing finishing pigs. Galipalli (2004) also

found that BSW extract supplementation increased liver weight and tended to increase kidney and gastrointestinal tract weights in goats (Table 13.4). Although microbial yield was not measured in that study, the antioxidant nature of BSW may have increased the microbial population and activity in the rumen. Microbial population increase results in increased digestible organic matter intake (DOMI) in ruminants (Goetsch 1998). The DOMI is correlated to the microbial nitrogen flow to the abomasum. Thus, higher DOMI and microbial nitrogen reach the small intestine (Kouakou et al. 1997; Goetsch 1998), and a greater array and quality of nutrients are absorbed by the small intestine and metabolized by the liver. To compensate for the increased nutrient load, the small intestine and liver enlarge and increase in weight as they try to metabolize and absorb the nutrients (Kouakou et al. 1997).

Heart, lungs, spleen, and mesenteric fat weights were not affected by BSW supplementation in goats (Galipalli 2004; Table 13.4). Carcass composition depends on diet, age, species, and breed of animal. Goats have a very low growth rate and have a tendency to store energy in the form of mesenteric fat if they consume more feed than required.

13.4.7 Carcass and Meat Quality Characteristics

Carcass composition, primal cut weights, or proximate composition of loin chops (*Longissimus dorsi*) were not affected by BSW supplementation in goats (Galipalli 2004; Table 13.5). Warner-Bratzler shear force (WBSF) values or cooking losses of chevon loin/rib chops were also not influenced by BSW supplementation in this study (Table 13.5). Increasing antioxidant content of muscles is unlikely to affect WBSF and cooking loss, as previous studies have shown that vitamin E supplementation in swine (Waylan et al. 2002) and cattle (Gardner et al. 1996) does not have a beneficial effect on these quality attributes.

Rosenvold et al. (2002) reported that vitamin E supplementation increased muscle glycogen content in pigs, which may lower the postmortem ultimate pH of meat. However, BSW supplementation does not influence muscle glycogen content and pH in goats (Galipalli 2004). Studies in cattle have shown that vitamin E supplementation does not influence pH of beef (Eikelenboom et al. 2000).

Meat color largely depends on the chemical status of myoglobin (Mb) pigment (Faustman and Cassens 1990). During storage, the different chemical forms (deoxymyoglobin, oxymyoglobin and metmyoglobin) can interconvert by oxidation and reduction reactions. Meat surface discoloration is mainly associated with the oxidation of myoglobin to metMb. The rate of discoloration depends on the rate of metMb formation in meat, which is controlled by the oxidation process of muscle pigment and enzymatic reducing systems.

The formation of metMb increased from days 1 to 7 of refrigerated display of chevon loin/rib chops in both control and BSW-supplemented groups in a study by Galipalli (2004); however, the mean metMb values pooled over all time periods were less in the BSW-supplemented group compared with the control group (Galipalli 2004). The percent metMb may have been the same in both treated and control goats

Table 13.5 Effect of dietary brown seaweed (*Ascophyllum nodosum*) extract supplementation on primal cut weights and composition and quality of chevon

Item	Seaweed extract	Control	*P*-value
Primal cuts			
Loin, kg	4.2 ± 0.16	4.0 ± 0.16	0.42
Leg, kg	6.2 ± 0.15	6.2 ± 0.15	0.98
Shoulder, kg	4.7 ± 0.15	4.6 ± 0.15	0.78
Ribs, kg	4.1 ± 0.15	3.7 ± 0.15	0.14
Neck, kg	2.4 ± 0.17	2.5 ± 0.16	0.77
Proximate composition			
Dry matter, %	25.9 ± 0.46	25.4 ± 0.44	0.46
Ash, %	1.1 ± 0.02	1.2 ± 0.02	0.51
Protein, %	22.8 ± 0.34	22.6 ± 0.33	0.71
Fat, %	0.5 ± 0.03	0.5 ± 0.03	0.57
Meat quality			
WBSF,[a] kg	4.9 ± 2.93	4.9 ± 2.83	0.87
Cooking loss, %	24.2 ± 0.76	24.7 ± 0.73	0.58
L^* value	39.9 ± 0.87	39.4 ± 0.85	0.66
a^* value	12.0 ± 0.33	11.8 ± 0.32	0.69
b^* value	15.5 ± 0.49	14.8 ± 0.48	0.89
Visual score	10.0 ± 0.29	10.0 ± 0.28	0.67
Metmyoglobin, %	18.7 ± 0.47	21.6 ± 0.46	0.01
TBARS[b]	2.2 ± 0.49	1.8 ± 0.47	0.17

Source: Galipalli (2004) and Galipalli et al. (2004b)
[a]Warner-Bratzler shear force
[b]Thiobarbituric acid reactive substances (mg melondialdehyde/kg of meat)

immediately after slaughter, since the carcasses would not have been exposed to atmospheric oxygen. The lower metMb levels at 24 h postmortem (d-1 sampling) in the BSW-supplemented group may have been due to the enhanced antioxidant content (Table 13.5), although vitamin E was not detectable in *L. dorsi* muscle or liver samples from BSW-supplemented goats (Galipalli 2004). Goats have less intramuscular fat compared to sheep or cattle; therefore, the color stability and antioxidant benefits of elevated α-tocopherols are likely to be more pronounced in lamb and beef than in chevon. Montgomery et al. (2001) reported that improvement in color stability of meat followed by BSW supplementation may be related to elevated antioxidant activity in steers. Elevated α-tocopherol can help extend the retail shelf life of meat cuts obtained from primals stored for extended time periods (Liu et al. 1995). Instrumental color (L^*, a^*, b^*, chroma, hue) values or visual scores of chevon were not influenced by dietary supplementation of BSW extract (Galipalli et al. 2004b).

In addition to color, odor also affects consumer acceptability of meat products. Lipid peroxidation is one of the major causes for meat quality deterioration due to the formation of hydroperoxide end products. These products are responsible for warmed overflavor in meat. The oxidative stability of meat depends on the degree of unsaturation of the phospholipids of the subcellular membrane (Coetzee and Hoffman 2001).

Lipid peroxidation in muscle starts in the highly unsaturated fractions of the subcellular membrane that include mitochondria and microsomes (Gray and Pearson 1987). The amount of polyunsaturated fatty acids in muscle is one of the most important factors that affect lipid peroxidation (Allen and Foegeding 1981). Chevon has been reported to have more unsaturated fat than other types of red meat (Park and Washington 1993). Dhanda et al. (1999) also reported that the saturated fatty acid concentration decreases and the unsaturated fatty acid concentration increases with increased age of goats. Dietary BSW supplementation in goats did not affect the thiobarbituric acid reactive substances, a measure of lipid oxidation, in chevon (Galipalli et al. 2004b). Goats may not have accumulated enough vitamin E, a fat-soluble vitamin, to influence the lipid peroxidation rate of meat because of their inherent tendency to deposit lower intramuscular and subcutaneous fat (Babiker et al. 1990). In addition to the amount of fat in muscles, the dosage and duration of antioxidant supplementation can also influence the lipid stability of the meat. The amount of α-tocopherol accumulated in subcutaneous fat depends on the length of time of vitamin supplementation in cattle (Arnold et al. 1993). At least 500 mg of α-tocopherol acetate/kg DM is required to be supplemented to lambs on dry pelleted feed to accumulate a sufficient amount (3.3 μg/g) in muscle (Kasapidou et al. 2001). Galipalli et al. (2004b) used BSW supplement at 2% of daily intake as recommended by the feed company.

The α-tocopherol contents in liver and muscle can also be affected by other factors. Nockels et al. (1996) reported that stress reduced the liver vitamin E content in both vitamin E-supplemented and non-supplemented heifers. Prolonged exposure to intense stressors may reduce the vitamin E content of the liver. The small amounts of muscle fat in goats (Babiker et al. 1990) may also be depleted due to antemortem stress, which may explain the undetectable amounts of muscle vitamin E in goats (Galipalli 2004). Based on the limited amount of data available, there is evidence that increasing the vitamin E content may not be the only mechanism by which BSW enhances antioxidant status in ruminants.

13.5 Perceived Downsides of Brown Seaweed Usage

Despite the overwhelming benefits of BSW usage in livestock feed supplements, as evidenced by scientific literature and various industry applications and related seasonal job creations, it is important to also be mindful of the perceived negatives of commercial overharvesting of seaweeds such as *A. nodosum*. Critics of commercial overharvesting believe that it can likely change the coastal ecological balance such that it may deprive certain marine organisms of feed. Several arthropods and birds that feed on benthic invertebrates cannot survive without the seaweeds (Makkar et al. 2016). There is also an ongoing debate on whether seaweed harvesting affects commercially important fish. In its natural habitat, *A. nodosum* could serve as a natural indicator of heavy metal pollution in seawater. Seaweeds generally take up inorganic elements and heavy metals from seawater, and the concentrations vary according to geographical regions and proximity to industrial wastes and mining operations (McHugh 2003).

13.6 Conclusions

Brown seaweed extract has several beneficial effects on farm animals. Goats are an important animal species for testing the BSW extract product since chevon is widely consumed throughout the world. The meat goat industry is steadily growing in the USA due to a growing ethnic population. Published data available on pre- and post-harvest strategies to improve profitability of meat goat enterprises is very limited. Furthermore, goats can be used as a model and the data derived can be applied to other farm animal species.

In the USA, goats are sometimes transported for long distances to abattoirs that accommodate goat processing, although 2–3-h journeys are common. After long journeys, meat goats may become extremely susceptible to respiratory infections during preslaughter holding, which makes them unfit for processing and human consumption. Transportation has been shown to be a potent stressor in small ruminants, which can adversely affect the immune function. Antioxidants are essential in reducing the formation of oxygen radicals, such as superoxide, and protecting the immune system. Similar to the effects on other livestock species, BSW extract supplementation improves antioxidant status and immune function in goats.

There is increased research attention for reducing the number of pathogens (such as *E. coli*) in live animals prior to processing them for food to minimize chances of food contamination. Similar to cattle and sheep, goats shed *E. coli* into the environment through feces without showing any clinical signs. The degree of visible contamination on the animal hide has been shown to affect contamination levels of the resultant carcass. Brown seaweed extract supplementation for 2 weeks prior to shipment of goats to processing facilities reduces *E. coli* counts in the rumen. Although the mechanism of action is not clear, BSW extract supplementation can be a viable preharvest food safety strategy in ruminants.

Brown seaweed extract supplementation improves meat quality in goats. Supplementation for 2–3 weeks prior to harvesting in goats results in improved color stability and shelf life of meat, thereby allowing higher profitability.

The additional cost incurred by the producer towards BSW extract feed supplement will be offset by the benefits, such as better disease resistance of animals and improved color stability and shelf life of meat, in addition to reduced fecal shedding of pathogens by animals. More studies on rumen ecology are required to better understand the biological mechanisms involved in changing the rumen microflora in BSW-supplemented animals.

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Chapter 14
Discovery of Green Tea Polyphenol-Based Antitumor Drugs: Mechanisms of Action and Clinical Implications

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14.1 Introduction

Cancer is an umbrella term for a group of diseases in which dysregulated cells proliferate. As of 2018, cancer was the world's second most prevalent and fatal disease (World Health Organization 2018). There was an estimated 1,735,350 new cases of cancer in the United States in 2018, and it was estimated that 609,640 deaths will occur due to cancer in the United States in 2018 (Siegel et al. 2018). Using global data, experts have estimated that there will be 21.7 million new cancer cases and 13 million cancer fatalities in 2030 (National Cancer Institute 2018). Conventional

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cancer therapies such as surgery, chemotherapy, and radiotherapy have significant limitations, including a high-cost development of resistance, and adverse side effects. Therefore, there is a pressing need for new drugs and new therapeutic strategies that are safe, selective, and effective for cancer treatment. In recent years, many physiological compounds and natural products, including steroid hormones, vitamin analogs, cytokines, and plant phytochemicals, have demonstrated the ability to induce differentiation, cellular death via apoptosis, and growth inhibition in human cancer cells (Wang et al. 2012).

In particular, the naturally occurring flavanols—polyphenols—which are found in green tea have been researched for such anticancer properties (Ahmed et al. 2016; Zhang et al. 2010a; Chen et al. 2011; Yang and Wang 1993; Dou et al. 2008; Dou 2009). A subgroup of polyphenols, catechins, is known for their powerful antioxidant properties. Studies by many groups have demonstrated that green tea catechins can act as cancer-preventative compounds by modulating multiple cell signaling pathways that are involved in carcinogenesis (Ahmed et al. 2016; Zhang et al. 2010a; Chen et al. 2011; Yang and Wang 1993; Dou et al. 2008; Dou 2009). The most abundant and potent of these green tea polyphenols is epigallocatechin-3-gallate (EGCG) (Fig. 14.1a). Several reports show that EGCG can reduce proliferation and growth of human tumors including breast, prostate, and leiomyoma (Ahmed et al. 2016; Zhang et al. 2010a). However, there are some identified limits to EGCG's therapeutic efficacy. EGCG has poor bioavailability especially when ingested. Not only will most of the EGCG be excreted, but it also has poor stability and is susceptible to biotransformation within the human body during digestion and absorption. Many researchers (including ourselves) have developed novel EGCG analogs and prodrugs to address these limitations (Chen et al. 2011; Yang and Wang 1993; Dou et al. 2008; Dou 2009).

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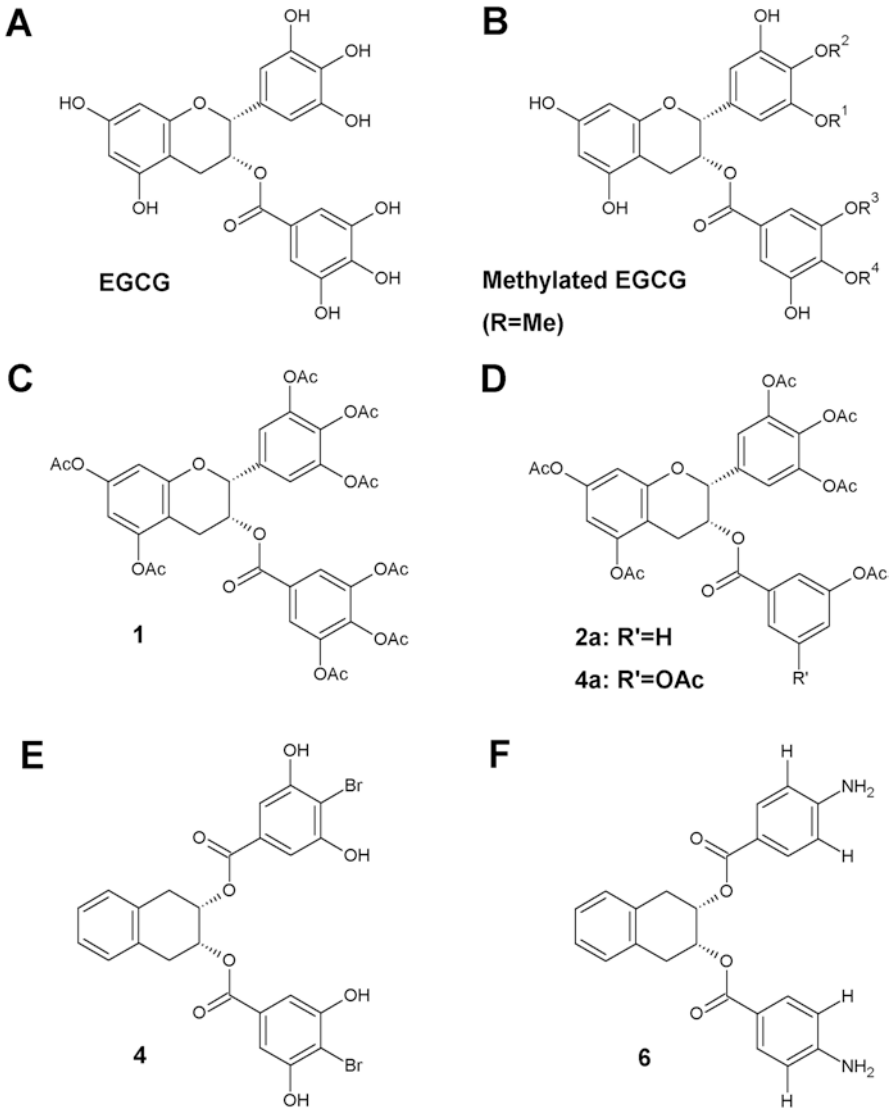

Fig. 14.1 Chemical structures of EGCG (**a**), methylated EGCG metabolites (**b**), EGCG-like prodrugs **1** (**c**), **2a**, and **4a** (**d**), as well as EGCG analogs **4** (**e**) and **6** (**f**)

14.2 Green Tea, EGCG, and Cancer

The origin of the tea plant, *Camellia sinensis*, is believed to be located somewhere in Asia; however experts cannot be entirely sure where, especially considering that the "wild" plant is extinct. Tea is thought to have originated as a medicine in ancient China. From sometime about 3000 BC to 2100 BC, tea leaves were chewed for stimulation and refreshment (Du et al. 2012). One ancient Chinese tells the story of

how a stray leaf fell from a wild bush and landed into a pot of water that was being boiled by the Chinese emperor Shen-Nung in 2737 BC. According to the legend, this led to the discovery of the beverage known as tea.

Historically, tea has been used in brews and meal preparation since 722 BC–221 BC. For the subsequent years there is evidence of a variety of teas' widespread consumption. In actuality, all "real" tea varieties originate from the herb *Camellia sinensis*; it is simply a matter of when the leaves are picked and how they are preserved. Any teas made from a different plant are called herbal teas. From China, tea migrated to Japan, and then along the trade routes to Tibet, the Arabic nations, the Turks, the nomadic tribes of the Indian Himalayas, and India. Finally, in the sixteenth century, tea crossed the continents to Europe. It is at this point that Europeans developed the process for black tea to increase the durability and thus the profit margin on tea. The increased hardiness allowed the beverage to become one of the world's most popular drinks after water.

Most research focuses on green tea's potential as a cancer preventative and cure, rather than black tea. This is because although they both come from the same plant they are not exactly the same due to the curing process. For example, green tea contains 30–40% catechins (like EGCG) whereas black tea contains only 3–10% (Hu et al. 2009). This high percentage of catechins largely explains why green tea has remained a focus of nutraceutical research. In recent years, green tea has become the focus of many research studies due to its potential for human health benefits, as well as in epidemiological and clinical settings as an anti-disease agent.

In one example, an investigation into the frequency of cancer across racial groups has shown that Asian and Pacific Islander populations have lower occurrences and mortality rates due to cancer when compared with other races. There may be a correlation between these low cancer statistics and the dietary intake of green tea, a popular beverage which is often consumed daily in Asian and islander societies (Dou et al. 2008). Green tea leaves may contain roughly 35–42% of catechins and flavonols upon extraction. Additionally, it is estimated that approximately one cup of green tea contains 300–400 mg of polyphenols (Chen et al. 2011). Catechins, a subgroup of polyphenols, are found in relatively high concentrations in green tea (Dou 2009), including epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and EGCG. EGCG has been identified as the most common polyphenol in green tea (Du et al. 2012). It has been well documented that EGCG has anticancer activity associated with its ability to inhibit key cancer-related targets including tumor necrosis factor alpha, the proteasome, and others (Yang and Wang 1993; Dou et al. 2008; Dou 2009). This suggests that EGCG could be used as an agent for treatment and prevention of cancer.

14.2.1 EGCG as a Chemopreventative

The diseases known as cancer occur where cellular homeostasis has been interrupted by hyperplastic, dysplastic, or regenerative changes. These changes are brought about by chemical, physical, biological, and/or genetic assaults on cells

inducing changes in the genome (Park and Dong 2003). Cancer essentially has two "phases," carcinogenesis and oncogenesis. Oncogenesis is the maintenance and evolution of the existing transformed cells and tumors. Carcinogenesis is the creation of those transformed cells and tumors. It causes the tumor initiation and growth within the host. While there are multiple theories of carcinogenesis, the *multistage theory* is the most widely held and accepted. This theory allows for three stages of carcinogenesis. The first two stages induce transformations of the cells, while the third stage is more involved in final transformations to malignancy.

The first stage of carcinogenesis is initiation. This stage occurs when DNA is targeted. These changes to genetic material are irreversible. They are subtle, and fast; initiation can occur from within minutes to within hours (Park and Dong 2003; Afaq et al. 2003). Initiation can be caused by mutations, transitions, deletions, and even changes in the gene function such as histone modification, transcription activity, and DNA methylation (Park and Dong 2003; Afaq et al. 2003). This first stage aligns with both the *genetic mutation* and *viral* theories of carcinogenesis. Cells can remain in the initiation stage for an unlimited amount of time and most initiated cells will never move to the next two stages remaining quiescent for the duration of their life cycle (Park and Dong 2003; Afaq et al. 2003). Unfortunately, cells at the initiation stages have no phenotype expression making it very hard to detect and target for treatment.

The second stage of carcinogenesis—promotion—is easy to identify. It acts on genetic programs of terminal proliferation and differentiation much like the *aberrant differentiation* theory of carcinogenesis suggests (Afaq et al. 2003). At this stage cells no longer recognize the differentiation signals that would usually remove them. Unlike initiation, promotion targets the cell membrane and is reversible by remediation and apoptosis (Park and Dong 2003; Afaq et al. 2003). It leads to the expression of the genome and is not an essential stage in carcinogenesis; promotion may be bypassed. Initiation can go directly to stage three, progression (Park and Dong 2003; Afaq et al. 2003).

Progression, the final stage of carcinogenesis, is when evident malignant neoplasms appear (Park and Dong 2003; Afaq et al. 2003). It is characterized by unstable karyotypes caused by major genetic alterations (Park and Dong 2003; Afaq et al. 2003). There are multiple types of evolving karyotypic instabilities caused by increasing chromosomal irregularities and cellular proliferation (Park and Dong 2003). These include (1) invasion, (2) metastatic growth, (3) anaplasia, and (4) increased proliferation and growth to ever-increasing malignancy, potentially leading to the death of the host (Park and Dong 2003; Afaq et al. 2003). Once cells have reached progression they must be treated.

Green tea polyphenols are of interest not only for their anti-oncogenesis capabilities, but also as possible chemopreventatives. Research has shown that green tea polyphenols can prolong the stages of carcinogenesis either individually or in tandem (Chen et al. 2011; Yang and Wang 1993; Dou et al. 2008; Dou 2009). While EGCG's mechanisms as a chemopreventative are not 100% understood they are assumed to include protecting the DNA from damage and methylation, which will stop carcinogenesis at its initiation stage. Similarly, it may inhibit cell proliferation and tumor promotion (Chen et al. 2011). EGCG inhibits oncogene expression and

uses its pro-apoptotic properties to induce apoptosis to reverse the promotion and progression stages of carcinogenesis (Park and Dong 2003). In the latter stages of carcinogenesis EGCG interferes with cell cycle regulation by disrupting signaling pathways. These affected pathways include (1) the ubiquitin proteasome system (UPS), (2) kinase signaling and activation (i.e., MAPKs), (3) EGFR-mediated pathways, and (4) insulin growth factor-I (IGF-I)-mediated signal transduction pathways (Chen et al. 2011; Yang and Wang 1993; Dou et al. 2008; Dou 2009). Studies have proven that decreased IGF-1 levels correlate to a decrease of the incidence of cancer (Chen et al. 2011; Yang and Wang 1993; Dou et al. 2008; Dou 2009). This supports the use of green tea polyphenols—EGCG in particular—as chemopreventatives.

Animal studies conducted using EGCG showed that green tea inhibits tumor occurrence, interfering with some stage of carcinogenesis, possibly initiation, through its antioxidant properties. A mouse study, using fluid ingestion of green tea, done on NDEA-involved lung tumors, found a 36–44% decrease of tumor incidence (Chen et al. 2011). The same study observed a decrease of 44–60% in tumor multiplicity (stage three). A different animal study showed reduced UVB-induced skin cancer incidence by 35%; the multiplicity was reduced by 63% and growth by 55% using oral green tea polyphenols (Chen et al. 2011). Even topical application demonstrated decreases in incidence, growth, and multiplicity in animal studies. These effects were demonstrated on numerous cancer sites including skin, lung, stomach, colon, liver, and mammary gland. All the while, they proved to be relatively safe (Chen et al. 2011; Yang and Wang 1993; Dou et al. 2008; Dou 2009).

14.2.2 EGCG Can Target Multiple Molecular Signaling Pathways Required for Cancer Cell Survival

It has been shown that EGCG exhibits antioxidant, anti-inflammatory, and antitumor activity in various types of cancer (Hu et al. 2009). These potent effects are related to EGCG's ability to regulate various signaling pathways involved in cancer cell growth and proliferation (Zaveri 2006). Studies have shown that EGCG is able to halt tumor cell growth at the G_1 phase (or Gap 1 phase, the first of four phases of the cell cycle that takes place in eukaryotic cell division) by inhibiting key enzymes known as cyclin-dependent kinases (CDKs), which prevents cells from continuing to mitosis and therefore inhibits tumor growth. EGCGs can also act by inducing other CDK inhibitors in breast, prostate, and other cancer cells (Park and Dong 2003). Additionally, EGCG has been shown to inhibit the DNA transcription factor NF-κB, a protein complex that controls cytokine production and cell survival (Afaq et al. 2003). Reactive oxygen species (ROS) can activate NF-κB, which subsequently activates the transcription of survival genes that may also be anti-apoptotic, thus creating hardier cells. NF-κB also cross talk to another cancer survival signaling pathway, the PI3-kinase/AKT pathway, ensuring cancer cell survival

(Hussain et al. 2012). EGCG possesses ROS-scavenging properties, which prevent NF-κB from initializing (Singh et al. 2011), and also inhibits PI-3K/AKT pathway (Van Aller et al. 2011).

When used with conventional cancer therapies, EGCG can increase their efficacy. In one study, breast cancer patients undergoing radiotherapy were split into two groups—one that was given 400 mg oral EGCG capsules thrice daily in addition to their therapy, and a control group that was not. The success of the EGCG treatment was determined by measuring cell proliferation factors in blood samples collected at various time points. The group who consumed the EGCG capsules followed by radiotherapy demonstrated lower serum levels of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and reduced activation of metalloproteinase-9 and metalloproteinase-2 (MMP9/MMP2) as compared with the group who only underwent radiotherapy (Zhang et al. 2012). In a different study that used pancreatic cancer cells, EGCG was shown to increase the effect of gemcitabine, a nucleotide analog, and CP690550, a small-molecule immunosuppressant. This study also showed that EGCG decreased expression and activation of STAT3, an oncogene transcription factor, while also inducing apoptotic pathways in the pancreatic cancer cells (Tang et al. 2012).

EGCG demonstrates specific discernment in its effect on tumors, or transformed cells. One study showed that when tested on normal and transformed NIH-pATM Ras fibroblasts, EGCG showed an inhibitory and pro-apoptotic effect on the tumor cells without any effect on normal cells (Wang and Bachrach 2002). The study analyzed various growth factors in response to EGCG treatment of normal and transformed fibroblasts. The cellular proliferation signal molecule, ornithine decarboxylase (ODC), displayed decreased levels in transformed cells, but not in normal cells. ECGC also caused a reduction in numerous kinase activities, including those involved in the MAPK pathway, and a decrease in the oncogenes Ras and Jun. These effects were also specific to transformed cells and were not in evidence in normal cells (Wang and Bachrach 2002).

14.2.3 EGCG as a Tumor 20S Proteasome Inhibitor

It has been reported that EGCG can act as a 20S proteasome inhibitor, and EGCG treatment of human cancer cells results in the accumulation of pro-apoptotic proteins, such as IκB and Bax, both of which are NF-κB inhibitors (Nam et al. 2001). Cells regulate their proteasome pathway as a survival strategy to respond to environmental changes and fluctuations in its growth needs. It is estimated that ~90% of proteins in the cell are degraded by the ubiquitin-proteasome system (UPS) (Fig. 14.2) (Shen et al. 2012). Many of the proteins involved in carcinogenesis and tumor growth are degraded through the UPS. The UPS consists of enzymatic ubiquitination followed by proteolysis. A cascade of enzymes—E1, E2, and E3—tag misfolded old or unneeded proteins with ubiquitin (Ub). The 26S proteasome is the executive end of the UPS. It is comprised of two 19S proteasome caps and a 20S

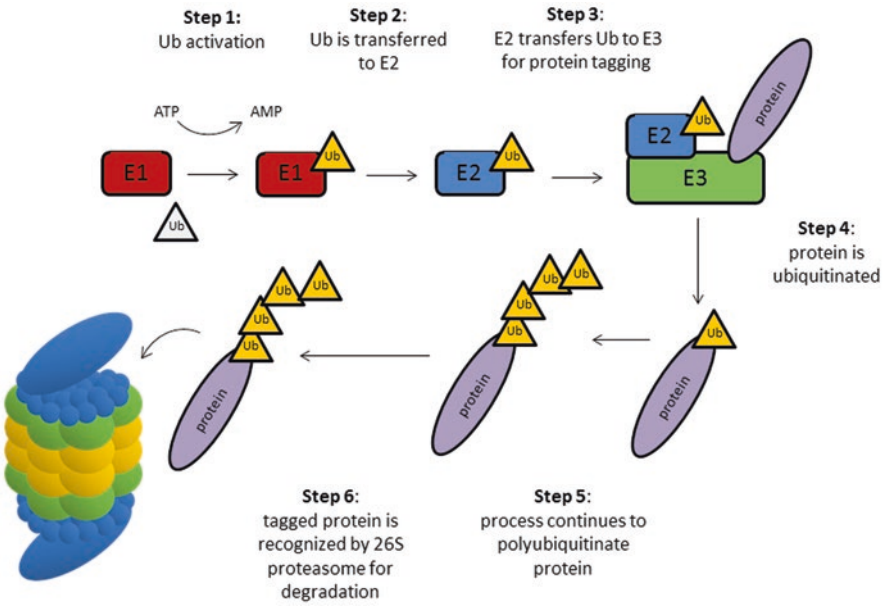

Fig. 14.2 The ubiquitin-proteasome system or UPS. Substrate proteins are tagged for degradation with ubiquitin molecules via an ATP-dependent pathway, facilitated by three enzymes (E1, E2, and E3)

proteolytic core (Fig. 14.3) (Shen et al. 2012; Lin et al. 2002). The 19S subunit recognizes Ub-tagged protein substrates; it can then release the protein or its deubiquitinating (DUB) enzymes remove the Ub tags and then the proteins are linearized in preparation for their cleavage by the 20S catalytic core particle. The 20S subunit is a cylindrical structure comprised of two identical outer α-rings and two identical inner β-rings. The α-rings have seven subunits and act as "gates," while the β-rings contain the active proteolytic sites. The β-subunits contain chymotrypsin-like (CT), trypsin-like, and post-glutamyl peptidyl hydrolytic-like (PGHP) activities (Shen et al. 2012; Lin et al. 2002; Adams 2004).

The first FDA-approved proteasome inhibitor was bortezomib (BTZ) also known by its trade name Velcade (Adams 2004; Chen et al. 2008; Dou and Zonder 2014). BTZ is administered to patients with multiple myeloma and mantle cell lymphoma intravenously. Bortezomib targets the β5 and β1 but not β2 active sites of the catalytic subunits of the 20S proteasome, leading to cancer cell growth inhibition (Adams 2004; Chen et al. 2008; Dou and Zonder 2014). Specifically, the boron atom in bortezomib can interact with the amino-terminus of β5 and β1 subunits, resulting in proteasome inhibition by preventing cleavage. Proteasome inhibition has been demonstrated as an effective cancer treatment strategy, and BTZ and future-generation proteasome inhibitors are considered to be important cancer therapies. Like all cancer therapies, BTZ has several limitations, including the following: (1) not all patients respond to treatment, and relapse occurs in many patients

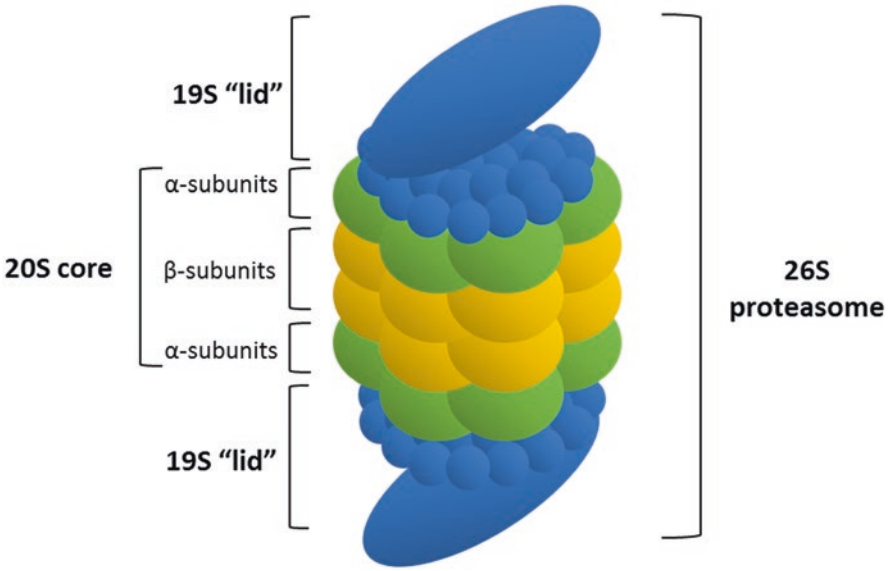

Fig. 14.3 The 26S proteasome, consisting of the 19S "lid" and 20S hollow core with α- and β-subunits

who initially responded; (2) solid tumors, in particular, are often resistant; and (3) treatment causes the induction of dose-limiting peripheral neuropathy. Several second-generation proteasome inhibitors have been developed to address these disadvantages. Carfilzomib (CFZ) can induce responses in a minority of multiple myeloma patients relapsed from or refractory to BTZ with less peripheral neuropathy (Dou and Zonder 2014). While the mechanism of BTZ resistance in human cancers remains unclear, targeting the immunoproteasome, ubiquitin E3 ligases, 19S proteasome, and deubiquitinases (DUBs) in preclinical studies represents possible directions for future-generation inhibitors of the UPS in the treatment of not only multiple myeloma but other cancers as well. Along this line, EGCG-based proteasome inhibitors may play an important role in this field whether individually or in conjunction with current proteasome inhibitors.

14.2.4 Development of Pro-EGCG to Improve EGCG's Stability, Bioavailability, and Activity

In our effort to increase the stability of EGCG, we converted its eight reactive hydroxyl groups into the corresponding acetates, named Pro-EGCG or **1** (Fig. 14.1c). In a previous study it was demonstrated that Pro-EGCG could be converted into EGCG, thus functioning as a prodrug of EGCG under in vitro and in vivo conditions (Lam et al. 2004; Landis-Piwowar et al. 2007a). It was also found that when breast

cancer MDA-MB-231 cells were treated with Pro-EGCG, the EGCG metabolite was produced and accumulated intracellularly, accompanied by increased levels of proteasome inhibition, growth suppression, and apoptosis induction, as compared to tumor cells treated with EGCG (Lam et al. 2004). To investigate whether Pro-EGCG can work as a novel prodrug that converts to a proteasome inhibitor and antitumor agent in vivo, MDA-MB-231 xenografts were induced in nude mice, followed by daily treatment with Pro-EGCG or EGCG for 1 month. From this in vivo study a significant inhibition of tumor growth by Pro-EGCG was observed, as compared to EGCG, associated with increased proteasome inhibition and apoptosis induction in tumor tissues (Landis-Piwowar et al. 2007a). It should be noted that independent research groups using various models have confirmed the increased stability, bioavailability, and biological activity of Pro-EGCG in vitro and in vivo as compared to EGCG (Lambert et al. 2006; Vyas et al. 2007; Lee et al. 2008; Chiou et al. 2012, 2013; Wang et al. 2013).

14.2.5 Novel EGCG Analogs Resistant to COMT-Mediated Methylation and Inhibition

One drawback to EGCG is its susceptibility to methylation by catechol-*O*-methyltransferase (COMT) (Fig. 14.1b) (Landis-Piwowar et al. 2007b). COMT is an enzyme found in many mammalian tissues. It carries an important function in catechol metabolism, especially amine hormones, and eliminates biologically active or toxic catechols. COMT's substrates include catecholamines and catecholestrogens. It catalyzes the transfer of a methyl group from *S*-adenosylmethionine to one of the hydroxy groups of the catechol ring (Axelrod and Tomchick 1958). With regard to EGCG, COMT seems to have a higher affinity for methylation of the 4′-hydroxy group of the D-ring rather than the hydroxyl groups of the B-ring (Lu et al. 2003). Methylation of EGCG results in decreased potency in terms of anticancer effects, possibly due to decreased proteasomal inhibition (Landis-Piwowar et al. 2007b).

The COMT protein is expressed in two forms: S-COMT, found in the cytoplasm, and MB-COMT, which is membrane bound. Both proteins are expressed by the same gene and are nearly identical in amino acid sequence, the only difference being an additional 50 amino acids on the NH_2-terminal of the MB-COMT protein, which allows it to anchor to the membrane. A single-nucleotide polymorphism has been found in the COMT gene, involving a methionine substitution in the place of valine. The addition of this allele (alternative forms of a gene that arise by mutation and are found at the same place on a chromosome) results in three genotypes: *Val/Val*, *Val/Met*, and *Met/Met* (Dawling et al. 2001). Each genotype results in a different level of COMT activity: the *Val/Val* genotype results in high COMT activity, *Val/Met* results in intermediate activity, and *Met/Met* results in low activity. Since EGCG is a substrate of COMT, EGCG potency is likely affected by COMT status.

One study specifically examined the relationship between tea consumption and breast cancer. The study found that the risk of developing breast cancer was significantly lower in women who drank green tea, but only in those with one or more low-activity COMT alleles. Those with the high-activity COMT allele (*Val*/*Val* genotype) had the same risk of breast cancer regardless of whether or not they consumed green tea (Wu et al. 2003).

There are several COMT inhibitors that are commercially available. Studies have shown that COMT inhibition can increase EGCG potency and thus allow for greater proteasome inhibition (Forester and Lambert 2014)]. Another approach to overcoming COMT methylation is to develop novel EGCG analogs and their corresponding prodrugs (**2a** and **4a**) (Fig. 14.1d), which are less susceptible to COMT methylation. Design and synthesis of EGCG analogs and prodrugs may be an effective way to overcome COMT methylation and therefore provide increased potency of anticancer effects (Chen et al. 2011). We hypothesized that EGCG analogs missing one hydroxyl at the 4′-position or two hydroxyl groups on the gallate ester moiety could result in compounds not having a catechol structure at the D-ring and would be less susceptible to COMT methylation than EGCG. With this in mind, two prodrugs (**2a** and **4a**) of such EGCG analogs were synthesized. Following this, we conducted a study using human leiomyoma cell lines and found that **2a** and **4a** have potent antiproliferative, antiangiogenic, and antifibrotic properties ((Ahmed et al. 2016); see below Sect. 3).

14.3 Activities of EGCG Prodrugs and Analogs in Uterine Fibroids

Uterine leiomyomas, or uterine fibroids, are the most common type of benign tumors seen in reproductive-age women (Styer and Rueda 2015). Women of African-American descent are at greater risk for developing uterine fibroids, with incidence rates in this population being 2–3 times higher than in white women of the same age (Wise et al. 2016). Other risk factors include obesity, age, early menarche, and increasing age up to menopause (Sparic et al. 2016). Though benign, uterine fibroids can cause pain, excessive bleeding, bladder issues, and complications during pregnancy (Downes et al. 2010; Sparic 2014). These tumors are the most common cause for hysterectomy in the United States, causing a negative impact on the lives of patients and resulting in billions of dollars per year in healthcare costs (Yang et al. 2016). Alternatives to hysterectomy, such as uterine artery embolization and magnetic resonance-guided focused ultrasound surgery, have many limitations, and new fibroids often recur after the procedure (Taylor and Leppert 2012). Currently, there are few noninvasive long-term treatment options for women with uterine fibroids, creating a need for development of other approaches.

It has been reported that the green tea polyphenol EGCG inhibits the growth of uterine leiomyoma cells in vitro and in vivo (Zhang et al. 2010a, b; Ozercan et al.

2008) and the use of a green tea extract (containing 45% EGCG) suggests potential clinical activity as a safe therapeutic agent for women with uterine fibroids (Roshdy et al. 2013). However, EGCG has a number of shortcomings, as stated above, including low stability, poor bioavailability, and rapid metabolic transformations under physiological conditions, presenting challenges for its development as a therapeutic agent (Johnson et al. 2010; Sang et al. 2011; Kanwar et al. 2012).

Most recently we have performed a study to expand our understanding of the mechanism of action of Pro-EGCG and Pro-EGCG analogs and assess whether Pro-EGCG and its analogs **2a** and **4a** display antiproliferative, antiangiogenic, and antifibrotic properties using human leiomyoma cell lines (Ahmed et al. 2016), as a prelude to advancing one or more compounds to human clinical trials.

14.3.1 Pro-EGCG and Its Analogs

Proliferation, fibrosis, and angiogenesis each plays a role in uterine fibroid growth and formation (Islam et al. 2014). Studies have shown that all of these processes can be inhibited by EGCG (Xu et al. 2009; Larsen and Dashwood 2010; Matsuzaki and Darcha 2014). We first studied EGCG in comparison to pro-EGCG and analogs **2a** and **4a** with regard to their effect on proliferation of human uterine leiomyoma cell lines (Ahmed et al. 2016).

UtLM-ht or UtLM cell lines were treated with the solvent DMSO, EGCG, Pro-EGCG (**1**), analog **2a**, or analog **4a**, followed by the MTT assay. It was found that growth of both cell lines was inhibited by each of the compounds, with **2a** and **4a** having the greatest antiproliferative effect (Ahmed et al. 2016).

The study also aimed to determine whether UtLM and UtLM-ht cell lines could form 3D spheres and if so whether EGCG, **1**, **2a**, and **4a** could inhibit proliferation of the spheres. 3D spheres were successfully grown for both cell lines and it was found that **4a** was able to significantly inhibit growth of the spheres, as well as **2a**, **1**, and EGCG to a lesser effect (Ahmed et al. 2016).

Additionally, western blots were performed to measure levels of S-phase markers PCNA and cyclin A as well as apoptosis-related proteins PARP and caspase-3 (Ahmed et al. 2016). Compounds **2a** and **4a** were able to significantly reduce S-phase markers, and they were also able to significantly reduce the apoptosis-related proteins. Western blots were also performed to look for biomarkers related to fibrosis and angiogenesis, both of which are critical to uterine fibroid development and growth (Islam et al. 2014). Compounds **2a** and **4a** were able to significantly reduce expression of fibrosis biomarkers α-smooth muscle actin (αSMA) and collagen type I (Col-I) in UtLM-ht cells, as well as expression levels of angiogenesis biomarkers vascular endothelial growth factor receptor 2 (VEGFR-2) and vascular endothelial growth factor C (VEGF-C). A scratch-wound-healing assay was performed next on both cell lines to measure migration of cells. While untreated control cells were able to migrate into the gap produced by the scratch after a 24-h recovery period, migration of cells treated with EGCG, **1**, **2a**, or **4a** was inhibited in both cell lines. Again, **2a** and **4a** were the most effective (Ahmed et al. 2016).

Next, proteasomal activity was measured in UtLM-ht cells. It was found that **4a** was able to most potently inhibit proteasomal activity, followed by **2a**, **1**, and EGCG, respectively (Ahmed et al. 2016). As EGCG has also been shown to inhibit tumor cell growth via Akt kinase activity inhibition (Tang et al. 2003; Zhang et al. 2006; Qin et al. 2007), a western blot was performed to measure pAkt and Akt levels in UtLM and UtLM-ht cells. pAkt expression was inhibited most successfully by **2a** and **4a** in both cell lines, followed by **1** and then EGCG. This indicated that **2a** and **4a** are capable of inhibiting the Akt pathway in this cell line (Ahmed et al. 2016).

Finally, it was hypothesized that **2a** and **4a** are more potent than **1** and EGCG due to the analogs' resistance to methylation by COMT. UtLM-ht cells were treated with dinitrocatechol (DNC), an inhibitor of COMT (Pérez et al. 1993), or a control solvent to test this. The cells were then treated with EGCG, **1**, **2a**, or **4a**. An MTT assay was then performed, showing that antiproliferative activity in cells treated with EGCG or 1 was significantly higher, while the DNC treatment had a much lesser effect on **2a**- or **4a**-treated cells. This indicated that EGCG and **1** are more susceptible to COMT methylation than the analogs (Ahmed et al. 2016).

14.3.2 Compounds **4** *and* **6**

Two EGCG analogs (compounds **4** and **6**) (Fig. 14.1e, f) (Chen et al. 2012) were studied alongside EGCG and **1** as a comparison to examine their effects on two human uterine leiomyoma cell lines, UtLM and UtLM-ht (Fig. 14.4). Previously, we reported that these synthetic EGCG analogs were more potent AMPK activators than metformin and EGCG in breast cancer cells (Chen et al. 2012).

Human UtLM-ht (Fig. 14.4a) or UtLM cells (Fig. 14.4b) were treated with DMSO, EGCG, **1**, **4**, and **6** at indicated concentrations (10, 25, 35, 50, or 75 μM) for 3 days (with a repeat dose of each drug given every 24 h), followed by an MTT assay to determine cell viability. We found that **4** and **6** inhibited growth of these two uterine leiomyoma cell lines in a dose-dependent manner, similar to **1** and more or equally potent to EGCG (Fig. 14.4).

Spheroid formation assay of UtLM-ht cells was then performed and the effects of **4** and **6** vs. EGCG and **1** were determined. As we found in our previous study (Ahmed et al. 2016), both uterine leiomyoma cell lines successfully formed spheroid masses (Fig. 14.5). When treated daily with 15 or 30 μM of EGCG, **1**, **4**, or **6** for up to 6 days, decreased number and size of uterine leiomyoma spheroids were observed, in the order of **1**, **4** > **6** > EGCG.

These results suggested that **4** and **6** can inhibit proliferation of uterine leiomyoma cells. Further studies were then conducted on the molecular level in order to validate the above results. The effects of **4** and **6** on the expression of cell cycle-specific proteins were analyzed using western blot analysis (Fig. 14.6). UtLM-ht cells were treated with DMSO or EGCG, **1**, **4**, or **6** at the indicated concentrations (25, 35, or 45 μM) for 24 h. A western blot was then performed, using antibodies to cell-specific proteins of interest. We found that in UtLM-ht line, the order of the

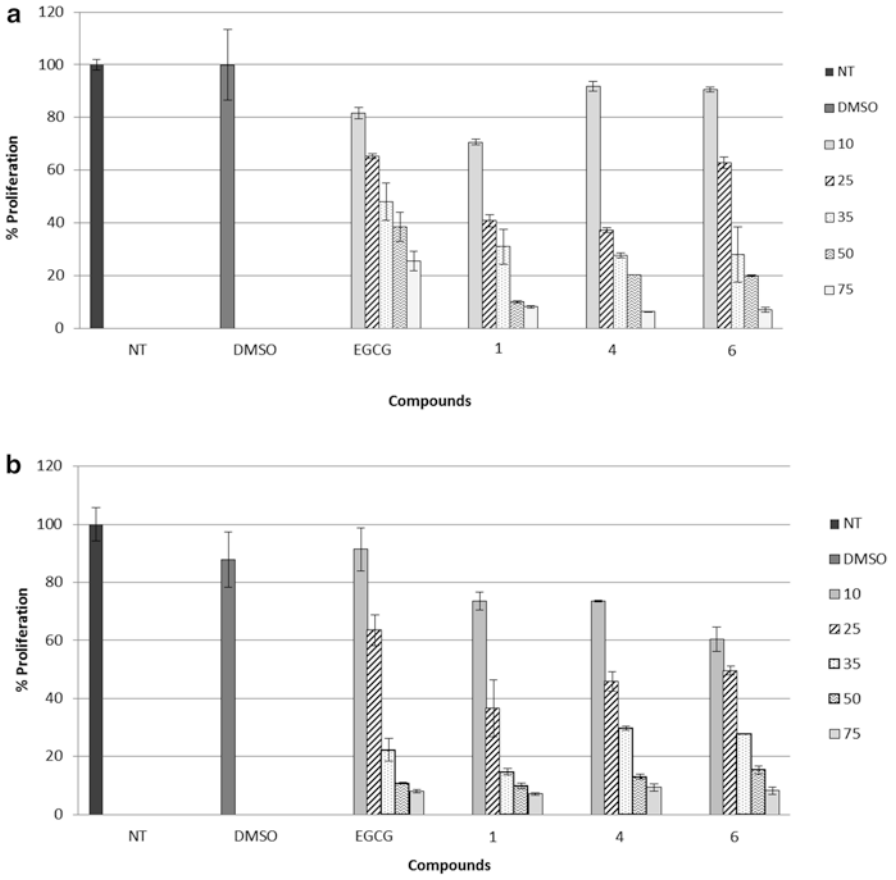

Fig. 14.4 Inhibition of human uterine leiomyoma cell growth. UtLM-ht (**a**) or UtLM (**b**) cells, grown in a 96-well plate, were treated with either the control solvent DMSO, EGCG, or Pro-EGCG **4** or **6** (at indicated concentration, μM) daily for up to 3 days (with each drug repeatedly added every 24 h), followed by the MTT assay

inhibition of PCNA expression, an S-phase marker, by these compounds was **6** > **4** > **1** > EGCG (Fig. 14.6).

We also found that Pro-EGCG potently reduced full-length caspase-3 protein levels, indicating caspase activation, in uterine leiomyoma cells and was more effective than EGCG, **4** and **6** (Fig. 14.6).

Angiogenesis is an essential component of uterine fibroid growth (Tal and Segars 2014). The effect of **4** and **6** on vascular endothelial growth factor-receptor 2 (VEGF-R2; MW 150 kDa), which is important for the process of angiogenesis, was investigated. UtLM-ht cell lines were either untreated (NT) or treated with the control solvent DMSO or 25, 35, or 45 μM of EGCG, **1**, **4**, or **6** for 24 h, followed by western blot analysis using a specific antibody to VEGF-R2 (polyclonal antibody 2). The order of inhibition of expression of VEGF-R2/p150 was **6** > **4** > **1** > EGCG (Fig. 14.6; we had no clear explanation for the effect of **4** at 35 μM in this experiment).

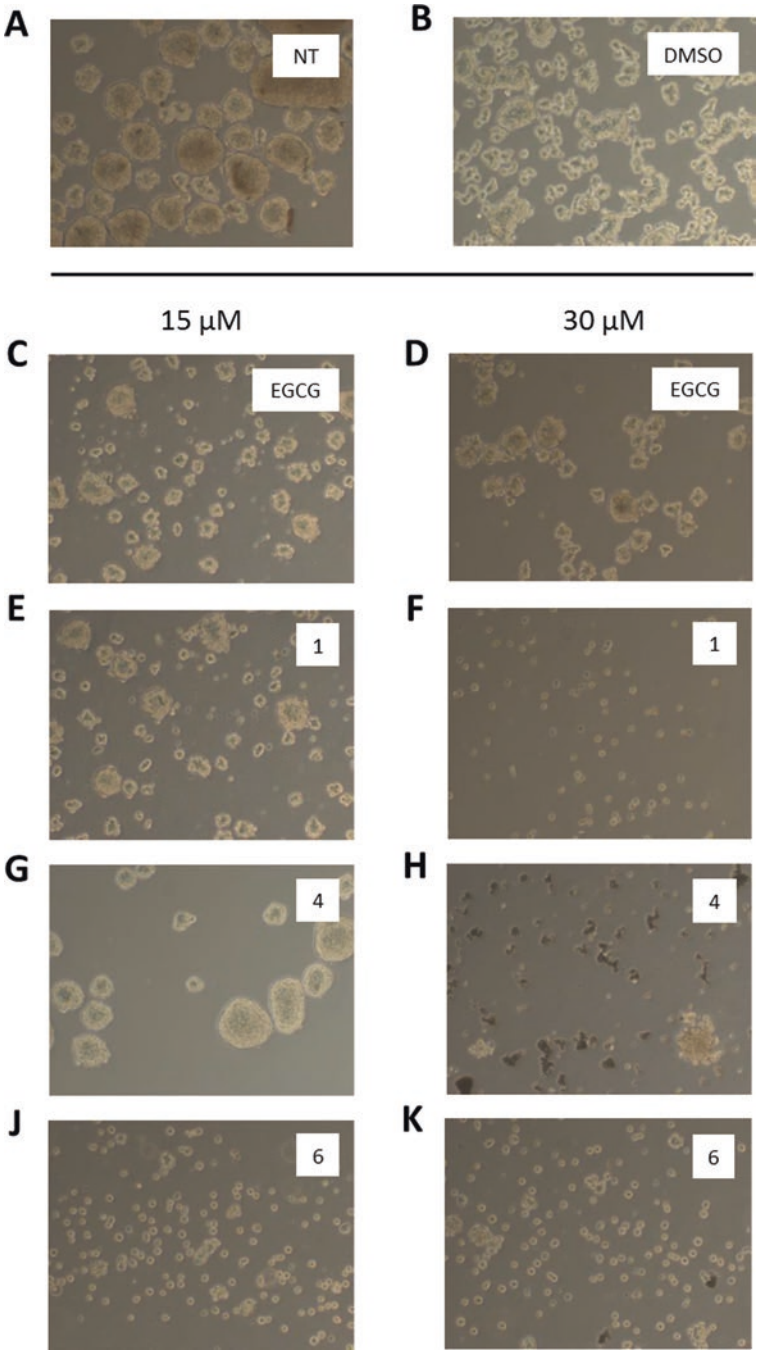

Fig. 14.5 Inhibition of spheroid formation and morphology in 3-D culture of human uterine leiomyoma cells. UtLM-ht cells were grown in a 6-well plate (in triplicates), followed by either no treatment (NT) or treatment with the control solvent DMSO or EGCG, **1**, **4**, or **6** (at 15 or 30 μM) daily for up to 6 days (with each drug repeatedly added every 24 h). The primary spheres were photographed at 200× magnification

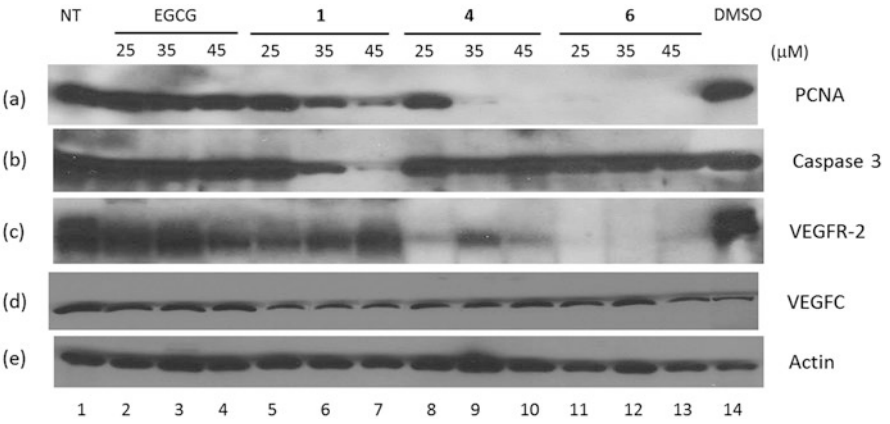

Fig. 14.6 Reduction of protein biomarkers of cell proliferation, apoptosis, and angiogenesis by Pro-EGCG analogs. UtLM-ht cells were either untreated (NT) or treated with the control solvent DMSO, EGCG, or Pro-EGCG **1**, **4**, or **6** (at 25, 35, or 45 μM) for 24 h, followed by western blotting using specific antibodies to PCNA (MW 37 kDa), caspase-3 (MW 32 kDa), VEGF-R2 (MW 152 kDa; the 200 kDa band might be a tetra-ubiquitinated form), VEGF-C (MW 46 kDa), and actin (MW 46 kDa)

The effect of **4** and **6** on vascular endothelial growth factor-C (VEGF-C), which is also essential for angiogenesis, was then studied (Fig. 14.6). Human UtLM-ht cells were either untreated (NT) or treated with the control solvent DMSO or 25, 35, or 45 μM of EGCG, **1**, **4**, or **6** for 24 h, followed by western blot analysis using antibodies specific to VEGF-C. Partial inhibition on VEGF-C was observed by **1** but not by others (Fig. 14.6).

The effect of **4** and **6** on migration of uterine leiomyoma cells was examined next by performing a scratch wound-healing assay (Fig. 14.7). A scratch was made in several dishes of UtLM-ht cells. The cells were either untreated or treated with DMSO or **4** or **6** at 25, 35, or 45 μM. The cells were then allowed to recover for 24 h. It was found that the untreated UtLM-ht cells could migrate into the gap left by the scratch. Cells treated with DMSO were able to migrate into the gap with 90% recovery. However, migration of UtLM-ht cells treated with **4** or **6** was inhibited. The order of inhibition potency was **6**, **1** > **4** > EGCG (Fig. 14.7).

14.4 Conclusions

Cancer is a prevalent and growing concern. Conventional treatments are not sufficient to address the problem. Research shows that green tea polyphenols have the potential as anticancer agents. The catechin EGCG is an especially effective anticancer compound. EGCG has demonstrated the potential to inhibit cancer cell growth and induce tumor cell death through a variety of different mechanisms.

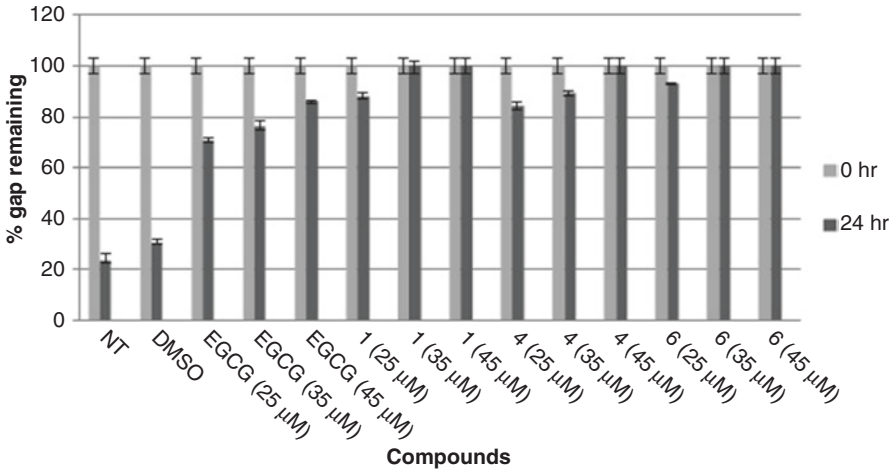

Fig. 14.7 Inhibition of human uterine leiomyoma UtLM-ht cell migration (wound-healing assay). Cells were grown in a 12-well Matrigel-coated plate until they reached ~80% confluence. A scratch was then generated by using a yellow tip in each dish, and cells were either untreated (NT) or treated with the control solvent DMSO or EGCG **1**, **4**, or **6** at indicated concentration for 24 h. The percentage of the gap remaining under each experimental condition was measured and the values were represented as the average of triplicate samples (±SD)

EGCG can disrupt cancer cell survival pathways such as CDK, or it can induce tumor cell death by proteasome inhibition and controlling the UPS. Despite its promise, EGCG has certain drawbacks that have hindered its use as a viable cancer therapy. There is no established delivery system that overcomes EGCG's poor bioavailability; and susceptibility to biotransformation via COMT methylation reduces its efficacy, which can vary based on genotype. Researchers are working on overcoming these hurdles through the design of new synthetic analogs and prodrugs. These analogs are designed to make the compound less susceptible to methylation by COMT. Studies have shown that prodrugs and synthetic analogs have performed better than natural EGCG in terms of effectiveness as well as stability demonstrating that they may be an effective way to circumvent EGCG's above problems.

Of all the green tea polyphenols EGCG and its analogs have shown the most promise as compounds for the treatment of cancer. Continued research on EGCG, particularly in design and synthesis of more stable analogs as well as their prodrugs, is strongly encouraged in hopes of developing new drug candidates for clinical research. In the future, EGCG analogs may be developed into effective new drugs for the treatment of many cancers.

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Chapter 15
Therapeutic and Medicinal Uses of Terpenes

Destinney Cox-Georgian, Niveditha Ramadoss, Chathu Dona, and Chhandak Basu

15.1 Introduction

15.1.1 What Are Terpenes?

Terpenes, also known as isoprenoids are the largest and most diverse group of naturally occurring compounds that are mostly found in plants but larger classes of terpenes such as sterols and squalene can be found in animals. They are responsible for the fragrance, taste, and pigment of plants.[1] Terpenes are classified on the basis of organization and number of isoprene units it contains (see footnote 1). An isoprene unit is a building block of terpenes that is a gaseous hydrocarbon that contains the molecular formula C_5H_8 (see footnote 1). Terpenes and terpenoids are terms that are often used interchangeably but the two terms have slight differences; terpenes are an arrangement of isoprene units that are naturally occurring, volatile, unsaturated 5-carbon cyclic compounds that give off a scent or a taste to defend itself from organisms that feed off of certain types of plants (see footnote 1). Terpenes have many functions in plants such as a thermoprotectant, signaling functions, and not limited to, pigments, flavoring, and solvents but also have various medicinal uses (Yang et al. 2012). Table 15.1 shows the different types of terpenes discussed in this chapter along with an example of that terpene.

[1] www.britannica.com/science/isoprenoid. Accessed 22 Jan 2018

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Table 15.1 Different types of terpenes and their properties

Classification	Carbon atoms	Species produced from	Medicinal uses	References
Monoterpenes	C_{10}	*Quercus ilex*	Fragrances, repellent	Loreto et al. (2002)
Sesquiterpenes	C_{15}	*Helianthus annuus*	Treat malaria, treat bacterial infections, and migraines	Chadwick et al. (2013)
Diterpenes	C_{20}	*Euphorbia, salvia miltiorrhiza*	Anti-inflammatory, cardiovascular diseases	Vasas and Hohmann (2014), Zhang et al. (2012)
Triterpenes	C_{30}	*Centella asiatica*	Wound healing, increases circulation	James and Dubery (2009)

15.1.2 Plants that Carry Medicinal Terpene

Terpene is a natural compound with various medical properties and found in both plants and animals (Gershenzon 2007). Among natural products that mediate antagonistic and beneficial interactions within the organism, terpene play a variety of roles (Gershenzon 2007). Terpene protects many living organisms like microorganisms, animals and plants from abiotic and biotic stresses (Gershenzon 2007). Terpene can ward off pathogens, predators, and competitors. Living organisms use terpene for multiple reasons like medicinal purposes and communications about food, mates, or enemies (Gershenzon 2007). It is impressive how different organisms use terpene for common purposes even though terpene contain many forms and varieties (Gershenzon 2007).

So far only a small percentage of terpene is investigated (Franklin et al. 2001). Cannabis is one of the most common sources for the medicinal terpene (Franklin et al. 2001). This plant contains many medicinal properties like anticancer, antimicrobial, antifungal, antiviral, antihyperglycemic, analgesic, anti-inflammatory, and antiparasitic (Franklin et al. 2001). Terpene is also used to enhance skin penetration, prevent inflammatory diseases (Franklin et al. 2001). Nowadays modern medication use large scales of terpene for various treatment drugs (Franklin et al. 2001).

There are commonly used plants like tea (*Melaleuca alternifolia),* thyme, Cannabis, *Salvia lavandulifolia* (Spanish sage), citrus fruits (lemon, orange, mandarin) etc. that provide wide range of medicinal values (Perry et al. 2000). Tea tree oil has increased in popularity in recent years when it comes to alternative medicine (Perry et al. 2000). Tea tree oil is a volatile essential oil and is famous for its antimicrobial properties, and acts as the active ingredient that is used to treat cutaneous infections (Carson et al. 2006) Apart from the flavor that gives to food, essential oil contain antimicrobial properties (Bound et al. 2015). Thyme is one of plants that synthesize terpene alcohols and phenols which contain powerful antibacterial and antifungal properties (Bound et al. 2015). Terpene synthesized from cannabis also long served as medicines (Perry et al. 2000). They also contain psychoactive properties and used against many infectious diseases (Perry et al. 2000). *Salvia lavandulifolia* is famous for anti-dementia (current memory-enhancing) drugs by enhancing

Table 15.2 Medicinal Properties of terpenes from different sources

Terpene	Medicinal properties	References
Tea tree	Contains the active ingredient to treat cutaneous infections	Carson et al. (2006)
Thyme	Possesses powerful antibacterial and antifungal properties	Bound et al. (2015)
Cannabis	Possesses psychoactive properties and used against many infectious diseases	Friedman et al. (2006)
Spanish sage	Enhances memory and is used in anti-dementia drugs	Lopresti (2016)
Citrus fruits	Drugs against pediculosis	Mehlhorn et al. (2011)
Citral	Antibacterial and antifungal effects	Silva et al. (2008)
Lemongrass	Insect repellent	Silva et al. (2008)

cholinergic activity via inhibition of cholinesterase (Perry et al. 2000). In vitro examination method was used to study the effects of constituent terpenes on human erythrocyte acetylcholinesterase (Perry et al. 2000). Some of the medicinal properties of terpenes are listed in Table 15.2.

15.1.3 Properties Associated with Terpene

Important properties associated with terpene are difficult to overstress (Franklin et al. 2001). There are many important uses with terpene and these include anti-insect properties, antimicrobial properties and anti-herbivore properties (Franklin et al. 2001). Terpene can be extracted through plants and thorough some insects (Franklin et al. 2001).

15.1.3.1 Anti-insect

Without using harsh chemicals that could potentially contain side effects, terpene is a healthy alternative to ward off insects (Franklin et al. 2001). There have been many pesticides made for killing domestic pests like lice, or mites (Franklin et al. 2001). In these cases, it is very important to make sure that these pesticides do not affect humans in harmful ways (Franklin et al. 2001). There are many options like shampoo, sprays, lotions that were manufactured against pests that include one or more terpenes that are employed in the instant invention (Franklin et al. 2001). These naturally occurring terpenes are generally not modified they were used in their raw form and the environment protection agency in the USA classified as "GRAS" which mean Generally Regards as Safe (Franklin et al. 2001).

Certain terpene is highly effective against both lice and lice eggs and there is a less than significant chance of resistance developing against this terpene based pesticides; reason for this is their observed modes of action (Franklin et al. 2001).

Table 15.3 Terpenes added in anti-insect formulations

Terpene type	Function	Features	References
Limonene	This is strongly preferred. Limonene enhances the properties of other terpenes	Redistilled limonene has less odor, more stable than D-limonene	Franklin et al. (2001)
Beta-ionone	Antibacterial and antifungal properties	Beta-ionone has prophylactic value.	Mikhlin et al. (1983)
Geraniol	Similar level activity like beta-ionone. Geraniol possesses antibacterial and antifungal properties.	Geraniol gives a pleasant fragrance.	Chen and Viljoen (2010)
Eugenol	This is also the active terpene in clove oil. This possesses anesthetic properties which help with the itching that comes with bug bites. Also contain antibacterial and antifungal properties	Contain a distinct fragrance which is like geraniol	Franklin et al. (2001)
Myrcene	Possesses antifungal, antibacterial properties	Famous for its fragrance properties	Filipowicz et al. (2003)

Unlike other types of pediculosis medication this terpene based instant inventions are not neurotoxins (Franklin et al. 2001) Terpenes are also used combined with terpene aldehyde called citral. Citral derives from an essential oil that is extracted from lemongrass (*Cymbopogon citratus*) (Franklin et al. 2001). Citral possesses antibacterial and antifungal properties, while lemongrass possesses anti-insect properties (Franklin et al. 2001).

A series of anti-insect formulation contain many terpenes (Franklin et al. 2001) Most of these pesticides are a mix of terpene and citral (Franklin et al. 2001). Table 15.3 consists of what these terpenes include.

15.1.3.2 Antimicrobial

Antimicrobial properties or the ability to kill or stop growth of a microorganism in terpenes are commonly used in traditional and modern medicine (Himejima et al. 1992). There are many terpenes with antimicrobial activities (Himejima et al. 1992). The following plants produce terpenes which have antimicrobial properties: *Pinus ponderosa* (Pinaceae), spices (sage, rosemary, caraway, cumin, clove, and thyme), *Cretan propolis*, *Helichrysum italicum, Rosmarinus officinalis*, and so on (Himejima et al. 1992). These antimicrobial terpenes can also be used against food borne pathogen like *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* (Himejima et al. 1992).

Pinus ponderosa cell extract contain wide-ranging antimicrobial activities (Himejima et al. 1992). After steaming and distillation from *Pinus ponderosa* cell extract, a distillate and a residue are obtained (Himejima et al. 1992). The distillate consists of monoterpenes and some sesquiterpenes while the residue consists of four diterpene acids

(Himejima et al. 1992). It was also reported that when a physical damage is caused to the pine tree or any other terpene containing tree from insect attacks, resin which contains terpene secret to protect the tree from further damage (Himejima et al. 1992).

Five different kinds of terpene can be isolated from *Cretan propolis*, they are, the diterpenes, 14,15-dinor-13-oxo-8(17)-labden-19-oic acid and a mixture of labda-8(17),13E-dien-19-carboxy-15-yl oleate, palmitate and triterpene (Popova et al. 2009). Spectroscopic analysis and chemical evidence has been used to establish the structures of the different compounds (Popova et al. 2009). These compounds that were isolated from terpene was tested for its antimicrobial activity against bacteria like gram positive and gram negative (Popova et al. 2009). It was all tested for human pathogenic fungi which has broad-spectrum antimicrobial activity (Popova et al. 2009).

Helichrysum italicum essential oil was analyzed using gas chromatography and mass spectrometry to fraction into terpene and terpenoid. Fifty two compounds, including hydrocarbons of the oil; α-pinene (10.2%), α-cedrene (9.6%) aromadendrene (4.4%), β-caryophyllene (4.2%), and limonene (3.8%), neryl acetate (11.5%), 2-methylcyclohexyl pentanoate (8.3%), 2-methylcyclohexyl octanoate (4.8%), and geranyl acetate (4.7%) were identified (Mastelic et al. 2017).

15.1.4 Monoterpenes

The smallest of terpenes are monoterpenes. They contain the compound $C_{10}H_{16}$, come from different flowers, fruits and leaves and are known as the main component of essential oils, fragrances and many structural isomers (see footnote 1). Monoterpenes are also the most fragrant of all the classes of terpenes (see footnote 1). Examples for the types of monoterpenes found in natural scents are α-pinene, which imparts scent to pine trees, and limonene from citrus plants (see footnote 1).

What is thought to be one of the main purposes of monoterpenes is to attract pollinators or to serve the purpose of repelling other organisms from feeding off of plants. They also may be related to the flowering process of the plants (Loreto et al. 2002). They are isolated from their plant sources by distillation with steam and have a boiling points in the range of 150 °C to 185 °C (see footnote 1). Monoterpenes are purified using fractional distillation at pressures that are reduced or use another process in order to form a crystalline derivative (see footnote 1).

15.1.5 Monoterpene Emission Under Heat Stress

Many studies test the hypothesis of high emissions of monoterpenes under high temperatures using the leaves of *Quercus ilex*, also known as evergreen oak (Table 15.1). The evergreen tree is native to the Mediterranean area where it has to survive under hot and dry conditions and synthesis of these monoterpenes may have been an adaptive mechanism for the plants to survive under heat stress.[2] This tree

[2] www.arborday.org/trees/treeguide/TreeDetail.cfm?ItemID=1094. Accessed 22 Jan 2018

does not emit isoprenes but it emits monoterpenes and is able to handle different environmental stresses such as drought, salt, and heat (see footnote 2). A particular study done by Loreto et al. (2002) were conducted to visualize monoterpene production in response to high temperatures and to see if thermotolerance is increased with monoterpenes (Loreto et al. 2002). In this study, the leaves were exposed in 5 °C intervals ranging from the temperatures 30 °C to 55 °C and leaves were kept under conditions in which inhibited or allowed monoterpenes to synthesize (Loreto et al. 2002). The results that were found in this experience was a discovery of seven most abundant monoterpenes which was emitted at the maximum temperature of 35 °C and decreased its abundance over time as the temperatures increased and α-pinene had the greatest abundance of emittance at 35 °C as well as other terpenes but greatly reduced over higher temperatures (Loreto et al. 2002). At 55 °C the monoterpenes, myrcene and limonene had higher emission rates compared to temperatures around 35 °C (Loreto et al. 2002). Photosynthesis was also decreased when the leaves were exposed to any temperature that was higher than 30 °C and at 55 °C showed a loss of CO_2 and recovery occurred around 30 °C (Loreto et al. 2002). Overall, the monoterpenes showed that their optimal temperature for emission was around 30–35 °C (Loreto et al. 2002). Researchers prove that the emission of monoterpenes is under enzymatic control due to their optimal temperatures (Loreto et al. 2002).

15.1.6 Sesquiterpenes

Sesquiterpenes, containing the chemical formula $C_{15}H_{24}$, are much larger compounds than monoterpenes and are much more stable in comparison.[3] They are isolated by distillation with steam or by extraction and purified by methods such as vacuum fractional distillation or gas chromatography (see footnote 1). Oxidation or rearrangement of isoprene units that are made to sesquiterpenes produce the corresponding sesquiterpenoids (see footnote 1). Sesquiterpenes are naturally occurring and found in plants, fungi, and insects and act as a defensive mechanism or attract mates with pheromones in insects (see footnote 1). Acyclic compounds of sesquiterpenes such as farnesans can be used as a natural pesticide for insects and also as pheromones for some insects and mammals such as elephants, to attract mates or to mark their territory (see footnote 1).

Sesquiterpenes have a vital role in plant growth hormones and signaling properties in response to its environment (Giraudat 1995). Abscisic acid has a role in plants such as development, germination, cell division, and synthesis of protein storage and signalling (Giraudat 1995). It also plays a role in plants in response to various environmental stresses. It regulates the closure of the stoma by regulating ion channels and exchange of water across the plasma membrane (Giraudat 1995). Cyclic ADP-ribose signals abscisic acid in response to drought-stressing conditions from the environment (Giraudat 1995). Abscisic acid is not unique to plants, it has

[3] www.cyberlipid.org/simple/simp00042.htm. Accessed 25 Jan 2018

shown to be present in the central nervous system of other organisms such as pigs and may play a role in humans as a pro-inflammatory cytokine and stimulator of insulin release in the human pancreas (Chadwick et al. 2013). Gossypol is a sesquiterpene that is found in cotton plants. It has anticancer properties and can potentially inhibit fertility in male humans which is why it must be removed from essential oils and various other products before human use or consumption. Avarol, a sesquiterpenoid that has shown to have antimicrobial and antifungal uses, is effective against the AIDS virus in humans (see footnote 3).[4]

The medicinal properties of sesquiterpenes typically come from flowering plants that are included in the Asteraceae family, which include, but not limited to sunflowers, marigolds, and daisies. This family of flowers is a significant resource for potent sesquiterpene lactones, which are usually found in the leaves and the flower portion of plants and are constantly being produced at high levels (Chadwick et al. 2013). The role of sesquiterpenes in these flowering plants are not solely made for human use but for the purpose of protecting the plant from predators and are produced de novo in response to microbial attack and ultraviolet ray protection (Chadwick et al. 2013). Their bitter taste is a defense mechanism against herbivores from feeding on them but some have sweet tastes or tastes that are pleasant to certain organism for the purpose of spreading their seeds and being fertilized in different areas (Chadwick et al. 2013). Sesquiterpenes have many uses in traditional, western medicine because they contain so many anticancer, antiplasmodial, and anti-inflammatory activities (Chadwick et al. 2013). Sesquiterpenes lactones are able to reduce stomach ulcers in some people and are also present in powerful antimalarial drugs (Chadwick et al. 2013). Artemisinin, a metabolite produced from *Artemisia annua*, which contains sesquiterpene lactone produced in the roots and shoots of the plants, is used in drugs to treat malaria (Chadwick et al. 2013). Other uses of this family of flowers is for treatment of bacterial infections, migraines, and to improve skin (Chadwick et al. 2013). Lettuce opium has been used for many years as a painkiller (Chadwick et al. 2013).

15.1.7 Diterpenes

Diterpenes are naturally occurring chemical compounds that contain the molecular formula, $C_{20}H_{32}$. Diterpenes have physiologically active groups such as vitamin A activity well as plant growth hormones that regulate germination, flowering and switch reproductive cycles (from asexual to sexual reproduction) of plants (Lee et al. 2015). They can also be classified as a phytol, which is an oxygenated acyclic diterpene. Over 650 diterpenoids have been isolated from *Euphorbia* plants, which is a very diverse genus of flowering plants (Popova et al. 2009). Diterpenes have many therapeutic benefits such as antitumor, cytotoxic, and anti-inflammatory

[4] www.cyberlipid.org/simple/simp00041.htm. Accessed 22 Jan 2018

(Vasas and Hohmann 2014). They are present in anticancer drugs such as taxol, and the tumor promoter, phorbol (Vasas and Hohmann 2014).

Tanshinones are a class of diterpenes that are isolated from dried roots or rhizomes of an herb in traditional Chinese medicine called *Salvia miltiorrhiza* also known as Danshen or Tanshen (Zhang et al. 2012). Tanshinones were first isolated in the 1930s, and since then, more than 90 chemicals have been identified and split up into two groups: 40 lipophilic and 50 hydrophilic compounds (Zhang et al. 2012). Tanshinones have recently been extensively researched for their anticancer properties in vitro and in vivo (Zhang et al. 2012). Their potential use as an anticancer drug comes from their broad range of activities such as anti-proliferation and inhibiting adhesion, migration, and invasion (Zhang et al. 2012). Analogues of tanshinone have been synthesized in many clinical trials because they have many anticancer attributes (Lee et al. 2015). This herb has been used in many Asian countries for preventative and therapeutic solutions to many diseases such as heart disease, vascular diseases, and arthritis (Zhang et al. 2012). Tanshinones may also reduce inflammation and increase immune responses (Zhang et al. 2012).

Cafestol and kahweol are diterpene alcohols that are found in the oil derived from coffee beans. These chemical structures are very similar but only differ by an extra double bond that is present in kahweol's chemical structure.[5] Researchers have reported that coffee lowers the risk of depression in women, prostate cancer in men, stroke, diabetes, and some cancers (see footnote 5). It is thought that the anti-inflammatory and antioxidant properties of these particular diterpenes are responsible for such events (see footnote 5). Coffee benefits the liver as well by lowering liver enzymes that are in response to inflammation and damage and may offer some protection against liver cancer as well (see footnote 5). The adverse result of these diterpenes is that they raise cholesterol level, but it seems to be limited to coffee that has been unfiltered and has oily droplets of cafestol and kahweol (see footnote 5). Filtered coffee may not have much impact on cholesterol levels (see footnote 5).

15.1.8 Triterpenes

Triterpenes are composed of three or six isoprene units and have the chemical formula $C_{30}H_{48}$ which includes steroids and sterols with squalene being the biological precursor of all triterpenes (see footnote 1). Triterpenes are produced by animals, plants, and fungi. They play a role as precursors to steroids in animal and plant organisms, and are derived from mevalonic acid (see footnote 1). Saponins come from the skins of many plants and have emulsion like properties that make them excellent detergents in the human digestive system (see footnote 1). Chemical structures of steroid saponins are similar to hormones that are produced in the human body (see footnote 1).

[5] www.health.harvard.edu/staying-healthy/what-is-it-about-coffee. Accessed 22 Jan 2018

The medicinal uses of triterpenes are not quite as recognized as other different types of terpenes but their uses are being continuously investigated by researchers. Their properties have been studied for anticancer, antioxidant, antiviral, and anti-atherosclerotic activities (Nazaruk and Borzym-Kluczyk 2015). Some studies have shown that there is promising potential for the use of triterpenes for people with diabetes by aiming to reduce glucose levels and also by reducing sweetness inhibitors in sweet and high calorie foods (Nazaruk and Borzym-Kluczyk 2015). Saponins have detoxification properties and act as a diuretic for the kidneys and wound healing properties (Nazaruk and Borzym-Kluczyk 2015).

15.1.9 Tetraterpenes

Tetraterpenes are also known as carotenoids that have the molecular formula $C_{40}H_{56}$ and can be in the category of terpenes because they are made from isoprene units.[6] Most carotenoids are highly unsaturated and for this reason, they are extremely difficult to isolate and purify (see footnote 1). They are found in all different types of fungi, bacteria, and plants and mainly responsible for red, yellow, or orange fat-soluble plant and animal pigments (see footnote 6). One of the most crucial and common tetraterpene is beta-carotene that contributes to the yellow pigment in carrots. It is important to mammals especially because it is a precursor in producing vitamin A and other important terpenoids for vision (see footnote 1).

Higher order terpenes have been shown to increase thermotolerance (Singsaas 2001). The permeability of the thylakoid membranes increase at higher temperatures and this happens by an increase in cyclic photophosphorylation around photosystem II (Singsaas 2001). When the temperature of the atmosphere continues to rise, the photophosphorylation system is not able to keep up with protons leaking, which causes the transmembrane gradient to drop and a reduction in ATP synthesis occurs (Singsaas 2001). All these events can potentially cause lowering in the Rubisco activation state due to an inhibition of RuBP regeneration (Singsaas 2001).

15.1.10 MEP Pathway

The MEP pathway, also known as the non-mevalonate pathway or methylerythritol phosphate pathway, is a metabolic pathway for isoprenoid biosynthesis that creates the products isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). This pathway occurs in the chloroplasts and produce monoterpenes, specific sesquiterpenes, diterpenes, and carotenoids (Zhang et al. 2012). The vital application of this pathway is to develop antimicrobial agents to target diseases such

[6] www.cyberlipid.org/simple/simp0002.htm#carotene. Accessed 25 Jan 2018

as malaria and sexually transmitted diseases (Hunter 2007). Since this pathway does not occur in humans, it is a valuable resource to develop antibacterial and antiparasitic drugs (Seemann et al. 2009).

The first steps of this pathway involve pyruvate and D-glyceraldehyde 3-phosphate to produce DOXP which is catalyzed by 1-deoxy-D-xylulose-5-phosphate (DXS) (Hunter 2007). 1-Deoxy-D-xylulose-5-phosphate reductoisomerase, otherwise known as IspC, coverts DOXP to MEP. From MEP, it reacts with CTP to create 4-diphosphocytidyl-2*C*-methyl-D-erythritol (Hunter 2007). A phosphate is released in this reaction and then reacts with ATP-dependent IspE to make 4-dipho sphocytidyl-2*C*-methyl-D-erythritol 2-phosphate and ADP and then reacts with the enzyme IspF to create 2*C*-methyl-D-erythritol 2,4-cyclodophosphate (Hunter 2007). The enzyme requires metal cations. Then finally, in the least understood step of the reaction, the two enzymes, IspG and IspH make the two products, IPP and DMAPP by using a two-electron reduction (Hunter 2007). The pathway is regulated by control of repression or activation of gene expression via feedback loops within the pathway or by effector molecules which target an enzyme or downstream activities (Hunter 2007).

15.1.11 MVA Pathway

The MVA pathway or mevalonic acid pathway occurs in the cytosol. It is responsible for the synthesis of sterols, specific sesquiterpenes, and also may play a role in the synthesis of transhinones (Zhang et al. 2012). In gram-positive bacteria, the genes in the metabolic pathways such as MVA are organized into operons and are thought to be regulated by transcription (Hunter 2007).

15.1.12 Cannabis

The use of cannabis is increasing for medicinal uses that commonly treat pain, the side effects of chemotherapy in cancer patients such as nausea, anxiety and depression, and its uses and benefits are continuously being researched by scientists (Cathcart et al. 2015). There are at least 80 compounds that come from the cannabis plant that are regarded as cannabinoids that cause psychotropic effects in the human brain due to CB_1 receptors (Klein et al. 2011). The main active ingredient, delta-9-tetrahydrocannabinol, otherwise known as THC, is a psychoactive agent and is a focus for controversy in society because it binds to the human endocannabinoid receptors in areas of the brain such as the hippocampus and the frontal cortex, which are responsible for memory, cognition and attention.[7] How THC works is by taking

[7] www.herb.co/2016/07/24/what-is-thc/. Accessed 22 Jan 2018

the place of endocannabinoids, naturally occurring chemicals in the human body (see footnote 7). One of the most common and well known molecules that THC replaces in the human body is called anadamide (see footnote 7). To this day, scientists are researching to discover the exact role of this molecule in the human body.

Cannabidiol, or CBD is also a common ingredient in cannabis but compared to THC, it is a non-psychoactive and it can potentially reduce the effects of THC (Klein et al. 2011). CBD does not bind to the same receptors as THC does in the human body and it works by inhibiting FAAH or the enzyme fatty acid amide hydroxyls (see footnote 7). This enzyme is responsible for degrading anadamide in the body and by inhibiting FAAH, CBD increases natural endocannabinoids already in the human system (Klein et al. 2011). CBD is thus an agent that works for depression, anxiety and neuroprotective effects (Klein et al. 2011).

What are major components in cannabis are the monoterpenes that are responsible for many different medicinal properties. One of the main uses for THC is the potential for cancer treatment and can play a role in reducing size of tumors (see footnote 7). THC can also reduce inflammation caused by certain diseases in patients. Other conditions that THC can help but are not limited to are ADHD, Arthritis, migraines, and glaucoma (see footnote 7).[8] It can also improve the symptoms in individuals that suffer from HIV by helping their appetite and thus causing weight again, improving their depression symptoms and their quality of life (Lutge et al. 2013).

15.1.13 Antiplasmodial Activity

Terpenes have been shown to have a favorable antiplasmodial activity. With the rising malarial infections and drug resistance, terpenes have gained more attention towards it through antiplasmodial activity (Nogueira and Lopes 2011). The interesting mechanism behind the terpene activity is that it binds to the hemin part of infected erythrocytes and kills the parasite just like the famous antimalarial drug chloroquine (Orjih et al. 1981; Kayembe et al. 2012). Hemin is made of iron which is necessary for the plasmodium development in the erythrocytes. Though hemin breaking enzymes are not yet found in plasmodium, it could be one reason why hemin binding accounts for parasite lysis (Ginsburg and Demel 1984). Another study suggests that drug-hemin complex binds to phospholipid layers thereby disrupting the respective membrane structure and causing cell lysis (Ginsburg and Demel 1984). Moreover, it is also known that hemin can affect the carbohydrate metabolism of the parasites, which could lead to lysis of parasites (Rodriguez and Jungery 1986). Thus, terpenes can be designed to be promising drugs for malaria.

Different kinds of terpenes show different effects on the parasites. For instance, beta-myrcene the most common terpenes, is proven to have in vitro antiplasmodial

[8] www.chem.libretexts.org/Core/Organic_Chemistry/Lipids/Properties_and_Classification_of_Lipids/Terpenes. Accessed 22 Jan 2018

activity (Kpoviessi et al. 2014). Beta-myrcene from *Cannabis sativa,* the plant which is high in terpenes, does not show an anti plasmodial effect but extracts from stem, leaves, and seeds of clove basil showed a good antiplasmodial activity (Small 2017; Kpoviessi et al. 2014). Additionally, it was also reported to have antitrypanosomal activity when tested against *Trypanosoma brucei brucei* (Habila et al. 2010). This data leads to the fact that terpenes are effective against pathogenic Protista.

Limonene regarded as the second most commonly found terpene, also possesses antiplasmodial activity against *Plasmodium falciparum*. Limonene achieves its goal by targeting the intermediates of the active isoprenoid pathway of the parasite. Isoprenoid pathway plays a major role in parasite survival by mediating cell signaling, protein translation and several other biological processes (Jordão et al. 2011). Specifically, the isoprenic products that are inhibited from being synthesized are dolichol and ubiquinone (Goulart et al. 2004). The isoprenoid pathway of parasites is distinct from that found in mammals, which makes limonene a reliable constituent of antimalarial drug (Goulart et al. 2004). Thus, the host cell pathway will not be affected by the administration of the drug.

Pinene, commonly found monoterpene in pine trees is composed of two classes—alpha-pinene and beta-pinene. Both the classes of pinene were reported to be effective against the W2 strain of *Plasmodium falciparum*, which is resistant to chloroquine (Boyom et al. 2010). Of particular interest is the increase in antiplasmodial activity of pinene in cumin seed oil with increase in the distillation time. The study concluded that the optimal distillation time for increased antimalarial activity is 0–5 and 5–7.5 min (Zheljazkov et al. 2015). Further investigation is needed to ascertain if distillation time is just increasing the yield of pinenes in the oil or improving the bioactivity of pinenes.

The next most abundant terpene, caryophyllene has the ability to both prevent and cure malaria. Caryophyllene is an active component of insect repellents especially for mosquitoes and other blood-feeding Diptera (Maia and Sarah 2011). Recent studies ensured that silver nanoparticles synthesized from caryophyllene are highly effective against *Plasmodium falciparum* (Kamaraj et al. 2017). Thus, terpenes could be a safer and a cost effective alternative for malarial treatment.

15.1.14 Antiviral Activity

The emerging viral diseases have necessitated the research for new effective antiviral agents such as terpenes. As a result, scientists evaluated various terpenes for their properties, among which monoterpenes showed a good result. Monoterpenes are terpene classes that possess two isoprene units. They form a major constituent of essential oils in plants which indicates monoterpenes play a major role in defense for plants (Grabmann 2005). A 2005 study evaluated the in vitro antiviral activity of several essential oils extracted from South American plants (Duschatzky et al. 2005). The oil

extracts were tested against three major human viruses—herpes simplex virus-1 (HSV1), dengue virus type 2, and Junin virus. The oils that were proved to be virucidal were mainly composed of monoterpenes, namely, carvone, carveol limonene, alpha- and beta-pinene, caryophylene, camphor, beta-ocimene, and one sesquiterpene which is germacrene (Duschatzky et al. 2005). A similar study in 2008 analyzed the essential oils of seven plants from Lebanon for in vitro antiviral activity (Loizzo et al. 2008). The viruses under investigation were HSV1 and severe acute respiratory syndrome corona virus (SARS CoV). The results were positive for antiviral effects, and the major constituents were alpha- and beta-pinene, beta-ocimene, and 1,8-cineole (Loizzo et al. 2008). Following this, a 2009 study on *Salvia cedronella* also had similar results which suggested 1,8-cineole, α-pinene, caryophyllene oxide, and sabinene to be the major components of virucidal oils (Alim et al. 2009). Functional data from these studies reveal that a few monoterpenes are shared by various plants for antiviral properties (Alim et al. 2009). These shared monoterpenes could be of importance as they are present universally.

Of particular interest is the single main monoterpene that is contributing to the virucidal activity. This was studied by Astani et al. (2009) using eucalyptus, tea tree, and thyme essential oil extracts (Astani et al. 2009). They suggested that monoterpene hydrocarbons have a slightly higher virulent activity compared to the monoterpene alcohols against HSV-1. The monoterpenes with the highest virucidal activity were identified to be alpha-pinene and alpha-terpineol (Astani et al. 2009). The mechanism behind the virucidal activity was suggested to be direct inactivation of free viral particles. However, the study concluded that more than isolated single monoterpenes, a mixture of monoterpenes are more effective and possessed lesser toxicity to host cells (Astani et al. 2009). This was further bolstered by another study which evidenced the virucidal property of a combination of monoterpenes obtained from *Melaleuca alternifolia* (Zamora et al. 2016). The activity was tested against a human flavivirus West Nile virus. The results were positive both in vivo and in vitro. The underlying mechanism was predicted to be induced cell cycle arrest at G0 or G1 phase. This indicates that a mixture of monoterpenes could act as a better antiviral agent rather than a single monoterpene (Zamora et al. 2016). Recent studies have shown that triketone-terpene adducts also exert antiviral, antimicrobial and antitumor activity (Chen et al. 2017). These adducts are obtained from Myrtaceae as secondary metabolites in the form of sesquiterpenes called myrtucomvalones A, B, and C. The terpene adducts successfully inhibited the respiratory syncytial virus (RSV) (Chen et al. 2017).

The bioactive terpenes present in various plants have shown various results for antiviral property. It would therefore be important to look for various plant source rather than various monoterpenes for therapeutic purposes. Researchers are also focusing on synthesizing terpene hybrid from fungal sources as they are presumed to have antiviral and UV protective properties (Yuan et al. 2017). Terpene synthesis from fungi can lead to cost effective and limited labor methods (Yuan et al. 2017).

15.1.15 Anticancer

The medicinal benefits of terpenes are not limited to pathogenic diseases. Terpenes are widely acclaimed for their anticancer activity too. An early 1997 study concluded that a combination of monoterpenes, diterpenes and sesquiterpenes can be effectively used to treat cancers that occur in colon, brain, prostate gland, and bones.[9] It also claimed that administration of terpenes in humans inhibited the growth of prostate cancer cells and sensitized the tumor in such a way it becomes susceptible to radiotherapy (see footnote 9). The major advantage of this treatment was that, the drug can be administered through several routes among which oral and topical were most preferred (see footnote 9).

Among the different kinds of terpenes, limonene is well recognized as an anticancer agent. Limonene is a bioactive food component found in citrus peels, orange peels, and several other citrus fruits (Jirtle et al. 1993). Studies have reported limonene to exhibit strong cancer inhibition activity both in vitro and in vivo. The mechanism behind limonene activity is still under investigation. A study by Jirtle et al. (1993) reported that limonene acts through induction of transforming growth factor B-1 and mannose-6-phosphate/insulin-like growth factor II receptors (Jirtle et al. 1993). In contrast a study by Bishayee and Rabi 2009) suggested that limonene eliminates cancer cells by induction of apoptosis (Bishayee and Rabi 2009). Structural studies on limonene reported that they are lipophilic and have the tendency to be deposited in fatty tissues when administered orally. This indicates that limonene can act as an excellent chemopreventive drug for cancer as it can be deposited in the body (Miller et al. 2010). Another study in 2013 concluded that limonene acts by suppressing the expression of breast tumor cyclin D1 (Miller et al. 2013). This lead to cell cycle arrest and mitigated proliferation of cancer cells in women with early stages of breast cancer (Miller et al. 2013). Recent study showed that limonene from pinecones can kill lung cancer cells in vitro by apoptotic mechanism that is activated through caspase-3 pathway (Lee et al. 2017). These findings indicate a novel application of limonene towards fighting and preventing cancer. Not just limonene, but also its metabolite perillyl alcohol is also said to exhibit antitumor activity in pancreatic cell lines through apoptotic mechanisms (Sobral et al. 2014; Dalessio et al. 2014).

Apart from limonene, the terpene thymoquinone has all been widely studied for its chemoprotective and chemotherapeutic activity. Thymoquinone is found to be an active constituent of the volatile oils of an annual herbaceous plant called *Nigella sativa* (black cumin) (Majdalawieh et al. 2017). The pathways affected by thymoquinone to exert its antitumor properties are p53, PPARγ, MAPK, NF-κB, PI3K/AKT, and STAT3 signaling pathways (Majdalawieh et al. 2017). Thymoquinone has been proved to be anticancerous against several cancers such as breast cancer, skin cancer, non–small cell lung cancer, bile duct cancer, and brain cancer. The basic mechanisms underlying the cancer inhibition is apoptosis and cell cycle arrest (Sobral et al. 2014;

[9] www.google.com/patents/US5602184. Accessed 23 Jan 2018

Khader and Eckl 2014). Most of the cancer related studies were performed using thermoquinone obtained from the *N. sativa* extracts. A 2012 study showed that thermoquinone can be obtained in larger amounts from the mint family, namely, *Monarda didyma* and *Monarda media* (Taborsky et al. 2012). Thus, thermoquinone from alternative sources has to be tested for its precious potential in cancer therapy.

Other terpenes that have reported cytotoxic effects on cancer cells include alloocimene, camphor, beta-myrcene, pinene, alpha- and gamma-thujaplicin, terpinene, thymohydroquinone, carvone, camphene, and cymene (Sobral et al. 2014). Terpenes being natural compounds are unlikely to affect the healthy cells or create a side effect, which attracts many researchers to exploit its capability in cancer treatment.

15.1.16 Antidiabetic

Diabetes is one of the widely prevalent diseases in the world. It is affecting both children and adults in both developing and developed nations (You and Henneberg 2016; Narayan et al. 2000). The social and economic burden of diabetes continues to grow and it is expected to rise rapidly in developing countries (Sarwar et al. 2010). In USA, diabetes is one of the leading causes for visual impairment, limb amputation, renal diseases, heart diseases and death (Saddinne et al. 1999). Diabetes can be of two types—type 1 (where the immune system of the body acts against the insulin-producing organs) and type 2 (where the insulin produced cannot be used by the body or insulin is produced in low amounts).[10] Although there are several medications available, their use is limited due to their adverse effects. Some of the commonly found side-effects include low blood sugar, vomiting, nausea, diarrhea, bloating, and weight gain.[11] This led to the research for natural products to be used as effective antidiabetic medication. Phytochemicals from the medicinal plants have been recommended for treating type 2 diabetes, of which terpene forms a major constituent (Jung et al. 2006).

Medicinal plants of Oriental Morocco were studied for their antidiabetic property in rats. The report showed that terpenes, terpene diols, and terpene diol glucosides form major components of the extracts of plants under study (Bnouham et al. 2010). A similar study on medicinal plant and their natural products that were reported from 2001–2005 was conducted by Jung et al. 2006. This study was focused on non–insulin-dependent diabetes mellitus (type 2), and it proved that terpenes along with few other secondary metabolites such as alkaloids and flavonoids exhibit antidiabetic potential (Jung et al. 2006).

The most promising terpene compound for treating diabetes is called andrographolide which is a diterpenoid lactone (Brahmachari 2017). This compound forms the major component of the leaves of the small herbaceous plant *Andrographis paniculata. A. paniculata* is an Asian plant that has already been reported to be used

[10] www.diabetes.ca/about-diabetes/types-of-diabetes. Accessed 23 Jan 2018

[11] www.diabetes.co.uk/features/diabetes-medication-side-effects.html. Accessed 23 Jan 2018

in traditional medicines for its therapeutic nature (Brahmachari 2017). The terpenoid acts by reducing the plasma glucose and increasing the utilization of glucose by the body in diabetes mellitus rats (Gupta et al. 2008). The actual mechanism by how it does this is it activates the alpha-adrenoreceptors to increase the release of an opioid peptide beta-endomorphin (Brahmachari 2017) which is reported to be secreted in low amounts in diabetic rats (Forman et al. 1985). This increased secretion in turn activates the opioid μ-receptors. These receptors can effectively curb the hepatic gluconeogenesis (glucose synthesis from non-carbohydrate precursors) and elevate the utilization of glucose by muscles. Finally, this results in a reduced plasma glucose concentration (Brahmachari 2017). Andrographolide is also observed to prevent the secondary complications of diabetes such as diabetic retinopathy, a condition that will lead to blindness (Brahmachari 2017). It significantly weakens the retinal angiogenesis and inflammation during the development of the disease (Brahmachari 2017). Moreover, it can also fix the impaired or extended estrous cycle in diabetic rats (Reyes et al. 2006). Andrographolide was orally administered in all the above studies. This indicates its efficiency for being used as a lead molecule in the future drugs designed for treating diabetes mellitus.

Another widely known terpene is curcumin obtained from *Curcuma longa* which commonly called turmeric (Nabavi et al. 2015). It exhibits high antidiabetic property and acts by quashing the oxidative stress and inflammation. By regulating the polyol pathway, it can also reduce the plasma glucose and levels of glycosylated hemoglobin (Nabavi et al. 2015). Moreover, curcumin is also reported to activate the enzymes present in the liver that are essential for glycolysis, gluconeogenesis, and lipid metabolism (Zhang et al. 2013). Alike andrographolide, curcumin is also reported to reduce the complications of diabetes (Nabavi et al. 2015), for example, liver disorder which is a common manifestation of diabetes type 2 (Zhang et al. 2013). Curcumin treats these disorders by reducing the liver weight and lipid peroxidation products. Further, it is also reported to normalize the levels of fetuin-A in serum that contributes to insulin resistance and fatty liver in diabetic rats (Zhang et al. 2013). Other complications that can be attenuated by curcumin include diabetes associated—retinopathy, microangiopathy, neuropathy, and nephropathy (Zhang et al. 2013). These findings confirm that curcumin is likely to be used in the future for diabetes treatment.

15.1.17 Antidepressant

Depression has become a serious health concern by contributing to the emerging mental and emotional disorders throughout the world. It is hitting both the developed and developing countries. Depression can pave way to various health issues from alcoholism to heart diseases (Holden 2000). It is also said to increase the rate of mortality significantly in breast cancer patients (Hjerl et al. 2003). Moreover, depression immobilizes its victims thereby leading to economic loss (Holden 2000). By analyzing the social and economic burdens caused by depression, researchers have stepped out towards finding novel stress-relieving drugs. Synthetic drugs have

serious side-effects and unintended interactions with the body that negatively affects the treatment outcome (Jawaid et al. 2011). Hence this necessitated the need for natural drugs. Terpenes serves as one of the most relevant bioactive compound for treating depression and therefore can open doors for designing natural or synthetic antidepressant drugs (Bahramsoltani et al. 2015).

Twenty-five percentage of antidepressant drugs prescribed by doctors are obtained from herbs through various extracts (Saki et al. 2014). To estimate the important compounds contributing to the antidepressant effect, Saki et al. (2014) performed an electronic database based study. The results revealed that terpenes formed a major part of the extracts of medicinal plants that exerted antidepressant effects (Saki et al. 2014). Thus, scientists focused on identifying the active principles of plant extracts contributing to the antistress effects. Different plant had different acting compounds.

Among the several terpenes, linalool and beta-pinene are commonly found to be active principles (both Guzmán-Gutiérrez et al. 2015; Guzmán-Gutiérrez et al. 2012). They were discovered from the extracts of medicinal plants *Litsea glaucescens* and *Tagetes lucida* and flowers of lavender (Appleton 2012; Guzmán-Gutiérrez et al. 2012; Guadarrama-Cruz et al. 2008). These monoterpenes act by interacting with the 5HT1A receptors of the serotonergic pathway. Serotonins are important in the fact that their release and re-uptake levels can be altered to overcome stress (Chaouloff 2000; Guzmán-Gutiérrez et al. 2012). They also interact with adrenergic receptors of the body that play a major role in stress-induced behavioral changes (Pandey et al. 1995; Guzmán-Gutiérrez et al. 2015). Another interesting finding is the interaction of beta-pinene with dopaminergic receptors namely D1 receptors. This is the mechanism followed by most of the antidepressant drugs available in the market (Guzmán-Gutiérrez et al. 2015). A more interesting study would be to examine the beta-pinene and linalool efficiency through inhalation tests. This is because these monoterpenes are aromatic compounds that generally have an enhanced activity when inhaled as they can directly hit the central nervous system (Guzmán Gutiérrez et al. 2014).

Apart from monoterpenes, sesquiterpenes also exhibit antidepressant effects. One striking example is beta-caryophyllene which was proved to ameliorate the depressive symptoms in mice (Bahi et al. 2014). The underlying mechanism of this compound is binding to a receptor called CB2 and activating it. CB2 is found in the brain and immune cells and plays a major role in regulating depressive-related disorders (Bahi et al. 2014). Thus beta-caryophyllene curbs depression by acting as a CB2 receptor agonist (Bahi et al. 2014).

Other terpenes that have effective antidepressant properties include hyperforin which is present in the extracts of *Hypericum perforatum* (Subhan et al. 2010). It has been shown that the extracts of *H. perforatum* differ in their antidepressant potential with the difference in concentration of hyperforin present in the extract (Laakmann et al. 1999). Similar to many other antidepressants hyperforin acts by inhibiting the neuronal uptake of mood regulators such as serotonin, dopamine and norepinephrine. In addition, it also has its own unique mechanism of controlling depression by inhibiting the neurotransmitters GABA and L-glutamate uptake (Müller et al. 2001).

Another fascinating antidepressant plant is *Valeriana wallichii,* which is a short perennial herb. This plant not only reduces the stress and anxiety levels but also improves the symptoms of depression in humans (Bhattacharyya et al. 2007). The major components of Valeriana extracts are terpenoids called maaliol, patchouli alcohol, and 8-acetoxypatchouli alcohol (Subhan et al. 2010). The terpenoid-less extract of Valeriana was found to be devoid of antidepressant activity which indicates that terpenes are the active components involved in reducing the depression (Subhan et al. 2010).

15.1.18 Uses in Folk Medicine

Folk medicine has always been an eye-opener for designing novel drugs for diseases. To be more specific, almost three-fourths of the plant-based drugs were created based on the knowledge of folk medicine (Table 15.4) (Efferth et al. 2008). Realizing this fact, western worlds are now turning back into old medicines and bioactive plant components to treat modern diseases (Efferth et al. 2007, 2008). This has boosted the export rates of Chinese medicinal products (based on traditional Chinese medicine) from China to other developed nations. Plants used in traditional Chinese medicine (TCM) are being extensively studied for their secondary metabolites and their therapeutic properties (Efferth et al. 2007). One of the active principles of TCM products is terpenes (Liu and Jiang 2012). Due to their large availability and diversity, terpenes contribute the most to industrial and medicinal applications among all the secondary metabolites of plants (Zwenger and Basu 2008).

Paclitaxel is one of the most successful terpenes available in the market today (Efferth et al. 2008). It is made out of yew trees which is a medicinal tree used in TCM.[12] Raw material from yew contains taxol (brand name of Paclitaxel) which is used in the treatment of cancers in breast, lung, ovary, pancreas, cervix, and blood (see footnote 12).[13,14] Two variations of this drug are used now in chemotherapy—conventional paclitaxel and albumin-bound paclitaxel (see footnote 13). The advantage of the latter is that concentration increases in tumor cells at a rate higher than that of the former (see footnote 13). The mechanism of anticancer activity is described as disruption of microtubules in the mitotic spindle, which will lead to incomplete chromosome separation thereby causing cell death (see footnote 13). In TCM and Ayurveda (herbal medicinal science mainly developed in India), healers used the twigs and barks of the tree to make a special kind of tea that can be given to patients suffering from cancer. However due to the slow growing nature of yew tree, paclitaxel nowadays is produced by coalescing the products of endophytic fungus that grows under the tree and the bark of the tree[15,16] (Heinig et al. 2013).

[12] www.yewbiopharm.com/about-us/. Accessed 29 May 2017

[13] https://www.drugs.com/monograph/paclitaxel.html. Accessed 21 June 2017

[14] www.dailymail.co.uk/health/article-3823690/Could-Chinese-medicine-cure-leukaemia-Taking-herb-alongside-treatment-helps-85-patients-enter-remission.html. Accessed 29 May 2017

[15] www.thepracticalherbalist.com/holistic-medicine-library/pacific-yew-pocket-herbal/. Accessed 29 May 2017

[16] https://nccih.nih.gov/health/ayurveda/introduction.html. Accessed 30 May 2017

Table 15.4 Uses of different terpenes in folk medicine

No.	Scientific name	Common name	Abundant terpene	Uses in ayurveda	Uses in TCM	References
1.	*Citrus limon*	Lemon	Limonene	Oral cavities, digestive problems, abdominal colic pain, and cough	Digestive problems and cleansing the body	[a,b]
2.	*Citrus reticulata*	Orange	Limonene	Digestive disorders, abdominal colic pain, and worm infestation	Stomach ache and cough	[c,d]
3.	*Juniperus communis*	Juniper	Limonene	Antiseptic, treat cellulite, pain and swelling	Treat cold and urinary problems	[e,f]
4.	*Phyllanthus emblica*	Indian gooseberry	Phyllaembicilins	Boost immunity Strengthen hair follicles Cure acne and pimples Improve circulatory system Cure diarrhea	Treat diarrhea, jaundice and inflammation	Zhang et al. (2000), Liu (2016)[g]
5.	*Panax sp.*	Ginseng	Humulene	Boosts energy Used to treat musculoskeletal problems such as rheumatism, arthritis, and so on.	Memory booster Reduce fatigue Reduce menopause symptoms	[h–j]
6.	*Cinnamomum verum*	Cinnamon	Alpha-pinene, caryophyllene, linalool,alpha-phelandrene, cymene, humulene	Cold, Diabetes, high cholesterol, digestive problems, bronchitis, sinus congestion	Cold, diabetes, high cholesterol, digestive problems; control sweating Chest pain	Ravindran et al. (2004)[k]
7.	*Lycium chinense*	Goji berry	Beta-carotene	Maintains kidney functions Improves eye-sight, fertility, circulation and increases lifetime	Improves eye-sight, fertility, circulation and increases lifetime	Bungheza et al. (2012)[l]

(continued)

Table 15.4 (continued)

No.	Scientific name	Common name	Abundant terpene	Uses in ayurveda	Uses in TCM	References
8.	*Zingiber officinale*	Ginger	Zingiberene	Digestive problems, Joint pain and air sickness	Cold, cough, wheezing, asthma	[m,n]
9.	*Allium sativum*	Garlic	Nerolidol, alpha-pinene, and terpinolene	Treat pimples, tumor, snakebites, wounds, headache, heart disease, gastric problems, ulcer, and measles	Food poisoning and digestive problems	[o,p]
10.	*Ocimum tenuiflorum*	Holy basil/ tulsi	Eugenol, β-elemene, β-caryophyllene and germacrene	Restores functions of nervous system Increases fertiltity Used to treat asthma and cold	Restores functions of the nervous system	Kousik and Baldev (2012)[q,r]

[a]http://easyayurveda.com/2012/11/14/health-benefits-of-lemon-ayurveda-details/. Accessed 1 June 2017
[b]http://limoneira.com/lemons-in-traditional-chinese-medicine/. Accessed 7 June 2017
[c]http://easyayurveda.com/2011/09/22/benefits-of-orange-fruits-traditional-and-modern-views/. Accessed 5 June 2017
[d]www.sacredlotus.com/go/chinese-herbs/substance/chen-pi-orange-peel-citrus-peel-tangerine-peel. Accessed 5 June 2017
[e]http://ayurvedicoils.com/tag/ayurvedic-uses-of-juniper-leaf-oil. Accessed 5 June 2017
[f]www.herbs-info.com/juniper.html. Accessed 5 June 2017
[g]http://homeofayurveda.org/the-indian-gooseberry-ayurvedas-wonder-fruit/. Accessed 5 June 2017
[h]www.organicfacts.net/health-benefits/herbs-and-spices/health-benefits-of-ashwagandha-or-indian-ginseng.html. Accessed 7 June 2017
[i]http://theleafonline.com/c/science/2014/11/terpene-profile-humulene/. Accessed 5 June 2017
[j]https://en.wikipedia.org/wiki/Ginseng#Uses. Accessed 5 June 2017
[k]https://classicalchinesemedicine.org/gpa/guizhi-cinnamon-twig-translations/. Accessed 5 June 2017
[l]https://trueayurveda.wordpress.com/2013/06/25/goji-berries-not-all-that-they-are-cracked-up-to-be/. Accessed 5 June 2017
[m]www.mapi.com/ayurvedic-knowledge/food-tips/the-healing-power-of-ginger.html. Accessed 5 June 2017
[n]http://bodymindwellnesscenter.com/ginger-root-in-ayurveda-and-chinese-medicine/. Accessed 5 June 2017
[o]www.meridian-acupuncture-clinic.com/support-files/garlic-in-tcm.pdf. Accessed 5 June 2017
[p]http://ayurveda-foryou.com/health_articles/garlic_benefits.html. Accessed 5 June 2017
[q]https://en.wikipedia.org/wiki/Ocimum_tenuiflorum. Accessed 5 June 2017
[r]www.consciouslifestylemag.com/tulsi-holy-basil-sacred-herb/. Accessed 5 June 2017

One more common terpene present in the drugs used in TCM is pinene (Wu et al. 2008). Pinene exhibits therapeutic properties such as anti-inflammatory, antiseptic, anticancer, and antibiotic properties.[17,18] The source for pinene is Eucalyptus and other related coniferous trees (see footnote 17, Sartorelli et al. 2007) In olden days, the juice from the bark of eucalyptus was collected and mixed in water, milk or wine to be used as a drug (see footnote 17). Currently, they are extracted in the form of oil and sold in the form of syrups and lozenges.[19] As eucalyptus oil contains several monoterpenes, a study analyzed the different constituents of eucalyptus oil for its effectiveness against bacteria. Here it was concluded that alpha-pinene is the best monoterpene with the highest inhibitory activity (Sartorelli et al. 2007). Recently scientists are studying another primary terpene in eucalyptus called cineole. Cineole is reported to improve the memory power, cognitive performance and attenuate the symptoms of Alzheimer's disease in humans (see footnote 19; Moss and Oliver 2012). In addition, studies also showed that cineole is capable of improving the health of bronchitis patients by reducing their cough (Fischer and Dethlefsen 2013). This is in agreement with the fact that eucalyptus oil was used as an expectorant in Ayurvedic medicine.[20] It is also known that local Brazilians used the Eucalyptus leaves to treat several human diseases such as cancer (Mathias et al. 2012). Further reports also suggest that eucalyptus oil has been involved in ancient Indian Ayurvedic and Greco-European medicine systems (see footnote 19).

Ayurveda is a popular medicine system which originated about 3000 years ago in India. The ayurvedic medicines are based on medicinal herbs, minerals, and metals (see footnote 16) along with diet regimes such as vegetarianism (Caldecott 2006). This system of medicine has proven to cure chronic disorders that could not be treated by western medicine (Sharma et al. 2007). Interestingly a lot of medicinal plants used by Ayurvedic practitioners owe their therapeutic property to their terpene contents. One good example is turmeric, a family of ginger which is regarded as "Golden Goddess" by medical practitioners (see footnote 18).[21] It has numerous therapeutic properties that includes anti-inflammatory, antioxidant, anticancer, antiseptic, antiplasmodial, astringent, digestive, diuretic, and many more (see footnote 18).[22] Recently, scientists discovered that most of the turmeric's properties are laid out by the yellow-colored terpene—curcumin (Kocaadam and Şanlier 2017). Studies are now trying to create curcumin analogues to improve the effects and activity of natural curcumin (Kocaadam and Şanlier 2017). Another popular example is clove which was used by both Ayurveda and TCM as a painkiller in dental cases. It was applied topically on cavities to relieve toothache and abdomen to treat digestive problems (Alqareer et al. 2006). The essential oil of clove is mostly composed of eugenol, a bioactive terpene that is responsible for clove's aroma (Alqareer et al. 2006). Eugenol

[17] http://ayurvedicoils.com/tag/health-benefits-of-a-pinene. Accessed 30 May 2017

[18] www.medicaljane.com/category/cannabis-classroom/terpenes/#terpenes-in-cannabis. Accessed 30 May 2017

[19] https://aromaticstudies.com/about-eucalyptus-globulus-and-18-cineole/. Accessed 30 May 2017

[20] www.eastwesthealingacademy.com/herbs/eucalyptus/. Accessed 30 May 2017

[21] www.ayurvedacollege.com/articles/students/turmeric. Accessed 6 June 2017

[22] http://articles.mercola.com/herbal-oils/clove-bud-oil.aspx. Accessed 6 June 2017

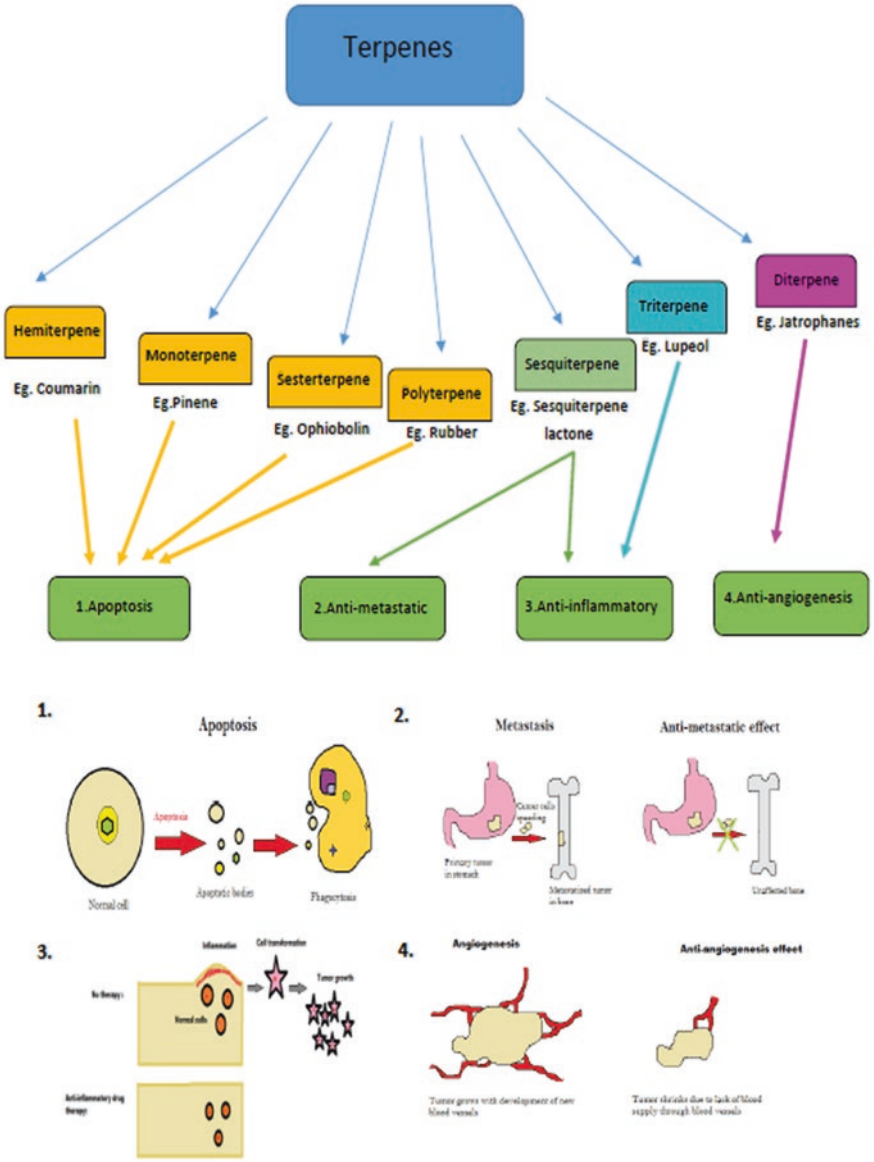

Fig. 15.1 A brief representation of terpenes and their medicinal uses. Based on (http://www.wise-geek.com/what-are-phagocytes.htm. Accessed 22 Jan 2018) (Zetter 2008; Keklikoglou and Palma 2014)

by itself is said to enhance the blood circulation in the body and improve metabolism (see footnote 22). Thus, based on the above data we can conclude that various terpenes have been in use even before their discoveries by modern science, due to their amazing medicinal properties. A schematic summary of different terpenes and their medicinal uses, that we discussed, is provided below in Fig. 15.1.

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Chapter 16
Unexplored Medicinal Flora Hidden Within South Africa's Wetlands

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16.1 Introduction

Aquatic plants (otherwise known as hydrophytes) are found across the globe in various ecosystems ranging from wetlands to oceans. These plants occur in areas where the land is either permanently or seasonally wet and have crucial roles in the functioning of the environment. Their most important biological role includes the energy fixation for other organisms by supplying oxygen through photosynthesis. They are incredibly diverse with regard to their structural adaptations, distribution and secondary metabolite production, each of which helps them survive within the flooded environments (Cronk and Fennessy 2001).

Wetlands are formed at the interface between terrestrial and aquatic ecosystems and are unique in that they have features of both these environments (Keddy 2010). While the various types of aquatic ecosystems exist across the world ranging dramatically in appearance, salinity, substrate composition and plant species, this chapter focuses on South African wetlands and the freshwater aquatic and marginal plants found within; however these plants are found growing in other parts of the world too.

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16.2 South African Wetlands

A particular broad definition of wetlands states that "wetlands are ecosystems which form when the influx of water produces soils dominated by anaerobic process, this then forces the biota (with special mention to rooted plants) to adapt to the flooding" (Keddy 2010). This characterisation focuses on three main concepts, highlighting the unique characteristics of a wetland. According to Mitsch and Gosselink (2007), wetlands are identified and differentiated from other habitats due to the presence of water (either on the surface or within the root zone) which can be either seasonal or permanent (Fig. 16.1). They have a unique soil composition enabling the support and growth of vegetation that thrives in wet conditions (hydrophytes). South Africa is home to many different types of wetlands including artificial, natural and estuaries which are located across the entire country. These habitats provide an environment which allows specific water-loving plants, known as hydrophytes or freshwater aquatic plants, to grow in abundance (Collins 2005).

The number of freshwater aquatic plants (macrophyte, whose photosynthetically active parts are submerged in freshwater or floating on the water surface either permanently or at least for several months each year) worldwide is estimated to be ~6000 species (based on 1–2% of *all* vascular plants being aquatic) (Darwall et al. 2009). However the approximated total number of freshwater aquatic and wetland species indigenous to South Africa, based on distribution data collected at various wetlands in South Africa, is ~2557 plant species (Sieben et al. 2015).

Fig. 16.1 A protected wetland located at the Pilanesberg National Park, South Africa, July 2017

16.3 The Types of Plants Found Within Wetlands

Freshwater aquatic plants can be described as plants associated with environments abundant in freshwater (lacking salt) and vegetation. This wet habitat may range from open water to regions where the soil is only seasonally or periodically waterlogged. The plants which grow in these environments may be found in different regions of the water (either on or under) with specific adaptations allowing them to survive in each situation. These unique aquatic adaptations were developed through evolution, as aquatic plants are believed to be phylogenetic descendants of terrestrial plants (Bornette and Puijalon 2009). However, submerged plants differ from other types of aquatic as well as terrestrial plants with regard to the photosynthetic medium in which the plants absorb sunlight (water and air, respectively). The site at which nutrients are taken up by the plants in both terrestrial and aquatic environments also differs. The free-floating receive their nutrients from the water, while soil provides the other types of aquatic and terrestrial plants with nutrients. These variations have an impact on the growing conditions required for each, but also provide evidence as to how terrestrial and aquatic plants are similar in terms of photosynthetic ability and nutrient uptake (Smart et al. 2005).

Different types of freshwater aquatic plants exist according to their adaptations to the water-filled environment. These include emergent, submerged, floating (floating-leaved and free-floating) and riparian or marginal plants.

16.3.1 Emergent

Freshwater aquatic plants that have majority of their structure beneath the water, rooted within the soil, while their photosynthetic parts, including their leaves, stems and reproductive organs, are above the water surface are known as emergent species (Cronk and Fennessy 2001). Their aerial leaves are very similar in structure and function to those typically seen in terrestrial environments (Arber 2010).

16.3.2 Submerged

Submerged plants have their entire structure submerged below the water surface where they spend their entire life cycle (Cronk and Fennessy 2001). These types of freshwater aquatic plants have special leaf and stem adaptations which allow them to move with the water current without damage. Adaptations include soft stems lacking lignin as well as highly divided or elongated leaves that are very thin, allowing increased flexibility (Taiz and Zeiger 2010).

16.3.3 Floating

There are two types of floating plants, namely free-floating and floating-leaved. Plants whose entire structure floats on the water are known as "free-floating plants". Their roots are not attached to any substrate but are instead hanging free within the water (Cronk and Fennessy 2001).

Freshwater aquatic plant species that have distinctive large circular leaves which float on top of the water surface while their roots are attached to a substrate are known as "floating-leaved plants" (Cronk and Fennessy 2001).

In both cases, the leaves are very broad, firm and leathery but flexible enough to withstand damage that may be caused by waves or movement occurring in the water (Arber 2010).

16.3.4 Riparian and Marginal

Riparian or marginal plants grow along the banks of rivers, streams and lakes, in a narrow strip of land that borders the water source. These plants are distinct from terrestrial plants as they grow in soils that are water rich or waterlogged (Freitag 2014).

These different types of freshwater aquatic and wetland plants produce a variety of secondary metabolites depending on their association with the water. The production of these secondary metabolites could potentially be used for medicinal and therapeutic purposes as the compounds possess the specific pharmacological properties described below.

16.4 Potential Medicinal Properties of Aquatic and Wetland Plants

While terrestrial plants have been extensively studied, the knowledge of the medicinal properties of freshwater aquatic and wetland plants has remained a relatively understudied field. This could be attributed to many reasons, ranging from the abundance of terrestrial plants already available to the concern of wetlands being rapidly destroyed and destructed (Macaskill 2010). However, due to the large abundance and variety of plant species available and relatively easy cultivation processes, this field holds much promise for the future of natural resource collection for medicinal usage and opens up many new opportunities for collaborations between the pharmaceutical industry and that of agriculture.

Like all plants, freshwater aquatic and wetland plants are sessile organisms and thus rely on many adaptations in order to survive various stresses including water pollution, herbivory, microorganism interaction and environmental cues (Taiz and Zeiger 2010). While aquatic plants vary in their structure and development, they also differ in the types of plant secondary metabolites they produce depending on their association with the water.

Many freshwater aquatic plants share their environment with not only other plants but microorganisms and wildlife as well. Like all plants, they have the ability to produce phytochemicals to help survive, grow and compete in such habitats. Phytochemicals, according to history and drug development, have been proven to be the medicinally important constituents of plants. These phytochemicals are used to treat human ailments due to their ability to initiate physiological effects (Bhowmik et al. 2013). Many studies have been conducted to investigate the bioactive compounds present within freshwater aquatic plants yet most of them rely on the plant-environment interactions in order to be produced.

According to Smolders et al. (2000), phenolic compounds within plant species are the most common secondary metabolites produced and have been investigated in various aquatic plant-herbivore interactions. It was discovered that in general, most submerged plants had less phenolics than emergent or floating-leaved types. This is due to the fact that plants found on the surface of the water or completely outside of the water are more susceptible to attack and thus require more phenolics than those that live below the water surface. Another plausible reason is that due to the lower ultraviolet light exposure, they do not require much protection that phenolics provide against light stress (Smolders et al. 2000).

When deterring herbivores, chemical compounds, although less visible than morphological adaptations, play an equally important role in plant defence. Compounds from the medicinally important family, coumarins, have been found in many plant families helping to deter feeders due to their bitter taste. They have also been found in many freshwater aquatic and wetland plant families including Cyperaceae, Araceae, Juncaceae and Poaceae (Keddy 2010).

A study conducted by McClure (1970) highlighted the important role that secondary metabolites play in adapting aquatic plants to water-filled environments. Flavonoids were noted to have a variety of functions within plants but mainly aided growing plants during physiological stress and provided photoprotection through antioxidant activities (Agati et al. 2012; Huang et al. 2015). Flavonoids were seen to be the most prominent secondary metabolite class in free-floating aquatic species while both flavonoids and phenols were found in high concentrations in emergent species. Alkaloids were the highest in floating-leaved species such as Nymphaeaceae while terpenoids were found to be more commonly present in plants that thrived in waterlogged soils (riparian). Both compound classes are important anti-herbivore compounds (Keddy 2010). In a more recent publication by Choi et al. (2002), it was found that some species of submerged plants, especially from the Haloragaceae family, had very high concentrations of hydrolysable tannins within their leaves that made up approximately 8–20% of their dry mass.

It can, therefore, be observed that freshwater plants have been reported to produce many structurally diverse yet novel bioactive compounds that aid in chemical defence. Compounds include antibiotics, alkaloids, mycotoxins and phenolic compounds which are all considered to be valuable sources of pharmaceutical compounds for the production of modern herbal remedies and drugs (Figs. 16.2, 16.3, 16.4, 16.5, 16.6 and Tables 16.1, 16.2, 16.3, 16.4, 16.5, 16.6) (Ramesh et al. 2013).

Fig. 16.2 Emergent (broad leaved) plants: (1) *Berula thunbergii* (Shebs 2008); (2) *Cyclosorus interruptus* (Eickhoff 2009); (3) *Floscopa glomerata* (Blittersdorff 2011); (4) *Gunnera perpensa*, Manie van der Schijff Botanical Garden, University of Pretoria, South Africa, March 2016; (5) *Ludwigia adscendens* (Harris 2005); (6) *Limosella maior* (Hyde et al. 2016); (7) *Marsilea schelpeana* (Rainwater Harvesting Garden, University of Pretoria, South Africa, March 2016); (8) *Persicaria senegalensis* (Wursten 2004); (9) *Plantago longissima* (Wildflower Nursery 2004)

Fig. 16.3 (10) *Carex austro-africana* (Rainwater Harvesting Garden, University of Pretoria, South Africa, March 2016); (11) *Cladium mariscus* (Du Rabuais 2008); (12) *Cyperus dives* (Braun 2013); (13) *Cyperus marginatus* (Rainwater Harvesting Garden, University of Pretoria, South Africa, March 2016); (14) *Cyperus rotundus* (Di Gregorio 2008); (15) *Cyperus sexangularius* (Rainwater Harvesting Garden, University of Pretoria, South Africa, March 2016); (16) *Eleocharis acutangula* (Milliken 2009); (17) *Juncus effusus* (Mittlehauser 2017); (18) *Juncus lomatophyllus* (Wildflower Nursery 2004); (18) *Phragmites mauritianum* (Gerber et al. 2004); (20) *Prionium serratum* (Porter 2005); (21) *Schoenoplectus brachyceras* (Gerber et al. 2004); (22) *Schoenoplectus paludicola* (Gerber et al. 2004); (23) *Typha capensis* (Massyn 2006)

Fig. 16.4 (24) *Lagarosiphon muscoides*; (25) *Lagarosiphon major*; (26) *Potamogeton schweinfurthii*; (27) *Potamogeton thunbergii*; (28) *Utricularia stellaris* (Gerber et al. 2004)

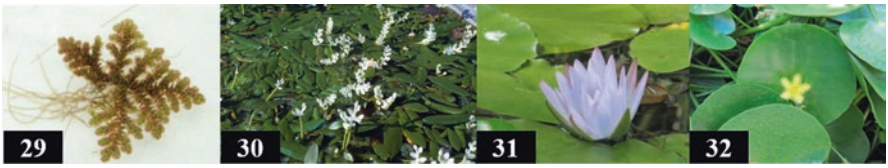

Fig. 16.5 (29) *Azolla pinnata var. africana* (Gerber et al. 2004); (30) *Aponogeton distachyos* (Howard 2017); (31) *Nymphaea nouchali var. caerulea* (Manie van der Schijff Botanical Garden, University of Pretoria, South Africa, March 2016); (32) *Nymphoides thunbergiana* (Rainwater Harvesting Garden, University of Pretoria, South Africa, March 2016)

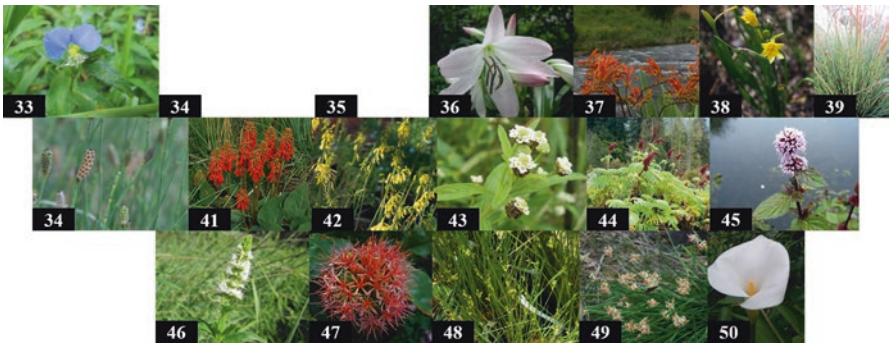

Fig. 16.6 (33) *Commelina benghalensis* (Manie van der Schijff Botanical Garden, University of Pretoria, South Africa, March 2016); (34) *Crinum bulbispermum* (Gerus 2011); (35) *Crinum campanulatum* (Manie van der Schijff Botanical Garden, University of Pretoria, South Africa, March 2016); (36) *Crinum moorei* (Khanyile 2004); (37) *Crocosmia paniculata* (Wildflower Nursery 2004); (38) *Cyrtanthus breviflorus* (Wildflower Nursery 2004); (39) *Elegia tectorum* syn. *Chondropetalum tectorum* (Rainwater Harvesting Garden, University of Pretoria, South Africa, March 2016); (40) *Equisetum ramosissimum* (Municipality of Sita, 2013); (41) *Erythrina zeyheri* (Wildflower Nursery 2004); (42) *Kniphofia pauciflora* (Wildflower Nursery 2004); (43) *Lippia javanica* (Nichols 2004); (44) *Melianthus major* (Shebs 2006); (45) *Mentha aquatica* (Calow 2008); (46) *Mentha longifolia* (Manie van der Schijff Botanical Garden, University of Pretoria, South Africa, March 2016); (47) *Scadoxus multiflorus* subsp. *katharinae* (Wildflower Nursery 2004); (48) *Scirpus ficinioides* (Wildflower Nursery 2004); (49) *Tulbaghia leucantha* (Wildflower Nursery 2004); (50) *Zantedeschia aethiopica* (Rainwater Harvesting Garden, University of Pretoria, South Africa, March 2016)

16.5 Conclusion

From the information summarised in Table 16.2, the importance of investigating the medicinal plants outside of terrestrial ecosystems, not only South Africa, but also the rest of the world, has been highlighted. In some cases, there have been literature reports on all aspects of the plant; however, the opposite can be noted for other species (Fig. 16.7). While the photochemistry and bioactivity of some aquatic plants

have been explored, numerous plants still lack comprehensive scientific data to validate the pharmacological effects of the medicinal plants and their respective bioactive compounds.

One of the largest gaps in research was noted for submerged and floating species. The scarcity and small size of the leaves as well as the troublesome locations for collecting these species have deterred ecologists and traditional healers from utilising these plants in scientific reports and traditional healing in South Africa, respectively. The botanical structures, specifically the leaves, of both submerged and free-floating species, are uniquely threadlike and small, respectively. Although important for their survival (freedom of movement and buoyancy), the lack of leaf biomass provides little benefit for medicinal uses. A bulkier plant is more sustainable as less plant material needs to be collected from the environment to yield a higher extract yield.

Contrary to free-floating plants, floating-leaved species have a much larger leaf biomass. A Nymphaeaceae species natively found growing in the Amazon, known as *Victoria amazonica*, produce leaves reaching 8 m in diameter. Due to its increased leaf size, these plants have been extensively studied in terms of their phytochemical constituents, pollination, flowering and economical importance as it is of ornamental significance (Prance and Arias 1975; Strack et al. 1992; Puchooa and Khoyratty 2004). The same can be seen for the South African Nymphaea *nouchali* var. *caerulea*. Although insignificant in size when compared to that of *V. amazonica*, *N. nouchali* var. *caerulea* has quite a sizable leaf biomass when compared to other freshwater species and even some terrestrial plants and thus is utilised by traditional healers for a variety of ailments (Table 16.5) and has been used in a few cosmetic products. The position of floating species on the water surface rather than below provides an additional advantage to its use, as it is easy to cultivate.

Riparian and emergent species tend to have been studied substantially and this could be attributed to their high similarity with terrestrial species. Riparian and emergent species are usually located near the water margin. The plants found in this area are easily accessible to animals and people, and therefore tend to have a similar chemical profiles as well as medicinal uses with families found in both aquatic and terrestrial ecosystems.

Of the 50 plants mentioned, majority of the traditional uses are related to skin disorders (wound healing, inflammation, acne, etc.) while the role of the extracts as bacterial growth inhibitors is highlighted in the biological activities. In conclusion, this highlights the important role that freshwater aquatic and wetland plants could be providing in terms of medicinal potential. These plants have been reported to be chemically diverse containing many phytochemical compounds of medicinal importance. The species which have been documented to be traditionally utilised in various communities have not been scientifically validated including *Berula thunbergii*, *Floscopa glomerata*, *Marsilea schelpeana*, *Persicaria senegalensis*, *Plantago longissima*, *Cyperus dives*, *Juncus lomatophyllus*, *Aponogeton distachyos*, *Nymphoides thunbergiana*, *Crocosmia paniculata*, *Cyrtanthus breviflorus* and *Tulbaghia leucantha*.

Table 16.1 Emergent (broad leaved) plant species, their reported traditional usage, biological activity and phytochemical constituents

	Emergent, broad leaved				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
1	*Berula thunbergii* H.Wolff (*Berula erecta* subsp. *thunbergii*) Apiaceae Toothache root	The rhizome (root) is chewed or held in the mouth to treat toothache in the Khoi-San communities of South Africa Small pieces of the boiled plant material are given to patients in Lesotho (a neighbouring country to South Africa), suffering from headaches	None reported	The essential oils of *B. erecta* was analysed using GC and GC-MS after being obtained through hydro-distillation. It was found that 125 components were identified which accounted for 96.2% of the total oil. The oil was characterised by the presence of (Z)-falcarinol (21.5%), β-sesquiphellandrene (17.2%), β-caryophyllene (14.9%) and γ-terpinene (14.8%). Terpenoids (66.2%) constituted the main fraction of the oil, with monoterpene (19.3%) and sesquiterpene hydrocarbons (39.2%) being among the most abundant compound class	Watt and Breyer-Brandwijk (1962), Lazarević et al. (2010), and Moteetee and Van Wyk (2011)
2	*Cyclosorus interruptus* (Willd.) H.Ito Thelypteridaceae Willdenow fern	The leaves are used widely in tropical regions as a treatment for coughs, skin burns and wounds, gonorrhoea, liver diseases and malaria	Acetone extracts of the leafy parts of the plant have been reported for antibacterial activity against *Staphylococcus aureus*. The antibacterial test was carried out by using the disc diffusion method and the highest activity was observed at an MIC of 31.255 μg/ml	Coumarins, furanocoumarins and a novel dioxocane derivative were isolated from *C. interruptus*	Quadri-Spinelli et al. (2000), Pauline Vincent et al. (2012), and Chai et al. (2015)
3	*Floscopa glomerata* Hassk Commelinaceae	The bark has been cited to possess anti-diabetic activity	None reported	None reported	Patel et al. (2012)

(continued)

Table 16.1 (continued)

	Emergent, broad leaved				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
4	*Gunnera perpensa* L. Gunneraceae River pumpkin	The roots are traditionally used as a remedy for the treatment and control of skin-related problems including pimples, eruptions and wounds. Other parts of the plant are used to treat gonorrhoea, syphilis and urinary tract infections The burnt leaves of *G. perpensa*, are crushed and smoked by patients living in Lesotho (a neighbouring country to South Africa), to relieve headaches	The MIC against clinically important bacterial strains was reported at 39 µg/ml (*Staphylococcus aureus*); 9.8 µg/ml (*Staphylococcus epidermis*); 18 µg/ml (*Bacillus cereus*); 39 µg/ml (*Enterococcus faecalis*) From the compounds isolated using dichloromethane, one of the 1,4-benzoquinones (2-methyl-6-(-3-methyl-2-butenyl)benzo-1,4-quinone) showed significant antimicrobial against *Staphylococcus epidermidis* with an MIC of 9.8 µg/ml, while the benzopyran (6-hydroxy-8-methyl-2,2-dimethyl-2H-benzopyran) had good anti-yeast activity against *Cryptococcus neoformans* (75 µg/ml) and *Candida albicans* (37.5 µg/ml)	Phytochemical screening of the rhizomes revealed the presence of alkaloids, flavonoids, steroids, saponins, tannins and glycosides A dichloromethane extract of the leaves and stems of *G. perpensa* contained simple 1,4-benzoquinones and a benzopyran, while a methanol extract of the same parts yielded phytol	Watt and Breyer-Brandwijk (1962), Steenkamp et al. (2004), Drewes et al. (2005), Buwa and van Staden (2006), Moteetee and Van Wyk (2011), and Simelane et al. (2012)
5	*Ludwigia adscendens* (L.) H.Hara Onagraceae Water primrose, willow herb	The whole plant is used as a poultice in the treatment of ulcers as well as skin and scalp diseases. It has also been used as an emetic, astringent, laxative, anthelmintic (anti-parasitic treatments), anti-dysenteric, diuretic (urine production stimulant) as well as for complaints regarding the eyes and throat	Extracts of the stems and leaves have shown to possess strong antimicrobial activities similar to those of commercially available antibiotics The flower petals exert a strong anti-inflammatory activity and causes dose-dependent inhibition of carrageenan-induced rat paw oedema with similar activity to that of hydrocortisone, a well-known anti-inflammatory drug	The leaves and stems have been reported to contain flavonoids, terpenes, triterpenoids, phenols, tannins, alkaloids and carbohydrates	Perry (1980), Yusuf et al. (1994), Ghani (1998), Kirtikar and Basu (1999), Ghani (2003), Selim (2003), and Ahmed et al. (2005)

6	*Limosella maior* Diels Scrophulariaceae	None reported	None reported	None reported	
7	*Marsilea schelpeana* Launert Marsileaceae Water clover	According to the ancient Materia Medica of China, the whole plant of *Marsilea* sp. is said to have anti-inflammatory and detoxifying properties More recently, however, a juice prepared from the leaves of *Marsilea* sp. is applied to the skin as a treatment for snakebites and can be used as a diuretic (urine production stimulant) and febrifuge (fever-reducing agent)	None reported	None reported	Stuart (1911) and Duke and Ayensu (1985)
8	*Persicaria senegalensis* (Meisn.) Soják Polygonaceae Snakeroot	The leaves and roots are pounded and applied to syphilitic sores and skin infections to reduce swelling	None reported	Flavonoids and phenols were isolated from the aerial parts of *P. senegalensis*; these compounds include apigenin (5,7,4′-trihydroxyflavone) (**1**), 3,6-dimethoxy-kaempferol (**2**), 3,7,4′-trimethoxy-kaempferol (**3**), calycopterin (5,4′-dihydroxy-3,6,7,8-tetramethoxy-flavone) (**4**), quercetin (3,5,7,3′,4′-pentahydroxy-flavone) (**5**), quercetin-3-*O*-glucopyranoside (**6**), quercetin-3-methoxy-3′-*O*-glucopyranoside (**7**), 5-hydroxy-7-methoxy-isoflavone (**8**), gentisic acid-5-*O*-(6′-*O*-galloyl)-glucopyranoside (**9**) and gentisic acid-5-*O*-(2′-*O*-glucopyranosyl)-rhamnoside (**10**)	Hussein et al. (2012) and Fern (2014a)

(continued)

Table 16.1 (continued)

	Emergent, broad leaved				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
9	*Plantago longissima* Decne. Plantaginaceae Lamb's tongue	Used in traditional herbal remedies due to its astringent, antitoxic, anti-histamine, demulcent (reduces pain and inflammation in membranes), styptic (blood coagulant agent), antimicrobial and diuretic properties. The leaves can be made into a poultice and used externally for the treatment of inflammation associated with insect bites, minor sores, boils and rashes caused by poison ivy When taken as a tea, tincture or syrup, the leaves are known to help with respiratory tract problems—relieving coughing and bronchitis as it acts as an expectorant (brings up mucous and other materials from the lungs, bronchi and trachea)	Ethanolic root extracts did not show any activity against methicillin-sensitive *Staphylococcus aureus* at the highest concentration tested (12.5 mg/ml)	None reported	Watt and Breyer-Brandwijk (1962), Heyman et al. (2009), and Grieve (2012)

Table 16.2 Emergent (narrow leaved) plant species, their reported traditional usage, biological activity and phytochemical constituents

	Emergant, narrow leaved				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
10	*Carex austro-africana* (Kük.) Raymond Cyperaceae Rushes	None reported	None reported	None reported	
11	*Cladium mariscus* (L.) Pohl Cyperaceae Saw grass	A decoction is made from the leaves to treat colic problems, colds and renal pain arising within the gastrointestinal tract by the local people of Beni Suef, Egypt	Extracts of the leaves were prepared using an 80% aqueous acetone solution (1:10, w/v), the results obtained were expressed as IC_{50} values; radical savaging ability: DPPH (0.23 ± 0.04 mg/ml) and ABTS (0.32 ± 0.00 mg/ml); metal chelating ability: iron (>1 mg/ml) and copper (>1 mg/ml); tyrosinase inhibition (>1 mg/ml) The extract was also tested on various cell lines to assess its cytotoxicity; the following results are indicated as percentage of viable cells (%): HepG2 (human hepatocarcinoma) = 99.2 ± 1.35; HeLa (human cervical adenocarcinoma) = 268 ± 5.02; THP1 (human leukaemia monocytes) = 64.4 ± 6.69 and S17 (murine bone marrow stromal) = 63.7 ± 0.8	The phytochemical profile of the extract is expressed as gallic acid equivalents (GAE) in milligrams per gramme of extract (dry weight, DW) for total phenolic content (TPC); as milligrams of rutin equivalents per gramme of extract (mg RE/g, DW) for total flavonoid content (TFC); as milligrams of catechin equivalents per gramme of extract (mg CE/g, DW) for total condensed tannin content (CTC) and as caffeic acid equivalents per gramme of extract (mg CAE/g, DW) for total hydroxycinnamic acid content (HAC) TPC = 254 ± 2.26 mg GAE/g DW TFC = 13.8 ± 0.20 mg RE/g DW CTC = 38.7 ± 2.21 mg CE/g DW HAC = 34.4 ± 0.78 mg CAE/g DW	AbouZid and Mohamed (2011) and Lopes et al. (2016)

(continued)

Table 16.2 (continued)

	Emergant, narrow leaved				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
12	*Cyperus dives* Delile Cyperaceae Paper reed, giant sedge	Within the Eastern Cape region of South Africa, the rhizomes of *C. dives* are ground to a powder and taken orally by patients suffering with diarrhoea	None reported	None reported	Madikizela et al. (2012)
13	*Cyperus marginatus* Thunb. Cyperaceae Water reed	None reported	None reported	None reported	
14	*Cyperus rotundus* L. Cyperaceae Purple nutsedge	Traditionally this plant is used to treat skin disorders (wounds, boils and blisters) while the rhizomes have been reported to treat stomach ailments (including dysentery, diarrhoea, indigestion and bowel irritations)	A study of the ethanol extract of *C. rotundus* showed a variety of effects including anti-inflammatory, antimicrobial, analgesic and antidepressant activity A methanol extract of the rhizome of *C. rotundus* rhizome was given orally to castor oil-induced diarrhoeal mice at doses of 250 and 500 mg/kg body weight. The results showed significant antidiarrheal activity The antimalarial constituents isolated from the rhizomes of *C. rotundus* exhibited antimalarial activity between a range of EC_{50} 10^{-4} and 10^{-6} M (patchoulenone, caryophyllene α-oxide and 4,7-dimethyl-1-tetralone), while the novel compound 10,12-peroxycalamenene exhibited the strongest activity of EC_{50} at 2.33×10^{-6} M	Phytochemical studies of the plant revealed the presence of alkaloids, flavonoids, tannins, starch, glycosides and furochromones, and many novel sesquiterpenoids The antimalarial activity guided fractionation of the rhizomes of *C. rotundus* yielded the following compounds: patchoulenone (1), caryophyllene α-oxide (2), 10,12-peroxycalamenene (3) and 4,7-dimethyl-1-tetralone (4)	Thebtaranonth et al. (1995), Uddin et al. (2006), Lawal and Oyedeji (2009), and Ahmad et al. (2012)

15	*Cyperus sexangularis* Nees Cyperaceae Matjiesgoed	None reported	None reported	None reported	
16	*Eleocharis acutangula* (Roxb.) Schult. Cyperaceae Sedge	None reported	None reported	The underground parts of this plant have been reported to contain three steroids: campesterol (1), stigmast-4-en-6β-ol-3-one (2), stigmast-22-en-3β,6β,9α-triol (3); and four pentacyclictriterpenes: lup-20(29)-ene-3β (4), 16β-diolbetulinic acid (5), fern-9(11)-en-3α-ol (6) and neohop-13(18)-en-3α-ol (7)—these compounds were isolated using detailed chromatography methods	Amaral et al. (2004)

(continued)

Table 16.2 (continued)

	Emergant, narrow leaved				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
17	*Juncus effusus* L. Juncaceae Common rush	Traditionally in China the dried pith of *J. effuses* has been used in the treatment of anxiety and insomnia and their associated symptoms while the Malay community use the pith to treat urinary problems In Vietnam, the whole plant is used as a diuretic	Ethanol extracts of the stems were reported to inhibit the production of inflammatory mediators: nitric oxide (IC_{50} = 1.98 μg/ml), prostaglandin E2 (IC_{50} = 5.5 μg/ml) and pro-inflammatory cytokines, IL-1β (IC_{50} = 4.74 μg/ml) and IL-6 (IC_{50} = 20.48 μg/ml) in lipopolysaccharide-stimulated RAW 264.7 cells as well as reduced the development of 12-O-tetradecanoylphorobol-13-acetate-induced ear oedema and carrageenan-induced paw oedema in mouse oedema models used for acute inflammation. These results suggest that *J. effusus* can be used as a herbal anti-inflammatory agent Dehydroeffusol, a phenanthrene compound, possesses anxiolytic and sedative properties. This was determined through a variety of behavioural tests in mice; the compound was found to reduce locomotive functions in certain tests while not impacting the general movement and coordination of the mice Photosensitizers are produced by higher plants; these types of compounds are of interest as they have been used for the treatment of skin diseases such as psoriasis and vitiligo The isolated compounds of *J. effusus*, dehydroeffusol juncusol, were shown to have photosensitizing activities against microorganisms (*Staphylococcus aureus* and *Candida albicans*) and DNA-binding activities with UVA irradiation. Although further investigations are necessary, these compounds could be considered as reagents to investigate cellular mechanisms such as DNA replication, transcription and repair system, and possibly for the treatment of some skin diseases	Phytochemical compounds including juncusol (1) (antimicrobial), betuline (2) (anticancer) and dehydroeffusol (3) (anxiolytic and sedative) were isolated from *J. effuses*, along with phytochemicals from the following classes, flavones, coumaric acid, coumaroyl glycerides, cycloartanes, dihydrodibenzoxepins and phenanthrenes	Watt and Breyer-Brandwijk (1962), Hanawa et al. (2002), Liao et al. (2011), and Park et al. (2016)

18	*Juncus lomatophyllus* Spreng. Juncaceae Leafy Juncus	The rhizome of most *Juncus* species is used to treat many ailments relating to the intestines in Lesotho (a neighbouring country to South Africa) communities	None reported	None reported	Moteetee and Van Wyk (2011)
19	*Phragmites mauritianum* Kunth Poaceae Thatching reed	None reported	None reported	None reported	
20	*Prionium serratum* Baill. Juncaceae Palmiet	Prior to flowering the young shoots can be eaten as a vegetable while the leaves are used for weaving baskets, hats and mats	None reported	None reported	Porter (2011)
21	*Schoenoplectus brachyceras* (A. Rich.) Lye Cyperaceae Water reed	Although not of medicinal value this plant is traditionally used to weave baskets and mats	None reported	None reported	Hyde et al. (2017)
22	*Schoenoplectus paludicola* (Kunth) Palla Cyperaceae Sedge	None reported	None reported	None reported	

(continued)

Table 16.2 (continued)

	Emergant, narrow leaved				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
23	*Typha capensis* (Rohrb.) Typhaceae Bullrush	A decoction of the rhizomes is used to treat venereal diseases, bleeding, diarrhoea, swelling and urinary problems. During labour the decoction can be either taken orally or applied externally to promote the removal of the placenta and strengthen uterine contractions to ensure an easy delivery *T. capensis* is also taken to promote fertility in women, enhance male potency and libido and improve circulation The narrow leaves are used as thatch for mats and baskets while the seeds are used as pillow stuffing	Leaf and rhizome extracts of *T. capensis* in various solvents exhibited antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli* with an average MIC value of 0.75 mg/ml (rhizome) and 0.21 mg/ml (leaves) for all four pathogens	Typhaphthalide (1), typharin (2), sitosterol (3), afzelechin (4), epiafzelechin (5), (+)-catechin (6) and (−)-epicatechin (7) were isolated among other flavones, phenolic compounds, long-chain hydrocarbons and triterpenoids	Hutchings et al. (1996), Shode et al. (2002), Voigt and Porter (2007), and Masoko et al. (2008)

Table 16.3 Submerged plant species, their reported traditional usage, biological activity and phytochemical constituents

	Submerged				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
24	*Lagarosiphon muscoides* Harv. HYDROCHARITACEAE Fine oxygen weed	None reported	None reported	None reported	
25	*Lagarosiphon major* (Ridl.) Moss HYDROCHARITACEAE Coarse oxygen weed	None reported	None reported	None reported	
26	*Potamogeton schweinfurthii* A.Benn. POTAMOGETONACEAE Broadleaved pondweed	None reported	None reported	None reported	
27	*Potamogeton thunbergii* Cham. & Schltdl. POTAMOGETONACEAE Floating pondweed	None reported	None reported	None reported	
28	*Utricularia stellaris* L.f. LENTIBULARIACEAE Star bladderwort	None reported	None reported	None reported	

Table 16.4 Free-floating plant species, their reported traditional usage, biological activity and phytochemical constituents

	Free-floating				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
29	*Azolla pinnata* R.Br. subsp. *africana* (Desv.) R.M.K.Saunders & K.Fowler AZOLLACEAE Red water fern	None reported	None reported	None reported	

Table 16.5 Floating-leaved plant species, their reported traditional usage, biological activity and phytochemical constituents

	Floating-leaved				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
30	*Aponogeton distachyos* L.f. APONOGETONACEAE Cape pond weed	The stems are used as a soothing treatment to reduce the redness associated with burns and wounds, due to their high juice content. Together with crushed flower petals, the juice of the stems can be applied to the skin for the treatment of acne and pimples For many years the flowers and the leaves of this plant were used in stews as a replacement for cabbage and is considered a South African delicacy	None reported	None reported	Roberts (2000), Schwegler (2015), and Kam et al. (2016)
31	*Nymphaea nouchali* Burm.f. var. *caerulea* (Savigny) Verdc. NYMPHAEACEAE Blue water lily	The whole plant is used as a poultice for healing wounds while the seeds are used as a remedy for diabetes. The flowers are used as a treatment for dysuria and coughing while also possessing narcotic and aphrodisiac effects. An infusion of the roots and stems is taken as an emollient and diuretic in treating gonorrhoea and urinary tract infections	Ethyl acetate extracts of *N. nouchali* were evaluated for antimicrobial activity against ten clinically important bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, *Enterococcus faecalis*, *Xanthomonas campestris*, *Streptococcus mutans*, *Lactobacillus casei*, *Lactobacillus acidophilus*) using the agar well diffusion method. The ethyl acetate leaf extracts were active against all the investigated bacterial strains	None reported	Watt and Breyer-Brandwijk (1962), Ammani and Kumar (2012), and Mabona and van Vuuren (2013)
32	*Nymphoides thunbergiana* Kuntze MENYANTHACEAE Floating hearts	An emollient plaster can be made using the stems, leaves and flowers, it is then applied to wounds received during hunting accidents to help extract the bullet fragments	None reported	None reported	Fern (2014b)

Table 16.6 Riparian plant species, their reported traditional usage, biological activity and phytochemical constituents

	Riparian Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
33	*Commelina benghalensis* L. COMMELINACEAE Benghal dayflower, Benghal wandering Jew	The whole plant has been used to treat leprosy, sore throats, skin burns, infantile thrush and irritations associated with the eyes and stomach During famine periods in India and the Philippines, the leaves and stems of the weed were chopped and cooked as a nutritional source for people and were also made into feed for livestock In southern Africa, the plant was used to combat infertility in various traditional communities	Various fractions of *C. benghalensis* (chloroform, petroleum ether, butanol and hydromethanol) were evaluated for their sedative and anxiolytic effects. At doses of 200 and 400 mg/kg, all fractions exhibited dose dependent suppression of motor activity, exploratory behaviour and prolongation of induced sleeping time in mice In another study, the methanolic extract of this plant was subjected to in vivo testing to evaluate its effect on cell growth and its ability to induce apoptosis on Jurkat T cells. The data recorded from this study showed that the extract was able to elicit a dose and time dependant inhibition of cell proliferation which was then followed by a dramatic decline in cell viability. This cytotoxicity can be linked to the ability of the extract to induce apoptosis	A brief phytochemical screening of the plant revealed the presence of phlobatannins, carbohydrates, tannins, glycosides, volatile oils, resins, balsams, flavonoids and saponins	van der Burg (2004), Mbazima et al. (2008), Hasan et al. (2009), and Ibrahim et al. (2010)

(continued)

Table 16.6 (continued)

	Riparian				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
34	*Crinum bulbispermum* (Burm.f.) Milne-Redh. & Schweick. AMARYLLIDACEAE Orange River lily	Used in Lesotho (a neighbouring country to South Africa) as a treatment for the common cold. The bulbs of the plant can either be roasted to treat rheumatism, varicose veins and backaches, or made into a poultices to treat septic sores The leaves and flowers are placed on sprains to reduce swelling	A review on the biological activities of *Crinum* species highlighted the role of *C. bulbispermum* in various biological assays The aqueous leaf extracts supplied to rats at a dosage of 1, 1.5 and 3 g/kg, orally significantly impaired the parameters of a rat hold-board test indicating that this plant possess sedative properties An antioxidant assay revealed that the extract had an EC_{50} of 203.76 μg/ml on DPPH radical scavenging The aqueous extract of the leaves were given to rats to assess its toxicity. Mild to moderate toxicity was observed in rats with symptoms ranging from diarrhoea, postural abnormalities, liver and renal toxicity and in some cases death. The authors attributed this toxicity to the alkaloids (lycorine and crinine) found within in the extract	Lycorine (**1**), crinine (**2**), 8α-ethoxyprecriwelline (**3**), *N*-desmethyl-8α-ethoxypretazettine (**4**), *N*-desmethyl-8-β-ethoxypretazettine (**5**), bulbispermine (**6**), crinamine (**7**), 6-hydroxycrinamine (**8**) and 3-O-acetylhamayne (**9**)	Elgorashi et al. (1999), Hankey (2001), Moteetee and Van Wyk (2011), Refaat et al. (2013), and Khumbula Nursery (2014)
35	*Crinum campanulatum* Herb. AMARYLLIDACEAE Water crinum, marsh lily	In the Zulu communities this genus is used widely to treat bodily swelling and problems relating to the urinary tract	The leaves, bulbs and roots of *C. campanulatum* were tested for their ability to inhibit acetylcholinesterase using a microtiter plate assay, the bulbs showed the greatest percentage inhibition of 68% at 0.1 mg/ml	None reported	Elgorashi et al. (2001) and Jäger et al. (2004)

36	*Crinum moorei* Hook.f. AMARYLLIDACEAE Natal lily	The bulbs are traditionally used as a skin treatment for infected sores and acne as well as blood cleansers	The leaves, bulbs and roots of *C. moorei* were tested for their ability to inhibit acetylcholinesterase using a microtiter plate assay, the roots showed the greatest percentage inhibition of 86% at 0.1 mg/ml	Alkaloid compounds previously isolated from this species includes: Bulbispermine (**1**), mooreine (**2**), crinine (**3**), undulatine (**4**), 3-O-acetylcrinine (**5**), powelline (**6**), cherylline (**7**), crinamidine (**8**), epibuphanisine (**9**), epivittatine (**10**), 1-epideacetyl-bowdensine (**11**), lycorine (**12**) and 1-O-acetyllycorine (**13**)	Elgorashi et al. (2001), Nichols (2002), and Jäger et al. (2004)
37	*Crocosmia paniculata* (Klatt) Goldblatt IRIDACEAE Zigzag Crocosmia	The corm is made into a decoction by Zulu, Xhosa and Sotho communities living in Kwa-Zulu Natal, South Africa and is used to treat dysentery and diarrhoea	None reported	None reported	McGaw et al. (2000)

(continued)

Table 16.6 (continued)

	Riparian				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
38	*Cyrtanthus breviflorus* Harv. AMARYLLIDACEAE Yellow fire lily	Zulu communities in South Africa use the bulbs to treat intestinal worms such as tapeworm and roundworm	None reported	Ethanol extracts of the bulb yielded four known isoquinoline alkaloids: haemanthamine (**1**), crinamine hydrochloride (**2**), lycorine (**3**) and tazettine (**4**) Hexane extracts of the bulb extract yielded five known lupane triterpenoids: lupeol (**1**), lupenone (**2**), glochidone (**3**), 3β,27-dihydroxylup-20(29)-ene (**4**) and betulinaldehyde (**5**)	Gerstner (1939) and Crouch et al. (2005)
39	*Elegia tectorum* syn. *Chondropetalum tectorum* (L.f.) Moline & H.P.Linder RESTIONACEAE Cape thatching reed	Not medicinally utilised but this plant, due to its structure makes a good thatching material for roofs	None reported	Flavonoids including procyanidin (**1**), and the myricetin derivatives, 3-galactoside-syringetin (**2**) and 3-galactoside-larycitrin (**3**) were found to be present within *E. tectorum*	Harborne (1979), Harborne et al. (1985), and Turner and Jamieson (2016)

40	*Equisetum ramosissimum* Desf. EQUISETACEAE Drill grass, branched horse-tail	In China, the whole plant is used to treat skin wounds and ulcers by making a decoction, while in India it is used as a cooling medicine for gonorrhoea and has, for many centuries, been used as a treatment for skin wounds It has been reported to be used as a liver and eye cleanser. It clears eyesight by reducing the reddening and swelling of the eye as well as the pterygium of the cornea The whole plant can be made into a decoction by adding 15–30 g of dried plant material to boiling water as a treatment for diarrhoea and jaunditic hepatitis In South Africa, infertile women drink a decoction made from the rhizome to aid in fertilization while the Zulu communities have been reported to use the sap from the plant to reduce pain of toothaches and heal wounds of tooth extractions	Various extracts (ethyl acetate, dichloromethane, hexane, methanol and aqueous) of *E. ramosissimum* were tested for their protective effects against oxidation, melanoma and melanogenesis From the results, the ethyl acetate extract exhibited effective DPPH scavenging activity (43.41 ± 7.68% at 200 μg/ml) and metal ion chelating activity (44.56 ± 1.32% at 200 μg/ml) Both the ethyl acetate and dichloromethane extracts inhibited melanoma cell growth but did not have an effect on the viabilities of normal dermal keratinocytes or fibroblast cells as their viabilities exceeded 65% after a 24-h treatment The extracts were also able to inhibit mushroom tyrosinase activity ranging from 23.27 ± 1.67 to 38.93 ± 3.09 percentage inhibition for 100 μg/ml of each sample	Seventeen compounds were isolated from the whole plant of *E. ramosissimum* including: 5α,6α-expoxy-β-ionone-3-O-β-D-glucopyranoside (**1**), loliolide (**2**), cycloart-24(30)-ene-3β-ol (**3**), cycloart-22(23)-ene-3β-ol (**4**), ergost-6,22-diene-3β,5α,8α-triol (**5**), friedelinol (**6**), apigene (**7**), genkwanin (**8**), genkwanin-5-O-β-D-glucopyranoside (**9**), apigene-5-O-β-D-glucopyranoside (**10**), luteolin (**11**), quercentin-3-O-β-D-glucopyranoside (**12**), kaempferol-3-β-D-glucopyranoside (**13**), kaempferol-3-O-β-D-glucose-7-O-β-D-glucopyranoside (**14**), adenine (**15**), β-sitosterol (**16**) and β-daucosterol (**17**)	Gerstner (1939), Jain and Srivastava (2005), Wang and Jia (2005), Banjamin and Manickam (2007), Mannan et al. (2008), Moteetee and Van Wyk (2011), and Li et al. (2016)

(continued)

Table 16.6 (continued)

	Riparian				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
41	*Erythrina zeyheri* Harv. FABACEAE Harrow-breaker, plough-breaker	The various plant parts of *E. zeyheri* are used to treat a wide variety of ailments. The seeds are used to treat asthma while the leaves are made into a tea for relief of tuberculosis. Powdered bark is used as a treatment for rheumatism while a tea of the bark is used for blood disorders	The aqueous extract of the leaves showed only a ratio of 0.9 inhibition when compared to that of the positive control, neomycin, against *Staphylococcus aureus* using the disc diffusion assay. The ethyl acetate, ethanol and aqueous extracts of the leaves did not show any inhibition towards the other microbes tested (*Staphylococcus aureus*, *Staphylococcus epidermis*, *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*) The extracts of the leaves showed between 65% and 82% (ethanol) 17% and 90% (ethyl acetate) cyclooxygenase inhibition at concentrations of 50 and 500 µg/ml, respectively	Phytochemical analysis of the seeds yielded several alkaloids including erysodine (**1**), erysopine (**2**), erysovine (**3**), erysothiopine (**4**), erysothiovine (**5**), erythraline (**6**) and hypaphorine (**7**)	van Rensburg (1982), Hennessy (1991), and Pillay et al. (2001)
42	*Kniphofia pauciflora* Baker ASPHODELACEAE Dainty poker	None reported	None reported	None reported	

43	*Lippia javanica* Spreng. VERBENACEAE Fever tea, lemon Bush	A tea made from the leaves and stems is cooled and applied to the skin as a lotion for the treatment against various skin disorders including rashes, scratches, stings, bites, lice and scabies Xhosa communities in South Africa make a tea or milk infusion using the leaves and stems. These infusions are then consumed for the treatment of bronchial problems. They also use the plant to disinfect meat that has been contaminated with anthrax The whole plant is used in the treatment of fevers associated with malaria, influenza and measles while the smoke of the burnt leaves and stems are effective against asthma, chronic coughing and pleurisy	The isolated compounds were tested against *Mycobacterium tuberculosis* and HIV-1 reverse transcriptase for bioactivity. It was found that compounds **2** and **4** inhibited the HIV-1 reverse transcriptase enzyme by 91% and 53%, respectively, at 100 µg/ml	Eight compounds were isolated from *L. javanica* during a phytochemical analysis, these compounds include, 4-ethyl-nonacosane (**1**), (E)-2(3)-tagetenone epoxide (**2**), myrcenone (**3**), piperitenone (**4**), apigenin (**5**), cirsimaritin (**6**), 6-methoxyluteolin 4′-methyl ether (**7**), 6-methoxyluteolin and 3′,4′,7-trimethyl ether (**8**)	Le Roux (2004) and Mujovo et al. (2008)

(continued)

Table 16.6 (continued)

	Riparian				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
44	*Melianthus major* L. MELIANTHACEAE Giant honey flower	Warm aqueous leaf infusions are prepared and either used a gargle or mouthwash for the treatment of oral ulcers, gum diseases, sore throats or applied as a lotion to the skin to treat sores and ulcers. A leaf paste is applied directly to the scalp for the treatment of ringworm while poultices of the leaves are used for backaches and rheumatic joints Local people in the Cape regions of South Africa make poultices from the leaves and apply it directly to heal wounds and bruises	In vitro antimicrobial activity of the aqueous extracts of the leaves were observed against *Candida albicans*, *Staphylococcus aureus* and *Mycobacterium smegmatis* using disc assays, however the inhibition of growth observed was not noteworthy An ethanol extract of *M. major* was tested for cytotoxicity on Vero cells using the XTT method, the sample exhibited an IC_{50} of 52.76 μg/ml. An ethanol extract of the leaves against methicillin-resistant *Staphylococcus aureus* (the bacterium known to cause pneumonia, mastitis, meningitis, urinary tract infections and post-operational infections) had an MIC of 0.781 mg/ml	Two flavonoid compounds namely, quercetin 3-O-β-galactoside-6-gallate (**1**) and kaempferol 3-O-α-arabinopyranoside (**2**), were isolated from the leaves using column chromatography	van der Walt (2000), Scott and Springfield (2004), and Heyman et al. (2009)

45	*Mentha aquatica* L. LAMIACEAE Water mint	In Indonesia, the volatile oils of the plant are used to treat headaches and cholera In Southern Africa, southern Sotho communities make a decoction of the plant to treat colds, bile, constipation and problems relating to the liver. In combination with *Trifolium burchellianum* or *Senecio asperulus*, the plants are used to treat hypertension and wash sore joints, respectively Basutoland communities in Southern Africa place the plant underneath a mat to treat chest complaints	The essential oil of *M. aquatica* was evaluated for its antimicrobial and antifungal activity against various microorganisms including, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enteritidis*, *Salmonella typhi*, *Shigella sonei*, *Sarcina lutea*, *Micrococcus flavus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton tonsurans*, *Microsporum canis*, *Epidermophyton floccosum* and *Candida albicans*; however, no noteworthy MICs were recorded against any of the microorganisms	The major constituents found in the essential oil of *M. aquatica* included, menthofuran (51.26–58.59%), limonene (5.94–12.06%), trans-β-ocimene (5.59–6.10%), ledol (3.01–4.06%) and β-caryophyllene (2.923–3.557%)	Watt and Breyer-Brandwijk (1962), Mimica-Dukić et al. (2003), Moteetee and Van Wyk (2011), and Boz et al. (2013)
46	*Mentha longifolia* L. LAMIACEAE Wild mint	Traditionally used for the treatment of pulmonary ailments (including tuberculosis and whooping cough—the leaves are boiled so that vapours can be inhaled to relive nasal and bronchial congestion), urinary tract infections, swelling of joints, minor skin wounds and sores, indigestion, menstrual disorders, flatulence, headaches, fever and colds	Antioxidant activity using the ABTS assay revealed IC_{50} values of 476.3 ± 11.7 mg/ml Menthol was tested for its anticandidial activity against *Candida albicans* using the disc diffusion method (zone of inhibition range: 7.1–18.5 mm) as well as the microtitre dilution assay (MIC = 125 μg/ml)	Pulegone (**1**), menthone (**2**), isomenthone (**3**), menthol (**4**), 1,8-cineole (**5**), borneol (**6**), and piperitenone oxide (**7**) were the major phytochemical constituents identified from *M. longifolia* essential oil	Watt and Breyer-Brandwijk (1962), van der Walt (2004), Al-Bayati (2009), Mkaddem et al. (2009), and Moteetee and Van Wyk (2011)

(continued)

Table 16.6 (continued)

	Riparian				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
47	*Scadoxus multiflorus* Raf. subsp. *katherinae* (Baker) M.R.Almeida AMARYLLIDACEAE Fireball lily, blood flower, katharine wheel, poison root	Due to their extremely toxic nature, *Scadoxus* species are used in conjunction with other plants in Cameroon, Gabon, Angola and the Central African Republic, as an arrow poison while in Guinea and northern Nigeria the bulbs are used to as a poison for fish In some instances the bulbs of this species are used traditionally to treat scabies and wounds that are taking long to heal	None reported	The tuber of this species is alkaloid rich which provides the plant with its toxic nature. Toxic compounds present include, lycorine (**1**), narciclasine (**2**), galanthamine (**3**) and a variety of tropane alkaloids Preliminary phytochemical screening of the aqueous extract of the plant included the presence of alkaloids, flavonoids, tannins, saponins and cardiac glycosides	Notten (2001) and Roberts (2013)
48	*Scirpus ficinioides* Kunth CYPERACEAE	None reported	None reported	None reported	

49	*Tulbaghia leucantha* Baker ALLIACEAE Wild garlic	The Khoi-San communities traditionally infuse the herb with milk. The milk is then given to patients to treat intestinal worms, fever, influenza, high blood pressure, tuberculosis The plant is also used mainly as a culinary herb to add flavour to food or recreationally to strengthen the taste of tobacco	None reported	The Alliaceae family is characterised due an absence of alkaloids. It is well known that among all *Tulbaghia* species, the characteristic garlic smell is due to the presence of S-(methylthiomethyl) cysteine-4-oxide known as marasmin, however, no specific chemical isolations have been performed on this *Tulbaghia* species	Watt and Breyer-Brandwijk (1962), van Wyk (2008), and Ranglová et al. (2015)

(continued)

Table 16.6 (continued)

	Riparian				
	Plant species, family and common name	Traditional usage	Biological activity	Phytochemical constituents	References
50	*Zantedeschia aethiopica* (L.) Spreng. ARACEAE White arum lily	In the Cape of Southern Africa, the washed leaves are heated and used as a dressing for wounds, boils, minor burns, insect bites and sores. Patients suffering from gout or rheumatism also use the warmed leaves as poultice to reduce the pain and inflammation The plant can be boiled and eaten by mixing it with honey or syrup as a treatment for asthma and bronchitis; it can also be gargled for the relief of sore throats. The plant must be boiled or cooked in some way as the raw plant material causes swelling of the throat due to the presence of microscopic calcium oxalate crystals	Ethanol and aqueous extracts of the rhizomes of *Z. aethipica* were tested for their ability to inhibit the growth of various bacteria known to human pathogens. Zones of inhibitions ranging from 2.00–4.13 mm were observed against *Staphylococcus aureus*, *Klebsiella pneumonia*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*	Bioactive phytochemcials such as tannins, alkaloids, saponins, steroids, phenols and glycosides were found to be present within the rhizome of *Z. aethiopica*	Watt and Breyer-Brandwijk (1962), Roberts (1990), Rood (2008), Wink and van Wyk (2008), and Pratush et al. (2013)

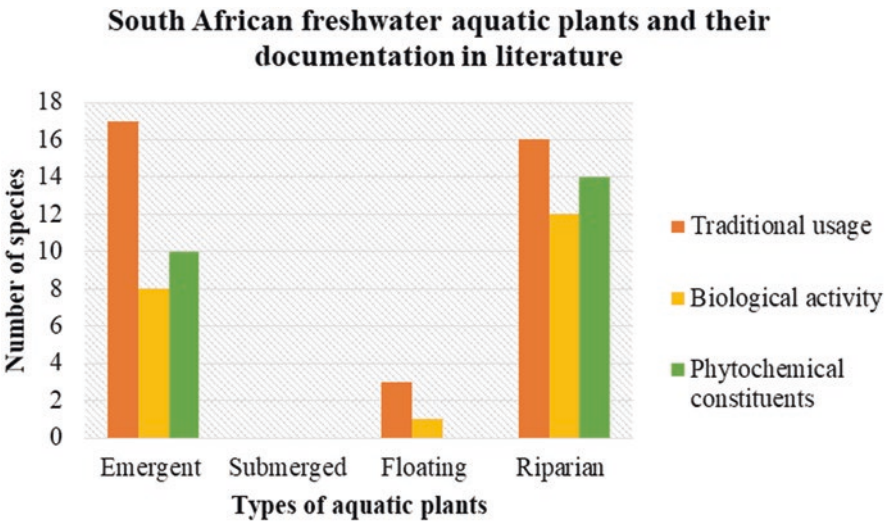

Fig. 16.7 A summary of the differences observed in the number of South African freshwater aquatic plant types and their citation in literature

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Chapter 17
Sea Buckthorn: A Multipurpose Medicinal Plant from Upper Himalayas

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17.1 Introduction

Sea buckthorn (*Hippophae* spp. L.) is an ecologically and economically important plant that belongs to the family Elaegnaceae. Sea buckthorn is known as *Shaji* in Chinese; *Duindoorn* in Dutch; *Sanddorn* in German; *Olivello Spinoso* in Italian; *Oblepicha* in Russian; *Tyrni* in Finnish; *Espino de Mar*, *Falso Espino*, *Espino Amarillo* in Spanish and *Havtorn* in Swedish (www.seabuckthornresearch.com). Every part of the plant, namely, fruit, leaf, twig, root and thorn has been traditionally used as medicine, nutritional supplement, fuel and fence, and therefore, sea buckthorn is popularly known as "Wonder Plant", "Ladakh Gold", "Golden Bush" or "Gold Mine". Recent research has supported and extended the traditional uses of the plant and several products are being produced for nutraceutical and medicinal values. It is said to have momentous economic potential and is predicted by some as the next major health food fad.

Sea buckthorn leaves are small, cuticle is thicker, leaf back densely covered with scales and star hairs to cover stomata. It has colourful red, orange or yellow berries that remain on the shrub throughout the winter. The plant is hardy and well adapted to extreme low temperatures of alpine and subalpine conditions of the mountains. Adult plants can withstand temperature as low as −40 °C during dormancy to +30 °C during summers and considered to be drought tolerant. It has an excellent

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ability to tolerate abiotic stresses like soil, moisture and nutrient. The shrub develops extensive root system having ability to fix atmospheric nitrogen. The root nodule of sea buckthorn has symbiotic association with bacterium belonging to the genus *Frankia* having the ability to fix 180 kg of nitrogen per ha per year (Jike and Xiaoming 1992). Sea buckthorn acts as an effective soil binder because of its extensive root system and help in checking soil erosion in those areas, which are very prone to erosion because of high wind velocities and flood. It is now in the UN Agenda 21 with focus on marginal areas and marginalized people.

The medicinal value of sea buckthorn was recorded as early as the eighth century in the Tibetan medicinal classic *rGyud Bzi*. As per literature available Chinese were the first to use this plant as medicine even before eighth Century. Genghis Khan, the Mughal conqueror, who established largest empire from China to Eastern Europe in thirteenth century, relied on three treasures, namely, a well-organized army, strict discipline and sea buckthorn. Pulp and seed oil of sea buckthorn makes soldier stronger as per recent research results. The Russian cosmonauts used sea buckthorn as a space food item. In Seoul Olympics, sea buckthorn squash was declared as the official health drink. Inspired by the ancient literature, scientists in the former Soviet Union carried out research on sea buckthorn from the 1930s onward. In 1940s, especially after the Second World War, nutritionists and pharmacologists analyzed the vitamin components and found that sea buckthorn could be used not only as food but also as medicine.

17.2 Classification

All the species of the genus *Hippophae* are called sea buckthorn. Sea buckthorn belongs to the family Elaegnaceae, which is in the major group *Angiosperms* (flowering plants). Genera in Elaegnaceae include *Elaegnus*, *Hippophae*, *Lepargyrea* and *Shepherdia*. Sea buckthorn believed to be originated from northwest Himalayas to the Hengduuan Mountains and middle or Mediterranean Asia (Zhuode et al. 1989). There are six species of sea buckthorn naturally distributed over the arid, semi-arid and high mountainous ecosystems of Europe and Asia including China, Mongolia, India, Nepal, Pakistan, Russia, Great Britain, France, Denmark, Netherlands, Germany, Poland, Finland, Sweden and Norway. The six species are *H. rhamnoides* L.; *H. salicifolia* D. Don; *H. tibetana* Schlecht; *H. neurocarpa* S.W. Lin et T. N. He; *H. gyantsensis* (Rousi) Lian and *H. goniocarpa* Lian (Yongshan 1988; Yongshan et al. 1995). The classification of genus *Hippophae* has been modified over the years. Originally it consisted of only one species, *H. rhamnoides*, with three subspecies, *rhamnoides, salicifolia* and *tibetana.* However, according to the latest systematic classification of the genus *Hippophae* L., the genus comprises of seven species, and the species *H. rhamnoides* circumscribes eight subspecies, namely, *sinensis* Rousi, *yunnanensis* Rousi, *turkestanica* Rousi, *mongolica* Rousi, *caucasia* Rousi, *carpatica* Rousi, *rhamnoides* Rousi and *fluviatilis* van Soest. The precise classification of the genus is still debatable due to the variations found in the Himalayas and

Table 17.1 Characteristics of *Hippophae* species found in India

		Species	
Characters	*H. rhamnoides* subsp. *turkestanica*	*H. salicifolia* D. Don	*H. tibetana* Schlecht
Plant height	4–7 m	4–5 m	7–60 cm
Leaf	Alternate, 2–4 mm wide, both surface silvery	Margin revolute, adaxial surface densely covered with stellate hairs or scale hairs of the hair part well developed, outwardly appearing as velvet-felted	Whorled, linear
Thorns	Branched thorns	Branched thorns are faintly developed	Thorny
Elevation	1200–3700 m	1500–3000 m	2700–5300 m
Type locality	Kazakhstan	Nepal	Xizang
Other features	Epidermis of branches is silvery, length of most fruits greater than breath, carpopodium is 3–7 mm long		Branches are pointing upward, usually broom-like, flower buds are ovate or ovate bifid, fruits are dark tangerine

the adjacent areas of Central Asia. All species are diploid ($2n = 24$), wind pollinated, and dioecious, and are restricted to the Qinghai Plateau and adjacent areas, with the exception of the species *H. rhamnoides* L. that occurs widely but sporadically in Asia and Europe. *H. rhamnoides*, *H. salicifolia* and *H. tibetana* are being grown naturally in India. Differences in their characteristics are presented in Table 17.1.

17.2.1 Taxonomic Status of the Genus

Morphological traits of seeds and fruits have been analyzed in *H. rhamnoides* L. collected from Turkey (Aras et al. 2007). Based on the ANOVA, it has been concluded that subsp. *caucasica* is not a subspecies but probably another taxon of *H. rhamnoides*. Furthermore, the hybrid features of pollen grains collected from Trabzon supported these results (Aras 1995). The differences in the fatty acid profile illustrated the chemotaxonomic relationships among the genotypes and demonstrated the importance and potential of fatty acids in delimitation of sea buckthorn genotypes (Ercili et al. 2008). The phylogenetic relationships among 15 taxa of *Hippophae* have been analyzed by comparing the sequences of the internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA (nrDNA) (Sun et al. 2002 and Shah et al. 2009) and chloroplast DNA (cpDNA) (Bartish et al. 2002). The consensus trees of parsimony analysis supported monophyletic origin of the genus *Hippophae*. Both the groups refrained from recognizing sections within the genus

as have been recognized by some other workers. However, the monophyletic origin of *H. rhamnoides* is supported by both the work groups. The ITS sequence data analysis supported the hybrid origin of *H. goniocarpa* and *H. litangensis* as proposed previously (Sun et al. 2002). Sequences of cpDNA have also been used to establish double maternal origin of diploid hybrid, *H. goniocarpa* (Wang et al. 2008).

17.3 Origin and Distribution

Sea buckthorn is native to Eurasia. The fossil pollens of this genus are well preserved in the lake sediments or deep soils and are usually used to indicate the past climatic oscillations (Tang and Shen 1996). Growing at an altitude between 60 and 5200 m, the distribution of *Hippophae* is extremely wide throughout various geographical areas of the world. It is naturally distributed over the arid, semi-arid and high mountainous ecosystems of Asia and Europe. The plant is found in China, Russia, India, Nepal, Bhutan, Pakistan, Afghanistan, Mongolia, Kazakhstan, Hungary, Switzerland, Romania, Germany, France, Britain, Finland, Sweden and Norway. In India, sea buckthorn is known by various names (Table 17.2) and it is widely distributed in Ladakh region of Jammu and Kashmir and in pockets of Himachal Pradesh, Uttarakhand, Arunachal Pradesh and Sikkim (Table 17.3).

17.3.1 Jammu and Kashmir

In Ladakh region of Jammu and Kashmir state around 11,500 ha area is reported under pure sea buckthorn (natural as well as planted; satellite imagery based finding) (Fig. 17.1) (Dwivedi et al. 2006). Although two species, namely, *H. rhamnoides* and *H. tibetana* are found in this region but majority of the area is under *H. rhamnoides*. Since 1960s State Forest Department is constantly extending the

Table 17.2 Local name of sea buckthorn in India

State	Ethnic group/ dialect	Local name
Jammu & Kashmir	Ladakhi	*tSer-Mang*, *tSer-Sa-Lu-Lu*, *Shib-Shu-Lu-Lu*, *sTar-Bu*
Sikkim (Basistha 2009)	Nepali	*Ghangaru*
	Hindi	*Charma*, *Kalabis*
	Sherpa	*Khurpu*
	Bhutia	*Torobo*
Uttarakhand (Yadav et al. 2009)	–	*Amesh*, *Chuk*, *Amil*, *Tarwar*
Himachal Pradesh	–	*Chharma*

Table 17.3 Distribution of sea buckthorn species in India (Dwivedi et al. 2009; Yadav et al. 2009; Basistha 2009)

Species	Ladakh	Himachal Pradesh	Uttarakhand	Sikkim
H. rhamnoides	Indus valley, Nubra valley, Suru valley, Changthang valley	Tinu, Gemur, Jispa, Darcha, Sumdoh, Shego-Lara, Kiato, Lingthi, Shichling, Kiamal, Morang, Sumling, Rangrik, upper Kinnaur	–	Nathula
H. salicifolia	–	Upper Kinnaur, Lahaul, Kaza	Gangotri, Harsil, Yamunotri valley, Har-Ki-Dum, Darma, Hanumanchatti, Budhi, Badrinath, Niti valley, Tambara-Gaurikund, Kali valley, Bogdiar, Gori valley, Byanse	Lachen, Zema I, Zema II, Zema III, Lachung
H. tibetana	Zanskar valley	Sangrum, Kibbar Takcha	Raini, Niti, Gomukh, Gori valley, Milam, shin-La	North Sikkim

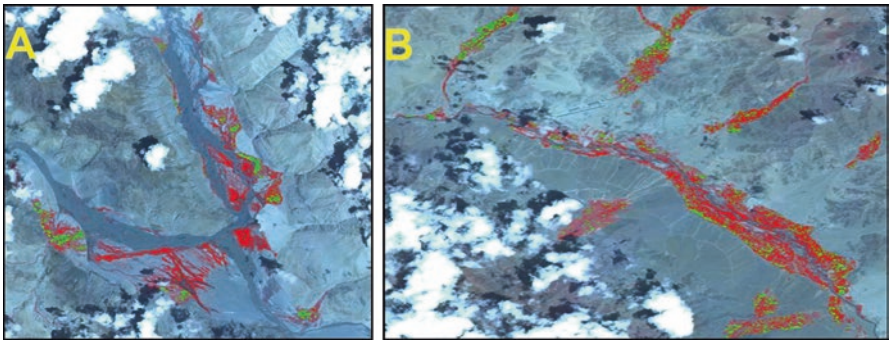

Fig. 17.1 Satellite images of sea buckthorn (red) in (**a**) Nubra valley and (**b**) Leh valley, Ladakh

area under sea buckthorn through root suckers essentially for soil conservation. Area covering more than 70 ha was planted with sea buckthorn in *Hunder* village of Nubra valley for fixing of sand dunes. Recently, plantations have been carried out in *Durbuk* and *Nyoma* villages in Changthang valley.

17.3.2 Uttarakhand

Sea buckthorn species found in Uttarakhand are *Hippophae salicifolia* and *H. tibetana. H. salicifolia* is found in large areas at high altitude regions (>2000 m AMSL), especially along the rivers or its tributaries originating from glaciers. The major

patches of *H. salicifolia* is identified in the regions, namely, Tons valley above *Naitwar*, Yamunotri valley above *Ranachatti*, Alaknanda valley above *Hanumanchatti*, Bhagirathi above *Harsil*, Mandakini above *Ghat*, Pindar valley above *Khati*, Saryu above *Munnar*, Gori Ganga above *Lilam*, Dhauliganga West above *Suraithota*, Dhauliganga East above *Sobala* and Kali valley above *Malpa*. On the other hand, *H. tibetana* is found in selected pockets, the major ones being about 100 ha patch on Burfu glacier and approximately 20 ha patch on Milam glacier. Small populations are also identified at *Bedang* in Darma valley. The 19.92 sq. km area of sea buckthorn is in reserved forests as per Uttarakhand Forest Statistics 2009–2010.

17.3.3 Himachal Pradesh

Wild populations of three species of sea buckthorn, that is, *Hippophae rhamnoides*, *H. salicifolia* and *H. tibetana*, have been recorded from Lahaul and Spiti and Kinnaur districts of Himachal Pradesh. As per preliminary assessment, the area of occupancy in the state is about 1000 ha, with geographical spread extending over about 1000 km^2 in the districts of Kinnaur and Lahaul & Spiti. More than 80% of the area under sea buckthorn is designated forest while the remaining 20% area is on private/community lands. Forest Department is currently raising sea buckthorn plants in eight nurseries in the State with annual production of about one Lakh plants. Over the past 3 years, sea buckthorn plantations have been extended over 105 ha in Lahaul & Spiti district.

17.3.4 Sikkim

Sea buckthorn is mostly confined to Lachen and Lachung valleys of North district and in few patches in East District. The approximate forest area covered under sea buckthorn is 800 ha. The species found in Sikkim is mostly *H. salicifolia* and it is distributed from 2430 to 3660 m above MSL. The Forest Department of Sikkim has planted 220 ha with sea buckthorn.

17.4 Cultivation Practices

17.4.1 Soil

Sea buckthorn is found growing profusely on a wide range of soil types, namely, sandy, rocky, saline and ravine soils. However, soil with light physical structure, rich in minerals, with a pH near neutral (pH 6.5–7.5) is found to be better. Best growth occurs in deep, well drained, sandy loam with ample organic matter.

17.4.2 Irrigation

Sea buckthorn can tolerate drought conditions but it is a moisture sensitive plant especially in the spring during flowering and fruit development stages. For economic reason, sea buckthorn orchard establishment should be restricted to areas receiving a minimum of 400 mm annual precipitation, unless irrigated. The optimum soil moisture content for mature plant is around 70%. Mulching should be practiced to reduce loss of soil moisture. Drip irrigation can be encouraged for optimal growth of plant and improved water use efficiency. The use of black plastic during first 3–4 years from seed has been found to improve moisture conservation and weed control. It is highly sensitive to water logging conditions, thus, water stagnation at any stage should be avoided.

17.4.3 Manure and Fertilizer

Sea buckthorn plants require little nitrogen, but it responds well to phosphorus fertilizer. Well-rotten farmyard manures (FYM) at 45 tonnes/ha should be used depending upon soil conditions (Li and McLoughlin 1997).

17.4.4 Propagation

Sea buckthorn can be propagated by various methods. Freshly harvested seeds have a short physiological dormancy and thus do not germinate immediately after harvesting and they remain viable for more than 2 years. Seed stratification for 20–25 days in alternate layers of moist sand yield over 85% germination. In Ladakh conditions, direct sowing of seeds in November also results in high germination rate. Cold water soaking of seeds for 6 days yields 93.33% germination and 73.33% survival rate. Treatment of seed with thiourea (100 mM) for 24 h has been reported to have highest germination percentage in case of *H. salicifolia*. Hard wood cutting is a method of choice for large scale propagation. Subjecting 1-or 2-year-old, pencil-thickness cuttings with two-thirds to half of the lower portion dipped in water for 2–3 days to 500 ppm IBA treatment results in 85% success in Ladakh condition. Planting of sea buckthorn cuttings in greenhouses, trenches and polyhouses results in even higher success rate in cold desert conditions. Micropropagation of sea buckthorn has been standardized in Murashige and Skoog media. For shoot proliferation 0.01 ppm IBA is found optimum while 2.0 ppm BA and 1.0 ppm NAA is optimum for rooting. Sea buckthorn has profuse suckering habit. A single plant produces 13–65 suckers which can be separated from the mother plant (Dwivedi et al. 2006). The individual suckers have been used as true to type planting material. Air layering has also been found useful in raising sea buckthorn nurseries.

17.4.5 Grafting

Grafting has been successfully demonstrated in sea buckthorn. Scions treated with heteroauxin gives better result in comparison with untreated scions. Cuttings can be prepared during autumn and stored at low temperature or under snow. Scion can also be prepared during early spring. Grafting is done on 1-year-old branch.

17.4.6 Nursery Management

Light and well drained soils are ideal. Soil should be at least 1.2 m deep. The site should receive sunlight and irrigation should be assured. Nursery bed should be ploughed deep before sowing. Well rotten FYM @ 4 kg/m^2 should be incorporated in the soil 20–30 days before sowing/planting depending on soil fertility status.

17.4.7 Orchard Establishment

Single or double hedge row system is recommended for sea buckthorn plantation under orchard system. In single hedge row system, the spacing between rows is maintained at 2–4 m for pure cultivation and at 4–5 m for intercropping (Dwivedi et al. 2006). The plants are planted in row at a distance of 1–2 m. Spacing is an important factor determining fruit yield. Method of plantation and spacing need to be modified based on cultivar, soil fertility status and climatic conditions to obtain good fruit yield (Table 17.4). For economic reasons, the ratio of male to female plant is important, as the number of female trees directly affects the total yield. Recommended male plants in an orchard vary from 6% to 12%. Weed control is very important in sea buckthorn planting, especially for promoting growth of newly planted seedlings.

Table 17.4 Effect of planting distance on sea buckthorn fruit yield (Khabarov 2003)

	Average yield (t/ha)	
Planting distance (m)	Chuiskaya variety	Jivko variety
4.0 × 1.0	18.5	14.5
4.0 × 0.8	22.2	16.2
3.5 × 1.5	15.9	12.7
3.5 × 1.0	21.7	16.0
3.5 × 0.8	25.0	20.0
3.0 × 1.5	17.3	14.5
3.0 × 1.0	25.1	19.0
3.0 × 0.8	26.2	16.9

17.4.8 Pruning

Moderate pruning increases the yield and reduces fluctuation of fruiting from year to year. The crown should be pruned annually to remove overlapping branches and long branches should be headed to encourage development of lateral shoots. Pruning has to be done every year, since the tree becomes older and fruiting zone moves upwards to gain umbrella shape, which needs to be avoided. Mature, fruiting plants should be pruned to allow more light penetration. March is the best month for pruning of in Ladakh, which is just before sprouting of sea buckthorn during spring. Pruning should not be done once the plant starts sprouting and autumn pruning is not advisable under the Ladakh conditions due to ultra-low temperatures and prolonged winters (Dwivedi et al. 2006).

17.4.9 Cultivars

There is no recommended cultivar so far for Indian cold desert conditions. Most of the cultivars known are of Siberian, Russian and German origin (Table 17.5). Production characteristics such as yield, ability to harvest, organoleptic test of fruit, nutrient profile of fruit and disease resistance need to be taken into consideration for selection of cultivar for a specific region.

Table 17.5 Promising cultivars and their important characteristics

Cultivars	Characteristics
Indian summer	Seedling variety, high yielding, multipurpose fruit bush, grows well in saline soils, average drought resistant, hardy and good for wind break
Krasny Fakel	Late ripening, abundant fruiting, high yielding, good for industrial processing, high in ascorbic acid and carotenoid contents. Fruits remain firm and retain majority of biological activity during freezing and defrosting
Botanica	Large sized fruit, excellent flavoured variety, heavy yielding tree with an upright growth habit
Tsherbonka-1	Dwarf in nature, frost hardy with big fruit size
Maslichnaya	Stable crop, high resistance to diseases and high yielding
Samarodok	High oil and carotene content
Frugana	Early ripening, upright growth habit, longer stems make hand harvesting of fruit easier
Hergo	Medium size and medium vigour plants. High productivity and high fruit quality
Capris	Early ripening, fruits are big, round-oval, orange colour, sweet pulp with pleasant fragrance. Fruits are good for fresh use and juice preparation
Russian Orange	Early maturing, vigorous bush with deep orange berries
Podruga	Medium ripening, high sugar content, mainly a table fruit variety, fruits are good for fresh use, making juice and jams
Parad	Fruits are big, red-orange colour, it's a universal use variety, suitable for mechanized harvesting, industrial uses of fruits and freezing
Siberian splendor	Fruit is large, sweet and easy to harvest. Good for fresh eating

17.4.10 Fruit Harvesting (Gupta and Singh 2003)

Harvesting of fruit starts soon after ripening. Fruit harvesting is the most time consuming operation in growing sea buckthorn. The relatively small fruit size, short pedicel, force required to pull off each fruit, density of fruit on the branch and the thorniness of the plant are the major factors affecting harvesting. Harvesting of fruit is comparatively easier in early morning hours. Fruits can be harvested by various direct and indirect harvesting techniques.

17.4.10.1 Hand Picking

Fruits are picked with hand either from the ground or from the plant. The method is labour intensive and time consuming. On an average, an individual can harvest 5–12 kg berries in 8 h.

17.4.10.2 Beat the Bush

In this method, the branches are beaten with a stick and the fruits are made to fall on the ground covered with polythene. Harvesting is easier during early morning hours before sunrise and causes less damage to the plant. This method is practiced for berry harvesting in Ladakh. Harvesting is also done during late winter in which the berries fall easily.

17.4.10.3 Cutting of Branch

Branches with fruit are cut from the plant and berries are harvested by manual or mechanical method. Cooling the branches makes berry harvesting easier. Harvesting by this method causes drop in fruit yield the next year.

17.4.10.4 Jaw-Tooth and Brush Harvester

This device consists of two jaws with teeth and brushes. The jaws are closed on to a fruiting branch and the fruits are rasped off and fall down through a fabric chute into a bag-type container. The device weighs 600 g and an individual can harvest 40–60 kg fruit in 8 h.

17.4.10.5 Fruit Comb

A simple method used in Mongolia in which a steel wire hook is used to harvest berries by combing the fruits in two directions at once. An individual can harvest 40–100 kg fruits in 8 h.

17.4.10.6 Use of Bioregulators

Spray of 200–250 ppm Ethrel (Ethephon) on sea buckthorn plant reduces the mechanized harvest time by half and ensures a complete crop harvest. Spray of the chemical cause decrease in the attachment force between sea buckthorn fruit and the branches.

17.4.10.7 Trunk and Branch Shaker

Mechanical harvester that shakes either the branch or the truck has been tried to harvest sea buckthorn berry. DIHAR has developed a hand held shaker that shakes the branch for berry harvesting. Trials conducted have shown that berry harvesting increased by 4–6 times as compare to manual harvesting, however, electric power supply under field condition is a major hurdle for its practical application. In comparison to beat the bush method, the mechanical harvester cause less damage to the plant.

17.4.11 Leaf Harvesting (Mann et al. 2003)

Harvesting of leaf has been a major problem for sea buckthorn industry. Harvesting by hand is time consuming and labour intensive. Efforts have been made to develop mechanical leaf harvester in Canada where manual labour is either not available or economically prohibitive.

17.4.11.1 Hand Held Prototype

A hand held prototype consisting of a pair of hydraulically powered, rotating brush heads at the end of a tubular, aluminium frame and other accessories has been developed. The best harvest rate observed was 10.5 g s^{-1} using 1.5 mm diameter nylon bristles rotating at 525 rpm. However, for practical application several modifications are needed.

17.4.11.2 Trailer-Mounted Prototype

The prototype has been developed to overcome the problem with the excessive weight of the hand-held prototype. The harvest rate ranged from 3.8 to 17.0 g s^{-1} using 1 mm diameter polypropylene bristles rotating at 500 rpm.

17.4.12 Yield

Fruit yield depends on species, cultivar, age of plantation, cultural practices and so on. Vegetatively propagated plants starts bearing fruits at the age of 4 years while seedling-raised plants take 5–6 years. Fruit yield under orchard system range from 10 to15 t/ha. Sea buckthorn cultivar Chuiskaya is reported to yield 26.2 t/ha when planted at a spacing of 3.0 × 0.8 m in Russia. Under Ladakh condition, where the plants are growing under natural condition without any management practices, the fruit yield varies from 0.2 to 8 kg per plant. Leaf yield also depends on a number of factors. Leaf yield of 5 years old nine Russian varieties ranges from 1.0 to 2.4 kg per tree (Morozov 2007).

17.4.13 Insect, Pests and Diseases

Very few insect, pests and diseases have been reported in sea buckthorn. Green aphid (*Capithophorus hippophae*) is reported to be the most damaging insect in sea buckthorn. These aphids cover the lower side of the leaves and suck the cell sap and thus leaves become yellow and die. Aphids can be controlled with insecticides or by biocontrol agents such as ladybugs.

Death Hawk Moth (*Acherontia styx)* is a prominent pest causing considerable damage to sea buckthorn plant at larval stage in Lahaul Spiti (Bhagat et al. 2003). Defoliating beetles belonging to subfamily Melolonthinae, namely, *Brahmina coriacea* (Hope), *Brahmina* spp. have also been found attacking sea buckthorn. Although the attack of these beetles is sporadic, they reduce plant vigour and fruit bearing. It has been observed that the Indian meal moth (*Plodia interpunctella* Huber) cause damage to berries of sea buckthorn in storage. Fruit fly (*Rhagoletis batava obscuriosa* Kol), defoliator brown tail moth (*Euproctis chrysorrhoea*), clear wing moth (*Synanthedon hippophae*), root borers (*Cossus* spp. and *Holotricha* sp.), Psyllido (*Cacopsylla* spp.), and trunk borer (*Asia's haladewdri*), have also been reported on sea buckthorn from various regions of the world. Root rot caused by *Rhizoctonia solani* Kuhn was reported from Uttarakhand (Singh et al. 2007). Mice and rats are other pests, which can destroy and girdle the trunk or chew up roots.

Few diseases have been reported in sea buckthorn and the most serious one is scab, which strikes both female and male plants, especially in a rainy spring-summer period (Dolgacheva and Aksenova 2003). Scab appears in the form of dark-brown tuberous spots, which gradually become black and glistening. The spots appear either on the leaves or the fruits.

Fusarium wilt has also been reported in sea buckthorn which is marked by tarnishing and falling of leaves. The fruits prematurely become brown and wither, but do not fall. An efficient method of plant protection is to cut off and burn the damaged branches.

Verticillium wilt also appears in the middle of summer. The leaves become yellow and fall off. The fruits prematurely dry up and the plant dries out very quickly. The shoots become black-cherry coloured with the swelling of the bark. The diseased plant should be uprooted and burnt.

17.5 Postharvest Handling and Storage

Sea buckthorn fruits are small, soft, delicate and juicy. It is highly perishable and cannot be transported over long distance. Sea buckthorn berries, when overripe, carry a strong musky odour with rancid taste, which can be detectable even in the fields. Therefore, berry must be harvested at the correct stage, quickly transported to the processing plant, and it should be cooled immediately to 4–6 °C to retard spoilage. If the berries are to be stored for more than few days, they should be frozen, preferably by quick freezing techniques. Pulp can be stored using preservatives such as KMS or benzoic acid.

17.5.1 Juice Extraction and Storage

Juice from ripe fruits is extracted by pressing or centrifugal techniques and can be stored under refrigeration for few days and requires pasteurization or freezing for long term storage. Aseptic filling at low temperature improves the storage of sea buckthorn juice. If fresh juice is allowed to stand 1 or 2 days, it separates into three phases: an upper floating particulate phase; a central liquid portion; and sinking particulate sediment. This separation is undesirable from consumer point of view. If pulp oil is not removed, it results in formation of an oily layer on juice surface. Centrifugal technique of reducing the oil below 0.1% eliminates the floating problem. For preservation purpose, it is necessary to sterilize/pasteurize the juice. High-temperature-short-time (HTST) processes of 80–90 °C for several seconds are preferred. Storage of juice beyond 6 months results in browning and shelf life of juice can be extended by storing at 4 °C.

During processing and storage some changes occur in the bioactive compounds. Phylloquinone (vitamin K) loss during industrial juice production has been reported about 36–54% due to technological processing of the berries (Gutzeit et al. 2007). Similarly, the total ascorbic acid loss during industrial juice production is about 5–11% (Gutzeit et al. 2008). The production of concentrated juice results in 50% depletion of ascorbic acid (Kallio et al. 2002). Storage of sea buckthorn juice for 7 day at ambient temperature (25 °C) results in 18% degradation of pantothenic acid. Therefore biochemical content of juice depends on the processing technologies and the equipment used.

Use of microwave has now been suggested for better bulk drying of sensitive biomaterials. In addition, a volumetric heat transfer mechanism coupled with drying in vacuum provides an ideal low-temperature drying technique resulting in better organoleptic quality. Therefore, use of vacuum with microwave results in drying in much lesser time (Bal et al. 2011).

17.5.2 Oil Extraction

Sea buckthorn contains pulp oil as well as seed oil. Pulp oil can be separated by centrifugal force or using organic solvent such as hexane. Since sea buckthorn oil is mostly used in cosmetic industry or as nutraceutical supplement, newer techniques such as supercritical carbon dioxide extraction under high pressure are preferred. Drying of pulp and seeds provide extended shelf life. Air-dried and freeze-dried seeds give a similar extraction yields with hexane, whereas air-dried pulps give better yield than freeze-dried pulp.

17.5.3 Pigment Extraction

A yellow pigment can be extracted from sea buckthorn waste after juice extraction with low concentration of alcohol. The pigment contains flavones, carotene and vitamin E. Pressure has the greatest influence on extraction with yield increasing with extraction pressure.

17.6 Nutritional Attributes

Sea buckthorn fruits are among the most nutritious of all berries. The berries generally consist of pulp (68%), seed (23%) and peel (7.75%). Fruit juice is rich in organic acids, amino acids, essential fatty acids, phytosterol, flavonoids, vitamins and mineral elements. Concentrations of vitamins A, B_2 and C are much higher than other fruits and vegetables such as carrot, tomato and orange (USDA 2008). Presence of these vitamins in high quantity along with flavonoids and tannins indicates its strong antioxidant property. Sea buckthorn berries also contain appreciable levels of vitamin B_1, and K. Sea buckthorn seed is a source of valuable oil characterized by high oleic acid content and one to one ration of omega-3 and omega-6 fatty acids. Sea buckthorn leaf is also a rich source of proteins. It serves as a valuable ingredient in animal feed, therapeutic agent having antiviral activity against a wide spectrum of viruses and can be used as a source of unconventional protein for human food.

17.6.1 Berry Pulp/Juice

Sea buckthorn from various parts of the world shows great variations in nutritional attributes. A brief comparison is presented in Table 17.6.

Table 17.6 Nutritional attributes of sea buckthorn pulp/juice from different regions

	India		China				
Parameters	Ladakh (Stobdan et al. 2010)	Uttarakhand (Dhyani et al. 2007)	China (Tong et al. 1989)	China (Zhang et al. 1989)	China (Kallio et al. 1999)	Finland (Kallio et al. 1999)	Pakistan (Sabir et al. 2005)
Calcium, mg/L	176.6	–	64–256	93.9–173	0.8–1.48 g/kg	0.27–0.74 g/kg	70–125
Iron, mg/L	30.9	0.703–1.127	5.93–161	–	64–282 mg/kg	22–33 mg/kg	40–170
Magnesium, mg/L	22.5	0.62–1.92	53.3–165	39.8–103	0.47–0.73 g/kg	0.56–0.79 g/kg	139–240
Phosphorous, mg/L	84.2	0.6–0.67 (%)	–	82.1–206	–	–	110–133
Sodium, mg/L	414.2	0.47–0.63(%)	18–89.9	17.7–125	–	–	20–80
Potassium, mg/L	647.2	10.12–14.87%	100–806	147–209	6.44–12.2 g/kg	10.3–14.84 g/kg	140–360
Zinc, mg/L	1.4	0.817–2.74	2.09–6.31	0.43–1.25	8.8–27 mg/kg	14–27 mg/kg	–
Copper, mg/L	0.7	0.09–0.133	–	–	3.8–12 mg/kg	6–9.5 mg/kg	–
Manganese, mg/L	1.06	–	0.81–3.86	1.17–2.6	8.7–15 mg/kg	8.1–17 mg/kg	–
Selenium, mg/L	0.53	–	–	7.96–11.3	–	–	–
Riboflavin, mg/100 g	1.45	–	–	–		–	–
Niacin, mg/100 g	68.4	–	–	–	–		–
Panthothenic acid, µg/100 g	0.85	–	–	–	–	–	–
Vitamin B_6, mg/100 g	1.12	–	–	–	–	–	–
Vitamin B_2, µg/100 mL	5.4	–	–	–	–	–	–
Vitamin C, mg/100 g	275	–	–	–	–	–	250–333
Vitamin A, IU/100 g	432.4	–	–	–		–	–

(continued)

Table 17.6 (continued)

Parameters	India		China			Finland (Kallio et al. 1999)	Pakistan (Sabir et al. 2005)
	Ladakh (Stobdan et al. 2010)	Uttarakhand (Dhyani et al. 2007)	China (Tong et al. 1989)	China (Zhang et al. 1989)	China (Kallio et al. 1999)		
Vitamin E, mg/100 g	3.45	–	–	–	–	–	–
Cadmium, mg/L	–	–	0.002–0.015	<0.05	0.016–0.055 mg/kg	0.044–0.105 mg/kg	–
Chromium, mg/L	–	–	0.47–1.0	0.108–0.287	–	–	–
Cobalt, mg/L	–	–	0.01–0.09	0.1	–	–	–
Lithium, mg/L	–	–	0.06–0.15	0.132–0.303	–	–	–
Molybdenum, mg/L	–	–	1.18	0.03–0.058	–	–	–
Nickel, mg/L	–	–	0.39–0.09	0.115–0.357	–	–	–
Strontium, mg/L	–	–	0.08–0.45	0.19–0.616	–	–	–
Tin, mg/L	–	–	–	0.045–0.259	–	–	–

17.6.1.1 Moisture and TSS

Moisture content varies from 20% to 87% depending on origin and climate. The content of soluble sugars ranges from 9.3 to 27.9° Brix. A wide range in the values suggests that both genotypic and environmental factors play important role in determining moisture and total soluble sugars in the berries.

17.6.1.2 Vitamins

Vitamin C represents a nutrient of major importance in sea buckthorn because of its presence in large quantities ranging from 200–2500 mg/100 g. Considering that fresh orange juice contains 35–56 mg/100 mL and Aonla contains 478.5 mg/100 mL, the value of sea buckthorn as a source of vitamin C is apparent. It is estimated that there is enough vitamin C in the berries of sea buckthorn plants across the world to meet the dietary requirements of the entire human population. Besides vitamin C, the juice also contains vitamin A, vitamin E, riboflavin, niacin, pantothenic acid, vitamin B_6 and vitamin B_2. Results of analysis of sea buckthorn growing in Leh valley of Trans-Himalaya showed the presence of high content of multivitamins

including vitamin C (275 mg/100 g), vitamin A (432.4 IU/100 g), vitamin E (3.54 mg/100 g), Riboflavin (1.45 mg/100 g), Niacin (68.4 mg/100 g), Pantothenic acid (0.85 μg/100 g), vitamin B_6 (1.12 mg/100 g), and vitamin B_2 (5.4 μg/100 g) (Stobdan et al. 2010). The high vitamin concentration makes sea buckthorn fruit highly suitable for the production of nutritious soft drinks. During Seoul Olympic Games in 1988, China designated its sea buckthorn sports drink as the official beverages for its athletes. Russian cosmonauts also were supplied with sea buckthorn beverages, to enhance their health and resistance to stress (Small et al. 2002).

17.6.1.3 Mineral Elements

There are 24 minerals in sea buckthorn juice, including calcium, magnesium, phosphorus, iron, manganese, sodium, potassium and aluminium, among others. Potassium is the most abundant of all the elements investigated in berries. Mineral composition of berries from Leh valley of Ladakh region revealed high amount of minerals including potassium (647.2 mg/L), calcium (176.6 mg/L), iron (30.9 mg/L), magnesium (22.5 mg/L), phosphorous (84.2 mg/L), sodium (414.2 mg/L), zinc (1.4 mg/L), copper (0.7 mg/L), manganese (1.06 mg/L) and selenium (0.53 mg/L) (Stobdan et al. 2010).

17.6.2 Seeds

Sea buckthorn seeds possess high amount of sterols. Total sterol content in seed varies from 1200 to 1800 mg kg^{-1}, of which 57–76% is sitosterol and 13–21% isofucosterol. Stigmastanol, citrostadienol and campesterol constitute 1–5, 2–5 and 2–3% of total seed sterols, respectively. The proportion of other sterols is typically 1–2% each. There is no significant difference in the level of most of the compounds or the total sterols between *sinensis* and *rhamnoides* subspecies. The level of most sterols in the seeds of ripe berries remained constant during the harvesting period (Yang et al. 2001).

17.6.3 Leaves

Sea buckthorn leaves contain many nutrients and bioactive substances such as carotenoids, free and esterified sterols, triterpenols, and isoprenols. It contains approximately 15–20% proteins. Flavonoids content in leaves ranges from 312 to 2100 mg/100 g of air-dried leaves. Lipids from sea buckthorn leaves have been recommended for antiburn and wound healing properties.

17.6.4 Bark

Sea buckthorn bark has not been studied in detail for its health promoting components. However, the same need to be studied since the plant grows fast and the bark can be used as source of active components with biological and therapeutic application. Bark of sea buckthorn branches contains natural lipids. The main components are the free fatty acids, which showed a high intensity of the metabolic processes taking places in the bark. Triacylglycerols also accumulate in the bark. It also contains a high level of cyclic and free alcohol and triterpene acids (Bekker and Glushenkova 1997).

17.6.5 Sea Buckthorn Oil

Sea buckthorn oil is a valuable product used in medicine, as nutraceutical supplement, and in cosmetic. The oil content of sea buckthorn ranges from 1.5% to 3.5% in fruit pulp and about 9.9–19.5% in seeds. Oil content depends on species, cultivar, part used, stage of maturity and method of extraction. Oil from juice and pulp is rich in palmitic (16:0) and palmitoleic (16:1) acids, while the oil from the seed contains the essential fatty acids, which are linoleic (18:2) and linolenic (18:3) acids. One of the many special features of sea buckthorn fruit is the exceptionally high content of tocopherols and tocotrienols (Kallio et al. 2002). Total content of tocotrienols varied from 1.5 to 8.1 mg kg^{-1} in berries and from 43 to 188 mg kg^{-1} in berry oil (Kallio et al. 2002). The content of these bioactive compounds are among the crucial criteria defining the quality of sea buckthorn. Oil derived from juice contains more vitamin E (216 mg/100 g of fruit) than seed (64.4–92.7 mg/100 g of seed). The tocopherol fraction consists of approximately 50% α-tocopherol, 40% β-tocopherol and 10% γ-tocopherol (Mironov 1989). Presence of high content of α-tocopherol has significant healthful effect which acts as natural antioxidant in the human body. Carotenoids content of sea buckthorn oil ranges from 314–2139 mg/100 g (Tong et al. 1989). It is indicated that the carotenoids consist of approximately 20% β-carotene, 30% γ-carotene, 30% lycopene and 15% oxygen-containing carotenoids. Phytosterol are also constituents of sea buckthorn oil which is capable of lowering plasma cholesterol on consumption by humans. The major phytosterol is sitosterol (β-sitosterol) followed by 5-avenasterol. A novel triglyceride, 1,3-dicapryloyl-2-linoleoyl glycerol has been isolated and its structure has been explained. The seed oil is characterized by its high oleic acid content (17%) and its one to one ratio of omega-3 (alpha linoleic) and omega-6 (linoleic) at approximately 34% and 31%, respectively. The relationship of equivalence between the two omegas is critical because they self-check each other in a delicate balance to regulate thousands of metabolic functions through prostaglandin pathways. Nearly every biological function is interconnected with balance between omega-6 and omega-3 (Bal et al. 2011).

17.7 Traditional Uses

Sea buckthorn has been judiciously used by people living in the cold deserts of Ladakh. Due to scarcity of resources, sea buckthorn has been used traditionally for a variety of purposes (Table 17.7). Every part of the plant, namely, fruit, leaf, twig, root and thorns, has been traditionally used as medicine, nutritional supplement, fuel and fence. The agricultural fields are valued the most in the region due to limited cultivable land. Traditionally the dense and thorny shrub is planted around agricultural field and plantation sites to protect against stray animals and pedestrian movement. The dried twigs and branches are also put along the boundary walls of residential houses and fields. Plantation of timber trees like willow and poplar is an important activity in cold desert of Ladakh. The cuttings during its initial years need utmost care especially from the stray animals. To prevent animals from damaging the plants, sea buckthorn branches are tied around the cuttings to serve as tree guard. This practice is effective in increasing survivability percentage of the plants during early stages. Cold deserts are characterized by high wind velocity leading to environmental degradation. Windbreaks made of sea buckthorn are effective at preventing wind erosion in open areas. The shrub can resist drying effect and physical injuries caused by the wind. The extensive root system of the shrub is ideal for soil binding. Due to extensive root system, sea buckthorn is planted around the water channel to check erosion due to water flow. A novel use of the shrub has been observed in Ladakh. Sea buckthorn plant is cut above the ground and placed horizontally on two opposite sides of river during winter months. This act as a support for floating ice blocks to form a stable ice cover. With due course of time the ice cover from the two opposite sides joins to form a long single piece ice connecting the two opposite river sides. The ice cover serves as route for crossing the river in situations when bridges are either far or does not exist in the area (Stobdan and Singh 2009).

Cold deserts have a typical problem of firewood. Cold regions require fuel wood to keep the houses warm during winter months. However, due to meagre forest

Table 17.7 Traditional uses of sea buckthorn in cold desert of Ladakh, India (Stobdan and Singh 2009)

Plant Parts	Traditional uses
Leaf	Fodder, tea
Berry	Treatment of common ailments, nutritional supplements, oil for household lightening
Twig and branches	Biological fencing, firewood, religious rituals, tree guard
Stem	Firewood, charcoal, handle for agricultural implements
Root	Firewood
Whole plant	Fencing, windbreak, protection against water erosion

Every part of sea buckthorn is being used traditionally in cold deserts

cover, availability of firewood is a major challenge. Under such circumstances, sea buckthorn stem and branches are used as firewood. The calorific value of dry sea buckthorn is 4785.5 calories per kg. The shrub is fast growing and can be stumped after every 3–5 years. Since the shrub grows fast and tolerates repeated cuttings, it reduces the harvesting pressure on other native woody plant species such as poplar, willow and juniper. A 6-year-old sea buckthorn plantation on 1 ha can produce 18 tonnes of firewood which is equal to nearly 12.6 tonnes of standard coal. The firewood is popular especially in monasteries and during ceremonies where high calorific firewood is required. Most monasteries maintain its own sea buckthorn growing areas to ensure regular supply of firewood. Sea buckthorn is therefore a popular green plant in the region. Charcoal that remains after burning sea buckthorn is often used by blacksmiths. Sea buckthorn stem is hard and often used as handle for agricultural implements (Stobdan and Singh 2009).

17.8 Socio-economic Benefits

Sea buckthorn has immense untapped potential which can change the livelihood of the local populace. Studies conducted using satellite imagery by DIHAR and Defence Electronic Application Laboratory has shown that about 11,500 ha of land is under pure sea buckthorn in Ladakh region (Dwivedi et al. 2006). As most sea buckthorn products are derived from natural forest and community land where application of chemical fertilizers and pesticides is not practiced, efforts are being made to certify sea buckthorn as organic in Ladakh region. The colourful sea buckthorn berries had little commercial value in Ladakh region till year 2001. However, after setting the first sea buckthorn processing unit in Leh, sea buckthorn collection is taken as an important activity as additional source of income. The collection period is short and the returns are high. Popularity of sea buckthorn as source of income can be judged from the increase in price of the berries from Rs. 8/kg in year 2001 to Rs. 23/kg in 2011. Sea buckthorn fruit worth Rs. 1.4 crore (approximately 400,000 USD) was sold in 2007 from Leh district of Ladakh region, which account for less than 5% of the region's total potential (Stobdan et al. 2008). Since collection and primary processing is being done by the locals, the revenue generated benefits the needy in the society.

In year 2007, 200 MT of sea buckthorn berries were collected from Leh district. In the Nubra and Leh Block, a total of 140.4 MT of sea buckthorn berries were collected from 39 villages and supplied to Cooperative Society for processing. In Nubra Block 69.4 MT of sea buckthorn berries were collected by 454 household from 17 villages. The village collection ranged from 10 kg to 19.8 MT. Average collections per household is over 152 kg per year resulting in net revenue of Rs. 3344 per household. Assuming that 20 days were involved in the harvesting and that about 1.2 persons were involved per household, the total employment generated by the activity comes to about 10,896 man days per year. Similarly, 606 household

from 12 villages in Leh Block have collected and supplied 71 MT of sea buckthorn berries to Cooperative Society. Number of household ranged from 22 to 80 in different villages. Average collection is over 117 kg per household per year resulting in net revenue of Rs. 2574 per household and generating employment to the tune of 14,544 man days per year (Stobdan et al. 2011).

17.8.1 Collection and Trading in Ladakh

Sea buckthorn is naturally distributed in Ladakh region and the area under the shrub largely belongs to either the nearest community or the Government Forest Department. Therefore, the local community or the Forest Department has the right over the natural sea buckthorn plantation. Collection of sea buckthorn berries is allowed only to the nearest community for which prior permission has to be taken from the Forest Department. Regulations have been made for scientific collection of berries for which time and method of collection has been standardized. Collection is allowed only during the morning hours when it is easy to harvest due to low temperature. The Forest Department takes utmost care not to damage the shrub while harvesting.

Primary processing of sea buckthorn is mainly done by the Cooperative Society since year 2004. The society fixes price of berry for a particular year before harvesting. Price fixation is done with consultation of the Ladakh Autonomous Hill Development Council and the local panchayat. Collection is done from villages and taken to sea buckthorn processing unit run by the society. The berry after primary processing is sold to a firm from outside the region for further value addition. The firm to which the pulp is sold has been selected based on highest bid through advertisement in national newspaper in the year 2004. Agreement has been made to sell the pulp to the highest bidding firm for few years with an annual increase in price of the pulp by 5% every year. The profit earned by the society is spent on creating mass awareness about scientific harvesting of sea buckthorn and creating infrastructure for social causes. Due to organized sea buckthorn collection and trading, the farmers are getting good returns. Since there is no middle man in the business, the entire amount goes directly to the farmers.

With increasing awareness and potential of sea buckthorn in Ladakh, several private entrepreneurs have entered into sea buckthorn business. However, the majority of them are limited to primary processing and sell the pulp to bigger firm located outside the region. DIHAR is organizing regular training to local entrepreneurs and Self Help Group (SHG) for development of value added products in the region. Sea buckthorn beverage technology has been formally transferred to a local entrepreneur and Non-Government Organization (NGO) to encourage value addition of pulp. Sea buckthorn products are few in number and new to the Indian market. There exist immense potential both in terms of diversified product development and income generation in the region.

17.9 Ecological Impact

Sea buckthorn brings many environmental benefits, including soil and water conservation, desertification control, land reclamation, erosion and water loss control, reforestation, and the establishment of wildlife habitats especially in fragile ecosystem, due to its extensive root system coupled with efficient nitrogen fixation. The shrub has been found growing well in sandy, rocky, saline and ravine soils. Since it is resistant to drought and tolerates soil salinity and low temperatures, it is suitable for conditions that are simply too challenging for most plants. Thorny and bushy growth of sea buckthorn provides a protective shelter for flora and fauna thereby maintaining the fragile ecosystem. Windbreaks made up of sea buckthorn are effective in preventing wind erosion in open areas. The spiny shrub has proven to be beneficial in acting as a barrier to pedestrian traffic, preventing sensitive vegetation from being trampled. Growing of sea buckthorn around the field and barren land improve soil fertility. Plant growth-promoting microbes have been isolated from sea buckthorn rhizosphere (Mundra et al. 2011).

17.9.1 Nitrogen Fixation

Root nodules of sea buckthorn have symbiotic association with bacteria belonging to the genus *Frankia* having the ability to fix nitrogen in non-leguminous woody plant species including sea buckthorn. It has been found that sea buckthorn can fix up to 180 kg of nitrogen per ha per year, which generally improves soil fertility (Jike and Xiaoming 1992).

Frankia strains make vesicles in nitrogen deficit culture. The vesicle's interior provides nitrogenase enzyme an anaerobic condition. Evidence from structural and physiological studies indicates that a variety of mechanisms exists for limiting O_2 diffusion to nitrogenase in symbiosis. The process of nitrogen fixation requires lots of energy in the form of ATP. The reaction for energy generation is carried by nitrogenase enzyme of *Frankia* where hydrogen is evolved as a by-product and at least one H is produced per N reduced to ammonia.

A number of physical and chemical parameters influence the nitrogen fixation capacity of *Frankia*. The effect of temperature on the nitrogen fixing capabilities in the excised root nodule of non-legume woody trees and shrubs including sea buckthorn showed maximum nitrogenase activity at 20 °C and no significant change was observed up to 30 °C. At 40 °C there was high temperature induced irreversible damage of nitrogenase. At temperature near 0 °C, the nitrogenase activity was found to be very low. The effect of nitrate-nitrogen on the nodule symbiosis of nodulated sea buckthorn using labelled N shows that applied nitrogen promotes plant growth in sea buckthorn while nodule growth and rate of nitrogen fixation per plant is depressed. The root nodular nitrogen fixation capacity in sea buckthorn fluctuates with stage of plant growth, plant age, plant variety, environmental factors, and

seasonal variation as well as between morning and evening. Plant sex did not influence the nitrogenase enzyme activity.

Nitrogen fixing capacity by *Frankia* is also influenced by infected root nodular ultrastructure. A very elegant relationship was established between nitrogen fixing capacity and nodular ultrastructure in sea buckthorn during early stage to multi-lobed root nodule nitrogenase development stage. The nitrogen fixing activity is proportional to the number of endophytic vesicles per unit cell area. Deficiencies of Molybdenum severely affect the formation of vesicles which reduces the nitrogen fixing activity.

17.9.2 Desertification Control

Sea buckthorn is an ideal plant for desertification control. The plant has been used successfully in Jianping County, Loess Plateau and Inner Mongolia of China (www.icrts.org). Jianping County has witnessed frequent disasters such as flood, drought and storm as a result of indiscriminate logging leading to only 1.9% forest coverage. However, plantation of sea buckthorn on more than 67,000 ha of land has resulted in creation of the largest sea buckthorn forest in the world. Similarly, in Chinese Loess Plateau, environmental degradation covering an area of 430,000 sq. km has resulted in an annual top soil losses of 1600 million tonnes. Search for a suitable plant species in terms of survival rate, multiplication and soil improvement coupled with economic benefits has resulted in identification of sea buckthorn as the most appropriate species. Sea buckthorn now covers more than 200,000 ha in Loess Plateau. Sea buckthorn plantations have been raised by the state forest department in *Hunder* village of Nubra valley (Ladakh) to control the desertification.

"Yikezhao Prefecture" in Inner Mongolia witnessed large-scale degradation and is often referred to as the "moon on earth" and "environmental cancer". Government initiatives to check environmental degradation has resulted in more than 50,000 ha of land under sea buckthorn. Plantation on difficult terrain like gully slopes, gully bottoms, elongated mounds, riversides and rocky areas were successful (www.icrts.org).

17.9.3 Soil and Moisture Conservation

Sea buckthorn develops extensive root system. In 10–12 year old shrub, the horizontal root extend up to 537 cm and vertically up to 127 cm deep thus making it ideal plant for soil binding (Bekker and Glushenkova 1997). Sea buckthorn plantation intercepts precipitation by lush crown layer which redistribute precipitation and control surface runoff. Plantation is done along water channel to check soil erosion. Plantation of sea buckthorn improves soil physical characteristics and fertility while

improving soil water holding capacity. It has been observed that soil moisture in sea buckthorn plantation areas is 3–4% higher than outside the forest (Chengjiang and Daiqiong 2002). Similarly, humidity is 10–20% higher in sea buckthorn forest.

17.9.4 Fencing and Windbreaks

Sea buckthorn is widely used for fencing around the house. Windbreaks made up of sea buckthorn are effective at preventing wind erosion in open areas. Plants that serve as windbreaks must be resistant to the drying effects and physical injuries caused by wind, and sea buckthorn is well suited to this task (Small et al. 2002).

17.9.5 Firewood

Cold desert is characterized by sparse vegetation and severe cold winter. This demands fast growing plant species to be used as firewood for heating houses and for cooking. Sea buckthorn has proved to be a popular green energy plant because of its quality biomass. In a 6-year old sea buckthorn forest, each hectare can produce 18 tonnes of firewood, equal to nearly 12.6 tonnes of standard coal. In monasteries, where hundreds of monks reside, the plant species is used as firewood to cook food. Since the shrub grows fast, it reduces the harvesting pressure on native woody plants thereby maintaining the fragile ecosystem.

17.9.6 Wildlife Habitats

It has been observed that a number of wildlife species depend on sea buckthorn stems, leaves, flowers, roots, fruit and seed. In the Loess Plateau of China, 51 bird species are entirely dependent and 80 bird species are relatively dependent on sea buckthorn for their food (www.icrts.org). Sea buckthorn berries remain on the bushes all winter, unlike most fruit that fall off the plant at maturity. In winter, the importance of sea buckthorn increases as it is almost the only food available for birds. In cold arid region of Ladakh, the endangered double-humped camel, yak, sheep, goat and so on survive on the plant mainly during winter months when no other fodder is available in the region (Dwivedi et al. 2006). Sea buckthorn thus provides long-term benefits in terms of maintaining the ecological equilibrium and improving the environment.

17.9.7 Improvement of Microclimate (Tan et al. 1994; Shun 1993; Sheng 2003)

Sea buckthorn interacts with its surroundings just like any other plant species. Plantation of sea buckthorn improves microclimate to a great extend within a given space and distance. Sea buckthorn canopy influence solar radiation, air temperature, soil temperature, humidity and wind velocity. The root system and the biomass improve physical properties and nutrient status of soil. Change in microclimate due to sea buckthorn plantation depends on geography, climate, scale of plantation and on many other factors. Studies conducted in China showed following microclimate changes:

17.9.7.1 Solar Radiation

Sea buckthorn foliage decrease solar radiation through absorption, transmittance and reflection. It has been observed that sea buckthorn canopy reduce solar radiation by 29.6% in leafless season (April) and 41% in summer (July).

17.9.7.2 Soil Temperature

The influence of sea buckthorn canopy on solar radiation and wind velocity influence soil temperature. The soil temperature (0–20 cm depth) in a sea buckthorn forest during full leaf season (July) remains 3.2 °C lower than outside.

17.9.7.3 Relative Humidity

The relative humidity within a sea buckthorn forest remains 18.9% higher than outside.

17.9.7.4 Evaporation

Evaporation of water within a stand reduced by 68.9%.

17.9.7.5 Wind Velocity

Wind velocity at a height of 1 m above ground reduced by 84.6%.

17.9.8 Improvement of Soil Physical Properties (Tan et al. 1994)

The root system and the biomass of sea buckthorn improve physical and nutrient status of soil. Root system and biomass of sea buckthorn improve texture and structure of soil. It changes the soil texture from clay to loam, soil structure changes from clod to granular. Change in soil physical properties is favourable to increase the porosity of non-capillary and decrease bulk density. Sea buckthorn plantations improve nutrient status of soil. Percent increase in organic matter, total nitrogen, total phosphorus, total potassium, available phosphorous and available potassium recorded in comparison to sandy soil was 13.29%, 4.76%, 15.38%, 1.0%, 30.3% and 90.3%, respectively.

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