Mohd Sayeed Akhtar
Mallappa Kumara Swamy *Editors*

Anticancer Plants: Natural Products and Biotechnological Implements

Volume 2

Springer

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This book is dedicated to

Maulana Abul Kalam Azad
(1888–1958)
A great visionary, scholar, social reformer, statesman of the twentieth century and first education minister of independent India

Foreword

Cancer is a cluster of diseases involving irregular cell growth with the possibility to invade or extend to other parts of the body. Like many other deadly diseases, it is involved in the killing of several thousands of humans every year. Pharmaceutical industry is struggling hard to find a permanent cure against this multi-disease, and there are many challenges in achieving this mammoth goal. The modern trend of the pharmaceutical industry has shifted from synthetic to natural treatments of using herbs. According to WHO, approximately about 80% of current world's human population rely upon plant-based drugs against several diseases. Like many other uses, many plants serve a pivotal role in the eradication and cure of so many deadly diseases and human ailments like cancer. Those plants which show anticancer activities are often rich in many secondary metabolites like various alkaloids, phenolics and antioxidant compounds.

This book is a wide collection of 23 well-articulated chapters on different aspects of medicinally important plants which show anticancer properties and are used as a conventional drug. Chakravarty and Gaur discuss the fungal endophytes as novel sources of anticancer compounds in Chapter 1, while Chapter 2 is the joint efforts by Malaysian and Thailand researchers focusing on current knowledge and future perspective on the potential of plant-derived phytobezoar in cancer treatment. In Chapter 3, Singh and Tripathi describe the potentiality of natural products for the prevention of oral cancer, while Chapter 4 by Siti-Syarifah and Nurhanan-Murni mentions the chemical structure, mechanisms of actions and other issues pertaining to the use of plant-based cardiac glycosides as a potential anticancer agent. Chapter 5 by Salma et al. mentions the conservation strategies towards *Catharanthus roseus*, a potent anticancer plant. Likewise, in Chapter 6, Roy et al. describe the significance of medicinal plants as a source of chemopreventive agents, and Chapter 7 by Indian investigators highlights the plant metabolites as new leads to anticancer drug discovery. Chapter 8 by Campos-Xolalpa et al. entails about the cytotoxicity and antitumoural effects of terpenes of the genus *Salvia*, while Chapter 9 by Angelini et al. discusses essential oils and their anticancer activity. In Chapter 10, the diversity and production of enzyme L-asparaginase is discussed for its fungal endophytes from anticancer plants as producers of the antitumour agent L-asparaginase by Chow et al., a group of Malaysian researchers. Chapters 11 and 12 by Indian scientists give an update on xylooligosaccharides and their anticancer potential and phytochemicals targeting endoplasmic reticulum stress to inhibit cancer cell proliferation.

Similarly, Chapter 13 by Ajumeera et al. explains the targeted remedy of targeting cancer and cancer stem cells using herbal plants and their extracts. Chapter 14 by Pandurangan and Mustafa discusses about several bioactive natural products and plant extracts from various parts of plants and mushrooms showing potential inhibitory effect against breast cancer cells. Subsequently, the chapter by Alves Cardoso Filho discusses about endophytic microbes as a novel source for producing anticancer compounds. Similarly in Chapter 16, Lee et al. discuss the therapeutic potential of traditional medicinal plants against major cancer types. In the next chapter, the Indian investigators beautifully describe the various dimensions of platelet-derived growth factor receptor (PDGF-R) as the target for herbal-based anticancer agents. Chapter 18 discusses an alternative for the production of anticancer compounds by plant cell and organ culture by joint venture of Indian and Korean researchers, while in Chapter 19, Lokhandwala and Jain discuss the utility of plant-derived extracts and compounds against breast cancer. Chapter 20 by Swamy et al. discusses the micro-propagation and conservation of endangered anticancer plants of Western Ghats of India. Moreover, Chapters 21, 22 and 23 by Gonçalves and Romano, Gajula et al., and Behera et al., respectively, highlights the application of modern biotechnological approaches to enhance the production of bio-active compounds from anticancer plants.

I believe this book will be helpful for the researchers in particular and also for graduate and undergraduate students studying the science of medicinal and aromatic plants for the cure of various diseases including cancers. Moreover, it is also helpful for teachers, healthcare professionals and biotechnologists interested in the natural products and the biotechnological aspects of anticancer plants.

I wish to thank the editorial board members, Dr. Mohd Sayeed Akhtar and Mallappa Kumara Swamy, and all the contributing authors for bringing the collection of such novel piece of work in the world for the first time and also for the grand success of this book.

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Khalid Rehman Hakeem

Preface

Cancer is one of the leading death causes of human population increasingly seen in recent times. Plants have been used for medicinal purposes since immemorial times. Though several synthetic medicines are useful in treating cancer, they are inefficient and unsafe. However, plants have proved to be useful in cancer cure. Moreover, natural compounds from plants and their derivatives are safe and effective in treatment and management of several cancer types. Anticancer plants such as *Catharanthus roseus, Podophyllum peltatum, Taxus brevifolia, Camptotheca acuminata, Andrographis paniculata*, *Crataeva nurvala*, *Croton tonkinensis*, *Oplopanax horridus*, etc., are important source of chemotherapeutic compounds. These plants have proven their significance in the treatment of cancer and various other infectious diseases. Nowadays, several well-known anticancer compounds such as taxol, podophyllotoxins, camptothecin, vinblastine, vincristine, homoharringtonine, etc., have been isolated and purified from these medicinal plants. Many of them are used effectively to combat cancer and other related diseases. The herbal medicine and their products are the most suitable and safe to be used as an alternative medicine. Based on their traditional uses and experimental evidences, the anticancer products or compounds are isolated or extracted from the medicinally important plants. Many of these anticancer plants have become endangered due to ruthless harvesting in nature. Hence, there is a need to conserve these species and to propagate them in large scale using plant tissue culture. Alternatively, plant cell tissue and organ culture biotechnology can be adopted to produce these anticancer compounds without cultivation. The proper knowledge and exploration of these isolated molecules or products could provide an alternative source to reduce cancer risk, antitumourigenic properties and suppression of carcinogen activities.

Anticancer Plants: Natural Products and Biotechnological Implements – Volume 2 is a very timely effort in this direction. This book volume with 23 contributions from Brazil, India, Italy, Malaysia, Mexico, Portugal, Republic of Korea, Romania, Thailand and the USA discusses the natural bioactive compounds isolated from plants as well as fungal endophytes, their chemistry and preventive measures to reduce the risk caused by cancer. Moreover, the genomics/proteomics approaches and biotechnological implements are also highlighted in this preceding volume. This book provides a solution to cope with the challenges involved in the cancer therapy. We hope that this book will be helpful for graduate students, teachers,

researchers and industry persons who are functioning in the fields of cancer biology, natural product chemistry, pharmacology, healthcare and medicinal plant research.

We are highly grateful to all our contributors for readily accepting our invitation for not only sharing their knowledge and research but for venerably integrating their expertise in dispersed information from diverse field in composing the chapters, and enduring editorial suggestions to finally produce this venture. We greatly appreciate their commitment. We are also thankful to Dr. Khalid Rehman Hakeem for his suggestions and writing the foreword for this volume. We also thank the team of Springer International, especially Dr. Kapila Mamta and Joseph Daniel, for their generous cooperation at every stage of the book publication.

Shahjahanpur, Uttar Pradesh, India — Mohd Sayeed Akhtar
Serdang, Selangor, Malaysia — Mallappa Kumara Swamy

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Fungal Endophytes as Novel Sources of Anticancer Compounds

1

Kashyapi Chakravarty and Smriti Gaur

Abstract

There has been an exponential rise in the world population having numerous health ailments as a result of carcinogens causing cancer. Therefore, newer arenas to combat this issue are being continuously explored, and endophytes constitute one such source of potential anticancer agents. Endophytes are microbes residing in viable plant tissues consisting of potential, substantial sources of natural bioactive agents. Endophytic fungi reside within the tissues of higher plants without causing any harmful symptoms. The anticancer activities displayed by these microbes against specific cancer cells have been due to the cytotoxic effects of their bioactive compounds. These organisms have been comparatively less explored, and their use in the pharmaceutical industry holds significant promise. Fungal endophytes form a reliable source of important secondary metabolites by employment of their biotransformation processes. They employ specific mechanisms by which they penetrate the tissues of host plants and live in mutualistic association with the plants. They can be genetically and physico-chemically modified to obtain higher yields of specific metabolites of interest. Unique analogues of active metabolites can also be generated using fungal endophytes. A critical balance maintained between the virulence by a fungal endophyte and the defence mechanism of the plant, by the release of endophytic metabolites, helps in sustaining its competition with plant pathogens and epiphytes. Therefore, the aim of this chapter is to highlight the new arena of research on fungal endophytes producing novel anticancer metabolites. Insights into this research would ultimately help in the production of safe, reliable and economical anticancer drugs.

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Anticancer compounds · Camptothecin · Cancer · Fungal endophytes · Taxol

1.1 Introduction

Cancer is one of the deadliest of all diseases, which involves a group of conditions wherein uncontrolled growth and division of cells affect the development of tumours which invade and damage healthy tissues around them (Chen et al. 2016). Cancerous cells have a tendency to spread to different parts in an individual's body producing tumours. Out-of-control and unregulated proliferation of cancerous tumours can result in the death of the patient (Kharwar et al. 2011). The World Cancer Report 2014 of World Health Organization's International Agency for Research on Cancer has stated that the incidence of cancer increased to an alarming number of around 14 million new cases globally in 2012, and it has been expected to increase up to 19.3 million individual cases annually by 2025 (Gulland 2014). The reason behind the rise in cancer cases has been attributed to population growth and ageing, along with the increase in risk factors like smoking, weight gain, lack of proper exercise and the altered reproductive patterns that have become the norm of urban, economically developed society (Torre et al. 2015).

Tumour suppressor genes, proto-oncogenes and the genes participating in DNA processes are majorly involved in the initiation of tumour formation (Berit and Rolf 2007). Principal cause of cancer is said to be damage to DNA (Wiseman et al. 1995). DNA damage occurs in a sequential manner which is brought about by a number of genetic as well as environmental factors leading to the activation of tumour suppressor genes and/or oncogenes. The target cells get transformed as a result of abnormal expression of genes regulating apoptosis and DNA repair genes (Knudson 2001; Croce 2008). Cancer arises from the conditions of metastasis and invasion resulting from a series of processes, which include cellular proliferation along with various mutations followed by the amplification of clones, leading to the formation of subclones having different traits (Fearon and Vogelstein 1990; Wood et al. 2007). The most commonly used chemotherapeutic drugs for clinical treatment of cancer aim at destroying malignant cells in tumours by the inhibition of mechanisms involved in cellular division. Mostly, the drug resistance and non-specific toxicity caused by anticancer drugs make chemotherapy of cancer a rather risky task (Nygren and Larsson 2003). The high costs of cancer research and anticancer drug development lead to a heavy financial burden on an individual's healthcare expenses and the government budget. Medicinal plant extracts and natural products, due to their resistance to drugs and low cytotoxicity, provide valuable aid in controlling the malignant nature of cancer (Mbaveng et al. 2011). Plant-derived drugs such as camptothecin and taxol are very beneficial in cancer treatment (Srivastava et al. 2005). By the process of altering the natural products or through the extraction of specific anticancer molecules from medicinal plants, new discoveries of anticancer agents have been made possible (Srivastava et al. 2005). However, conservation of endangered plants

of medicinal value has become essential as excessive harvesting has led to substantial damage to the ecosystem (Kala 2000). Therefore, research into the development of alternative sources of anticancer molecules has become the need of the hour.

The limitations associated with the production of anticancer compounds of plant origin led scientists to tap a new arena of novel bioactive resources, and that is microorganisms. These microscopic entities especially fungi constitute an inexhaustible and renewable storehouse of a plethora of novel bioactive compounds having potential pharmaceutical properties (Chandra 2012). Bioactive metabolites, with antibacterial, antiviral as well as antimycotic properties obtained from fungi (especially fungal endophytes), have been a growing area of research for quite some time. Microbial source of a product of potential interest has many benefits. Indefinite availability of most microbes of interest can be ensured by their perpetual storage under favourable conditions (Okami 1986). An inexhaustive supply of desirable compounds can be maintained by large-scale cultivation of microorganisms in tank fermenter (Okami 1986). Routine techniques for culturing microbes have shown favourable results with respect to increase in yield of products of interests. This chapter mainly focuses on the remarkable world of fungal endophytes which provide a plethora of novel secondary metabolites having potential anticancer properties. It gives an overview of fungal endophytes and their interaction with host plants. Furthermore, it presents a comprehensive account of the latest research dealing with the production of anticancer secondary metabolites from endophytic fungi along with the chemical structures of several major types of anticancer compounds. Efficient methods for the large-scale production of anticancer compounds of fungal endophytic origin have also been discussed. The aim of this chapter is to highlight the new arena of research on fungal endophytes producing novel anticancer metabolites.

1.2 Fungal Endophyte

Novel compounds can be discovered by the investigation of secondary metabolites obtained from microbes of unique and unusual niches. In 1866, the term 'endophyte' was first defined by Anton de Bary as all microorganisms (namely, fungi, cyanobacteria, actinomycetes and bacteria) which take up residence inside living plant tissue without causing any harm (De Bary 1866). Fungal endophytes have earned very little limelight as compared to their pathogenic counterparts as they usually reside without showing any symptoms inside the living hosts (Swamy et al. 2016). Therefore, they constitute an untapped resource in the research of novel compounds from unknown microbial species (Kharwar et al. 2011). They form a major proportion of the micro-ecosystem of plant community (Tan and Zhou 2001; Zhang et al. 2006; Rodriguez et al. 2009). After the discovery of the "gold" bioactive agent paclitaxel (taxol) from the fungal endophyte *Taxomyces andreanae* (Stierle et al. 1993), the interest in research on bioactive and novel metabolites from fungal endophytes has increased manifold among scientists. During the last few decades, a number of potential bioactive agents with several beneficial properties including

anticancer activities have been isolated from fungal endophytes (Swamy et al. 2016). They help the host plant in the promotion of its growth and in the synthesis of secondary metabolites which aid in plant defence (Chandra et al. 2010; Swamy et al. 2016). There is a constant interaction between a fungal endophyte and its host plant at the metabolic level. Secondary metabolites originating from fungal endophytes are produced through many metabolic pathways (Tan and Zhou 2001). Sometimes, endophytic fungi are capable of synthesizing the same biologically active compound which the plant host produces. An example of such is the isolation of phytohormones 'gibberellins' from *Fusarium fujikuroi* during the early 1930s (Kharwar et al. 2008). Mosses, ferns, algae and many other vascular plants are found to contain bacterial or fungal endophytes. The diversity of fungal endophytes is very high in tropical regions. Certain inducing factors from the host plant as well as the fungal endophyte when combined reveal a marked increase in the amount of secondary metabolites in both the entities (Li et al. 2009; Zhang et al. 2009). Research into the biosynthetic pathways has shown that the fungal endophytes and the host plant have quite similar but unique metabolic pathways that produce secondary metabolites (Jennewein et al. 2001).

Fungal endophytes are microbes which are inhabitants of living host plants during certain stages of their life cycle which do not cause any apparent damage to the host. Certain saprophytic fungi, mycorrhizae and latent pathogenic fungi are also included in this group which also reside in living host plant during various life cycle stages (Petrini and Fisher 1990; Akhtar and Siddiqui 2008; Akhtar and Panwar 2011). Endophytic fungi synthesize many novel biologically active secondary metabolites with distinct structures which include alkaloids, chinones, phenolic acids, benzopyranones, quinones, steroids, tetralones, flavonoids, xanthones, terpenoids and others (Tan and Zhou 2001; Swamy et al. 2016). A variety of applications of biologically active metabolites include antiparasitics, anticancer agents, agrochemicals, antibiotics, immune suppressants and antioxidants (Gunatilaka 2006). Fungal endophytes have gained considerable attention as potential source of anticancer bioactive compounds for the treatment of many types of cancer (Schulz et al. 2002; Strobel et al. 2004; Kharwar et al. 2011).

1.3 Fungal Endophyte-Host Symbiosis

Special mechanisms are developed by endophytes for the purpose of penetration in order to reside in the host species' tissues. Exoenzymes are released by the endophytes which help in colonizing their hosts and in growth inside the apoplastic washing fluid material of the host (Chandra 2012). Mutualistic association with the host is maintained when the roots of the host species are colonized. The mutualistic association of the endophytes and host species helps in the growth of the plant species, and the endophytes receive nutrients for survival and colonization in the host species' roots (Chandra 2012). Pathogenic invasion of a host plant has demonstrated lower values of metabolic agents of plant defence as compared to the control of noninfected plant, whereas the presence of endophytic fungi inside the host plant

has shown no such dip in plant defence metabolites (Schulz et al. 2002). A state of equilibrium is noted between the plant defence mechanisms and fungal virulence. Development of disease occurs if an imbalance is created and when virulence increases and defence mechanism of the plant is lowered (Chandra 2012). Synthesis of metabolites is done by the endophyte which helps in competition with epiphytes and pathogens for the process of colonization of the host species and for the regulation of host metabolism in a well-balanced association (Chandra 2012).

For the selection of the most suitable host plants and utilization of potential endophytes, studies of plant diversity, fungal taxonomy and ethnobotany are required. When endophytes interact with host in metabolic interactions, synthesis of secondary metabolites may occur (Preeti et al. 2009). Long-term symbiotic relationship is experienced between endophytes and their host plant species, and as a result bioactive substances are often produced. There is an exchange of genetic material between the endophyte and host species because of coexistence and direct contact for a long period of time (Wang and Dai 2011; Nadeem et al. 2012). Several defence mechanisms are developed by floral species which help them in adapting to the environmental stresses such as microbial diseases; these mechanisms include production of toxic substances. In few of the healthy plants, certain defence mechanisms are present, and in some other plants, synthesis of toxic substances in times of pathogenesis occurs (Chandra 2012). Endophytes are known to exhibit tolerance towards host species' unique metabolites. Many endophytes have the capability to detoxify these bioactive compounds of plant defence which is the determining factor of the colonization rate of these endophytes (Wang and Dai 2011). The endophytes are able to biologically transform the toxic metabolites produced by the host species into novel secondary metabolites that are bioactive in nature (Zikmundova et al. 2002; Saunders and Kohn 2009). These biotransformation abilities of the endophytes allow them to survive in adverse environmental conditions. Endophytes are known to produce active compounds that are more developed and structured than those produced by the host plants (Wang and Dai 2011; Swamy et al. 2016). Endophytes have been recognized as good sources of novel secondary metabolites of biological origin. Novel metabolites can be utilized by incorporating the concerned endophytes in drugs currently used in order to improve their efficacy. The endophytic fungi with the help of their efficient biotransformation enzymes are employed to modify the three-dimensional structure of many compounds (Chen et al. 2016). Few scientists have explored the possibility of using fungal endophytes to synthesize substances with even higher activity. Studies demonstrated that various types of fungal endophytes can be used to obtain a variety of metabolites and the production processes involved showed stereo-selectivity (Agusta et al. 2005; Borges et al. 2008; Verza et al. 2009; Swamy et al. 2016).

Novel compounds, which cannot be produced by chemical processes, can be synthesized from endophytic fungi by using stereo-selective and region-selective reactions. Hence, natural drugs obtained from microbial bioactive metabolites demonstrate many characteristics making them attractive candidates for production processes in industries (Tejesvi et al. 2007). Improvement in endophytic fungal strains results in comparatively higher yield of secondary metabolites under culture

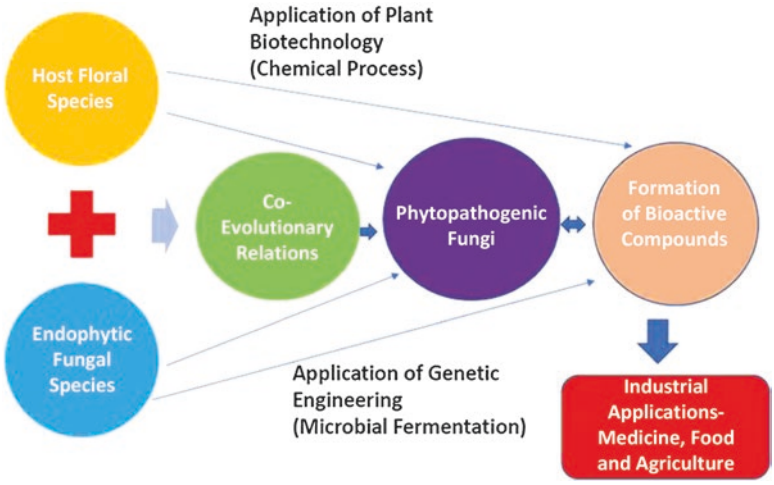


Fig. 1.1 Overview of sequence of bioactive metabolites production from fungal endophytes and their host plants and their applications (Modified after Zhao et al. 2011)

conditions (Penalva et al. 1998). Figure 1.1 elucidates the sequence of bioactive metabolites production from fungal endophytes and their hosts as well as their applications (Zhao et al. 2011). The fungal endophytic world still remains largely unexplored, and plant species harbouring them also need further research into their geographic distribution, taxonomy and microbial and chemical profile.

1.4 Fungal Endophytes: Novel Source of Anticancer Metabolites

1.4.1 Alkaloids and Nitrogen-Containing Heterocycles

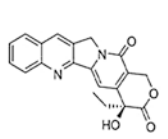
Alkaloids derived from plants demonstrate many bioactivities of toxicity, medicinal properties and recreational purposes. A variety of alkaloids obtained from plants have been studied for their use as potential agents against cancer, and many such compounds have been isolated from fungal endophytes (Kharwar et al. 2011) The antitumour properties of various alkaloids have been explored for use as commercially potent drugs like camptothecin (CPT) and vincristine. Many novel alkaloids have been obtained from fungal endophytes with anticancer properties during the last few years. *Fusarium oxysporum* was utilized to isolate beauvericin, which displayed cytotoxic behaviour towards A549 (IC_{50} 10.4 ± 1.6 μM), PC-3 (IC_{50} 49.5 ± 3.8 μM) and PANC-1 (IC_{50} 47.2 ± 2.9 μM) (Wang et al. 2011a, b). A fungal endophyte, *Eurotium rubrum*, isolated from tissues of the plant *Hibiscus tiliaceus* showed the presence of variecolorin grand alkaloid E-7 which are alkaloid compounds along with the presence of a dioxopiperazine alkaloid (Wang et al. 2007).

An example of a quinoline alkaloid is CPT (Fig. 1.2 [1]), which aids in the inhibition of eukaryotic topoisomerase I (Chandra 2012). The fungal endophytic strain, *Fusarium solani* MTCC 9667, isolated from the tree *Apodytes dimidiata* (Icacinaceae), was able to produce the alkaloids CPT and 9-methoxycamptothecin in its mycelia (Shweta et al. 2010).

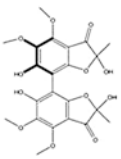
Seed and fruit portions of *Miquelia dentata* (Icacinaceae) showed the presence of fungal endophytes, *Alternaria alternata*, *Fomitopsis* sp. and *Phomopsis* sp. These fungal endophytes are responsible for the production of 9-methoxy CPT (9-MeO-CPT), CPT and 10-hydroxy CPT (10-OH-CPT), respectively. Cytotoxicity against colon and breast cancer cell lines has been reported to be observed on application of these fungal species extracted using ethyl acetate and methanol (Shweta et al. 2013). *Chaetomium globosum* TY1, a fungal endophyte isolated from *Ginkgo biloba*, was used to obtain novel alkaloid chaetomugilides A–C, azaphilone alkaloids and chaetoviridin E. A substantial degree of cytotoxicity was observed when chaetomugilide A was applied against HepG2 (IC_{50} 1.7 μM), and a medium amount of cytotoxicity (IC_{50} 19.8–53.4 μM) was reported upon application of chaetomugilides B and C and chaetoviridin E against the same HepG2 (Li et al. 2013). *Desmotes incomparabilis* has been reported to contain the fungal endophyte *Mycoleptodiscus* sp. from which a novel alkaloid compound mycoleptodiscin B was isolated. Proliferation of cancer cell lines with IC_{50} values ranging from 0.60 to 0.78 μM was markedly inhibited by mycoleptodiscin B (Ortega et al. 2013). The fungal endophyte *Penicillium citrinum* proliferating on bean and rice media was used to obtain the metabolite citriquinochroman. Young stems of Moroccan plant *Ceratonia siliqua* were used to isolate the endophytic fungi *P. citrinum.* The metabolite citriquinochroman displayed cytotoxic activity towards murine lymphoma L5178Y cells (IC_{50} 6.1 μM) (El-Neketi et al. 2013). The fungal endophyte *Pestalotiopsis* sp. showed the presence of the metabolite pestalactam A and pestalactam B which displayed moderate *in vitro* effect during experimental analysis against MCF-7 and NFF mammalian cell lines (Davis et al. 2010). Inhibition of HeLa cells by cytochalasin analogues, aspochalasin D and aspochalasin J (IC_{50} 5.72 μM and 27.4 μM, respectively), was observed. These two compounds were extracted from *Trichoderma gamsii* culture, a fungal endophyte residing in medicinal plant *Panax notoginseng* (Ding et al. 2012).

1.4.2 Coumarins

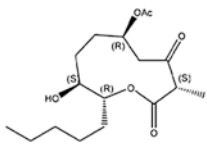
A novel furanocoumarin compound derived from a mangrove fungal endophyte *Penicillium* sp. ZH16 was called 5-methyl-8-(3-methylbut-2-enyl) furanocoumarin. This active metabolite was found to be cytotoxic towards KB cells (IC_{50} 5.0 μM) and KBV200 cells (IC_{50} 10.0 μM) (Huang et al. 2012). *Microsphaeropsis arundinis*, a fungal endophyte, was used to obtain arundinone B (Fig. 1.2 [2]), a polyoxygenated benzofuran-3(2H)-one dimer (Chen et al. 2016). This compound was cytotoxic towards T24 and A549 cell lines (Luo et al. 2013).



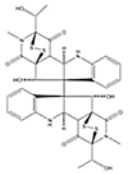
[1] Camptothecin - Alkaloid
(Chandra 2012)



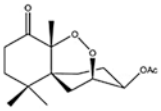
[2] Arundinone B - Coumarin
(Chen et al. 2016)



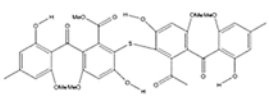
[3] Cytospolide P – Lactone
(Chen et al. 2016)



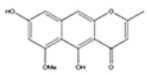
[4] Verticillin D – Peptide
(Chen et. al 2016)



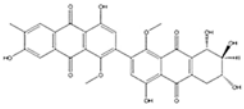
[5] Talaperoxide B – Peroxide
(Chen et al. 2016)



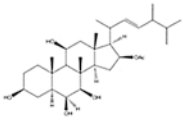
[6] Guignasulfide – Polyketide
(Chen et al. 2016)



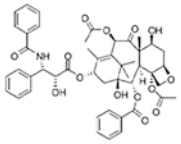
[7] TMC 256 A1 – Pyrone
(Chen et al. 2016)



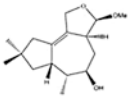
[8] Alterporriol L – Quinone
(Chen et al. 2016)



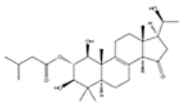
[9] Penicisteroid A – Steroid
(Chen et al. 2016)



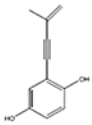
[10] Taxol– Diterpene
(Chandra 2012)



[11] Ceriponol G – Sesquiterpene
(Chen et al. 2016)



[12] Xylariacin A – Triterpene
(Chen et al. 2016)



[13] Siccayne – Alkyne
(Chen et al. 2016)

Fig. 1.2 Structures of novel anticancer metabolites from fungal endophytes

1.4.3 Lactones

A nonanolide metabolite cytospolide P (Fig. 1.2 [3]) was obtained from a fungus *Cytospora* sp. living inside the plant *Ilex canariensis* (Chen et al. 2016). Experimental analysis of cell cycle indicated that the metabolite cytospolide P was responsible for a marked arrest of G1 phase in A549 cancer cells, thus proving the major function

of C-2 methyl in the inhibitory effect of growth of the tumour cell line (Lu et al. 2011). The plant *Pandanus amaryllifolius* was reported to be home to a fungal endophyte of *Colletotrichum* sp. which was used to extract a novel macrolide compound, colletotriolide. The endophytic fungi *Chaetomium globosum* showed the presence of the compounds cytosporone and dothiorelone C. The three metabolites dothiorelone C, cytosporone and colletotriolide were reported to be inactive upon application to HT29, HCT116 and A549 cancer cell lines (Bungihan et al. 2013). Fungal endophytes belonging to the *Streptomyces* sp. 211,726 were utilized to extract seven novel azalomycin F analogues. *In vitro* bioassays showed that the seven metabolites had a broad range of antimicrobial as well as anti HCT-116 properties having IC_{50} 1.81–5.00 μM (Yuan et al. 2013).

1.4.4 Peptides

The endophytic fungi *Bionectria ochroleuca* isolated from inside the leaf tissues of *Sonneratia caseolaris* displayed the presence of depsipeptides pullularin A, pullularin B and verticillin D (Fig. 1.2 [4]) (Chen et al. 2016). Verticillin D was observed to exert potent cytotoxic effects towards L5178Y cell line. Prevalence of antiproliferative characteristics was also observed among the cyclic depsipeptides, pullularin A, pullularin C and chloroderivative of pullularin E which had IC_{50} 0.1 to 6.7 μM (Ebrahim et al. 2012). The branches of the mangrove *Bruguiera gymnorhiza* showed the presence of a fungal endophyte, *Aspergillus terreus* (No. GX7-3B), which contained a depsipeptide, beauvericin (Deng et al. 2013). This compound has been earlier extracted from various other fungal species (Kharwar et al. 2011). Beauvericin was reported to exert a medium degree of cytotoxicity towards HeLa (IC_{50} 1.14 μM), A549 (IC_{50} 0.82 μM), MCF-7 (IC_{50} 2.02 μM) and KB (IC_{50} 1.10 μM) cell lines (Chen et al. 2016).

1.4.5 Peroxides

A fungal endophyte, *Talaromyces flavus*, residing in the mangrove, *Sonneratia apetala*, was utilized to obtain the nor-sesquiterpene peroxides, talaperoxide B (Fig. 1.2 [5]) and talaperoxide D (Chen et al. 2016). On evaluating the cytotoxicity during *in vitro* analysis, it was revealed that the two metabolites were cytotoxic against a number of human tumour cells (PC-3, HepG2, MCF-7, MDA-MB-435 and HeLa) having IC_{50} 0.70–2.78 μM (Li et al. 2011).

1.4.6 Polyketides

The young leaves of *Hopea hainanensis* plant, home to the fungal endophyte *Guignardia* sp. IFB-E028, showed the presence of guignasulphide (Fig. 1.2 [6]) (first natural sulphur-containing benzophenone dimer) (Chen et al. 2016).

Guignasulphide was actively cytotoxic towards HepG2 (IC_{50} 5.2 ± 0.4 μM) liver tumour cells in humans (Wang et al. 2010). A polyketide, chaetoglobosin (family cytochalasin), has been reported to display a strong affinity towards actin filaments. The central structure of this compound resembles a C_{18} polyketide having an amino acid side chain. The medicinal plant *Curcuma wenyujin* containing the fungal endophyte *Chaetomium globosum* was used to extract the metabolite, chaetoglobosin X, which displayed potent cytotoxicity towards MFC and H22 tumour cells (Wang et al. 2012).

1.4.7 Pyrans and Pyrones

The stems of *Aquilaria sinensis* contained the fungal endophyte *Nodulisporium* sp. A4 which showed the presence of a novel benzopyran, (2R*, 4R*)-3,4-dihydro-4-methoxy-2- methyl-2H-1-benzopyran-5-ol. This metabolite displayed a relatively mild cytotoxicity towards the cell line SF-268 having 100 mg/ml concentration as compared to cisplatin (positive control) (Wu et al. 2010). The fungal endophytic genus *Aspergillus* forms the major source of the metabolites of pyrones and its derivatives (Liu et al. 2011a). Cytotoxic inhibitions against cancer cells of MCF-7, Hep3B, SNB19, MDA-MB-435, Huh7 and U87 MG (IC_{50} 19.92–47.98 μM) were observed during *in vitro* analysis upon the application of naphtho-gamma-pyrone, TMC 256 A1(Fig. 1.2 [7]) (Chen et al. 2016), a monomeric compound, derived from the mangrove fungal endophyte, *A. tubingensis* (GX1-5E) (Huang et al. 2011b).

1.4.8 Quinones

The mangrove *Aegiceras corniculatum* showed the presence of the fungal endophyte, *Alternaria* sp. ZJ9-6B, from which anthraquinones, alterporriol L (Fig. 1.2 [8]) and alterporriol K, were extracted (Chen et al. 2016). A medium degree of cytotoxicity was observed against MDA-MB-435 and MCF-7 cells (IC_{50} 13.1–29.1 μM) upon the application of alterporriol K and alterporriol L during experimental analysis (Huang et al. 2011a). A fungal endophyte, *Eurotium rubrum*, from the plant *Hibiscus tiliaceus* was used to extract a new derivative compound of anthraquinone, 9-dehydroxyeurotinone, and another anthraquinone compound, emodin. Cytotoxicity towards SW1990 and Du145 cell lines was observed when these two compounds were applied (Yan et al. 2012). Cytotoxicity was also seen towards A549 lung cancer cells and human chronic myeloid K562 leukaemia by altersolanol A, a product from fungal endophyte, *Stemphylium globuliferum*. The isolation was made from *Mentha pulegium* (Lamiaceae), a medicinal plant. The lowering of the anti-apoptotic protein expression causes the cleaving of caspase-3 and caspase-9 which consequently leads to the destruction of cells through apoptosis induced by altersolanol A (Teiten et al. 2013).

1.4.9 Steroids

A marine red alga, *Laurencia*, containing the fungal endophyte *Penicillium chrysogenum* QEN-24S was used to extract two novel polyoxygenated steroidal compounds penicisteroid A (Fig. 1.2 [9]) and penicisteroid B (Chen et al. 2016). Preliminary experimental analysis of penicisteroid A reported substantial antifungal activity and cytotoxicity (Gao et al. 2011). The fungal endophyte *A. niger* MA-132 residing in the mangrove plant *Avicennia marina* was used to obtain two novel sterols, nigerasterol A and nigerasterol B. Nigerasterol A (IC_{50} 0.30 μM) showed a stronger activity towards HL60 tumour cell line than nigerasterol B (IC_{50}1.50 μM). These two metabolites displayed potent cytotoxicity towards A549 cell line (IC_{50} 1.82 and 5.41 μM) (Liu et al. 2013). The fungal endophyte *A. terreus* (No. GX7-3B) isolated from *Bruguiera gymnorhiza* was explored, and the presence of phytoecdysteroids was detected. This metabolite displayed potent cytotoxicity towards MCF-7 (IC_{50} 4.98 μM), HeLa (IC_{50} 0.68 μM), A549 (IC_{50} 1.95 μM) and KB (IC_{50} 1.50 μM) (Deng et al. 2013).

1.4.10 Diterpenes

Taxol (Fig. 1.2 [10]) and taxane were produced by *Taxomyces andreanae* isolated from Pacific yew (Stierle et al. 1993). Fungal endophyte *Paraconiothyrium* sp. MY-42 was used to isolate two isopimarane diterpenes which displayed a medium degree of cytotoxic effect towards promyelocytic HL60 cells in human leukaemia (Shiono et al. 2011). The viable photosynthetic cells of a moss, *Ceratodon purpureus*, home to fungal endophyte *Smardaea* sp. AZ0432, were reported to be responsible for the production of the diterpenes sphaeropsidin A and sphaeropsidin D. These two metabolites were explored for potent anticancer properties along with 6-O-acetylsphaeropsidin A (sphaeropsidin A derivative) by employing various cancer cell lines and normal primary fibroblasts derived from human. It was inferred that the three diterpenes displayed a substantial amount of cytotoxicity. Interestingly, it was observed that sphaeropsidin A was selective towards a particular cell type in its cytotoxic activity and at subcytotoxic quantities, and it resulted in the inhibition of metastatic adenocarcinoma (MDA-MB-231) migration in breast cells (Wang et al. 2011a, b).

1.4.11 Sesquiterpenes

A medicinal plant, *Huperzia serrata*, harbours in its stems the fungus *Ceriporia lacerate*, from which the sesquiterpenes, ceriponol F, ceriponol G (Fig. 1.2 [11]) and ceriponol K, were extracted (Chen et al. 2016). Moderate cytotoxic activity was observed against HeLa, HepG2 and SGC 7901 (IC_{50} 32.3 ± 0.4–173.2 ± 1.5 μM) by ceriponol F and ceriponol K, whereas a somewhat improved cytotoxic effect towards HeLa cell line by ceriponol G was observed (Ying et al. 2013).

1.4.12 Triterpenes

Fungal endophyte *Xylarialean* sp. A45 was used to extract fermentation triterpene metabolites xylariacin A (Fig. 1.2 [12]), xylariacin B and xylariacin C (Chen et al. 2016). The *in vitro* analysis of their cytotoxic effect was carried out against HepG2 (human tumour cells), which displayed a moderate cytotoxicity (Lin et al. 2011). Fungal endophyte *Periconia* sp. residing in the plant *Annona muricata* was reported to contain two novel terpenoids, namely, (+)-(3S,6S,7R,8S)-periconone A and (−)-(1R,4R,6S,7S)-2-caren-4,8-olide. *In vitro* experiments revealed that these two compounds had a slight cytotoxicity against human tumour cell lines (HCT-8, Bel-7402, BGC-823, A549, A2780 and MCF-7) (Ge et al. 2011).

1.4.13 Miscellaneous

An alkyne compound siccayne (Fig. 1.2 [13]) has been isolated from a fungal endophyte *Pestalotiopsis fici* (Chen et al. 2016). Siccayne was reported to exert cytotoxicity towards HeLa (IC_{50} 48.2 μM) and HT29 (IC_{50} 33.9 μM) cancer cells in humans (Liu et al. 2013). *Botryosphaeria rhodina*, living in the medicinal plant *Bidens pilosa*, showed the presence of the metabolites botryorhodine A and botryorhodine B. These compounds exerted a medium to slight degree of cytotoxicity towards HeLa cell lines (IC_{50} 96.97 and 36.41 μM, respectively) (Abdou et al. 2010). The fungal endophyte *Pestalotiopsis fici* has been utilized to extract pestalofones F, G and H and pestalodiols C which are isoprenylated epoxy derivatives. Cytotoxic behaviour was observed in these four compounds towards MCF-7 and HeLa cell lines (Liu et al. 2011b). An isoflavone called cajanol was derived from the fungal endophyte *Hypocrea lixii* residing in *Cajanus cajan*. This metabolite was found to possess a very high cytotoxic effect against A549 cell line which was dependent on the time duration and the dose (Zhao et al. 2013). The anticancer drugs such as teniposide, etoposide and etopophos phosphate usually contain an aryl tetralin lignin called podophyllotoxin as precursor. A fungal endophyte, *Fusarium solani*, has been newly discovered from *Podophyllum hexandrum* root cells for the production of podophyllotoxin (Nadeem et al. 2012). A moderate cytotoxicity was observed against cancer of the oral cavity and kidney fibroblast (Vero) cells in African green monkey due to the presence of guignarenone A, a novel tricycloalternarene derivative, from the fungal endophyte *Guignardia bidwellii* PSU-G11 (Sommart et al. 2012).

1.5 Large-Scale Production Strategies

The initial step of research into the anticancer properties of the fungal endophytes involves the screening and isolation of the microorganism. However, in order to make the large-scale production of the targeted anticancer compounds economically feasible, it is important to implement these research findings. In order to

increase the large-scale production of the anticancer metabolites, the process parameters of the culture need to be optimized. Two widely used processes for anticancer metabolite production from fungal endophytes are solid-state fermentation (SSF) and pulse fed-batch method. Fermentation can be categorized into two types, namely, submerged fermentation (SmF), a method which involves microbial cultivation utilizing a liquid medium enriched with nutrients (Pandey 2003; Chen et al. 2016), and solid-state fermentation (SSF), a method which employs the cultivation and metabolite production on solid nutrient medium which may contain little water or might be completely devoid of water, although the moisture content of the substrate will be sufficient for the growth and metabolism of the microbe (Thomas et al. 2013, Chen et al. 2016). Biologically active metabolites produced by fermentation are an attractive alternative to using chemical process since pure and highly active extracts, without the generation of toxins, can be obtained through this process unlike the chemical production involving organic solvents (Chen et al. 2016). Biologically active substances can be extracted as secondary metabolites obtained after the microbe has attained its full growth in culture. It has been observed in liquid culture conditions that the essential nutrients like nitrogen, phosphate and carbon form the rate-limiting substance in the production process of the secondary metabolites since depletion of any of these nutrients affects their production process (Barrios-González et al. 2005). SSF has been preferred over SmF in the recent years owing to the fact that SSF has resulted in higher yields, improved product quality and enhanced productivity, as per studies. Further, the cost of operation is lower than in SmF because cheap agricultural and industrial by-products can be utilized as substrate in production by SSF (Chen et al. 2016). The SSF process is also more economical as low water utilization helps in the reduction of the size of the fermenter, downstream processing along with decreased stirring and sterilization expenses (Raghavarao et al. 2003; Hölker and Lenz 2005; Thomas et al. 2013; Chen et al. 2016). Research in developing new and improved bioreactors is being done in order to overcome the disadvantages involving the scaling up of production process by SSF which is mostly because of homogeneity problems in culture and poor heat transfer (Mitchell et al. 2000; Di Luccio et al. 2004; Thomas et al. 2013; Chen et al. 2016). For many fungal endophytes, SSF is not a suitable option to extract anticancer compounds, and pulse fed-batch method forms a better option. Few filamentous fungi have a rich reservoir of novel biologically active metabolites (Schulz et al. 2002), but their large-scale extraction can be hindered due to insufficient supplies (Couto and Toca-Herrera 2007).

A new anthraquinone derivative named 1403C (also called SZ-685C) was extracted from a filamentous fungal endophyte *Halorosellinia* sp. (No. 1403) residing in marine mangroves and studied to increase its yield. Not much of 1403C is available for research involving preclinical trials even though it is found to be a potent Akt (protein kinase B, PKB) inhibitor and antitumour metabolite (Xie et al. 2010). Kang et al. (1999) observed that the inhibition caused by increased levels of glucose during 1403C production could be circumvented, and yield of 1403 could be increased by the usage of lower levels of initial glucose concentration and feedings of glucose in pulses during the production process.

1.6 Conclusions and Future Prospects

Fungal endophytes residing in the viable tissues of young and healthy plants display unique and intricate interactions with the host. Various remarkable and novel properties are inculcated by the fungal endophyte during their existence inside their hosts. The proliferation and colonization of the endophytic fungi and the stability of the symbiotic association are maintained by the secretion of many enzymes by the fungal species. The fungal endophytes are potent sources for the biosynthesis of active natural metabolites because their unique niches in the ecosystem and selectivity in bioconversion processes prove to be advantageous. The conjecture that, during the coexistence of fungal endophytes with their hosts throughout evolution, the endophytes were able to adapt to their unique microenvironments by specific variations in the genes, involving the uptake of the host plant DNA and incorporating them into their own genomic material, can be supported by the studies, which show that few similar natural, biologically active secondary metabolites are synthesized by both the host plants and their fungal endophytes. It has been reported that numerous fungal endophytes can be isolated from a single host plant and at least one of these endophytes usually display host specificity.

Being a novel alternative source of anticancer metabolites for the production of antitumour drugs, fungal endophytes form an essential key to controlling the mortality rates caused by cancer and decreasing the expenses involved in cancer therapy. The studies on fungal endophytes have involved an extensive range of host plants such as ferns, gymnosperms, lichens and flowering plants from which numerous novel anticancer compounds have been extracted. Several anticancer novel metabolites have been isolated from fungal endophytes derived from marine microorganisms, which has awakened considerable interest among researchers. Exploring the fungal endophytic community of the ocean bodies seems to be very promising since the knowledge about the huge oceanic ecosystem is currently limited. Most of the studies reported about the anticancer properties of fungal endophytes have been limited to *in vitro* analysis, and *in vivo* animal and human trials are required to search potent anticancer compounds. Researchers need to focus their studies on the molecular makeup of endophytes and strategies for optimizing fermentation process for the scale-up of production of anticancer metabolites from these fungal agents. New insights into the discovery of novel anticancer drugs and their clinical utility hold great promise through the continued research on fungal endophytes and their biologically active secondary metabolites which can play a potent role in cancer therapy and its prophylaxis.

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2 Gastrointestinal Bezoar Stones: Current Knowledge and Future Perspective on the Potential of Plant-Derived Phytobezoar in Cancer Treatment

Peng Nian Yew, Bey Hing Goh, Yau Yan Lim, and Wai Leng Lee

Abstract

Bezoar stone formation is an infrequent event in the gastrointestinal (GI) system due to the chemical reaction between stomach bile and the high intake of nondigestible food materials. There are mainly four types of GI bezoars: trichobezoar, lactobezoar, pharmacobezoar, and phytobezoar, and formations of these bezoars in humans are physical disorders which require clinical treatments. Intriguingly, phytobezoars such as *Calculus bovis* obtained from ox/cattle have been used in China as medicine since 2000 years ago. Modern science found *C. bovis* possesses antioxidant and anti-inflammatory properties, and identification of its chemical constituent led to successful synthesis of the artificial medicinal bezoar. Phytobezoars, major type of GI bezoar, can also be found in animals besides ox/cattle, and these bezoars have been traditionally used to treat against poison, and more recently those obtained from porcupines are used by Asian Chinese to treat cancer. However, to date, medicinal values of porcupine bezoars lack scientific proof. Therefore, understanding on the factors that lead to bezoar formation in the GI tract, identification of their chemical composition, and elucidation of underlying physiological mechanism modulated by phytobezoars are important to provide guidance in their usage in treating various ailments including cancer. In this chapter, the development of different bezoar stones in the GI tract is summarized on the pharmacological action of phytobezoars *C. bovis*, and porcupine

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bezoar is presented on the medicinal values of these phytobezoars. Moreover, distinct composition and classification of plant materials found in the phytobezoars and their potential benefits in cancer treatment are also discussed.

Keywords
Bezoars formation · Porcupine bezoars · Phytobezoars · Anticarcinogenic · Cancer

2.1 Introduction

Gastrointestinal (GI) bezoar stones are agglomerates of partially digested or undigested inorganic/organic materials formed within calcareous concretions in the GI tract of animals as well as human. In humans, bezoars are found mostly in the stomach and gastrointestinal tract as product of undigested fiber or hairs. However, the incidence is low in humans and has been reported as 0.4% in the general population (McKechnie 1972; Kadian et al. 1978). The incidence rates of bezoar formation in animals were not reported, but it was correlated with their respective diets. For instance, herbivores such as porcupine and cow that consume high amount of fiber-rich plants tend to have higher chance of developing gastrointestinal bezoar. In a primate order, the incidence of bezoar formation reported in nonhuman omnivore primates is very limited, where there are two in baboons (*Papio hamadryas* and *P. cynocephalus anubis*) (Butler and Haines 1987; Gillin et al. 1990), one in chimpanzee (Nolan et al. 1988), and two in saddleback tamarins (*Saguinus fuscicollis*) (Gozalo et al. 1990). However, an endemic species of monkey *Presbytis hosei* in Southeast Asia which is dominantly herbivore was found to have higher incident of developing gastrointestinal bezoars. A repeat census on the population of *P. hosei* in East Kalimantan, Indonesia, has showed their population declined for 50–80% from year 1996 to 2003 due to extensive hunt for their bezoar stones by local villagers, as they are promised a guarantee purchase by a profitable merchant (Nijman 2005).

The word bezoar is derived from the Arabic context of "badzehr" which literally represents the meaning of counterpoison antidote. Its alexipharmic usage was recorded since 968 AD (Christopher 2013; Raju et al. 2013). In the eleventh century, animal bezoars were promoted by Islamic physicians such as Avicenna (Ibn Sina) and Ibn al-Baitar as effective counterpoison against poisonous plants and animals, for instance, scorpion bites and stitches. The bezoars were also used to relieve violent fevers as well as to strengthen the emotion of patients who suffered in grief (Stark 2003). To remove poisons, bezoar stones were chewed in the mouth; otherwise, they were mounted on signet ring or necklace worn by the people with high social status. A century later, the medieval Arab beliefs in bezoar stones were passed into the early modern European medical practice. However, the function of bezoar stone as efficacious counterpoison was not perceived through celestial force but more toward the removal of poison through sympathetic attraction, where the European hypothesized that the bezoar stones were composed of poisonous herbs and are able to draw poisons into themselves (Stark 2003).

In Europe, animal bezoars from cows, sheep, monkeys, and apes became very prominent in the early modern period between the twelfth and eighteenth centuries. It is ubiquitous among the rich and famous in early modern period as they were allegedly used as antidotes against poisons, plague, snake poison, leprosy, and epilepsy (Khattak and Asghar 2004; Stephenson 2008). In this period, these people were exposed to threat and resentments of others who were eager to climb the social ladder. Lacing wines or foods with poison such as arsenic is commonly used as the insidious means of assassination. Therefore, many high-status families armed themselves with preventive measures to avoid being the victim of poisoning. The most effective and common practice was holding a bezoar stone as an amulet, which was immersed into suspected beverages with the belief that it could inactivate the poison. Other methods include mounting the bezoar stone on the interior of drinking vessels to absorb any poison from the drinks it held (Malcom 1998).

In the nineteenth century, modern scientist Gustaf Arrhenius and Andrew A. Benson of the Scripps Institution of Oceanography performed a scientific investigation on the anti-poison properties of bezoar stones. The study showed the stone could remove poison when immersed in an arsenic-laced solution. The two toxic compounds in arsenic-laced solution, arsenate and arsenite, were found to be effectively neutralized using bezoar stones via different mechanisms. The phosphate of mineral brushite found in the bezoars could effectively exchange and remove arsenate in the solution. On the other hand, the sulfur-rich proteins found in the degraded hair from bezoars are able to bond and deactivate the toxicity of arsenite (Malcom 1998). Following the development of modern Western medicine, the magical healing power of bezoars had gradually been perceived as nonscientific folklore medicine although there were some evidences shown on their pharmacology efficacy. Within a few years, bezoars were almost completely replaced by more effective clinical drugs and medicine in Europe. However, bezoars are still used in Eastern folk medicine especially in Traditional Chinese Medicine, a common medical practice in Chinese community both from mainland China and Southeast Asia. One of the most common bezoar that can be found in various Chinese herbal formulations in China today is the *C. bovis* (ox/cattle bezoar). They are used in combination with other herbal components to treat various ailments, including but not limited to fever, heat, inflammation, and cancer. Following the advancement of science toward the twentieth century, the medicinal values of *C. bovis* were scientifically proven, and their various medicinal potentials were unveiled. However, the scientific evidence for the medicinal values of many other types of bezoar stone is scarce. The present chapter highlighted the chemical and mechanical formation of different types of bezoar stones with a focus on the potential medicinal values, in particular the anticancer properties of the *C. bovis* and porcupine phytobezoars.

2.2 Classification of Gastrointestinal Bezoar Stones

Bezoars are generally categorized into four different groups: trichobezoars (composed of hair and some food fibers), lactobezoars (composed of milk proteins and mucus), pharmacobezoars (conglomeration of medication remnants), and

phytobezoars (composed of indigestible food materials/fibers). Among all, phytobezoars are the most common type of bezoars, accounting for approximately 40% of all the reported bezoars (Sanders 2004).

2.2.1 Trichobezoar

Trichobezoar is a concrete ball of hairs which gradually increase in size over the period of years, causing gastrointestinal obstruction, epigastric pain, and GI bleeding, ulceration, and/or perforation. Unlike the phytobezoar, the incidence of trichobezoar is very rare in humans, and the recurrent rate is almost unseen (Henn and Chen 2015). The formation of trichobezoars in humans is strongly associated with Rapunzel's syndrome which is an extremely rare condition due to subconsciously ingestion of hair (trichophagia). This syndrome is often associated with trichotillomania disorder (Bouwer and Stein 1998) where patients undergo hair loss from continuous self-pulling of hair (Sah et al. 2008). Due to the entangled nature of hairs, they are gradually accumulated in the GI tract which eventually reached a size big enough to cause complications.

In most cases, complications such as gastric ulceration, mucosal erosion, stomach perforation, and intestine perforation are observed (Mehta and Patel 1992). In addition, severe complications including intussusceptions, protein-losing enteropathy, pancreatitis, obstructive jaundice, and fatality as a result of trichobezoar have been reported in the literature (Hossenbocus and Colin-Jones 1973; Mehta and Patel 1992). Most GI bezoars can be removed by endoscopic method. However, up to 95% removal of the trichobezoars are extremely difficult due to its enormous size, high density, and hardness (Gorter et al. 2010). In addition, removal of trichobezoar by endoscopic fragmentation elevates the risk of esophagitis, ulceration, perforation, and intestinal obstruction caused by distal migration of satellites (Phillips et al. 1998; Gorter et al. 2010). Collectively, these undesired complications result in deregulated homeostasis and might potentially lead to the onset of cancer in a patient.

2.2.2 Lactobezoar

Lactobezoars are acid-insoluble gastric bezoar stones that occur as a result of coagulation between undigested milk and mucous protein (Castro et al. 2014). It predominantly occurs in neonates, especially in infants with low birth weight who are fed with highly concentrated milk (Heinz-Erian et al. 2012). However, the incidence of pediatric lactobezoar is rare, with less than 100 cases reported in literature since 1975 (Towery and Chan 2004; Heinz-Erian et al. 2012). In adults, the factors that predispose an individual to the formation of lactobezoar include inhibition of gastric secretion and motility, gastric dehydration, and delayed gastric emptying (Heinz-Erian et al. 2012). Usage of medication that counteracts diarrhea or reduce gastric secretion was associated with the increased risk of lactobezoar formation in

adults (Sippell et al. 1977; Schreiner et al. 1979). Apparently, disturbed gastric function in the stomach that increases the coagulation of milk and mucous protein is the major cause. Further, the size of lactobezoar increases when gastric deficiency coupled with elevated calcium, phosphorus, and fat (Corzine 2011; Heinz-Erian et al. 2012).

There are several treatment options for patients with gastrointestinal lactobezoars. In most cases, gastric lavage was utilized. This treatment may take up to several weeks to effectively resolve the lactobezoar (DuBose et al. 2001; Heinz-Erian et al. 2010). Surgical and endoscopic removals of lactobezoar were reported in some cases (Silva et al. 2002). However, these invasive approaches may increase the risk of negative health consequences for diabetic patients who are prone to complication as well as those who suffer from immunosuppression. Recently, administration of N-acetylcysteine (NAC) through nasogastric route was used as a noninvasive treatment for gastrointestinal lactobezoar (Bajorek et al. 2012; Sparks and Kesavan 2014). The mucolytic property of NAC cleaves the disulfide bonds of mucoprotein in the lactobezoar which subsequently reduces its viscosity (Sparks and Kesavan 2014). Complete dissolution of symptomatic gastric lactobezoar was reported in two cases after nasogastric administration of NAC (Schlang 1970; Usmani and Levenrown 1989). Collectively, NAC is a good candidate as primary treatment option for medically complex patients with lactobezoars.

2.2.3 Pharmacobezoar

Pharmacobezoar is an uncommon concretion of various medications in the stomach after taking pharmaceutical prescription, tablets, insoluble drug delivery vehicles, or concentrated drug formulas such as cholestyramine and kayexalate (Ertugrul et al. 2012). Similar to other types of bezoar, the primary consequence of pharmacobezoar is causing gastric obstruction. While obstructing the GI tract, the pharmacologic properties of the medication in the pharmacobezoar might be released when there is a change in GI environment such as pH, which might further result in drug intoxication. Medication such as aluminum hydroxide gel, sucralfate, cholestyramine, enteric-coated aspirin, psyllium preparations, guar gum, nifedipine XL, meprobamate, enteral feeding formulas, and pharmaceutical components such as tablets and insoluble drug delivery vehicles are thought to contribute to the formation of pharmacobezoar (Stack and Thomas 1995; Simpson 2011).

For example, nifedipine XL tablet consists of an osmotic active bilayer core surrounded by a water-permeable cellulose acetate shell. The shell is designed to withstand gastric transit and to regulate the constant rate of content release. Under normal circumstance, the depleted tablet shell should be excreted in the stool. However, several cases of gastric obstruction by pharmacobezoar formed from the fragments of nifedipine tablets were reported (Shepherd 1993; Stack et al. 1994; Georgopoulos and Gerdes 1995). The interaction of different orally ingested medication with the GI tract and the possible mechanisms that cause pharmacobezoar formation were recently reviewed and discussed by Simpson (2011). However,

various studies showed that there is no consistent risk factor that predisposes an individual to pharmacobezoar formation. Pharmacobezoars can form regardless of ingesting whether sustained-release or immediate-release tablets, soluble or nondigestible formula, and highly overdose or standard dosage (Simpson 2011).

2.2.4 Phytobezoar

Phytobezoars are the most common type of bezoars formed in humans and those animals with high intake of herbal nutrients and plant materials. The common materials that contribute to its formation are indigestible fibers, cellulose, lignin, and tannins, which can be found in celery, grape, pineapple, prune, and persimmon (Ertugrul et al. 2012). Diospyrobezoars, for example, is a subset of phytobezoars, primarily formed by consumption of *Diospyros* persimmon (Zhang et al. 2008). *Diospyros* is a genus of over 700 species which is commonly known as persimmon trees. Some *Diospyros* trees bear fruits that are edible such as *Diospyros lotus* (date-plum), *D. kaki* (Asian persimmon), *D. peregrina* (Indian persimmon), and etc. Most of these persimmon fruits especially the skin part are rich in tannins, phytochemicals that possess properties of high agglutination and protein binding. Besides fruit skin, the amount of tannins in the fruit flesh is also high when unripe, giving it the astringent taste. Upon digestion, agglutination occurs between tannins and dietary proteins which led to the formation of phytobezoar stones in the gastrointestinal tract, causing complication such as small bowel obstruction or gastric obstruction (Zhang et al. 2008; de Toledo et al. 2012). In addition, consumption of *Diospyros* persimmons could increase the risk of phytobezoar formation in the patients who have undergone gastric operation (Krausz et al. 1986).

The formation of phytobezoars is much more rapid as compared to trichobezoar (Eng and Kay 2012; Wyllie et al. 2015). It explains why phytobezoar is the most common type of all bezoars. The rapid formation of phytobezoar is mainly due to intake of high-fiber food from plants. Although high-fiber diets are often recommended for cancer prevention especially against colorectal cancer (Shankar and Lanza 1991; Aune et al. 2011), it has been reported that high-fiber diet might not be suitable for some individuals predisposed to phytobezoar formation especially those who previously experienced gastrointestinal obstruction (Emerson 1987). Many dietary fibers are resistant to digestive enzymes. These fibers are frequently trapped in agglomerates together with various dietary nutrients and minerals (Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, etc.) (Wright and Lenard 2001); and the agglomerates are also readily bound to dietary phytochemicals such as tannins, through covalent interaction (Le Bourvellec et al. 2015). These interactions progressively hasten and induce the formation of phytobezoar in predisposed patients. On the other hand, recent study showed that soluble fibers can delay gastric emptying which is a key factor that promotes the formation of phytobezoar (Yu et al. 2014).

Under normal circumstances, gastric emptying is a physiological process where the stomach gradually digests and ejects the food contents within few hours of eating. In humans, taking medication that contains acid suppressant generally

suppresses serum gastrin level. As a result, solid foods consumed are unable to be completely hydrolyzed, causing delays in gastric emptying (Parkman et al. 1998). Under this condition, food particles start to accumulate in the stomach, and further delay in gastric emptying enables the particles to interact and form bonds with each other, subsequently leading to the formation of phytobezoar. The condition could be further complicated by high-protein intake. There was a study which showed high-protein intake increases the duration of gastric emptying and causes protein precipitation in the stomach promoting the formation of phytobezoar (Klair et al. 2015). Therefore, high-protein diet might not be suitable for patients who are undergoing acid suppression medication.

There are several treatment options for patients bearing phytobezoar. It depends on the size and structure consistency of phytobezoar. In general, phytobezoars with small to moderate sizes are susceptible to enzymatic dissolution such as cellulase enzyme due to its fiber-rich content (Kramer and Pochapin 2012). However, tannin-mediated agglutination in phytobezoars such as diospyrobezoars decreases their susceptibility to enzymatic dissolution and mechanical fragmentation possibly due to the astringency of tannins protecting the rock-solid structure of the bezoars (Lee et al. 2009). Recently, ingestion of commercial Coca-Cola was found to be an economical and effective way for the treatment of phytobezoar due to its acidity, penetrating effect of CO_2 bubbles, and mucolytic properties of sodium bicarbonate content (Elzouki et al. 2012; Kramer and Pochapin 2012). The acidity of Coca-Cola, coming from its phosphoric acid and carbonic acid contents, resembles gastric acid which is important for digesting dietary fibers. Interestingly, Coca-Cola was shown to clear diospyrobezoar effectively, which is less susceptible to enzymatic dissolution. Recent studies suggested that combination of carbonated sodas and endoscopic removal can effectively treat patients with diospyrobezoar (Ertugrul et al. 2012; Ogawa et al. 2016). Formation of phytobezoar does not only occur in humans but also in animals such as porcupine, monkey, goat, and ox. As mentioned earlier, phytobezoar stones from animals have been used since centuries ago and still being used in some folk's medication. Today, phytobezoars from animals such as the porcupine and ox/cattle are used to treat various disorders including cancers and inflammations. However, there is lack of in-depth scientific investigation in analyzing the pharmacological mechanism of most of these phytobezoars. In the following sections, recent studies carried out to investigate the medicinal properties of phytobezoars found in ox/cattle and porcupines are summarized.

2.3 Ox/Cattle Bezoar *Calculus bovis*

Calculus bovis are gastrointestinal bezoars that are found in ox or cattle. According to the Classic of Herbal Medicine and Shen-nung Pen-tsao Ching, it is a rare medicinal material that has been clinically used in China for more than 2000 years, since approximately 200–250 AD (Winder 1988; Xiao 2002; Jin et al. 2011). They were mainly used to cure poison from laced drink and to treat various toxications in those days. Some believe consumption of the bezoar can maintain perpetual youth and

prolonged the life-span (Barroso 2013). Today, these bezoars are still prescribed by doctors in the hospitals practicing Traditional Chinese Medicine. *C. bovis* was once thought to be a phytobezoar composed of indigestible plant materials coming from the diet of ox/cattle (Yang et al. 1996). However, recently it has been found that the main bioactive component in *C. bovis* was not of plant materials but rather the product of bile acid conglomerate from the stomach of an ox (Zhou 2010).

Nowadays, *C. bovis* is more commonly used as an active ingredient in "Angong Niuhuang (AN) pills," a popular herbal formulae widely used in China and other Asian countries to treat disease associated with the central nervous system (CNS), and some were reported to be effective against cardiovascular, liver, and several other diseases (Wang et al. 2014; Fu et al. 2017). The main components of AN pills include *C. bovis*, *Radix curcumae*, *R. scutellariae*, *Cornu rhinocerotis*, realgar, *Rhizoma coptidis*, *Fructus gardeniae*, cinnabaris, and *Borneolum syntheticum*. The pills have been shown to improve various clinical conditions. The reported *in vitro* and *in vivo* mechanisms mediated by AN pills containing *C. bovis* in improving different clinical conditions are summarized in a tabular form (Table 2.1).

In the treatment of diseases associated with the CNS, AN pills are able to relieve stroke and acute cerebral infarction in clinical patients. A major cause of stroke and cerebral infarction is due to cerebral vascular disturbance, where the arteries supplying oxygen to the brain are disturbed. The antioxidant effects of AN pills were

Table 2.1 Clinical conditions modulated by AN pills containing *C. bovis*

Clinical condition	Mechanisms	Effects	References
Stroke	Decrease C-reactive protein antioxidant	Increase curative effect and treatment efficacy. Increase number of distal red blood cells	Wu et al. (2012) and Guo et al. (2014)
Coma	Inhibits platelet aggregation	Lower blood viscosity and reverse narcosis, prevent dehydration, maintain hemostasis, reduce intracranial pressure, and prevent infection	Li (2002), Qiu and Wu (2003), and Guo et al. (2014)
Centric fever	N/A	Increase recovery of consciousness and reduce body temperature	Zhang and Wang (2001)
Virus encephalitis	Decrease NO and TNF-α levels	Reduce inflammation of the central nervous system	Wang and Dong (2013)
Atherosclerosis	Decrease C-reactive protein, LDL, malondialdehyde, troponin I, and lactate dehydrogenase	Decrease aortic membrane thickness and platelet aggregation rates	Fu et al. (2017)
Cerebral ischemia	Upregulated Bcl-2 and downregulated Bax, caspase-3 activation	Induction of apoptosis	Wang et al. (2014)

shown to increase the number of distal red blood cells, to relieve spasms caused by cerebral vascular disturbances, and subsequently to reduce risk of stroke (Guo et al. 2014). In patients suffering from coma caused by blockage of cerebral circulation, AN pills significantly improved the condition by inhibiting platelet aggregation and lowering blood viscosity to counter the state of stupor/drowsiness caused by treatment drugs (Li 2002). For centric fever, AN pills demonstrate significant antipyretic effects by lowering the body temperature and promptly recovery of consciousness of the patients (Zhang and Wang 2001). AN pills are also effective in the treatment of virus encephalitis, an acute intracranial inflammation caused by viruses which usually results in organ lesion and damages. Several studies showed that *C. bovis* in the AN pills suppresses cytokine TNF-α regulating the inflammatory pathway and improves the recovery of organ lesion caused by virus infection. In some cases, the administration of AN pills allows complete recovery from brain damage (Yao et al. 2009; Wang and Dong 2013). Besides those CNS-related diseases, AN pills were shown to exert cardioprotective effects in cardiovascular diseases. In a recent study, administration of AN pills in high-fat diet rats effectively reduced the risk of developing atherosclerosis, a common cause of heart attack. The mechanism may involve reducing aortic membrane thickness and reducing platelet aggregation and the ratio of low-density lipoprotein (LDL) to high-density lipoproteins (HDL) (Fu et al. 2017).

Recently, the bioactive composition of *C. bovis* was successfully characterized and identified. The bezoar was found to contain cholic acid, deoxycholic acid, cholesterol, and bilirubin. By knowing the bioactive compounds, Zhou (2010) successfully produced a synthetic *C. bovis* that is highly similar to the natural bezoar through chemical stimulation using calcium bilirubinate as the main component, added with a large amount of conjugated bile acids, proteins, and lecithin. The synthetic *C. bovis* is commonly referred to as *C. bovis sativus* or artificial *C. bovis*. A number of studies showed artificial *C. bovis* can function as good as the natural one. For instance, a mice study demonstrated the protective effect of artificial *C. bovis* against the development of ulcerative colitis through scavenging reactive oxygen species and anti-inflammatory properties (Li et al. 2015). In another study, artificial *C. bovis* was shown to restore normal biliary transport in patients undergoing intrahepatic cholestasis (uncontrolled release of bile from the liver) (Wu et al. 2013), by upregulating PDZK1 scaffold protein that regulates the multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP) (Xiang et al. 2017).

Several other Chinese medicinal pills were then commercialized by proportional mixing either natural or synthetic *C. bovis* with other herbal components such as *Moschus*, *Olibanum*, *Myrrha*, and realgar. These herbal formulae are used to treat different symptoms such as anti-poison, antitumor, antipyretic, gingivitis, reducing swelling, and clearing heats (Miao et al. 2011). The pharmacological effects of *C. bovis* synergistic with different herbal compounds are summarized in Table 2.2. Some of these commercial mixtures and preparations were extensively studied and patented (Feng et al. 2015; Picard et al. 2016). For example, Feng et al. (2015) patented the nanopreparation of an antitumor herbal compound by mixing solid lipid

Table 2.2 Pharmacological effects modulated by *C. bovis* in synergy with other herbal compounds in different hosts

Pharmacological effects	*Synergized compounds	Host	Pathway modulated	References
Reducing brain edema	Angong Niuhuang pill (a, b, c, d, e, f, g, h, i, j)	–	Increase the levels of catalase and glutathione peroxidase	Zhao et al. (2002) and Zhou et al. (2012)
		Rats	Regulate the matrix metalloproteinases	
Antipyretic	Angong Niuhuang pill (a, b, c, d, e, f, g, h, i, j)	Mice	Reduce heat and inflammation	Ye et al. (2003)
Anti-inflammatory	Angong Niuhuang pill (a, b, c, d, e, f, g, h, i, j)	Rats	Inhibit the expression of TNF-α mRNA and protein	Yang et al. (2009), Liu et al. (2011), Miao et al. (2011), and Yin (2011)
	Niu Huang Jie Du Pian (c, f, l, m, n)		Reduce nitric oxide and nitric oxide synthase	
Neuroprotective	Natural *C. bovis*	*In vitro*	Prevent mitochondrial dysfunction and endoplasmic reticulum stress	Kumari et al. (2013), Hu et al. (2014), and Wang et al. (2015)
			Negative modulation of NMDA receptors	
Hepatoprotective	Natural *C. bovis*	Rats	Elevate aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, alkaline phosphatase, and lactate dehydrogenase	Wan et al. (2013)
Cholestasis	Artificial *C. bovis*	Rats	Upregulated the PDZK1 scaffold protein	Liu et al. (2014) and Xiang et al. (2017)
Antipyretic	Niu Huang Jie Du Pian (c, f, l, m, n)	Mice	Reduce heat and inflammation	Miao et al. (2011)
Gingivitis		Cell line	Cytotoxicity effects	

**Calculus bovis* was synergized with different herbal compositions to mediate the pharmacological effects. These herbal compositions include (a) *Radix curcumae*, (b) *Cornurhino cerotis*, (c) realgar, (d) *Moschus*, (e) margarita, (f) *Radix scutellariae*, (g) *Rhizoma coptidis*, (h) *Fructus gardenia*, (i) cinnabaris, (j) *Borneolum syntheticum*, (k) frankincense oil, (l) *Radix glycyrrhizae* uralensis, (m) gypsum, and (n) *Platycodon grandiflorum*

nanoparticles of frankincense and myrrh with nano-realgar and micro-powder of *C. bovis*. The compounds are suggested to exhibit anti-inflammatory effect for effective treatments of acute mastitis, scrofula, and breast cancer (Feng et al. 2015).

Together, these studies implicate the profound pharmacological effectiveness of *C. bovis* alone or in combination with other herbal ingredients. More importantly, significant amount of evidences demonstrated that artificial *C. bovis* is produced as a result of a better understanding of its composition and mechanisms. We can envisage

the synthetic bezoars eventually replace the limited source of natural *C. bovis* to meet the demands and to avoid massive sacrifice of animals for their bezoars. In a nutshell, the studies of *C. bovis* have increased the availability of once limited medication and opened a new path to the discovery of potential anticancer agents through the modulation of various physiology pathways.

2.4 Phytobezoar: Porcupine Bezoars/Dates

Various pharmacognostic properties of ox bezoar *Calculus bovis* discussed in the previous section imply that the medicinal values of bezoar stones are more than a myth, implicating that the medical uses of other types of bezoars, for instance, phytobezoar stones, once used in folklore medical remedies, should bear the pharmacologic actions which yet to be investigated. Animal phytobezoars were extensively used in the early modern era, and some were recorded in ancient pharmacopeia. However, following the development of medical research on human pathology, the concerns for phytobezoars are mainly focused on their negative implications such as causing gastrointestinal/bowel obstruction for several decades. Little have been done to reveal the bioactive composition of the phytobezoars and their potentials in treating various diseases. For example, porcupine bezoars, previously named as *Lapis malaccensis* by Caspar Bauhin, are phytobezoars found in the gall bladder/stomach of Himalayan porcupine (*Hystrix brachyura*) (Christopher 2013). They were recorded in the early medical text for usage as alexipharmic (against snake/insect bites) by hill tribes in Southern China and Southeast Asia (Barroso 2013; Christopher 2013). Porcupine bezoars gained popularity among Asian-Chinese in the twentieth century. There are testimonies stating that it can cure various illnesses including cancer. Lately, it was recommended as a last resort medicine by some Asian-Chinese who are practicing Traditional Chinese Medicine (TCM) and obsessively believe the bezoars can treat various forms of cancer, dengue fever, meningitis, herpes, throat infection, pneumonia, and diabetes (Borschberg 2006; Gan 2014; Wong and AbuBakar 2013). Thus far, most of the medicinal values of porcupine bezoars were registered based on patient testimonies (http://www.soonhingcheong.com.my/en/testimonial). The common claim is that consumption of porcupine bezoars could help to recover from meningitis and early stage of cancer. The bezoars were also used to palliate the pain of cancer patients receiving chemotherapy. In some cases, these bezoars were claimed to delay the metastasis of cancer cells. In diseases related to infection, porcupine bezoars were employed to clear the infection from bacteria and virus which cause pneumonia and dengue fever, respectively.

In TCM, porcupine bezoars are believed to help in modulating the body homeostasis by regulating the inner "chi" of the body which involves reduction of inflammation-related "heat" in the body, to normalize blood glucose level in diabetic patients, and to increase platelet count of a dengue patient (http://www.soonhingcheong.com.my/en/testimonial). Interestingly, despite all the claims and testimonies, unlike the *C. bovis*, there are limited researches to provide scientific

evidence for the pharmacological actions underlying the medicinal values of porcupine bezoar. This represents a critical area to be investigated in order to build a comprehensive medicinal knowledge on porcupine bezoars to prevent misprescription of bezoar-based treatments for patients. The latest studies on porcupine bezoars are summarized, and the prospective research direction is discussed below.

2.4.1 Structures and Chemical Constituents of Porcupine Bezoars

As previously mentioned, phytobezoars are bezoar formed by the conglomeration of plant materials including indigestible fibers and bioactive phytochemicals. To date, the common phytobezoars identified are the diospyrobezoar, porcupine bezoars, and goat bezoars. Diospyrobezoars refer to phytobezoars that are agglutinated from tannins from the *Diospyros persimmon* catalyzed by dilute hydrochloric acid inside the stomach. Porcupine bezoars are further divided into several subtypes depending on their structure and appearance. The common types of porcupine bezoars identified include grassy date, powdery date, black date, kernel date, and blood date. In general, phytobezoars are brown/dark in color with rock-like structure. For example, the diospyrobezoar (Fig. 2.1) shows brownish appearance and is hard enough to cause stomach perforation (de Toledo et al. 2012). However, porcupine bezoars have distinct appearance depending on the subtypes (Fig. 2.2 A-E). The structure of black date and powdery date resembles that of diospyrobezoar (tannin-agglutinated phytobezoar), which is rock solid and appears brownish in color mainly due to the tannin composite. Undigested fruit shell is visible on the surface of the black date but not the powdery date. Another unique feature of powdery date is that it consists of multiple layers with powdery surfaces.

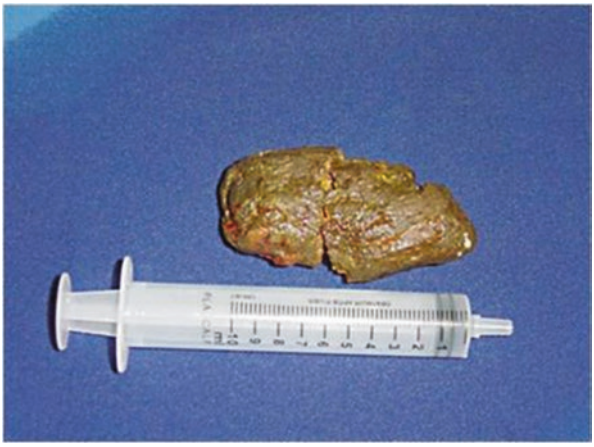

Fig. 2.1 Structure of diospyrobezoar extracted from the distal ileum of a patient

Fig. 2.2 Classification of porcupine bezoars based on appearances. (**a**) Blood date; (**b**) grassy date; (**c**) powdery date; (**d**) black date, and (**e**) kernel date (www.soonhingcheong.com.my/en/porcupine-dates; Assessed on May 15, 2017)

Fig. 2.3 Internal structures of the *GD* grassy date, *BD* black date, and *PD* powdery date

The appearance of blood date resembles the color of blood which is reddish-black in color. On the other hand, grassy date shows the presence of hairlike structure which resembles the trichobezoar but was characterized as phytobezoar due to the entanglement with solid structures which are composed of phytochemicals. In our laboratory, studies are focused on grassy date, black date, and powdery date (Fig. 2.3). In a qualitative analysis of chemical components of these bezoars, phytochemicals such as hydrolyzable tannins, cardiac glycosides, and terpenoids were found in all three porcupine bezoars, while flavonoids were only present in black date and powdery date. Further, we found the majority of phytoconstituents of porcupine bezoars are made up of hydrolyzable tannin compounds, while flavonoids, terpenoids, and glycosides only make up the remaining percentage (Yew et al. 2017).

2.4.2 Bioactivities and Anticancer Properties of Phytobezoars

In the previous section, the antioxidant properties of *C. bovis* which improve the treatment efficacy of stroke have been discussed. Besides, anti-inflammatory properties of *C. bovis* which involve the reduction of NO and TNF-α level in the brain were reported to cure virus encephalitis. These studies implicate that antioxidant and anti-inflammatory properties of phytobezoars may play an important role in pharmacological action of these bezoars in treating various ailments. In several antioxidant assays, porcupine bezoars were found to scavenge free radical *in vitro* and to suppress intracellular radical scavenging activity in mouse macrophage cell line RAW264.7. In addition, the stimulation of nitric oxide (NO), a key mediator of inflammatory pathways in the same cell line, was effectively inhibited by porcupine bezoars, showing its potential anti-inflammatory properties (Yew et al. 2017). The study provided evidences on the antioxidant and anti-inflammatory potential of porcupine bezoars that, similar to *C. bovis*, may contribute to their efficacy in treating various physiological disorders including cancers.

Recent studies suggested that oxidative stress and inflammation may alter several physiological conditions which subsequently led to cancer development (Kim and Chang 2014; Hussain et al. 2016). Oxidative stress can initiate the production of a variety of transcription factors including NF-κB, AP-1, p53, HIF-1α, PPAR-γ, β-catenin/Wnt, and Nrf2 which govern the expression of more than 500 genes regulating inflammatory responses and cell division (Reuter et al. 2010). In a clinical study, patients diagnosed with inflammatory bowel disease were found to have increased risk of developing colorectal cancer due to multiple interrelated pathways regulated by mucosal inflammatory mediators and oxidative stress (Kim and Chang 2014). Therefore, many studies are seeking for effective therapeutic intervention on these pathways, and in some of these studies, the antioxidant and anti-inflammatory properties of phytochemicals such as tannins were shown to prevent the development of colon and breast cancers (Booth et al. 2013; Yildirim and Kutlu 2015; Afrin et al. 2016). In our laboratory, preliminary tests demonstrated that the crude extract of porcupine bezoars exerts good cytotoxic activity against colon cancer cell lines (HT-29 and HCT-116). The extracts induced apoptosis as well as cell cycle arrest in tested colon cancer cells (unpublished data). Along the line of these findings, tannins as the major components of most of the medicinal phytobezoars may play indispensable role in their anticancer activities.

2.4.3 Tannins: The Major Component of Phytobezoar

Tannins have been known to be one of the major compounds that can cause the formation of phytobezoar, for instance, in diospyrobezoar. Tannins are astringent and large biomolecules that have abundant hydroxyl groups which readily form strong complex with various macromolecules and precipitate proteins (Noferi et al. 1997; Melone et al. 2013). Tannins can briefly be categorized into hydrolyzable tannins (susceptible to hydrolysis, producing gallic acid and pyrogallol) and condensed

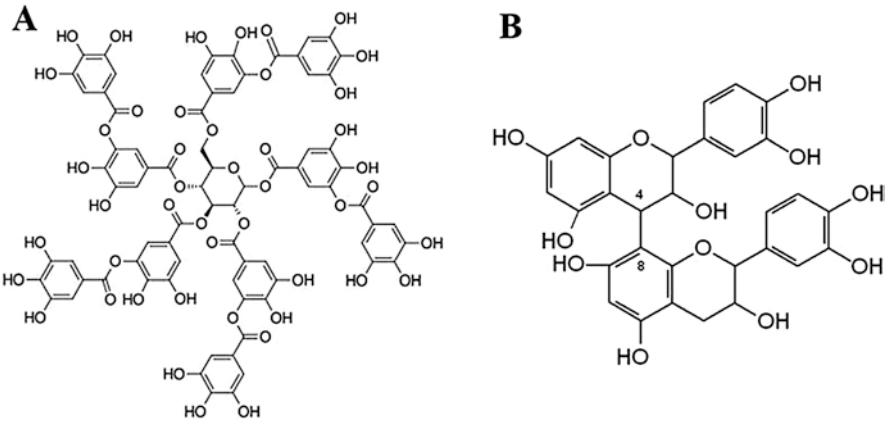

Fig. 2.4 Structure of tannins (**a**) tannic acid (hydrolyzable) and (**b**) dimers of proanthocyanidin (condensed tannins)

tannins (resistant to hydrolysis). Chemically, hydrolyzable tannins have a core polyol structure esterified with numerous hydrolyzable moieties such as gallic and ellagic acids which contribute to the high antioxidant properties (Noferi et al. 1997), while condensed tannins are made up of polymer of proanthocyanidins that have distinct interflavanyl coupling and substitution pattern (Melone et al. 2013) (Fig. 2.4 A and B).

Previously, high consumption of dietary tannins was thought to be the root cause of esophageal cancer in high-risk areas of the world (Warner and Azen 1988). However, Chung et al. (1998) reported that the carcinogenic effects might be related to components associated with tannins rather than tannins itself. Interestingly, the interaction between salivary proline-rich proteins and tannins was reported to protect the esophagus from carcinogenic effect (Warner and Azen 1988). Recently, *in vitro* and *in vivo* studies showed the anticarcinogenic and antiproliferative effects of tannins (Li et al. 2013; Yildirim and Kutlu 2015). That tannins can inhibit prostate cancer metastasis and growth (Karakurt and Adali 2016), exert antiproliferative and anti-inflammatory effects on colon cancer (Afrin et al. 2016), and prevent the development of breast cancer are also investigated (Booth et al. 2013; Yildirim and Kutlu 2015). In a nutshell, more studies demonstrated that tannins are potentially protective against cancer rather than induce carcinogenic effects. In general, tannins are a group of various derivatives of gallic acids which varied in chemical properties and bioactivities. Recently, it is revealed that hydrolyzable tannins and related derivatives are responsible for tannin-induced cytotoxicity in the liver and ovary, fibrosarcoma, and leukemia cancer cells (Li et al. 2013), while condensed tannins and tannin compound with an open-chain glucose moiety showed minimal antitumor activity (Miyamoto et al. 1987). Moreover, tannic acids are responsible for the reduction of cardiotoxic effect of doxorubicin antibiotic during cancer treatment (Tikoo et al. 2011).

Several *in vivo* studies also demonstrated the selective anticancer activity of hydrolyzable tannins/gallotannins in preclinical model (Hu et al. 2008; Kuo et al.

2009; Zhang et al. 2009). As an example, penta-O-galloyl-beta-D-glucose (5GG) suppresses NF-kappaB nuclear translocation and c-jun N-terminal kinase (JNK) activation by epidermal growth factor which led to the suppression of matrix metalloproteinases (MMPs), the key regulators in prostate cancer metastasis (Kuo et al. 2009), while Tanimura et al. (2005) showed that punicafolin (a hydrolyzable tannin) significantly inhibits MMP activity which regulates the invasiveness and metastasis of tumor cells. These studies may provide important evidences on anticancer activities of hydrolyzable tannins through modulation of MMP-mediated pathways. In-line with these findings, the hydrolyzable tannins were found to be the major phytochemicals present in the porcupine phytobezoars tested with anticancer activities. Hydrolyzable tannins are varied in the oxidative cross-linked hydroxyl groups. There is distinct formation of monomeric, dimeric, to oligomeric structure, which may affect their antitumor activities. Increase in galloyl groups within a monomeric structure showed higher *in vivo* antitumor activity in sarcoma-induced mice (Miyamoto et al. 1987), and most of the dimeric/oligomeric tannins demonstrate higher antitumor activity than monomeric tannins. On the other hand, it also proved that substituting the hydroxyl group from hexahydroxydiphenoyl to gallic acid could improve the potential of tumor suppression (Tanimura et al. 2005). Taken together, the antitumor activity of hydrolyzable tannins does not solely increase with molecular weight but may be greatly affected by their hydroxyl side group.

2.5 Conclusions and Future Prospects

The formation of different bezoar is dependent on the ingestion of distinct diet composition or medication. Bezoar stone such as *C. bovis* has medicinal values in modulating the physiological mechanism such as antioxidant and anti-inflammation related to tumorigenesis. The identification of the active compound in *C. bovis* allows artificial and cost-effective production of this precious medicine. Other phytobezoars such as the porcupine dates are investigated in its infant stage. It is also revealed that tannins as the major phytochemicals agglomerated in the porcupine phytobezoars may potentiate their therapeutic intervening action against various forms of diseases, including cancers. Furthermore, studies are needed to confirm the underlying mechanisms of porcupine phytobezoars that may modulate cancer development.

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Potential of Natural Products for the Prevention of Oral Cancer

3

Aditi Singh and Parul Tripathi

Abstract

Cancer is the second leading cause of mortality and morbidity all over the world, after cardiovascular diseases, resulting in high economic burden. Oral cancer comprises 85% of all head and neck cancers which includes cancers of the cheeks, lips, tongue, hard and soft palate, floor of the mouth, and pharynx. Its incidences are particularly high in India as people here have a frequent habit of chewing tobacco and betel nut. As early as the diagnosis of oral cancer is possible, the specific treatments are initiated which may enhance the survival of the patient and minimize the need for extensive surgery. A combination of treatments is generally practiced and consists of surgery with radiation and chemotherapy, hormone therapy, targeted therapy, or immunotherapy. However, these therapies generally cause many undesired mild to severe, short- to long-term side effects. Therefore there is a great need to find out alternative plans which not only cure the malignancy but have minimum or no side effects. Since ancient times, plants and their metabolites have been explored and were found to have great potential against various acute diseases as well as chronic disorders. Currently, many novel bioactive compounds from different plants have shown potential as therapeutic agents because of their high activity and low toxicity. Studies have already been initiated in some plants like *Azadirachta indica*, *Brucea javanica*, *Curcuma longa*, *Typhonium flagelliforme*, etc. with good results. The aim of this chapter is to provide a detailed account of the various plants and natural sources which have potential for oral cancer therapeutics. Moreover, recent developments in the physicochemical and biological properties of these plants is also highlighted.

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Anticancer agents · Bioactive compounds · Phytochemicals · Natural resources · Oral cancer

3.1 Introduction

Cancer is the second most common cause of death after heart diseases in developed countries and the third leading cause of mortality following heart and diarrheal diseases in developing countries (George et al. 2011). Cancer is defined as the uncontrollable growth of cells that invade and cause damage to surrounding tissue. Governments of different countries, under the guidance of WHO, maintain a National Cancer Registry, in which the epidemiological data on cancer is recorded. The National Cancer Registry program becomes a significant campaign in understanding the incidence and magnitude of the problem (Sarode et al. 2017). Among the various types of cancer, oral cancer is the sixth most common cause of cancer and related mortality in developing countries. Globally, the 5-year survival rate after diagnosis ranges from 50% for oral squamous cell carcinoma (OSCC) to 90% in lip cancer. Oral cancer appears as a growth or sore in the mouth that does not go away. Oral cancer, which includes cancers of the lips, tongue, cheeks, floor of the mouth, and hard palate, can be life threatening if not diagnosed and treated early. Oropharyngeal cancer is the sixth most common cancer among men and 12th most common in women (Mortazavi et al. 2014). However, carcinoma of the head and neck is most common in Central Asia and at fifth position worldwide (Parkin et al. 1999). The present chapter contains a detailed account of the various plants and natural sources which have potential for oral cancer therapeutics along with the physicochemical and biological properties of these plants.

3.2 Types of Oral Cancer

The major types of oral carcinoma and oral lesions are described below.

3.2.1 Squamous Cell Carcinoma

More than 90% of cancers that occur in the oral cavity and oropharynx are squamous cell carcinoma (Vyas et al. 2013). It is a severe problem all over the world. Normally, the throat and mouth are lined with so-called squamous cells, which are flat and arranged in a scale-like way. Squamous cell carcinoma means that some squamous cells are abnormal. More than 300,000 new cases of oral squamous cell carcinoma are diagnosed annually (Markopoulos 2012). Oral squamous cell carcinoma, a major cause of morbidity and mortality, may develop from oral preneoplastic lesions (Foy et al. 2017).

3.2.2 Verrucous Carcinoma

About 5% of all oral cavity tumors are verrucous carcinoma, which is a type of very slow-growing cancer made up of squamous cells. This type of oral cancer rarely spreads to other parts of the body but can invade the tissue surrounding the site of origin.

3.2.3 Minor Salivary Gland Carcinomas

This category includes several kinds of oral cancer that can develop on the minor salivary glands, which are found throughout the lining of the mouth and throat. These types include adenoid cystic carcinoma, mucoepidermoid carcinoma, and polymorphous low-grade adenocarcinoma.

3.2.4 Intraoral Carcinoma

The most common site for intraoral carcinoma is the tongue, which accounts for around 40% of all cases in the oral cavity proper. Tongue cancers most frequently occur on the posterior-lateral border and ventral surfaces of the tongue. The floor of the mouth is the second most common intraoral location. Less common sites include the gingival, buccal mucosa, labial mucosa, and hard palate.

3.2.5 Lymphomas

Oral cancers that develop in lymph tissue are known as lymphomas. The tonsils and base of the tongue both contain lymphoid tissue. Hodgkin lymphoma and non-Hodgkin lymphoma come under this category.

3.3 Types of Benign and Premalignant Tumors

3.3.1 Benign Oral Cavity and Oropharyngeal Tumors

Several types of noncancerous tumor-like conditions (benign tumors) can arise in the oral cavity and oropharynx. Though benign tumors do not recur, they may have a chance of developing into cancer; therefore these tumors are usually surgically removed. The types of benign lesions include eosinophilic granuloma, fibroma, granular cell tumor, keratoacanthoma, leiomyoma, osteochondroma, lipoma, schwannoma, neurofibroma, papilloma, condyloma acuminatum, verruciform xanthoma, pyogenic granuloma, rhabdomyoma, and odontogenic tumors.

3.3.2 Leukoplakia, Erythroplakia, and Lichen Planus

These are also noncancerous conditions of the mouth or throat. In leukoplakia, a white area can be seen, and in erythroplakia, there is a red flat or slightly raised area that often bleeds when scraped. Many studies have shown that both these conditions may be precancerous and can develop into different types of cancer (Feng et al. 2012; Yardimci et al. 2014). When these conditions occur, a biopsy or another test is done to determine whether the cells are cancerous. About 25% of cases of leukoplakia are either cancerous when first discovered or become precancerous. Erythroplakia is usually more serious, with about 70% of cases cancerous either at the time of diagnosis or later. Feng et al. (2012) reported that the expression of podoplanin and ABCG2 in oral erythroplakia correlated with oral cancer development.

Oral lichen planus is a cell-mediated immune disorder, the clinical features of which are variable. Oral lichen planus and lichenoid lesions have also been studied as risk factors for oral squamous cell carcinomas, and in a retrospective study of 323 OSCC confirmed cases, 18% patients had a history of oral lichen planus, and almost 4% had oral lichenoid lesions as precursor lesions (Ruokonen et al. 2017). Thus, these precursor lesions become very important in patients who do not have alcohol use or smoking as etiological factors, and an active follow-up in such cases is a must.

3.3.3 Epidemiology of Oral Cancer

Oral cancer (OC) accounts for 2–4% of all cancer cases worldwide (Ferlay et al. 2015). OC represents the majority of head and neck cancers with more than half million patients being affected each year worldwide (Haddad and Shin 2008; Corso et al. 2016) and accounts for approximately 3% of all malignancies diagnosed annually worldwide (Mortazavi et al. 2014). According to the American Cancer Society, OC is higher in developed countries when compared to developing countries, but the mortality rates remain higher in developing countries. In developing countries, the incidence of OC is 107,700 in males, and the estimated deaths are 61,200 (ACS 2016). But according to the latest GLOBOCAN estimates, 14.1 million new cancer cases and 8.2 million deaths occurred all over the world in the year 2012. Developing countries accounted for 57% of all cases and 65% of all cancer deaths, with the fear of shifting the disease burden to these less developed countries (Torre et al. 2015). In South Central Asia, OC is one of the most frequent types of cancer. The incidence rate also remains high in several developed countries such as Denmark, Poland, Germany, Scotland, Australia, Japan, New Zealand, and the USA. The age group of 55–64 years has the highest incidence of oral cancer in the USA (Petersen 2003).

Oral cancer remains one of the major serious health problems in the Indian subcontinent. India has one of the highest incidences of oral cancer (age-standardized rate of 20 per 100,000) (Coelho 2012) and the most common cancer among men (men/women ratio approximately 2:1), accounting for about 30% of all new cases

annually (More and D'cruz 2013). A recent survey of cancer mortality in India shows cancer of the oral cavity as the leading cause of mortality in men and is responsible for 22.9% of cancer-related deaths (Sankaranarayanan et al. 2005; Dikshit et al. 2012). Several studies indicate that increasing incidence correlates with delayed presentation of oral cancer (about 60% patients diagnosed at stage III or IV) (Lingen et al. 2008). The Indian National Cancer Registry data show an increasing incidence as per age. However, the incidence among women is lower than among men. This can be related to differences in lifestyle and behavioral pattern between the two genders (Thorat et al. 2009). The incidence of oral cancer has significant local variation, and this high prevalence of oral and pharyngeal carcinomas is attributed to the influence of carcinogens and region-specific epidemiological factors, especially tobacco and chewing betel quid. An increase in the prevalence of oral cancer among young adults is a cause of special concern. There has been a 60% increase in the number of under 40 years old with tongue cancer over the past 30 years (Boffetta et al. 2008).

3.3.3.1 Survival Rate in Oral Cancer

According to the surveillance and epidemiology studies, the overall 5-year relative survival rate is 62.2%. The 5-year survival rate of late-stage OC is only 20%, whereas is approximately 82% for early-stage oral cancer. In the USA from 1983 to 2006, the 5-year survival rate has increased from 52.5% to 60.8% within the time period (Ferlay et al. 2010).

3.3.3.2 Risk Factors of Oral Cancer

Men account for 70% of all oral cancers with those over the age of 50 to be at greatest risk (Corso et al. 2016). The most important risk factor for the development of oral cancer is use of tobacco (Warnakulasuriya et al. 2005) and alcohol (Ogden 2005). More than 90% are squamous cell carcinomas, which are mostly attributed to exogenous factors such as tobacco smoking and heavy alcohol consumption (Scully and Bagan 2009). Tobacco chewing in the form of betel quid or khaini/gutkha and smoking of "bidis" and cigarettes have been reported as important etiological factors (Khandekar et al. 2006). The use of smokeless tobacco products such as gutkha and betel quid in Asian countries is responsible for a high percentage of oral cancer cases (Jeng et al. 2001; Tanaka et al. 2011). Although alcohol is less potent than tobacco in causing oral cancers, the combination of alcohol with tobacco results in a much higher risk of developing oral cancers, compared to either agent alone (Moreno-Lopez et al. 2000). Family history of cancer, excessive sun exposure for lip cancer, and poor dietary habits have also been correlated with higher risk.

3.3.3.3 Diagnosis and Evaluation

A detailed history and physical examination are critical for the comprehensive evaluation of patients with oral cancer. The most common presentation is a patch or a non-healing ulcer with a history of tobacco and alcohol consumption. Pathological diagnosis should confirm with tissue biopsy from the most representative non-necrotic area of the lesion. A fine-needle aspiration (FNA) should be done of

suspected regional cervical metastases. CT scan is preferred in buccal mucosa cancer, while MRI is favored for tongue cancer. Ultrasound is also preferred for close observation and follow-up of the neck in patients who are lymph node negative. The role of newer imaging modalities such as PET-CT scan in pretreatment assessment lacks evidence. However, it is useful in assessing post-treatment residual/recurrent disease (More and D'cruz 2013).

3.3.3.4 Treatment Available

The local control of the primary tumor is the key to a successful outcome. Early-stage tumors (I/II) are managed with a single treatment modality such as surgery or radiotherapy (RT). Surgery is often the treatment of choice as transoral resection is possible with complete removal of the tumor and adequate margin control. It is cost-effective, well tolerated, repeatable, and of a relatively shorter duration as compared to 6–8 weeks of RT.

3.4 Chemoprevention of Oral Cancer

It is the use of natural or synthetic substances to halt, delay, or reverse malignant progression in tissues at risk for the development of invasive cancer (Tanaka 1995; Tanaka 1997a, b). Apoptosis induction in OSCC cells is being viewed as one of the most effective strategies for oral cancer control. Retinoids are the extensively studied agents for chemoprevention of oral cancer (Tanaka et al. 2011). Studies with retinoids in patients with oral premalignant lesions have confirmed clinical and pathologic response rates, though toxicities remain a concern in them (Papadimitrakopoulou et al. 1997). However, translational studies have indicated that cancer development may be delayed rather prevented by retinoid therapy (Mao et al. 1998). In oral leukoplakia also, clinical trials have been done with compounds for their chemopreventive studies. Such compounds include vitamin E, Bowman-Birk inhibitor concentrate (BBIC) derived from soybeans (Armstrong et al. 2000), curcumin (Cheng et al. 2001), and green tea polyphenol gallate. Attention is currently focused on the development of agents targeted to specific steps in the molecular progression from normal to oral premalignancy and to invasive carcinoma. Examples of these agents, which have shown promise *in vitro*, in animal models, or in early clinical trials, include cyclooxygenase 2 (COX-2) inhibitors and epidermal growth factor receptor (EGFR) inhibitors (Lippman et al. 2005; Zhang et al. 2005). COX-2 is overexpressed in head and neck squamous carcinoma, and its inhibitors prevent oral cancer development in animal models (Nishimura et al. 2004). The future of COX-2 inhibitors as chemoprevention agents will also depend on determining the extent of risk for cardiac toxicities associated with this class of agents. EGFR is a receptor tyrosine kinase that is overexpressed in oral dysplasia and invasive cancer and associated with poor prognosis in patients with head and neck squamous carcinoma (Shin et al. 1994). EGFR inhibitors, alone or in combination with chemotherapy and radiotherapy, showed activity against head and neck squamous

carcinoma in clinical trials and were well tolerated (Pomerantz and Grandis 2004). Evidence has suggested that combination therapy targeting COX-2 and EGFR may become more significantly effective than alone (Choe et al. 2005; Lippman et al. 2005).

3.5 Plants Against Oral Cancer

A large percentage of population all over the world uses plant products, herbs, and traditional medicines as a cure for various ailments and illnesses. The use of plants and phytomedicines has increased dramatically in the developed world (Ekor 2013). A lot of attention is being given to the natural way of treating illness. Scientists are continuously studying the vast variety of plants for their anticancer activity against different types of carcinomas and malignancies. In a meta-analysis of 16 different studies to assess the effect of fruits and vegetables intake on risk of oral cancers, the authors (Pavia et al. 2006) have found 49% reduction in risk of oral cancers by citrus fruits consumption and a significant 50% reduction in overall risk of oral cancer by green vegetable consumption. However, reduced risk to oral cancer is strongly influenced by the type of fruit consumed. Many of the natural products have been studied to induce apoptosis in various cancer cells of human origin. It is important that these findings are available for others to take lead. The following are some significant plants which have been studied and showed good response against oral cancer.

3.5.1 *Allium sativum*

Garlic (*A. sativum*) root bulb has been used for thousands of years for medicinal purposes. Allicin is an active component of garlic consisting of a high concentration of sulfur-containing amino acids, which is formed when allicin comes in contact with enzyme alliinase (Tattelman 2005). Garlic has important antineoplastic property. Various studies have shown that consumption of high levels of garlic decreases the risk of colon cancer, stomach cancer, and melanomas by inhibiting the growth and proliferation of cancer cells (Anand et al. 2008; Tattelman 2005). A previous study demonstrated that water extract of fresh garlic had apoptotic effect on cancerous cells and prevented the inception of oral carcinoma (Balasenthil et al. 2002). Another study using 7,12-dimethylbenz(a)anthracene (DMBA)-induced buccal pouch cancer model reported that garlic caused apoptosis of malignant cells (Hsu et al. 2004). It can act as an anticarcinogenic agent by scavenging the free radicals, increasing glutathione levels, increasing the activities of enzymes such as glutathione S transferase and catalase, inhibiting cytochrome p450 enzyme, and inducing DNA repair mechanisms; at the same time, it can prevent chromosomal damages (Anand et al. 2008). Garlic therefore is an alternative therapeutic agent for primary as well as invasive cancer (Balasenthil et al. 2002).

3.5.2 *Aloe vera*

A. vera is one of the most widely explored folk medicines, and its use for skin disorders dates back to thousands of years. The *Aloe* plant-derived medicinal and cosmetic products are among the biggest natural product-based industries all over the world. This succulent herb belongs to the family of Alliaceae. Many a times also referred as *Aloe barbadensis* Miller, it is native to Southern and Eastern Africa. In India and China, since ancient times *Aloe* has been an important medicine for its cathartic, stomachic, and emmenagogue properties (Grindley and Reynolds 1986). The therapeutic and medicinal uses of *A. vera* have been reviewed in detail by Sahu et al. (2013), which describe the anti-inflammatory, antioxidant, antimicrobial, antifungal, anti-helminthic, antiseptic, emollient, purgative, laxative, and aphrodisiac properties of the plant's leaves. *Aloe vera* gel is known for its healing properties for skin injury and as a remedy for sunburns, burns and cuts, acne, injury to epithelial cells, and even skin cancer (Shelton 1991). Its antiviral, antidiabetic, and stress-reducing effects are also well documented (Noor et al. 2008; Sahu et al. 2013). Among the various active components of *Aloe* are anthraquinones; chromones; monosaccharides; polysaccharides; vitamins B1, B2, B6, and C; niacinamide; choline and enzymes like acid and alkaline phosphatase; amylase; lactate dehydrogenase; lipase; and many inorganic ingredients, but most important among them is the long chain of acetylated mannose (Hayes 1999; Djeraba and Quere 2000).

The anticancer activity of *A. vera* gel is also being explored. The glycoproteins present in the gel have antiulcer and antitumor activities (Yagi et al. 2003). *Aloe vera* gel was tested for its efficacy in the management of oral submucous fibrosis (OSMF), a potentially malignant disorder frequently associated with gutkha and betel nut chewing. The topical application of gel for 3 months significantly improved the clinical symptoms in all studied subjects with reduced burning and improved mouth opening or cheek flexibility (Sudarshan et al. 2012). The gel has also shown promising results in reducing oral mucositis, which is a frequent complication in radiation therapy in head and neck cancer patients (Ahmadi 2012). Because of its antifungal properties, topical application of *A. vera* gel is also effective in oral candidiasis, another risk factor in such patients. In another study, the gel polysaccharides were tested on oral ulcer animal models for its antioxidant activity. Results, as described by investigators, demonstrated enhanced innate immune response and suppressed oxidative injury as compared to control group animals (Yu et al. 2009). The gel polysaccharide has inhibitory effect on ornithine decarboxylase activity in Balb/3T3 mice and on tyrosine kinase activity in human leukemic cells (Kim et al. 1999).

3.5.3 *Artemisia annua*

The genus *Artemisia* of the family Asteraceae is comprised of more than 500 species which are found all over the world. Many members of the genus are used in various traditional therapies including East Asian medicine and Ayurveda. Some

important species which have been studied for their various therapeutic potentials are *A. asiatica* for inflammation, infection, and ulcerogenic disorders; *A. annua* for fevers specially malaria; *A. afra* for cough, cold, headache, dyspepsia, colic, diabetes, and kidney disorders; *A. judaica* for gastrointestinal disorders; *A. tripartite* for sore throat, tonsillitis, cold, headache, and wounds; *A. vulgaris* as analgesic, anti-inflammatory, and antispasmodic; and *A. verlotorum* for hypertension (Bora and Sharma 2011).

Artemisia annua is an aromatic herb native to Asia and has been used in traditional oriental medicine since thousands of years. The major and important phytocompounds are (i) essential oils, containing cineole, α-pinene, camphene, camphor, and germacrene D; (ii) sesquiterpene lactones comprising of artemisinin, artemisinic acid, and other artemisinin derivatives; and (iii) flavones and phenolics (Bora and Sharma 2011). Artemisinin, however, is the major bioactive compound, which is rich in mono- and sesquiterpenes, and is a new class of potential antimalarial drug used throughout the globe. The combination therapies of artemisinin are considered to be the best treatment for *Plasmodium falciparum* malaria (He et al. 2009). Apart from antimalarial activity, the oil has antibacterial and antifungal (Bilia et al. 2014), immunosuppressive, anti-inflammatory, antioxidant (Cavar et al. 2012), and antiviral (Alesaeidi and Miraj 2016) activities. *A. annua* has also been studied against diabetes, heart diseases, arthritis, eczema, and cancer.

In vitro and *in vivo* studies on artemisinin have given good evidence of its anticancer activity. The mechanism of action of its antineoplastic activity has also been exhaustively studied and reviewed. Artemisinin is described to induce oxidative stress and nitric oxide production; cause DNA damage and repair; induce apoptosis, autophagy, and necrosis; and inhibit angiogenesis and mitogen-activated protein kinases (MAPK) pathway, metastatic pathway, etc. (Efferth 2017). Phase I and II clinical trials for the molecule have also been done; but hepatotoxicity caused by artemisinin combination therapy is a limitation as of now. The anticancer activity of artemisinin has been studied in breast cancer, in lung cancer, and in prostate carcinoma (Lai and Singh 2006; Sun et al. 2014; Michaelsen et al. 2015).

The essential oil artemisinin has also shown good potential against oral cancer in various studies. Ricci et al. (2011) have studied the anticancer activity of novel artemisinin-glycolipid hybrid and reported that the molecule at a very low concentration of 20 μM has shown antineoplastic activity which is better than artemisinin and five times more than that of paclitaxel or cisplatin (Ricci et al. 2011). In another study, artemisinin and its derivatives were found to induce apoptosis through caspase-3 and growth inhibition on oral cancer cells (Nam et al. 2007). The essential oil of *A. capillaris* was studied in human oral epidermoid carcinoma cells and KB cells in which the essential oil induced cell death through apoptosis, which in turn is activated via mitochondrial stress and caspase activation (Cha et al. 2009a). Similar results were obtained with essential oils of *A. iwayomogi*, which mediated apoptotic cell death via inducing mitogen-activated protein kinases (MAPKs; Cha et al. 2009b). Thus the plant seems to have good potential against various forms of cancer as well as in other ailments. It is therefore important that all

such studies are combined and more rigorously validated so that the potential of traditional therapeutic plants is exploited in chemotherapeutics and chemoprevention of oral cancers.

3.5.4 *Aster tataricus*

A native of subalpine regions in Central and South Japan, this perennial plant is found from Eastern Asia (China, Japan, and Korea) to Siberia. It belongs to the family Asteraceae and is used in traditional Chinese medicine since ancient times. Known for its antibacterial, antifungal, and antitussive activity, the roots are used to treat chronic bronchitis. The plant's anticancer activity has been attributed to triterpene epifriedelinol. The ethanolic extract of *A. tataricus* was tested on SCC-9 oral squamous carcinoma cells and was found to have significant cytotoxic and clonogenic activity in a dose-dependent manner. The plant extract had significantly increased the number of cells in G2/M phase (Wang et al. 2017a). However, prolonged use of *A. tataricus* extract can cause liver toxicity (Peng et al. 2016).

3.5.5 *Camellia sinensis*

The shrub is the source of one of the most popular beverages, tea, made from its leaves. The plant is native to India and China. Apart from being at the center of social gatherings, tea is also highly appreciated for its stimulant properties and health benefits. There are mainly two varieties – *Camellia sinensis* var. *sinensis* (Chinese tea) and *C. sinensis* var. *assamica* (Assam tea or Indian tea). For many decades, black and green teas were thought to be coming from different plants, but now it is known that they are the same species; only difference is that black tea is fermented. Tea is known to contain antioxidants, boost immunity, help in weight loss, and reduce serum cholesterol levels. The brewed beverage made from fresh and fermented leaves of *C. sinensis* has also attained a lot of attention for its potential to reduce risk of heart attack, stroke, neurological disorders, and lung damage. The leaves contain polyphenols or flavonoids, catechins, caffeine, and theanine, of which the catechin (-)-epigallocatechin-3-gallate (EGCG) is found to be having the highest biological activity (Suzuki et al. 2016). Green tea catechins if taken for long period can be beneficial against diet-induced obesity (Suzuki et al. 2016) and type II diabetes and may help in reducing the risk of coronary disease. Other described benefits of green tea are lowered risk of cardiovascular diseases (Crespy and Williamson 2004) and its anti-inflammatory (Ohishi et al. 2016), antiarthritic, antibacterial, antioxidative, antiviral, and neuroprotective (Chacko et al. 2010) properties.

Green tea is also thought to help in the prevention of cancer (Kavanagh et al. 2001) and may interfere with the growth of bladder, breast, stomach, pancreatic, and colorectal cancers. A number of studies describe the potential of tea polyphenols in oral cancer also. Green tea polyphenol-induced apoptosis in OSCC cell line has

been studied by Hsu et al. (2004), in which green tea polyphenol-induced apoptosis was found to be a mitochondria-targeted, caspase-3-executed mechanism. The polyphenol of black tea, theaflavin-3,3'-digallate, has caused elevation of reactive oxygen species (ROS) production, thereby inducing apoptosis in HSC-2 cells (Schuck et al. 2008).

3.5.6 *Curcuma longa*

C. longa is a golden herb consisting of many medicinal properties and is an effective source of treatment for various diseases since ancient times (Chaturvedi 2009; Zhang et al. 2012). This perennial plant is a member of the family Zingiberaceae. The rhizomes are the source of yellow dye and spice, "turmeric." The yellow-colored pigment in turmeric is curcumin. *C. longa* has exhibited anti-inflammatory activity in animal models, and curcumin is majorly responsible for that. Curcumin acts as an antioxidant agent because of its phenolic structure. This compound alters serum glutathione and peroxidase activity, reduces lipid peroxidation, and scavenges the reactive oxygen species. Curcumin is established as an antioxidant, antimicrobial, and anti-inflammatory agent and effective against inflammatory bowel disease, pancreatitis, arthritis, cough, and cold (Jurenka 2009). Though chemopreventive efficacy of turmeric is established in experimental systems, its mechanism of action is not fully elucidated *in vivo*. Garg et al. (2008) have investigated in detail the mechanism of turmeric-mediated chemoprevention in buccal pouch carcinogenesis in hamsters. Dietary turmeric (1%) led to decrease in tumor burden by causing a decrease in cell proliferation, enhanced apoptosis, decrease in inflammation, and aberrant expression of differentiation markers, the cytokeratins. These biomarkers are proving to be helpful in monitoring clinical trials and evaluating drug effect measurements.

The most significant role of curcumin is its anti-tumorigenic and chemopreventive role (Anand et al. 2008). Curcumin shows growth inhibitory effect on cell lines of various cancers like those of the large intestine and bone, leukemia, and oral malignant epithelium (Sharma et al. 2005). In different studies on the cell lines of various cancers, curcumin inhibited proliferation of cells and accumulated cells at G2/M cell cycle (Sharma et al. 2005). A study on turmeric and oral cancer has reported that curcumin taken either in diet or applied locally could significantly reduce DNA adducts (Ganjre et al. 2015). Curcumin effectively repaired the broken DNA strands in the peripheral cells (Gupta et al. 2013a, b). It also deactivates tobacco carcinogens (Chaturvedi 2009). The augmented effect of curcumin and green tea is also studied on oral carcinogenesis in hamsters. Green tea and curcumin treatment significantly decreased oral visible tumor incidence from 92% to 69% and that of squamous cell carcinoma from 77% to 42%. Also curcumin alone and along with green tea significantly inhibited angiogenesis in papilloma and squamous cell carcinoma (Li et al. 2002).

Pathogenesis of cancer has been associated with various pro-inflammatory molecules like nuclear factor kappa (NFκ) B, interleukin (IL)-6, IL-8, and vascular endothelial growth factor (VEGF; Bielak-Zmijewska et al. 2000). At the molecular

level, curcumin suppresses tumor promoter transcription factor NFκB and apoptotic protein (AP)-1 (Navadagi 2005). Significant inhibitory activity of curcumin on kappa-B kinase (IκBK), an enzyme responsible for NFκB activation, was obtained in head and neck squamous cell carcinoma (HNSCC; Gupta et al. 2013a, b). Curcumin inhibits cell-signaling pathways including serine/threonine kinase (Akt), NFκB, AP-1, or Jun amino-terminal kinases (JNK; Lin et al. 2009) and upregulates genes related to cell growth arrest and downregulates genes related to cancer cell proliferation such as EGR-1 (early growth response–1), c-Myc, Bcl-xL (B-cell lymphoma extra-large), and mutated tumor suppressor gene p53. Gene expression profiling by cDNA array in total RNA extract from curcumin-treated and untreated oral epithelial cancer cells has shown fourfold increase in the pro-apoptotic activating transcription factor 3 (ATF3; Sharma et al. 2005). Curcumin has a chemopreventive effect on oral precancerous lesions like oral leukoplakia (OL), oral lichen planus (OLP), and oral submucous fibrosis (OSMF). The study using OL lesion showed that curcumin reduced the size of a lesion by 10% in the treated 62 patients (Sharma et al. 2005). In OSMF, curcumin minimized micronuclei formation in exfoliated mucosal cells and in circulating lymphocytes and help to prevent onco-transformation (Navadagi 2005). It can prevent and reduce the formation of fibrosis by acting as an antifibrotic agent in OSMF (Zhang et al. 2012).

3.5.7 *Imperata cylindrica*

Imperata cylindrica, also known as thatch grass, is a perennial tufted tall grass and commonly found throughout the Indian subcontinent, Southeastern Asia, and the subtropics of Africa and Australia (Parvathy et al. 2012). It is used as an anti-inflammatory and diuretic in traditional Indian medicine or Ayurveda. The plant has also been known as an important antioxidant and immunomodulator (Pinilla and Luu 1999; Pragya et al. 2015). The extract of *I. cylindrica* has significant apoptotic and cell proliferation inhibition activity (Kuete et al. 2013). In another study by Keshava et al. (2016), the methanolic leaf extract of *I. cylindrica* has caused morphological changes in oral squamous cell carcinoma cell line SCC-9 in a concentration-dependent manner. The leaf extract has cytotoxic effect only on SCC-9 carcinoma cells and not on normal cells. The plant's potential as anticancer agent is further established by significant anti-clonogenic activity in this *in vitro* study. DNA 7 and arrest of cell cycle at G2/M phase, characteristic features of anticancer therapeutic agents, have been observed in leaf extract of *I. cylindrica*. Since the plant is easily available in most parts of the world, it should be explored more for its therapeutic potential against oral squamous cell carcinoma.

3.5.8 *Kochia scoparia*

The herb belongs to the family Amaranthaceae and is also called summer cypress or kochia. It is found all over China, Japan, and Korea. The fruit of the plant is used for skin diseases and malignant tumors of the breast, head and neck in traditional

Chinese medicine (Kim et al. 2006). The plant has anti-inflammatory and anti-allergic properties also (Lee et al. 2011). The active compounds isolated from it are saponins, triterpenoid glycosides (Wen et al. 1995), and flavone glycosides (Xu et al. 2014). The anticancer activity of *K. scoparia* has been studied in immortal neuroblastoma cells (Mazzio and Soliman 2009) and human hepatocellular carcinoma HepG2 cells (Wang et al. 2014).

The anti-carcinoma potential of *K. scoparia* is also investigated on human OSCC cell lines, and it is reported that methanol extract of mature fruit inhibited cell proliferation in OSCC via inducing apoptosis by activation of caspase-3 and caspase-9 in a dose-dependent manner. The inhibitory effects of *K. scoparia* were also seen in *in vivo* studies, where tumor growth inhibition and tumor cell apoptosis were observed in mice model (Han et al. 2016).

3.5.9 *Panax quinquefolius*

Ginseng is a group of plants of genus *Panax* which belongs to the Araliaceae family. *P. quinquefolius* or American ginseng or ginseng herb is a perennial herb, native to Northeastern America. The herb has been used as alternative medicine since ancient times, and its documented records in Native American medicine date back to over 5000 years. It is one of the most widely used traditional Chinese herbal medicines. *P. ginseng* or Asian ginseng is another important plant of the group. Ginseng root is an elixir and considered a wonder drug due to its innumerable health benefits and a promoter of well-being. The major constituents in ginseng are triterpenoid saponins, acetylenic compounds, and sesquiterpenes. The herb root is anti-inflammatory, sedative, cardiotonic, hepatoprotective and helps in digestion (Jiao et al. 2016). It has remarkable ability to help the body relieve of mental and emotional stress, fatigue, insomnia, heat, cold, and even hunger. This powerful herb also stimulates the nervous system and secretion of hormones, improves stamina, and lowers blood glucose and cholesterol levels (Bing et al. 2016).

Ginseng and its ginsenosides have been observed to play a modulating role in many stages of angiogenesis, an important factor in progression of cancer. American ginseng has anticancer properties and has shown good effects in treating colorectal cancer (Yu et al. 2013). Reduced production of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 was observed after ginseng treatment in lipopolysaccharide (LPS)-stimulated rat microglia cultures (Dai et al. 2017). In an angiogenesis model for gastric cancer, treatment of endothelial cells with Korean red ginseng extract *in vitro* resulted in significantly lower expression levels of inflammatory mediators like iNOS, COX-2, IL-8 and IL-1β, angiogenic factors like IL-6, VEGF, platelet-derived growth factor, and MMPs, with decrease in endothelial cell tube formation and cell proliferation (Choi et al. 2012). Ginseng has the potential to modulate the angiogenesis at the transcriptional, translational, and protein-signaling level via various different mechanisms of action; therefore it is surely more effective than products which target only a single mechanism.

The ginseng polysaccharide (GPS) is tested for its anticancer activity on human nasopharyngeal cancer cells (CNE-2) by Fan et al. (2013). GPS has shown remarkable inhibition in proliferation of CNE-2 cells in a dose-dependent manner. It promotes apoptosis via decreasing expression of beta-catenin and Bcl-2, at the same time increasing expression of Bax, an apoptosis-promoting protein. In a recent study on effect of protopanaxadiol (PPD), a product of *P. ginseng* on cytotoxicity in human laryngeal carcinoma cell lines, significant inhibition on cell proliferation and induced apoptosis was observed. Activation of caspase-3 and Bax and reduced expression of Bcl-2 were indicative of PPD-induced apoptosis. The anticancer effect of PPD ginsenoside is attributed to downregulation of m-TOR-signaling pathway in laryngeal carcinoma cell lines (Teng et al. 2017).

3.5.10 Proanthocyanidin

Proanthocyanidins (PACs) are flavonoids or polyphenolic compounds which are present abundantly in grapes, blueberries, cranberries, almonds, green tea, cocoa beans, and chocolates (Gu et al. 2004). They are very good antioxidants and have shown protection against tobacco-induced DNA damage. PACs have been demonstrated to have cytotoxic effects on breast cancer, lung cancer, prostate cancer, and gastric cancer (Kim et al. 2004; Seeram et al. 2004; Vayalil et al. 2004). The flavonoids were studied for cytotoxic activity in human oral cell lines, and most of them were found to be cytotoxic in oral squamous cell carcinoma and salivary gland tumor cell lines. They induced apoptosis with DNA fragmentation and caspase activation in these tumor cell lines (Sakagami et al. 2000). PAC suppressed the cellular proliferation and inhibition of OSCC CAL27 (OSCC cell line) in a dose-dependent manner. Their administration may induce apoptosis in OSCC and cervical cancer cell lines (King et al. 2007). In the study, PAC when used at higher concentrations caused changes in cell morphology and appearance. Cell clustering and rounding were observed in cervical carcinoma cell lines as well as OSCC cell lines. It had caused fragmentation of total DNA and some other alterations in CAL27 and Cal-TF16 OSCC cell lines (King et al. 2007).

The proliferative effect of proanthocyanidins may depend on the source from where they are derived, i.e., the type of fruit or vegetable. An example is the flavonoids from raspberry which have been found to be cytotoxic on oral tumor cell lines. Compounds like PACs are of much interest as they may result in more effective chemotherapeutic agents, because of multiple apoptotic pathways and less chances of drug resistance in contrast to conventional drug therapies. The bioavailability of PACs is also established. The compounds are significantly available in serum and tissues without any toxicity, when taken orally, thereby becoming a strong adjuvant candidate for people undergoing oral cancer chemotherapy (Manach et al. 2005). In another study, ethanol extract of black raspberries was demonstrated to induce apoptosis and terminal differentiation, suppress the proliferation of OSCC cell lines without affecting viability of normal cells, and inhibit

production of vascular endothelial growth factor, thus being described as a promising candidate for use as a chemopreventive agent for oral epithelial dysplasia (Rodrigo et al. 2006).

3.5.11 *Rheum palmatum*

R. palmatum belongs to family Polygonaceae and is a native plant of China, Tibet, and Mongolia. Also called as rhubarb or Da Huang, it is one of the few herbs which are still being used for its immense therapeutic benefits. The root of the plant is frequently used in traditional Chinese medicine for gastrointestinal and renal disorders as well as hyperlipidemia. It is also a very good astringent and laxative and has antibacterial, antiviral, and antifungal properties. The most active components in Da Huang roots are anthraquinones, emodin, aloe-emodin, and rhein (Wang et al. 2008). It also contains flavonoids, phenolic acids, and tannins. Emodin, aloe-emodin, and rhein, the major active compounds of the herb, have been described to have antitumor properties. They have caused cell cycle arrest and apoptosis in many human cancer cell lines, including human tongue cancer SCC-4 cells. Aloe-emodin, the root and rhizome anthraquinone of *R. palmatum*, has been shown to inhibit human oral cancer KB cells by causing cell cycle arrest at G2/M phase in a dose-dependent manner (Xiao et al. 2007). Chen and his co-workers have demonstrated that these bioactive compounds inhibit migration and invasion of SCC-4 cells via preventing gene expression of matrix metalloproteinase-9 (MMP-9), an important enzyme for tumor migration (Chen et al. 2010a). The molecules have also been shown to induce DNA damage and inhibition of DNA repair gene expression in SCC-4 human tongue cancer cells (Chen et al. 2010b).

3.5.12 *Salvia miltiorrhiza*

The plant is also known as red sage and is a member of Salvia family. The dried root of *S. miltiorrhiza* (Danshen) is extensively used in Chinese medicine to promote blood flow and treat vascular diseases. Danshen is described to have anti-atherosclerotic, antihypertensive, antiplatelet aggregation, anti-inflammatory, and antioxidative effects (Lin and Hsieh 2010). It enhances activity of antioxidative enzyme, nitric oxide synthase, and may scavenge free oxygen radicals. Triterpenoid-enriched extract of the root reduces serum cholesterol and triglyceride (Zhang et al. 2008). Danshen has neuroprotective effects via its various anti-inflammatory activities, and studies have reported reduction in brain edema after treatment with Danshen and an increase in catalase, superoxide dismutase (SOD), and glutathione (GSH) enzyme level of the cerebral cortex and hippocampus region (He et al. 2012). Danshen has shown promising effects in inhibiting tumor cell proliferation and inducing apoptosis in breast cancer, hepatocellular carcinomas, promyelocytic leukemia, and ovary carcinomas. The alcohol extract of Danshen has been recently studied on the human oral

squamous carcinoma (OSCC) cell lines HSC-3 and OC-2. It significantly inhibited the proliferation of OSCC cell lines by activating the caspase-3 apoptosis, thereby demonstrating its chemopreventive potential on human oral cancer cells. *In vivo* studies have shown that HSC-3 tumor xenograft growth was suppressed by 40% and 69% following treatment with Danshen alcohol extract at 50 and 100 mg/kg, respectively (Wang et al. 2017b). Salvianolic acid B (Sal B) is the water-soluble and most abundant and bioactive compound in Danshen. It is an antioxidant, hepatoprotective, and anti-inflammatory agent and platelet-aggregation inhibitor (Kastrup et al. 2005). Sal B has been shown to decrease squamous cell carcinoma incidence from 65% to 17% (Zhou et al. 2006). In another *in vitro* study, Sal B was investigated on two OSCC cell lines, CAL27 and SCC4, as well as premalignant leukoplakia cells. It induced significant inhibitory effect on OSCC cell growth by inhibiting TNF-α, MMP9, and many other angiogenesis regulator genes, thus inducing anti-angiogenesis in these cell lines (Yang et al. 2011). Sal B shows promise to be an important anticancer natural product against oral squamous cell carcinoma.

3.5.13 *Solanum lycopersicum*

Solanum lycopersicum (tomato), one of the most important vegetable plants, belongs to the nightshade family Solanaceae. An important guideline recommended by the American Cancer Society for prevention of cancer points out that eating five or more servings of fruits and vegetables each day can reduce the risk of cancer significantly (Pavia et al. 2006). Lycopene is the red carotenoid pigment found in high amounts in tomatoes and also in watermelon, apricot, pink guava, etc. Lycopene's ability as quencher of singlet oxygen is twice as high as β-carotene and ten times higher than vitamin A. It also inactivates free radicals which are responsible for lipid peroxidation process, thus preventing tissue damage (Lu et al. 2011).

Lycopene has been found to be a significant anticancer agent by its various mechanisms. Many studies have been done for tomato lycopene and oral cancer. In case control studies done in China, Italy, and Uruguay, tomato and tomato-rich food intake was associated with reduced risk for cancer of the oral cavity, pharynx, and aerodigestive tract (Franceschi et al. 1991; Zheng et al. 1993). Lycopene has inhibited the proliferation of KB-1 human oral tumor cells in a dose-dependent manner (Livny et al. 2002). The pigment was demonstrated to confer its anticancer effect by suppressing proliferation of G0/G1 phase and enhancing gap junction between KB-1 cells (Livny et al. 2002; Cheng et al. 2007). Thus, non-oxidative mechanism for anticarcinogenic effects of lycopene is through regulation of gap junction communication (Agarwal and Rao 2000). It acts as an antiproliferative agent by inhibiting insulin-like growth factor and thus reduces proliferative capacity of cells. In an animal study by Bhuvaneswari et al. (2004), administration of lycopene solution and tomato paste has significantly minimized incidence of hamster buccal pouch carcinogenesis. Lycopene has been shown to exert its anticarcinogen effect by modulating lipid peroxidation, enhancing antioxidants in the target organs and liver, as well as suppressing cell proliferation (Lu et al. 2011).

3.5.14 *Toona sinensis*

Also known as Chinese cedar, this deciduous tree is a member of Meliaceae family. The plant is indigenous to Southeastern Asia, including India, China, and Thailand. The decoction of bark has astringent, carminative, and febrifuge properties and is used for diarrhea, flatulence, bloody stool, leukorrhea, gonorrhea, etc. (Edmonds and Staniforth 1998). Many active compounds have been isolated from *T. sinensis*, such as gallic acid, quercetin, catechin, oleic acid, palmitic acid, linoleic acid, stigmasterol, and β-sitosterol-glucoside (Park et al. 1996). The aqueous leaf extract has been shown to cause apoptosis in human ovarian cancer cells and inhibit tumor growth in xenograft model (Chang et al. 2006). Gallic acid from *T. sinensis* showed anticancer activity on oral squamous carcinomas (Inoue et al. 2000). Chia et al. (2010) have studied the molecular mechanism of this antineoplastic effect on different oral squamous carcinoma cell lines, UM1, UM2, SSC-4, and SCC-9, and demonstrated that 3,4,5-trihydroxybenzoic acid (or gallic acid) has upregulated pro-apoptotic genes (TNF-α, TP53BP2, GADD45A) and downregulated the anti-apoptotic genes (survivin, CIAP1), thus resulting in cell death. Gallic acid induces generation of reactive oxygen species including hydrogen peroxide intracellularly, eliciting early response of apoptosis (Inoue et al. 2000). Gallic acid has also suppressed growth of lung cancer by inducing apoptosis in an *in vivo* study (Kawada et al. 2001).

3.5.15 *Vaccinium myrtillus*

Also known as European blueberry or huckleberry, the bilberry plant belongs to the large genus *Vaccinium.* It is a low-growing shrub native to northern Europe and also found in some parts of North America and Asia. The other close members of this plant are blueberry (*Vaccinium corymbosum*) and cranberry (*V. macrocarpon*). Bilberry is one of the richest sources of anthocyanins, the polyphenolic components which give bilberry its blue/black color and high antioxidant content. These anthocyanins are the major bioactive compounds responsible for many health benefits of this fruit. Bilberry is known to improve vision; it also lowers blood glucose and lipid levels and is an anti-inflammatory, antioxidant, and antimicrobial. This berry has been studied in prevention of inflammation, dyslipidemia, hyperglycemia, increased oxidative stress, cardiovascular disease (CVD), cancer, diabetes, dementia, and other age-related diseases (Chu et al. 2011). The anticancer property of bilberry anthocyanins has been studied and well documented. Various *in vitro* and animal studies have demonstrated that these anthocyanins have cancer-preventive and suppressive property, the mechanism for which is its antioxidant activity, and effects like antiproliferative, apoptotic, anti-angiogenic, and anti-inflammatory (Seeram 2009; Benzie and Wachtel-Galor 2010; Matsunaga et al. 2010). In a recent study on soft tissue changes in oral cavity by Swedish smokeless tobacco, bilberries and an extract from the common milk thistle were found to exert a significant inhibition of cell proliferation induced by tobacco in the experimental rat forestomach epithelium, indicating a potential protection with respect to soft tissue changes in the human oral cavity (Nilsson et al. 2016).

3.5.16 *Zingiber officinale*

Ginger, one of the most widely used condiments, is the rhizome of *Zingiber officinale Roscoe* of Zingiberaceae family. Since the old ages, it has been an important ingredient in Ayurvedic herbal medicines for the treatment of rheumatism, neuronal diseases, gingivitis, toothache, diabetes, etc. (Minaiyan et al. 2008). Ginger consists of numerous pungent but active agents (Ali et al. 2008). Gingerol is the major compound of ginger and has shown to exert important antioxidant, anti-inflammatory, anticancer, anti-angiogenic, and anti-atherosclerotic pharmacological properties (Habib et al. 2008). Another pungent phenolic compound, paradol, has also been described to induce apoptosis in OSCC cell lines and KB cells, via caspase-3-dependent mechanism (Keum et al. 2012). Ginger inhibits NFκ B-signaling pathway and thus represses COX-2 expressions (Anand et al. 2008). It has a dose-dependent cytotoxicity on oral carcinoma cells by activating caspase-3-mediated apoptosis (Ghasemzadeh et al. 2010).

3.6 Conclusions and Future Prospects

Oral malignancy is associated with severe morbidity and reported long-term survival of less than 50% of affected patients. Cancer management generally gets complex because of the resistance of cancer cells toward conventional treatment and due to many of those factors which govern the effectiveness of chemotherapy. In this regard, phytocompounds are getting too much attention for cancer therapy worldwide due to their minimum toxicity and resistance of cancer cells toward conventional regimes. These phytocompounds may illicit their response by arresting cell cycle, initiating apoptosis, or regulating autophagy leading to cell death and could be used to develop novel, cost-effective, anticancer therapeutic molecules with less side effects and systemic toxicity in humans. Curcumin, ginseng, lycopene, artemisinin, and anthocyanins are some of the potential compounds which have shown promising results against OSCC, head and neck carcinoma, and other such tumors.

With an increased interest in traditional medicines in developed as well as developing countries, molecules derived from such natural sources are giving important leads for the development of drugs against many human diseases, including cancer. The natural compounds showed promising apoptosis-inducing activity; this may not always guarantee antitumor activity. Enough evidences have been gathered to ascertain the ability of plant cell to synthesize antigens, which are effective in cancer chemotherapies till date. Non-Hodgkin lymphoma (NHL) vaccines have successfully been evaluated in clinical trials and are in most advanced stages of development. Apart from NHL, plant-based vaccines for breast cancer, cervical cancer, and hepatitis B virus infection have been also evaluated in preclinical trials. These promising results provide a wide array of effective plant-based anticancer biopharmaceuticals with less adverse effects on patients.

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Therapeutic Potential of Cardiac Glycosides Against Cancer

4

Mohd Mutalip Siti-Syarifah and Yunos Nurhanan-Murni

Abstract

Cardiac glycosides represent a group of naturally derived compounds isolated from several plants and animal species. It is generally used in the treatment of cardiac congestion and various types of cardiac arrhythmias. The compounds of the cardiac glycoside group have been well characterised in inhibiting Na^+/K^+-ATPase pump and are responsible for the Na^+, K^+ and Ca^{2+} ion level exchange that resulted in the ionotropic activity that is useful for the treatment of various heart conditions. The therapeutic effect of cardiac glycosides as anticancer agents was revealed in the eighth century; however, the mechanisms of action by cardiac glycosides remain largely unknown. The aim of this chapter is to discuss the chemical structure, mechanisms of actions and other issues pertaining to the use of cardiac glycosides as potential anticancer agents.

Keywords

Cancer · Cardiac glycosides · Natural products · Plants · Therapeutics

4.1 Introduction

Cardiac glycosides (CG) are secondary metabolites that are naturally derived from plant species and also from the venom of a toad species (Steyn and Heerden 1998). A few examples of CG such as digitoxin, digoxin, oleandrin, neriifolin, cerberin, ouabain, thevetin and proscillaridin can be found in the plant families of *Scrophulariaceae*, *Liliaceae*, *Apocynaceae* and *Asparagaceae* (e.g. *Digitalis*

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purpurea, *D. lanata*, *Nerium oleander*, *Strophanthus gratus* and *Urginea maritima*); some of them can also be found in few amphibians and mammals, for instance, digoxin, ouabain, bufalin, marinobufagenin and telecinobufagin (Steyn and Heerden 1998; Lòpez-Lázaro 2007). The use of CG for medicinal purposes was first reported more than 1550 years ago in ancient texts. Traditionally, CG was used as arrow poisons, as abortifacients, as emetics, as diuretics and as heart tonics. Notable use of the plants containing CG in the treatment of congestive heart failure and as anti-arrhythmic agents has been documented (Braunwald 1985). At present, digitoxin has been recognised as a primary compound used in the treatment of heart failure (Lòpez-Lázaro et al. 2006; Lòpez-Lázaro 2007; Elbaz et al. 2012), and its mode of action is believed to be through inhibition of Na^+/K^+-ATPase pump. Studies have also suggested on the possible benefits of CG as anticancer agent. It is believed that earlier use of CG in treating cancer was practised by Arab physicians in the eighth century. In addition, an ancient Chinese medicine for cancer treatment made from extract of *Bufo bufo* toad known to contain CG has also been reported (Newman et al. 2008). However due to the toxicity effects of CG, its use has been banned. In the late 1970s, it was reported that women receiving CG had tumour cells with more benign characteristics as compared to patients who did not receive CG treatment (Kometiani et al. 2005). Since then, the interest in the studies on the anticancer effects of CG has substantially increased. The aim of this chapter is to discuss the chemical structure, mechanisms of actions and other issues pertaining to the use of cardiac glycosides as potential anticancer agents.

4.2 Structures of Cardiac Glycosides

The structure of cardiac glycosides (CG) has been acknowledged as "unique" due to two distinct structural features, the sugar which is known as glycone and the nonsugar which consists of steroid and lactone ring known as aglycone moieties (Kokate et al. 2008). CG having the lactone 2-furanone is known as cardenolides, and those having the lactone 2-pyrone are known as bufadienolides (Fig. 4.1). A variety of sugar types including glucose, galactose, mannose, rhamnose and digitalose are commonly attached to CG. Although the sugar moiety alone possesses no biological activity, the presence of this moiety at position 3 on the steroidal ring enhanced severalfold the pharmacokinetic activity of CG. This is because glycone is more water soluble than aglycone; hence, the attachment of glycone to the aglycone ring increases the hydrophilicity which eventually improved the uptake, distribution and binding stability of the compound (Křen and Martínková 2001). The core structure, the aglycone, consisting of a steroidal framework is considered important and responsible for the activity of these compounds especially with the additional binding of R group (lactone ring) at position 17 that defines the class of CG (Mijatovic et al. 2007; Prassas and Diamandis 2008). The presence of the glycone moieties attached to these compounds has demonstrated severalfold improvement in the treatment of various heart diseases. Therefore, the binding of the sugar moiety to the aglycone moiety plays a great importance to the pharmacokinetic and

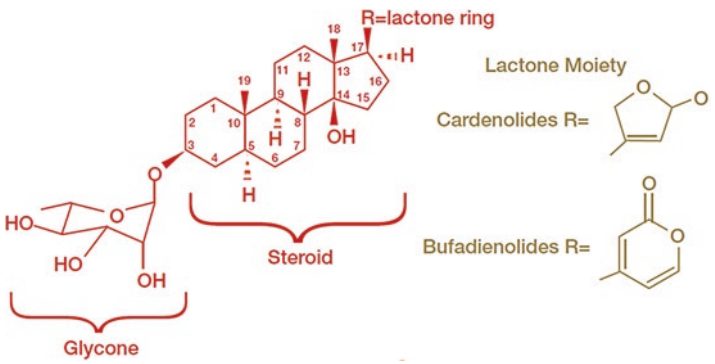

Fig. 4.1 Chemical structure of cardiac glycosides (CG) with two basic skeletons of cardenolides and bufadienolides

pharmacodynamic effects and increases the biological activity of the compounds (Křen and Martínková 2001). This effect was not only reported in the treatment of congestive heart failure and as antiarrhythmic agent but also as potential therapeutics in various cancer treatments (Prassas and Diamandis 2008).

4.3 Reported Studies on Cardiac Glycosides As an Anticancer Agent

The discoveries on the potential therapeutic roles of CG in various cancer types have been documented since the eighth century; however due to the toxicity effects of these compounds, their use has been halted. Later in the year of 1979, a new interest on the anticancer properties of these compounds re-emerged following the observation of lower mortality rates of cancer patients receiving CG (Kometiani et al. 2005). Following the report in 1979, numerous subsequent *in vitro* and *in vivo* studies have reported that a few of CG compounds such as digoxin, ouabain and oleandrin selectively killed the cancer cells but not the normal cells (Therien and Blostein 2000; Yeh et al. 2001; Laphookhieo et al. 2004; López-Lázaro et al. 2005). An earlier study on bufalin reported the selective killings of leukaemia cells with no effects on the normal leukocytes (Zhang et al. 1992). Another study on the use of ouabain in radiotherapy found that it was feasible due to a number of cervical (HeLa) and lung carcinomas (A549), as well as one transformed fibroblast cell, dying in response to the radiation following ouabain pretreatment, without having any damaging effects on the normal cells (Verheye-Dua and Böhm 1998).

There were also reports claiming on the capability of CG in blocking the tumour growth in mice xenotransplanted with human cancer cells, thus supporting the idea to assess the effectiveness of using of these compounds in patients (Zhang et al. 2008). In addition, several investigations on the effects of CG including bufalin, digoxin and UNBS1450 in human tumour xenografts have been successfully evaluated (Newman et al. 2008). The effects of bufalin introduced intraperitoneally in

human hepatocellular carcinoma cell implanted orthotopically (i.e. transplantation of cells or tissue into its normal anatomical site) showed significant reductions in tumour volumes and a prolongation in life span of the nude mice without any marked toxicity observed in myocardial, hepatic or renal tissues. In a separate study, UNBS1450 was reported to decrease the tumour growth in mice xenotransplanted with A549 lung cancer cells and human NCI-H727 lung cancer cells (Mijatovic et al. 2006). Moreover, a study by Zhang et al. (2008) reported on the effects of digoxin in reducing tumour growth and at the same time inhibiting the metastasis of human breast cancer cell (MDA-MB-231) in the lungs of xenograft mice without having any signs of toxicity. The evidence in human tumour xenografts is limited; however, as emphasised, the great potential of CG as an anticancer agent in humans has been shown due to compounds including digitoxin and digoxin, semisynthetic CG UNBS1450 and two extracts from *Nerium oleander* successfully entering the phase I clinical trials (Newman et al. 2008; Prassas et al. 2011; De et al. 2016). In addition, Anvirzel, the aqueous extract of *N. oleander* that contains mainly oleandrin compound, has been approved for phase I study by the US Food and Drug Administration (FDA) in April 2000, and it was found to be well tolerated by solid tumour patients with mild-to-moderate side effects (Prassas and Diamandis 2008; De et al. 2016). Besides these reports, the anticancer effects of CG on various types of cancer cells (breast, lung, prostate, cervical, oral and skin) are summarised in tabular form (Table 4.1).

4.4 Mechanisms of Action of Cardiac Glycosides

The understanding on the mechanisms of the pharmacological activities of CG has increased significantly ever since the discovery of its antiarrhythmic effects. Members of CG family have been well characterised in inhibiting Na^+/K^+-ATPase pump and are responsible for the Na^+, K^+ and Ca^{2+} ion level exchange. These resulted in the ionotropic activity that is useful in the treatment of various heart conditions which eventually increases the force of the myocardial contraction in congestive heart failure. The pump is involved in transporting potassium ions inside and sodium ions outside of cells in a 2:3 stoichiometry (Kaplan 2002). Such activity plays an important role in keeping the intracellular sodium levels low, thus initiating and sustaining adequate electrochemical gradient in the plasma membrane of all mammalian cells. This pump is important in regulating cell volume, cytoplasmic pH and Ca^{2+} levels through the Na^+/H^+ and Na^+/Ca^{2+} exchangers, respectively, and in driving a variety of secondary transport process such as Na^+-dependent glucose and amino acid transport (Scheiner-Bobis 2002). CG is also involved in the regulation of major cell signalling pathways that contribute to the prevention and/or treatment of cancers. CG was found to exhibit anticancer effects through apoptotic cell death mechanisms. However, the detailed information on the mechanisms of actions remains largely unknown. CG at low doses may activate the downstream proapoptotic pathways in few types of cancer cells. Several reported mechanisms showed that the proapoptotic effects might cause the preferential cytotoxicity in cancer

Table 4.1 Potential cardiac glycosides with anticancer properties against various cancers

Plant/animal species	Family	Cardiac glycoside(s)	*In vitro* anticancer cell lines	References
Apocynum cannabinum	Apocynaceae	Apocannoside, cymarin	Human nasopharynx carcinoma (KB)	Kupchan et al. (1964)
Asclepias curassavica	Asclepiadaceae	Calotropin, 16α-acetoxycalotropin, 15β-hydroxycalotropin, calactin, 15β-hydroxycalactin, asclepin, 16α-hydroxyasclepin, uscharidin, uscharin, uzarigenin	Human lung carcinoma (A549), breast carcinomas (MCF-7 and MDA-MB-231) and hepatoma (HepG2)	Roy et al. (2005)
Beaumontia brevituba	Apocynaceae	Digitoxigenin, oleandrigenin, digitoxigenin, α-L-cymaroside, digitoxigenin β-gentiobiosyl-α-L-cymaroside, Δ^{16}-digitoxigenin β-D-glucosyl-α-L-cymaroside	Human breast carcinoma (BC1), colon carcinoma (Col2), fibrosarcoma (HT-1080), nasopharyngeal carcinoma (KB), vinblastine-resistant KB (KB-V1), lung carcinoma (Lu1) and melanoma (Mel2)	Kaneda et al. (1992)
Bufo bufo gargarizans		Bufalin, cinobufagin	Prostate carcinomas (LNCaP, DU145, PC3, Paca-2, BxPC-3, Panc1, CF-PAC1, Capan 1, Su-86.86) and hepatoma (PLC/PRF/5)	Kamano et al. (1998), Yeh et al. (2003), and Prassas et al. (2011)
Calotropis procera	Asclepiadaceae	Calotropin, calactin, uscharin, voruscharin, 2″-oxovoruscharin	Human non-small cell lung carcinoma (A549), human glioblastomas (Hs683 and U373), human colon carcinomas (HCT-15 and LoVo), hepatoma (Huh7), non-hepatoma (COS-1) and colorectal carcinoma (COLO320)	Smit et al. (1995) and Choedon et al. (2006)

(continued)

Table 4.1 (continued)

Plant/animal species	Family	Cardiac glycoside(s)	*In vitro* anticancer cell lines	References
Cerbera odollam	Apocynaceae	2′-*O*-Acetyl cerleaside A, 17α-neriifolin, 17β-neriifolin, cerberin	Human oral epidermoid carcinoma (KB), breast carcinoma (BC), small cell lung carcinoma (NCI-H187), colorectal carcinoma, ovarian carcinoma	Chang et al. (2000), Laphookhieo et al. (2004), and Siti Syarifah et al. (2011)
Coronilla varia	Fabaceae	Hyrcanoside	Human lymphocytic leukaemia (P-388) and nasopharynx carcinomas (9KB)	Hembree et al. (1999)
Crossopetalum gaumeri	Celastraceae	Securigenin-3β-*O*-β-6-deoxyguloside, 19-hydroxy-sarmentogenin-3β-*O*-β-6-deoxyguloside, sarmentogenin-3β-*O*-α (-allosyl-(1 → 4)-β-6-deoxyalloside), securigenin-3β-*O*-(α-allosyl-(1 → 4)-β-6-deoxyalloside)	Human oral epidermoid carcinoma (KB)	Ankli et al. (2000)
Digitalis purpurea Digitalis lanata	Scrophulariaceae	Digoxin, digitoxin, gitoxin	Human prostate carcinomas (LNCaP, DU145, PC3), renal adenocarcinoma (TK-10), breast adenocarcinoma (MCF-7), malignant melanoma (UACC-62) and chronic myelogenous leukaemia (K-562), colorectal carcinoma, human pancreatic carcinoma (Paca-2, BxPC-3, Panc1, CF-PAC1, Capan 1, Su-86.86), cervical carcinoma	Johansson et al. (2001), Yeh et al. (2001), López-Lázaro et al. (2005), Felth et al. (2009), Prassas et al. (2011), and Vaklavas et al. (2011)

Elaeodendron sp.		Elaeodendrosides	Human ovarian carcinoma (A2780)	Cao et al. (2007)
Euonymus alata	Celastraceae	Acovenosigenin A 3-*O*-α-L-rhamnopyranoside, euonymoside A, euonymusoside A	Human oral epidermoid (KB), promyelocytic lymphoma (HL-60), non-small cell lung carcinoma (A549) and cervical carcinoma (HeLa)	Kitanaka et al. (1996)
Euonymus sieboldianus	Celastraceae	Euonymoside A	Human lung carcinoma (A549) and ovarian adenocarcinoma (SK-OV-3)	Baek et al. (1994)
Maquira calophylla	Moraceae	Maquiroside A	Human oral epidermoid carcinoma (KB)	Rovinski et al. (1987)
Nerium oleander	Apocynaceae	Oleander, oleandrin, cardenolide N-1, cardenolide N-4, 3β-*O*-(β-D-sarmentosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20-(22)-enolide, 16β-acetoxy-3β,14-dihydroxy-5β,14β-card-20-(22)-enolide	Human Jurkat leukaemia (T-cell), histiocytic lymphoma (U-937), promyelocytic lymphoma (HL-60), cervical carcinoma (HeLa), breast carcinoma (MCF-7), prostate carcinomas (LNCap, DU145, PC3), malignant fibroblast (VA-13) and liver carcinoma (HepG2)	Zhao et al. (2007) and Vaklavas et al. (2011)
Nierembergia aristata	Solanaceae	17-*epi*-11α-Hydroxy-6,7-dehydrostrophanthidin-3-*O*-β-boivinopyranoside; 6,7-dehydrostrophanthidin-3-*O*-β-boivinopyranoside; 6,7-dehydrostrophanthidin-3-*O*-β-oleandropyranoside	Human breast carcinoma (BC1), fibrosarcoma (HT), lung cancer (LU1), melanoma (Mel2), colon carcinoma (Col2), oral epidermoid (KB), drug-resistant KB with and without vinblastine, epidermoid carcinoma (A-431), prostate carcinoma (LNCaP)	Gil et al. (1995)

(continued)

Table 4.1 (continued)

Plant/animal species	Family	Cardiac glycoside(s)	*In vitro* anticancer cell lines	References
Ornithogalum umbellatum	Hyacinthaceae	Convallatoxin	Human oral epidermoid carcinoma (KB), colorectal carcinoma, human pancreatic carcinoma (Paca-2, BxPC-3, Panc1, CF-PAC1, Capan 1, Su-86.86), lung carcinoma (H460, Calu-3)	Kelly et al. (1965), Felth et al. (2009), Prassas et al. (2011), and Kaushik et al. (2017)
Pergularia tomentosa	Asclepiadaceae	3′-*O*-β-D-Glucopyranosylcalactin, 12-dehydroxyghalakinoside, 6′-dehydroxyghalakinoside, ghalakinoside, calactin	Kaposi's sarcoma (KS)	Hamed et al. (2006)
Periploca graeca	Asclepiadaceae	Periplocin isomers	Human prostate carcinoma (PC-3)	Spera et al. (2007)
Rohdea japonica	Liliaceae	Rhodexin A	Human leukaemia (K562)	Umebayashi et al. (2003)
Saussurea stella	Asteraceae	3-*O*-β-D-Fucopyranosyl strophanthidin, 3-*O*-β-D-quinovopyranosylperiplogenin, 3-*O*-β-D-glucopyranosyl-(1 → 4)-α-L-rhamnopyranosylcannogenin, 3-*O*-β-D-xylopyranosylperiplogenin, 3-*O*-β-D-quinovopyranosyl-strophanthidin, 3-*O*-β-D-xylopyranosyl-strophanthidin, 3-*O*-β-D-fucopyranosyl-periplogenin, 3-*O*-α-L-rhamnopyranosyl-cannogenol, convallatoxin, 3-*O*-α-L-rhamnopyranosyl-acovenosigenin A	Human gastric cancer (BGC-823) and hepatoma (Bel-7402)	Wang et al. (2007)
Streblus asper	Moraceae	Stebloside, mansonin	Oral human epidermoid carcinoma (KB)	Fiebig et al. (1985)

Streptocaulon juventas	Asclepiadaceae	Periplogenin digitoxoside, periplocymarin, digitoxigenin 3-*O*-(*O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-digitoxopyranoside, echujin, corchorusoside C	Human fibrosarcoma (HT-1080)	Ueda et al. (2003)
Streptocaulon griffithii	Asclepiadaceae	3-*O*-(β-Glucopyranosyl) acovenosigenin A	Human gastrointestinal cancer (HCG-27), lung carcinoma (A549), breast carcinoma (MCF-7) and cervical carcinoma (HeLa)	Huang et al. (2004)
Strophanthus gratus		Ouabain	Human prostate carcinomas (LNCaP, DU145, PC3), cervical carcinoma, breast carcinoma, human pancreatic carcinoma (Paca-2, BxPC-3, Panc1, CF-PAC1, Capan 1, Su-86.86), lung carcinoma	Yeh et al. (2001), Johansson et al. (2001), Vaklavas et al. (2011) and Prassas et al. (2011)
Thevetia ahouai	Apocynaceae	Neriifolin, 3′-*O*-methylevomonoside, 2′-acetylneriifolin	National Cancer Institute's human disease-oriented 60-cell line tumour screening panel	Decosterd et al. (1994)
Thevetia peruviana	Apocynaceae	Thevetin A and B, thevetoside	Human hepatoma (SMMC-7721), gastric carcinoma (SGC-7901) and cervical carcinoma (HeLa)	Decosterd et al. (1994)
Urginea maritima	Liliaceae	Proscillaridin A, scillaren A	Human breast carcinoma (MCF-7), colorectal carcinoma, human pancreatic carcinoma (Paca-2, BxPC-3, Panc1, CF-PAC1, Capan 1, Su-86.86)	Jha and Sen (1983), Decosterd et al. (1994), Johansson et al. (2001), Lizuka et al. (2001), Bielawski et al. (2006), Felth et al. (2009), Vaklavas et al. (2011), and Prassas et al. (2011)

cells, including the cancer-specific overexpression of Na+/K+-ATPase pump subunits, inhibition of glycolysis and inhibition of N-glycan expression. Details on these mechanisms of action by CG will be discussed in the following paragraphs.

The anticancer effects of CG were shown to possibly associate with their ability in inducing apoptosis at nontoxic concentrations in different malignant cell lines *in vitro*. For example, oleandrins including digoxin and digitoxin have been reported to induce apoptosis in non-small cell lung cancer cells (Frese et al. 2006). Besides, the apoptosis-inducing effects of oleandrin in cervical cancer cells have been reported by Gan et al. (2012), in which this compound acts by triggering the cleavage of caspases including caspase-3/7, caspase-6 and caspase-9 and by upregulation of the proapoptotic factor Bim. On the other hand, digitoxin was not only demonstrated to inhibit the cancer cell growth but also reported to induce mitochondrial apoptotic pathway via caspase-9 activation (Elbaz et al. 2012). Besides that, ouabain has been reported to trigger apoptosis in prostate cancer cells by interfering with the mitochondrial functions and induce apoptosis by TRAIL-mediated lung cancer cell death through Mcl-1 downregulation (Chanvorachote and Pongrakhananon 2013). The binding of CG to the Na^+/K^+-ATPase pump has been reported to inhibit the pump activity which in turn triggers the formation of a signalosome complex by the activation of some associated downstream signalling pathways. The inhibitory effects of Na^+/K^+-ATPase pump by CG, namely, ouabain, were reported by Daut (1983) in guinea pig ventricular muscle, and subsequent studies on the ability of CG in inhibiting the Na^+/K^+-ATPase pump have been extensively conducted. Later in year 1985, CG was known as specific inhibitor of Na^+/K^+-ATPase pump (Barry et al. 1985). Although not all anticancer effects caused by CG were through the inhibition of the plasma membrane Na^+/K^+-ATPase pump (Olej et al. 1998), the blocking of this pump was found to exhibit anticancer activity through the activation of signalosome that may activate multiple downstream signalling pathways leading to apoptotic cell death (Elbaz et al. 2012).

The exact mechanisms underlying the effects of CG in inhibiting Na^+/K^+-ATPase pump are not yet fully understood; however there were few possible mechanisms that have been reported. Inhibition of Na^+/K^+-ATPase by the binding of CG activates multiple signal transduction cascades that eventually inhibit cancer cell growth and induce apoptosis through inhibition of TNF/NF-κβ pathway (Frese et al. 2006), inhibition of glycolysis (López-Lázaro 2007), inhibition of topoisomerase 1, impairment in N-linked glycan expression (Zavareh et al. 2008) and alterations in the homeostasis of K^+, Na^+ and Ca^{2+}. In line with that, Newman et al. (2008) hypothesised that CG might possibly regulate multiple pathways in controlling the proliferation of malignant cells despite their narrow therapeutic index. The report suggested that the binding of CG to the Na^+/K^+-ATPase may trigger a complex signalling cascade that is distinct from the ion-pumping activity. Administrations of CG were shown to increase the expression of death receptors (DR4, DR5) and activate caspase activity that resulted in increased intracellular calcium concentrations. The increase in Ca^{2+} decreases the expression of transcription factors such as

activator protein-1 (AP-1) that may alter many genes and related factors that are involved in autophagic process and apoptotic cell death in cancer cells (Newman et al. 2008).

Inhibition of Na^+/K^+-ATPase pump by CG has also been reported to activate different signalling pathways by regulating the expressions of a number of genes that mediate the control of malignant cell proliferations (Xie and Cai 2003; Prassas and Diamandis 2008). Apart from that, it was also hypothesised that the inhibition of Na^+/K^+-ATPase pump by CG is associated with the inhibition of glycolysis that perhaps is related to the anticancer effects of CG (López-Lázaro 2007). Another study on prevention of distant tumour formation by digoxin using two mouse models of metastatic prostate cancer showed that CG contributed in the impairment of N-linked glycan expression and functions of cancer cells (Prassas and Diamandis 2008). In a study by Elbaz et al. (2012), two theories (old and new) on the effects of digitoxin on Na^+/K^+-ATPase pump were summarised. The old theory was based on the study by Xiao et al. (2002) and López-Lázaro (2007) on the effects of CG at micromolar (0.5–5 μM) concentration. The study results showed decrement in the intracellular potassium and increment in the intracellular sodium following inhibition of Na^+/K^+-ATPase pump which were the early key steps in apoptosis. For instance, the binding of CG such as ouabain, digitoxin, oleandrin and digoxin has been shown to inhibit the Na^+/K^+-ATPase pump and subsequently increase the intracellular levels of sodium through $Na^+/Ca2^+$ exchanger that induces apoptosis in human prostate cancer cell lines (Xiao et al. 2002; López-Lázaro 2007).

The old theory was attempted to be replaced with a new theory following a report on the possible anticancer effects of CG at nanomolar (10–100 nM) concentrations (Elbaz et al. 2012). They suggested that digitoxin triggers apoptosis by inhibiting Na^+/K^+-ATPase pump through multiple signalosome including MAPK, proto-oncogene tyrosine-protein kinase Src (SRC), Akt and phospholipase C (PLC) signalling. In addition, ouabain was also found to trigger apoptotic effect by multiple signalling through activation of mitogen-activated protein kinase 1 (MAPK) that caused the increased of sodium and reactive oxygen species (ROS) and activation of transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappaβ (NF- κβ) (Xie and Cai 2003). Besides the reported studies, our recent findings (Siti Syarifah et al. 2014) stated that a CG compound (17βH-neriifolin) induced apoptotic cell death in ovarian cancer cells via multiple pathways. Our study employed proteomic techniques including two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS) and proteomic database search using Mascot and Ingenuity Pathway Analysis (IPA) as the tools to search for the affected proteins (i.e. upregulated or downregulated) following treatment with the compound. The protein-protein interaction network predicted by IPA has found a few key proteins that may interact with other apoptotic-related proteins including topoisomerase 1 (TOP1), reactive oxygen species (ROS), tumour protein (p53), ubiquitin C (UBC) and caspase 3(CASP3). It is also predicted that the apoptotic cell death of ovarian cancer cells was through multiple signalling pathways,

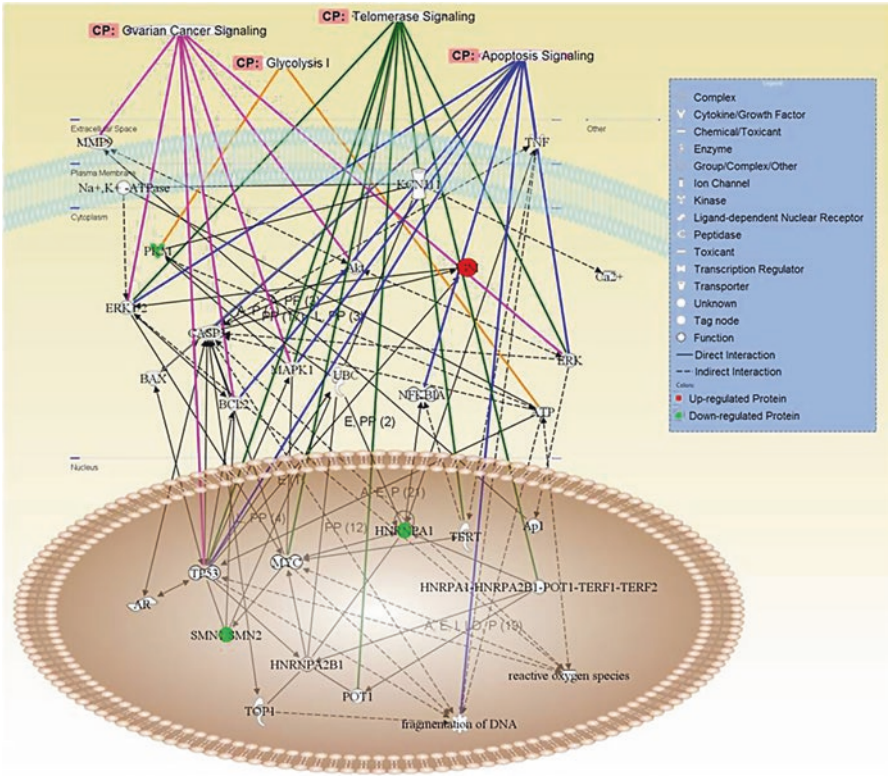

Fig. 4.2 IPA graphical representation showing multiple signalling pathways involved during the binding of 17βH-neriifolin to Na^+/K^+-ATPase pump leading to cancer cell death in ovarian cancer cells (Siti Syarifah et al. 2014)

including apoptotic signalling, ovarian cancer signalling, telomerase signalling and glycolysis (Fig. 4.2). From these findings, it is hypothesised that the proteins involved in these multiple signalling pathways may be responsible in transferring signals from Na^+/K^+-ATPase pump to the DNA fragmentations leading to apoptosis of ovarian cancer cells. This also showed that 17βH-neriifolin is able to inhibit the Na^+/K^+-ATPase pump and activate the downstream protein activities leading to apoptosis – a new finding on the potential of 17βH-neriifolin as an anticancer compound (Siti Syarifah et al. 2014).

4.5 Issues Pertaining to CG as an Anticancer Agent

The potential effects of CG as anticancer agent are being continuously reported, and many future studies are warranted to tackle on two main issues of CG, including the selectivity of the targeted cancer cell and the narrow therapeutic windows. For instance, the effects of CG are more susceptible to cancer cells (skin, breast, oral,

human small cell lung and pancreas) as compared to the normal cells (Therien and Blostein 2000; Yeh et al. 2001; Laphookhieo et al. 2004; Lòpez-Lázaro et al. 2005; Raghavendra et al. 2007; Zhao et al. 2007). The therapeutic windows of CG are still controversial, and studies are being conducted to determine the optimal CG concentration to inhibit the proliferation of cancer cells. The risks of cardiovascular toxicity of CG appear to be manageable as the concentrations that are needed to induce anticancer are very small (in nanomolar) and below the concentrations that could produce cardiac toxicity (Gupta et al. 1986; Lòpez-Lázaro et al. 2005); however, whether they are similar to the therapeutic plasma, concentrations found in cardiac patients treated with these drugs need to be determined. In addition, the inhibition of cancer cell proliferations by CG at low concentrations could not be taken as a sole factor to confirm the therapeutic potential of a drug candidate (Calderón-Montaño et al. 2014). As such, clinical trials are demanded to investigate the required plasma concentrations of CG that are sufficient to inhibit tumour growth and metastasis.

Clinical trials are highly important as the therapeutic level of a drug in the plasma should be within the required range where the drug is expected to be effective without causing any side effects to the patients. The importance of plasma drug concentration level is based on the concept that pharmacologic response is closely related to the drug concentration at the site of action (Kang and Lee 2009). As such, the plasma concentrations above the therapeutic level can lead to digitalis (CG) toxicity, which is marked as arrhythmias and could be life threatening (Hauptman and Kelly 1999). In current practice, digitoxin is the only CG compound that has been approved for clinical use in treating cardiac patients and had been shown to inhibit cancer cell viability at concentrations of 10–100 nM (0.01–0.1 μM – below the toxic concentrations) (López-Lázaro 2007; Elbaz et al. 2012). A study conducted in more than 9000 patients in the year 2001 revealed that digitoxin inhibits the proliferation of leukaemia and renal cancer cells at concentrations that are similar to the therapeutic plasma concentrations found in cardiac patients receiving this drug (Haux et al. 2001). This study's results were also supported by Lòpez-Lázaro et al. (2005). In the year 2012, Nilubol et al. reported on the maximum plasma levels of digitoxin, digoxin and ouabain in adrenocortical cancer (NCI-H295R) which were 0.05–0.2, 0.003 and 0.128 μM, respectively. Since these results indicated the requirement of low CG concentration for effective anticancer effects, these compounds also could be potentially considered for use in the development of CG-based anticancer drugs.

4.6 Conclusions and Future Prospects

Cardiac glycosides have been used in the treatment of cardiac congestion and a few types of cardiac arrhythmias since ancient times, but there are also reports on their role as potential anticancer agents. However, the fear on the toxicity effects and their narrow therapeutic windows has limited the subsequent investigations towards developing their potential as anticancer agents. Lack of information on the required

dosage and specificity of CG has been the cause of poisoning and mortality in patients. Throughout the years, scientific studies have provided evidence on the low concentration required by CG to treat cancer than that being used in the treatment of cardiac disorders. Although CG exert their anticancer potential, there is still a lot of work that needs to be done to ensure their safety of use. In addition, investigations on the pharmacodynamics and pharmacokinetic properties of CG are also essential in order to understand the therapeutic values of CG compounds, before they can be used in the future CG-based anticancer drug development.

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5 Conserving Biodiversity of a Potent Anticancer Plant, *Catharanthus roseus* Through *In Vitro* Biotechnological Intercessions: Substantial Progress and Imminent Prospects

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Abstract

In vitro interventions are exceedingly advantageous for large-scale propagation and conservation of plant biodiversity, involving endangered plant species as well as elite genotypes that produce commercial products. The importance of *Catharanthus roseus* in the treatment of several kinds of cancers such as skin cancer, breast cancer, lymph cancer, leukemia, and Hodgkin's disease warrants persistent attention for the biotechnological improvement of this plant. Therefore, the present chapter provides an overview of the state of knowledge on the current use of biotechnological tools applied on propagation, genetic enhancement and conservation of *C. roseus* besides its implications to improve the plant in the future. Explants from this clonally propagated species can be easily harvested under field conditions using *in vitro* approaches. *In vitro* micropropagation methods affirm the accelerated duplication of disease-free material. Medium-term conservation can be attained by slow growth of plant material leading to the increased time interval between subsequent cultures. Synthetic seeds are also considered for short- to mid-term conservation and germplasm exchange. For long-term conservation, cryopreservation (in liquid nitrogen at −196 °C) permits storing of *C. roseus* germplasms for extended periods exclusive of any clonal variation. Besides micropropagation and conservation, the enhancement of sec-

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ondary metabolites through hairy root culture and cell suspension culture and the use of molecular markers to detect somaclonal variation in *C. roseus* are also highlighted in this chapter.

Keywords
Conservation · Cryopreservation · Micropropagation · Secondary metabolites · Synthetic seed

5.1 Introduction

Research in the field of plant biotechnology is marking a key role worldwide for the conservation and manipulation of plant-based resources. But in view of this fact, exhaustion of biodiversity is also a matter of great concern that includes more than 21,000 plant species evidenced for their therapeutic importance (WCMC 1992). Modern medicine makes use of various pharmacological and phytochemical properties, mostly available in flowering plants (Harnischfeger 2000). As medicinal plants are advantageous on account of their ethnobotanical characteristics since long past, their indigenous cultures can come in quite useful for conservation of biodiversity, traditional cultures, drug development, and community health care (Ajaib et al. 2010). In contrast to synthetic medicines, herbal drugs are much popular due to their lower side effects (Gupta et al. 1998). As a consequence of this popularity of plant-based drugs, the environmentalists are deeply concerned over reducing population of endangered medicinal plants in their natural habitat, illegitimate collection, and overexploitation. Various advancements in the traditional propagation system (in spite of its associated demerits) in the last decade have resulted considerable development of pharmaceutical industries. Nevertheless, biotechnological tools provide a lucrative platform to overcome the obstacles during conventional breeding due to less manufacturing cost together with meeting the need for linear increase in productivity.

Already, more than 130 compounds have been extracted and identified, including terpenoid indole alkaloids (TIAs), from *Catharanthus roseus* (a very potent medicinal plant) (Samuelsson 1999), among which vinblastine and vincristine have powerful anticancerous properties. Currently, India, being the third largest producer of vinblastine and vincristine worldwide, exports these alkaloids mostly to European nations. As such *C. roseus* is a significant model to study the biotechnological aspects of secondary metabolism in plants on account of its pharmaceutical importance due to the fact that it contains lesser quantities of vinblastine and vincristine. Metabolite enhancement using biotechnology currently involves two processes: (a) *in vitro* intervention of direct or indirect organogenesis, callus, or suspension and (b) genetic transformation and over-synthesis of enzymes involved in alkaloid biosynthesis pathways. However, hairy root cultures and synthetic seed production that involves advanced biotechnological methods are yet to be explored. In this chapter, an overview on the *in vitro* propagation, conservation, and enhancement of secondary metabolites through biotechnological intercessions in *C. roseus* is presented.

5.2 *Catharanthus roseus* at a Glimpse

Catharanthus roseus (L.) G. Don. is an important dicotyledonous medicinal plant, popularly known as Madagascar periwinkle. It belongs to the family Apocynaceae. This perennial plant, synonymously also known as *Ammocallis rosea*, *Vinca rosea*, or *Lochnera rosea*, is prevalent throughout the tropics and is distributed across America, Africa, Australia, Asia, Southern Europe, and the Pacific Islands. This plant grows annually in temperate regions on account of the frosty conditions. It can endure the summer extremes, drought as well as heavy rainfall. An evergreen herbaceous plant, *C. roseus* grows around 1 m long having opposite, glossy leaves (2–3 inch length). The plant produces dark pink to white-colored flowers having a red dark center; its basal tube is 2.5–3 cm long, whereas corolla consists of five petallike lobes and is about 2–5 cm in diameter. Its fruit is a follicle pair, 2–4 cm long and 3 mm broad (Cononer and Litz 1978). Due to a wide range of TIAs present in it, this plant has been used traditionally for curing different types of ailments around the globe. The shoots contain alkaloids such as vinblastine, antineoplastin-dimeric, and vincristine, while the roots or basal stem contains vinceine, ajmalicine, raubasine, vincamine, and reserpine (Metha et al. 2013).

5.3 Medicinal and Pharmaceutical Importance

In comparison to *in vivo* propagated plants, *in vitro* micropropagated plants provide a resourceful and steady production of ameliorated secondary metabolites as well as additional bioactive compounds. Since long past, this plant has been used to cure numerous diseases such as diabetes in Europe (Swanston et al. 1989), as wasp sting treatment using leaf juice in India, as a bleeding deterrent in Hawaii, and as a diuretic, astringent, and anti-cough tonic in China (Farnsworth 1961). Europeans considered it as a magic plant; especially the French referred to it as the "violet of the sorcerers" as they believed that it would keep away evil spirits. *C. roseus,* comprising potent pharmacological features has thus raised profound interest among the medical and scientific communities. Its root barks contain maximum alkaloid ranging from 0.15 to 1.34% and sometimes up to 1.79% (in some genotypes) (Singh and Jagdev 1996). On account of two vital antitumor agents, i.e., bisindole alkaloids, vincristine and vinblastine, *C. roseus* has been the most comprehensively studied medicinal plant. Vinblastine sulfate (trade name Velban) has been employed to cure Hodgkin's disease along with choriocarcinoma, neuroblastoma, lymphosarcoma, carcinoma of the breast and lungs, and acute and chronic leukemia. The same compound (trade name Oncovin) is used to cure lymphocytic leukemia and acute leukemia in children by arresting mitosis in the metaphase stage of cell division (Aslam et al. 2010). A modified form of vinblastine known as desacetyl vinblastine amide or vindesine has at present been introduced with the trade name Eldisine for the purpose of treating acute lymphoid leukemia in children (Aslam et al. 2010). Besides vinblastine, serpentine and ajmalicine are used to provide relief from hypertension and associated cardiac disorders. Catharanthine, tetrahydroalstonine,

lochnerine, vindoline, leurosinesulfate, and vindolinine control sugar level in blood. The plant is also known for curing ailments like diabetes, high blood pressure, constipation, and asthma as well as menstrual problem (El-Sayed and Cordell 1981; Aslam et al. 2009). Major active phytoconstituents present in *C. roseus* have shown different pharmacological activities, viz., antihypertensive (Chopra et al. 1959), cytotoxic (Misawa 1976), antitumor (Chattopadhyay and Das 1990), anti-inflammatory (Chattopadhyay et al. 1992), antidiabetic (Chattopadhyay et al. 1991; Chattopadhyay 1994), antiulcer (Babulova et al. 2003), antioxidant (Singh et al. 2004; Halliwell 2012), and antimicrobial (Patil and Ghosh 2010) activities.

5.4 Conventional Propagation Practices

C. roseus, besides being propagated for herbal medicine, also has ornamental utilities such as borders, beddings, and mass effect. In subtropical gardens, during low-fertility and dry seasons where temperatures rarely drop below 7 °C, it flowers all year round (in warm temperate conditions from spring to autumn) and is propagated using seeds or vegetative cuttings. Due to its varied flower colors such as (white, peach, scarlet, mauve, and reddish orange) and resistance to cold and temperate climates, its different cultivars became ornamentally important too; some notable cultivars among them are Albus (white flowers), the Ocellatus group (various colors), Grape Cooler (rose pink, cool tolerant), and Peppermint Cooler (white with a red center, cool tolerant) (Griffiths and Huxley 1992).

5.5 *In Vitro* Mass Propagation of *C. roseus*

Plants can be quickly multiplied under protected and controlled conditions; this fact has been made possible through micropropagation by which the whole plant can be regenerated from any meristematic tissue (Vasil and Vasil 1980). This technique is convenient over traditional methods of vegetative propagation since the plant multiplies faster and those species, which show less response to cloning, can be efficiently propagated (Harbage 2001). Owing to the fact that the climate and soil of the European nations do not prefer *C. roseus* cultivation, it can be grown annually in greenhouses using plastic tunnels, though production of dimeric indole alkaloids is less (Kohlmunzer and Tomczyk 1967). As a source for obtaining a better quantity of secondary metabolites, various *in vitro* methods can be employed. Scores of *in vitro* techniques have been reported in *C. roseus* by quite a few researchers employing multiple explant sources and regeneration pathways (Bakrudeen et al. 2013; Mehta et al. 2013). Many physical as well as chemical factors are accountable for successful *in vitro* regeneration as reviewed by Gantait et al. (2016a). *In vitro* biotechnology-based research achievements on *C. roseus,* integrating the key factors affecting the regeneration and conservation of this species have been summarized in tabular form (Tables 5.1 and 5.2). In addition, the biotechnological research areas that are already exploited and yet to be exploited for *in vitro* cell, tissue, and organ culture as well as conservation of *C. roseus* germplasm are displayed in figure form (Fig. 5.1).

Table 5.1 Outline of work on direct organogenesis in *Catharanthus roseus*

Explant	Basal medium	Carbon source	PGRs	Responses	Acclimatization (% survival)	References
Auxiliary node	MS	3% sucrose	4.0 mg l^{-1} BA + 3.0 mg l^{-1} Kin	Increased number of shoot/explant	Acclimatized in garden soil, sand, and vermiculite (1:1:1)	Bakrudeen et al. (2011)
	½MS		4 mg l^{-1} IBA	Enhanced rooting		
Nodal segments	MSL	3% sucrose	5.0 μM BA	Increased number of shoot/explant	Acclimatized in sand and garden soil (1:1)	Pati et al. (2011)
			5.0 μM NAA	Enhanced rooting/explant		
Axillary node	MS	–	4 mg l^{-1} BA + 3 mg l^{-1} Kin	Enhanced multiple shooting	Acclimatized in garden soil, sand, and vermiculite (1:1:1)	Bakrudeen et al. (2013)
	½MS	–	4 mg l^{-1} IBA	Increased rooting		
Auxiliary bud	MS	3% sucrose	1 mg l^{-1} BA + 0.2 mg l^{-1} NAA	Increased number of shoot/explant	Acclimatized in polythene bags in the garden (80%)	Kumar et al. (2013)
			1 mg l^{-1} IBA + 0.25% charcoal	Enhanced rooting/explant		
Nodal segments	MS	3% sucrose	0.5 mg l^{-1} BA + mg l^{-1} NAA	Increased number of shoots/explant	Acclimatized in culture room conditions (90%)	Mehta et al. (2013)
	¼MS		5.0 mg l^{-1} IBA	Enhanced number of roots/explant		
Nodal segments	MS	3% sucrose	6.0 mg l^{-1} BA	Enhanced multiple shoot	–	Rajora et al. (2013)
			10.0 mg l^{-1} IBA	Increased rooting		
Shoot tip	MS	3% sucrose	2 mg l^{-1} BA + 0.2 mg l^{-1} NAA	Increased number of shoot/explant	–	Al-Oubaidi and Mohammed-Ameen (2014)
Nodal segments	MS	3% sucrose	0.5 mg l^{-1} BA + 1 mg l^{-1} NAA + 350 mg l^{-1} tryptophan	Increased number of shoot/explant	–	Rahmatzadeh et al. (2014)
	½MS		0.1 mg l^{-1} IBA + 350 mg l^{-1} tryptophan	Enhanced rooting		
Shoot tip	MS	3% glucose	2 mg l^{-1} BA + 1 mg l^{-1} Kin	Increased multiple shooting	–	Sandhya et al. (2016)

BA N^6-benzyladenine, *IBA* indole-3-butyric acid, *Kin* (kinetin) 6-furfurylaminopurine, *MS* Murashige and Skoog's medium, *MSL* Murashige and Skoog's liquid medium, *NAA* α-naphthalene acetic acid, *NS* nodal segment, *PGRs* plant growth regulators, "-" denotes "not reported"

Table 5.2 Outline of work on indirect organogenesis through somatic embryogenesis in *C. roseus*

Explant	Basal medium	Carbon source	PGRs	Responses	Experimental findings	References
Mature embryo	MS	3% sucrose	7.5 µM TDZ	Somatic embryo	Hypocotyl and cotyledon did not induce somatic embryogenesis and organogenesis in TDZ-containing medium but gave a maximum percentage of shoots in supplemented MS medium	Dhandapani et al. (2008)
Somatic embryo			2.5 µM TDZ	Shoot	88% of shoot regeneration with an average of 11 shoots per explant	
Shoot	½MS	1–2% sucrose	2.2 µM IBA	Root	–	
Internode	Woody plant medium	2% sucrose	5 µM BA + 5 µM NAA.	Plant regeneration	73.3% regeneration via callus	Swanberg and Dai (2008)
			20 µM BA + 10 µM NAA.	Plant regeneration	56.7% regeneration via callus	
Shoot tip	MS	3% sucrose	1 mg l^{-1} 2,4-D + 1 mg l^{-1} Kin	Callus	2.25 g callus were produced	Taha et al. (2008)
Embryogenic callus			1 mg l^{-1} BA + 1mg l^{-1} NAA	Plant regeneration	Maximum number of shoots	
Leaf	MS	3% sucrose	6.96 µM 2,4-D	Callus	Vincristine content was very high in leaf callus and germinated embryos	Aslam et al. (2009)
Embryogenic callus		3% sucrose	5.37 µM NAA + 6.72 µM BA	Somatic embryo		
Somatic embryo		3% maltose	2.60 µM GA_3	Mature somatic embryo		
Mature somatic embryo		3% sucrose	2.24 µM BA	Plantlet		

Hypocotyls	MS	3% sucrose	4.52 µM 2,4-D	Callus	Appearance of fragile and white callus in 0.25 ml liquid overlaying	Siddiqui et al. (2011)
Embryogenic callus			5.37 µM NAA + 6.72 µM BA	Somatic embryo	Maximum number of embryos was formed in 0.5 ml liquid overlaying with an increased value of 23.89%	
Somatic embryo			2.60 µM GA_3	Mature embryo	Decreased fresh weight was observed in liquid overlaying. In 0.25 ml liquid overlaying the embryos were well developed, while in 0.5 ml liquid overlaying embryos were green in color as compared to control	
Mature embryo			2.24 µM BA	Plant regeneration	0.5 ml liquid overlaying embryos showed maximum germination (55.55%)	
Hypocotyl	MS	3% sucrose	1.0 mg l^{-1} BA + 1.0 mg l^{-1} NAA	Callus	Callus induction was found to be the earliest in hypocotyl explants	Singh et al. (2011)
Embryogenic callus			1.5 mg l^{-1} BA + 1.0 mg l^{-1} NAA	Shoot	72.4% shoot regeneration from hypocotyl-derived calli was observed	
Shoot	½MS		2.5 mg l^{-1} IBA + 0.5 mg l^{-1} NAA	Root	Regeneration of roots (90%) varies in quality from hard, thick, and hairy to thin	
Leaf	MS	3% sucrose	5 mg l^{-1} NAA + 2 mg l^{-1} BA	Callus	–	Kaya and Aki (2013)

(continued)

Table 5.2 (continued)

Explant	Basal medium	Carbon source	PGRs	Responses	Experimental findings	References
Hypocotyls	MSL	3% sucrose	4.52 µM 2,4-D	Callus	Callus biomass was 1.65 g in solid, while it was 1.95 g in agitated liquid medium. However, in bioreactor the callus attained a mass of 2.11 g after 45 days of incubation	Mujib et al. (2014)
Embryogenic callus	MS		6.62 µM BA + 5.53 µM NAA	Somatic embryo	The numbers of normal somatic embryos were more in solid medium (99.75/50 mg of callus mass) compared to agitated liquid medium (83.25/callus mass) and bioreactor (84.88/callus mass)	
Somatic embryo	MS		2.60 µM GA_3	Mature embryo	The embryos turned green and elongated	
Mature embryo			2.24 µM BA	Regeneration	Germinated embryos had well-developed shoot and root ends. Shoot length (11.25 mm) was higher in bioreactor	

2,4-D 2,4 dichlorophenoxy acetic acid, *BA* N^6-benzyladenine, *GA_3* gibberellin *A_3*, *IBA* indole-3-butyric acid, *Kin* (kinetin) 6-furfurylaminopurine, *MS* Murashige and Skoog's medium, *MSL* Murashige and Skoog's liquid medium, *NAA* α-naphthalene acetic acid, *PGR* plant growth regulator, *TDZ* thidiazuron, "-" denotes "not reported"

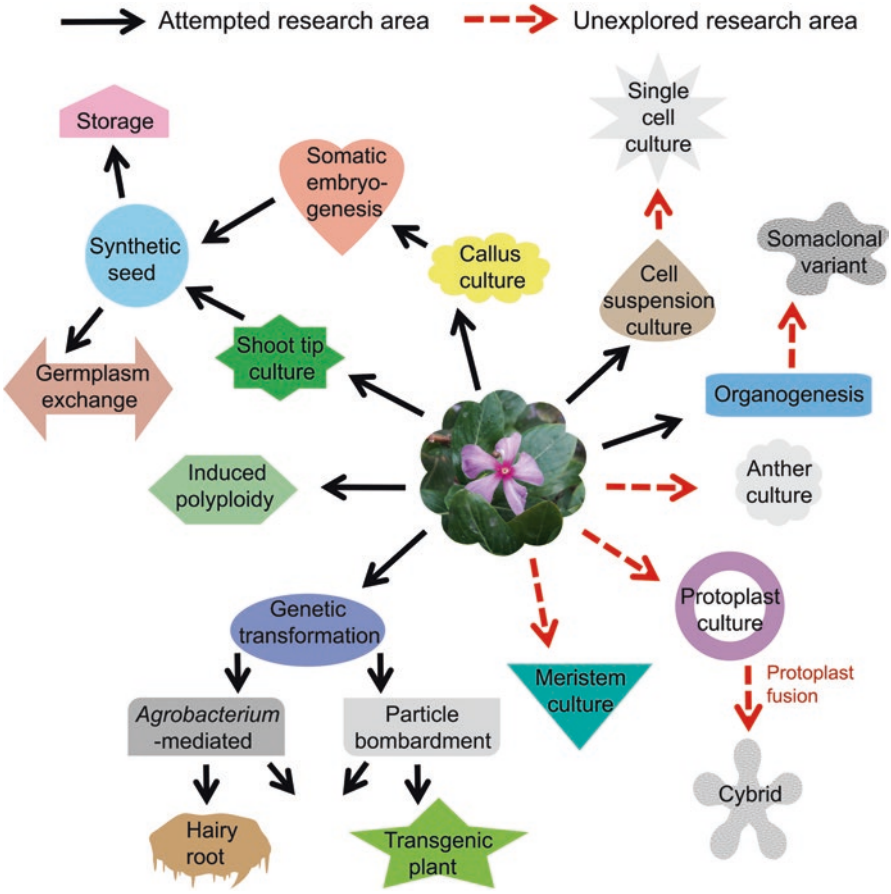

Fig. 5.1 Diagrammatic representation of exploited and unexploited biotechnological research areas for *in vitro* cell, tissue, and organ cultures and germplasm conservation of *C. roseus*

5.5.1 Role of Explant Source in Organogenesis

The accurate selection of explant source is generally an imperative initial factor for establishing a micropropagation procedure, and the selection may differ on the accessibility of material, seasonality, level of contamination, and/or abundance of tissue [shoot tips (STs), nodal segments (NSs), flowers, leaves, or roots]. A variety of explants have been used for effective initiation of *in vitro* direct organogenesis in *C. roseus* (Table 5.1), but NSs have been the most accepted explant (Bakrudeen et al. 2011; Pati et al. 2011; Bakrudeen et al. 2013; Mehta et al. 2013; Rajora et al. 2013; Rahmatzadeh et al. 2014). There are accounts where comparable experiments have been carried out between ST and NS as explant sources and found NS to be quicker in terms of response and production of more number of multiple shoots (Bakrudeen et al. 2011; Bakrudeen et al. 2013). In case of indirect organogenesis or

somatic embryogenesis in *C. roseus* (Table 5.2), a range of explants, involving leaf (Aslam et al. 2009), ST (Taha et al. 2008), internode (Swanberg and Dai 2008), mature embryo (Dhandapani et al. 2008), and hypocotyls (Mujib et al. 2014; Siddiqui et al. 2011), were employed. Dhandapani et al. (2008) reported that the highest frequency of regeneration through the intervention of somatic embryogenesis was attained from matured zygotic embryos in 7.5 μM of thidiazuron-supplemented Murashige and Skoog's (MS; Murashige and Skoog 1962) medium. On the other hand, hypocotyl and cotyledon failed to produce somatic embryogenesis or subsequent organogenesis.

5.5.2 Role of Chemical Factors in Direct Organogenesis

Even though the formulation of the mineral media was not the chief factor, MS culture medium was the mostly employed basal nutrient media for direct propagation. Any other media types have not been reported for the micropropagation of *C. roseus* except Nitsch and Nitsch's (NN; Nitsch and Nitsch medium 1969) medium that was used by Furmanowa et al. (1994). Interestingly, the reduction in the MS nutrients to one half assisted in better root induction (Bakrudeen et al. 2011; Mehta et al. 2013; Rahmatzadeh et al. 2014). Use of liquid MS medium was reported by Pati et al. (2011), for its cost-effectiveness at commercial scale. A comparative shoot proliferation data was recorded in solid and liquid medium. It was found that liquid medium displayed considerably superior shoot proliferation in comparison to the agar-gelled medium; moreover, shoots cultured in agar-gelled MS medium resulted in signs of chlorosis. The presence of cytokinin as plant growth regulator (PGR) in the culture medium was found to be crucial for shoot multiplication. N_6-benzyladenine (BA) was the majorly used cytokinin for micropropagation. However, Bakrudeen et al. (2011) found higher proliferation rate when 6-furfurylaminopurine (kinetin) was used along with BA. Additionally, the occurrence of low levels of auxin in the multiplication medium improved the proliferation rate. Inclusion of α-naphthalene acetic acid (NAA) (Kumar et al. 2013; Al-Oubaidi and Mohammed-Ameen 2014) showed superior response in respect to shoot emergence from axillary buds and multiplication of shoots. In the rooting step, isolated shoots were inoculated in media containing indole-3-butyric acid (IBA), which was superior for root induction. Kumar et al. (2013) reported that the rooting efficiency was quite proportionate to the levels of IBA, and a greater occurrence was detected once the *in vitro* shoots were inoculated on MS medium containing both IBA (1 mg l^{-1}) and charcoal (0.25%) in comparison to IBA alone.

5.5.3 Role of Chemical Factors in Indirect Organogenesis and Somatic Embryogenesis

Somatic embryogenesis is another potent system for upgrading and propagating any plant species. En masse propagation, transgenic development, and cryopreservation of long-term conservation of exclusive germplasms/lines are significant functions of

somatic embryogenesis. In addition, somatic embryogenesis may sometimes be the lone way for regeneration of difficult-to-propagate plant species. For callus induction and subsequent somatic embryogenesis, majority of the researchers suggested semi-solid full-strength MS medium (Dhandapani et al. 2008; Taha et al. 2008; Aslam et al. 2009; Siddiqui et al. 2011; Singh et al. 2011). Further, Mujib et al. (2014) deduced that the frequency of somatic embryo (SE) induction was more in case of agar-solidified MS medium in comparison to the liquid medium. Moreover, PGRs are the chief factor for manipulating growth as well as morphogenesis in a plant tissue. Predominantly, auxin supports callus initiation and holds back tissue differentiation, and then the decline in concentration of auxin induces SE formation. Table 5.2 presents the overall research that has been pursued to examine the role of PGRs on indirect organogenesis in *C. roseus*. Callus induction or direct SE development was stimulated by various auxins but 2,4-dichlorophenoxy acetic acid (2,4-D) being the most successful (Taha et al. 2008; Aslam et al. 2009; Siddiqui et al. 2011; Mujib et al. 2014). Contrastingly, other auxins, such as indole-3-acetic acid (IAA) and IBA, had not been reported. On the other hand, NAA had been used in association with low dose of BA by Kaya and Aki (2013) and found effective results. Likewise, Singh et al. (2011) found 1.0 mg l^{-1} BA plus 1.0 mg l^{-1} NAA to be efficient in callus induction and subsequent regeneration. Cytokinins have proved to play an inducible role on the induction of SE. Dhandapani et al. (2008) accounted the highest rate of regeneration through SE, which was attained in MS medium having 7.5 μM of thidiazuron. A blend of high concentration of BA with low concentration of NAA was found more advantageous in SE initiation (Aslam et al. 2009; Siddiqui et al. 2011; Mujib et al. 2014). Even conversion of SE into complete plantlet is reliant on media enriched in cytokinins as the primary PGR in combination with low levels of auxins (Singh et al. 2011; Mujib et al. 2014) and gibberellin A_3 (GA_3) in some instances (Siddiqui et al. 2011).

5.5.4 Role of Substrates in Acclimatization

The success of micropropagation is entirely dependent on the transfer process of *in vitro* regenerants to the ex vitro soil. Accomplished field transfer of *in vitro*-grown plantlets has been reported in *C. roseus* using various substrates, with differing success rates. Most of the successful acclimatization had been achieved by relocating the *in vitro*-grown plantlets to a blend of garden soil, sand, and vermiculite (1:1:1; *v/v*) (Bakrudeen et al. 2011; Bakrudeen et al. 2013). Nevertheless, Pati et al. (2011) and Kumar et al. (2013) successfully hardened the plantlets in sand and garden soil (1:1). On the other hand, Mehta et al. (2013) transferred the plantlets to culture bottles with one fourth-filled soilrite mixture (soil/sand/peat moss).

5.6 Cell Suspension Culture

Plant cell suspension culture has proved to be a boon for ameliorating secondary metabolite that substitutes the need for traditional field cultivation. Cell suspension culture has no dependency on agronomic and abiotic concerns since the technique

is completely laboratory based. The precious active ingredients in *C. roseus* are TIAs, involving the anticancerous vinblastine and vincristine and the antihypertensive ajmalicine and serpentine. Nonetheless, it is mandatory to note that vinblastine and vincristine are not accumulated in cell suspension cultures owing to the lack of one precursor's biosynthesis, i.e., vindoline. On the other hand, ajmalicine and serpentine though can be accumulated to high content; still their levels are too low in comparison to field cultivation methods. Namdeo et al. (2002) produced cell suspension cultures and added with fungal elicitors including cell wall fragments of *Aspergillus niger*, *Fusarium moniliforme*, and *Trichoderma viride*. The influence of elicitor concentration, exposure duration, and subculture age was tested for ajmalicine accumulation. Accumulation of ajmalicine was found to be increased by three times when treated with *A. niger*, *F. moniliforme*, and *T. viride.* Later on, He et al. (2011) analyzed the impacts of methyl jasmonate (MJ) and light on the transcription efficiency of biosynthetic genes as well as the gathering of catharanthine and vindoline in *C. roseus* C20hi cell cultures. The gene coding for tabersonine 16-hydroxylase (*t16h*) could be influenced by MJ and light, whereas the gene corresponding to deacetoxyvindoline 4-hydroxylase (*d4h*) could only be stimulated by light. Estimation by UPLC-MS exhibited that light notably enhanced the production of vindoline in C20hi cells almost by 0.49–5.51 times more than that of controls. The production of catharanthine however was not increased by either MJ or light. The outcome revealed that MJ and light could induce vindoline production in C20hi cells but not catharanthine. Mustafa et al. (2009) investigated the impact of salicylic acid (SA) on metabolism of *C. roseus* suspension culture cells employing NMR spectroscopy plus multivariate data analysis. In comparison to the control, SA-elicited cells demonstrated a high concentration of sugars, amino acids, phenylpropanoids, and tryptamine. Moreover, the compound, 2,5-dihydroxybenzoic-5-O-glucoside was solely detected in SA-elicited cells. Shukla et al. (2010) also reported that the antineoplastic bisindole alkaloids and the precursor, vindoline, were not accumulated in cell suspension cultures. Their research aimed at analyzing the impact of MJ as well as *Pythium aphanidermatum* homogenate on cell cultures that demonstrated enhanced level of alkaloid production and transcript abundance of strictosidine β-D-glucosidase (*SGD*) along with acetyl-CoA: 4-O-deacetylvindoline 4-O-acetyltransferase (*DAT*) genes, representing intermediate and late steps of TIA biosynthesis, correspondingly. Elicitation of cell suspension cultures leads to upregulation of *SGD* and greater production of total alkaloids, yet did not accumulate vindoline, since *DAT* transcripts remain absent in cells of suspension culture. Vindoline was only accumulated upon the addition of fungal homogenate elicitor for 24 h that also caused maximal DAT transcription. Lately, Pandiangan et al. (2013) studied cell culture employing MS medium supplemented with tryptophan 50–250 mg l^{-1}. The outcome exhibited the maximum amount accumulated on day 14 following 150 mg l^{-1} tryptophan treatment with 75% long cells (specialized cells) with 50.96 μg g^{-1} dry weight of catharanthine.

5.7 Artificial Polyploidy

One of the key events discovered during evolution of eukaryotes is polyploidy, which is found commonly in several fungi, plants, and animals. Polyploidy plays a chief role in speciation and diversification because of greater genome number multiplication unlike in the normal sets of diploid (Soltis et al. 2009). The method of non-natural mitotic polyploidization was successfully applied in agriculture during the 1930s by Blakeslee and Avery (1937). Antimitotic chemicals can provide for *in vitro* chromosome doubling, such as colchicine, an alkaloid found in *Colchicum autumnale* (Nilanthi et al. 2009). Unlike their diploid progenies, polyploids frequently show some morphological characteristics that are better in form. Induced chromosome doubling is a step-by-step procedure consisting of an induction phase, regrowth phase, and a confirmation phase for assessing the frequency of success (Salma et al. 2017). Few published reports on induced polyploidy in *C. roseus* are available. Resistance to *Pythium aphanidermatum* was studied in four induced autotetraploid lines and in seven diploids of *C. roseus* by Kulkarni and Ravindra (1988). When compared to diploid lines, the tetraploid ones showed greater biotic stress resistance with respect to infection rate, plant mortality, and area under disease curve. These tetraploid lines would produce around four to five times more root and leaf as well as total alkaloids on a regular basis for over 3 years. *C. roseus* tetraploid production was achieved from seeds by means of colchicine induction, and flow cytometry was applied to assess the ploidy level (Xing et al. 2011). Treatment using ideal concentration of colchicine solution at 0.2% for 24 h increased the rate of induction up to 30%. The tetraploids had more leaves and branches along with larger stoma when growth habits and morphological characteristics were compared with that of the diploid. The increased concentration of TIAs is due to tetraploidization as seen by HPLC analysis of *C. roseus*, thus providing a technique for higher alkaloid production commercially. Even enzyme expression for TIA production increased as deduced by QRT-PCR results. Autotetraploidy was induced using colchicine at true two-leaf stage of *C. roseus* by Hosseini et al. (2013), and their identification was achieved by flow cytometry, morphology, and various cytogenetic techniques after which the plants developed under favorable conditions in greenhouse up to seed production stage. Seed germination rate of both line seedlings was calculated. The results revealed that the seed weight content of tetraploid plants increased substantially when ploidy level was increased, but percentage of seeds and their rate of germination were decreased. However, the root and shoot length were shorter in tetraploid than in diploid seedlings. But as the diameter of both root and stem in case of tetraploid seedling increased, their fresh and dry weight also increased.

5.8 Genetic Transformations

Genetic transformations methods put forward several improvements for the value-added crops (Wang and To 2004), and the transmission of foreign genes into plants has helped to analyze the developmental regulations and their biosynthetic activities

(Guillon et al. 2006). Overall, the explant type, cocultivation conditions, and selection as well as regrowth of transformed plant tissue influence the efficacy of true transformation in *C. roseus*. The present study portrays the diverse procedures of plant transformation in *C. roseus*, with the help of *Agrobacterium tumefaciens*, *A. rhizogenes*, protoplast fusion, and particle bombardment.

5.8.1 *Agrobacterium tumefaciens*

Wide-ranging researches have been performed with *A. tumefaciens*-mediated transformation in *C. roseus* suspension cultures developed following over-expressing either with tryptophan decarboxylase (*Tdc*) or strictosidine synthase (*Str*) encoding genes (Canel et al. 1998). The Str-stimulating cultures exhibited ten times advanced Str activity, which resulted in higher TIA accumulation. In contrast, *Tdc* over-expression seemed detrimental to *C. roseus* growth and did not induce higher alkaloid accumulation. However, utilizing the same transgenic cell lines, a long-term unsteadiness was reported and even declining their ability to produce TIAs (Whitmer et al. 2003). Furthermore, the expression of *Tdc* was also investigated by Whitmer et al. (2002) in a transgenic *C. roseus* suspension culture. After precursor feeding, especially loganin and secologanin, the culture showed a higher tryptamine level with respect to controls. A ternary transformation system that is a new approach to genetic transformation has been productively used in *C. roseus*. Cocultivation of *A. tumefaciens* LBA44040 strain containing a constitutive virG mutant gene (*vir GN54D*) in a compatible ternary plasmid, jointly with cells of *C. roseus* suspension culture, resulted in a competent and improved T-DNA transfer which led to steady rates of transformation (van der Fits et al. 2000). Later on, Begum et al. (2009) employed *A. tumefaciens* C58 strain for induction of shooty teratoma in *C. roseus* using epicotyl and nodal explants, for which confirmation was done by nopaline assay. Growth kinetics was maximum during 21–24 days of transformed culture, and the dimeric alkaloid vincristine was present at a tenfold higher concentration as compared to untransformed control cultures. Srivastava et al. (2009) employed *A. tumefaciens* LBA4404/pBI-S1-mediated transformation in leaf-derived callus. Almost 98% of the explants showed GUS expression under optimal cocultivation conditions. Moreover, PCR-based amplification of the selected and transformed callus indicated the presence of *uidA*, *Gly I*, and *nptII* genes. Verma and Mathur (2011) produced *A. tumefaciens*-mediated transgenic plants, by harboring the plasmid pBI121 with GUS gene *uidA* and kanamycin resistance gene *nptII* via direct shoot bud organogenesis. Maximum transformation competence of 1.4 transgenic shoots/explants was achieved when pre-plasmolyzed leaves were preincubated for 10 days on shoot bud induction medium and sonicated for 30 s before transformation. Rai et al. (2013) reported the enhancement in vindoline content when *A. majus* geranyl diphosphate smaller subunit (*AmGPPS.SSU*) was transiently expressed in *C. roseus* leaves. The research specified that the geranyl diphosphate larger subunit (*CrGPPS.LSU*) works together with over-expressed *AmGPPS.SSU* and led to amplified GPPS activity that

enhanced GPP accessibility. Verma et al. (2015) reported the complete transformation protocol of *A. tumefaciens*-mediated transgenic plant development in *C. roseus*. *A. tumefaciens* strain LBA4404 containing the gene construct *pBI121* with GUS and ntp II as well as with *p35SGUS-INT* (containing GUS introns) was successfully employed. The procedure included a 60-min pre-plasmolysis treatment of explants by means of 13% mannitol. The organogenesis might be influenced through exosmosis by lowering the concentration of cytotoxic TIAs or creating osmotic stress in and around dividing cells involved in the process of shoot regeneration. Function of different transcription factors such as *ORCA2*, *ORCA3*, and *CrWRKY* was also investigated (Pan et al. 2012; Li et al. 2013; Yang et al. 2013). Following transformation, two types of plants were attained, one containing *ORCA3* over-expression alone (OR line) and the other with co-over-expression of *G10H* and *ORCA3* (GO line). The transcripts of AS, TDC, STR, and D4H were enhanced in this procedure. Nevertheless, Pan et al. (2012) could not detect any influence on *CRMYC2* and *G10H* transcription in OR lines. At the same time, GO lines demonstrated increase in the accumulation of ajmalicine, catharanthine, strictosidine, and vindoline but without any effect on anhydrovinblastine and vinblastine synthesis. Furthermore, Li et al. (2013) accounted that the transcripts of TIA transcriptional repressors and some of the TIA regulators were considerably improved by *ORCA2* over-expression. The *CrWRKY1* promoter can induce the expression of β-glucuronidase (GUS) reporter gene in indigenous *C. roseus* protoplasts and hairy roots as well as heterologous (transgenic tobacco seedlings) systems (Yang et al. 2013). Alam et al. (2017) standardized the procedure for improved transformation and recovery to minimize the time duration through *Agrobacterium* and sonication-assisted transformation (SAAT) technique. In this report, hypocotyl explants were highly responding with maximum rate of transformed shoots. The expression of GFP was evidently confined in leaf tissue after transformation of *pRepGFP0029* construct. Accordingly, accomplishment of transformation was based on GFP localization. The transformation competence of SAAT method was 6%, which was much higher than conventional method that lies at 3.5%. Moreover, integration of the *nptII* gene in the transformed plantlets of *C. roseus* was confirmed by PCR analysis.

5.8.2 *Agrobacterium rhizogenes*

It is well known that *A. rhizogenes*-mediated hairy root culture is able to accumulate higher levels of secondary metabolites than cell suspension culture. Further, if an inducible promoter is devised, it would control the intensity of gene expression; for this reason, an inducible promoter mechanism was established on account of hairy root culture of *C. roseus* (Hughes et al. 2002). In this procedure, a glucocorticoid-inducible promoter regulating the expression of green fluorescent protein (GFP) was developed in hairy roots by infecting the shoot tips by means of *A. rhizogenes* 15,834 containing the gene construct. The mechanism exhibited a controlled as well as responded in a dosage-dependent manner to the production of glucocorticoid

dexamethasone. Whereas, upon exclusion of the inducer, GFP expression was reduced, suggesting the controlling capability of this inducer for gene expression in *C. roseus* hairy roots. Ongoing with the unchanged approach, the same group also experimented on the influence of *Arabidopsis* feedback-resistant anthranilate synthase alpha subunit (ASa) in *C. roseus* hairy roots, an enzyme component of shikimate pathway producing TIAs, and showed increased levels of tryptamine and tryptophan (Hughes et al. 2004a). Furthermore, expression of two transgenes, i.e., ASa along with Tdc or Tdc alone, controlled by the same inducible method, was also conducted (Hughes et al. 2004b), where TDC enzyme assays resulted in 48% and 87% enhancement in the Tdc or ASa-Tdc, respectively. The TDC single line did not produce tryptamine, contrasting the double ASa-Tdc line where tryptamine accumulation was increased to six times. Conversely, there are studies on TIAs in employing wild strains of *A. rhizogenes* (strain A4)-mediated hairy roots in *C. roseus*. Therefore, a hairy root culture accumulating catharanthine and vindoline, the two monomer intermediates for the biosynthesis of vincristine and vinblastine, the bisindole alkaloids, has been reported (Palazón et al. 1998). In another report by Morgan and Shanks (1999), hairy root cultures were developed employing *A. rhizogenes* 15,834, to examine inhibitors of tabersonine metabolism into hörhammericine and lochnericine. The effect of buffered media was analyzed upon the growth and alkaloid production of *C. roseus* hairy root culture. The production of lochnericine was lower in response to the addition of buffers, despite the fact that tabersonine was considerably higher. An investigation by Hong et al. (2003) on the non-mevalonate pathway (DXP or MEP) in *C. roseus* hairy roots was also conducted. Hairy roots were induced by means of *A. rhizogenes* 15,834, which were added with different precursors such as loganin or 10-hydroxygeraniol, 1-deoxy-D-xylulose, as well as a DXP inhibitor like fosmidomycin at late exponential phase of growth. Addition of the precursors resulted in the enhancement of lochnericine and tabersonine content by 33–56% above controls but not the ajmalicine level. In addition, inhibition by fosmidomycin was also reliant on the hairy root's growth phase, where accumulation of ajmalicine (93%), tabersonine (81%), and lochnericine (62%) was reduced at exponential phase, but result was unchanged when inhibition was conducted at stationary growth phase. Later on, the Ri T-DNA gene transfer in *C. roseus* was tested in response to growth and TIA production (Batra et al. 2004). *In vitro*-grown 1-month-old plantlet's leaves were infected with wild-type *A. rhizogenes* A4 strain and incubated in the dark. The hairy root culture showed single and multiple copies of T-DNA, and considerable amounts of ajmalicine and serpentine were accumulated. Verma et al. (2012) studied 15 hairy root lines of *C. roseus* and compared for alkaloid accumulation along with growth potential with regard to the pattern of *rol* gene incorporation. Among them 12 hairy root lines exhibited the occurrence of *rolB* along with *rolC* and only 2 showed the incorporation of *rolC*. Genes of *rolA*, *B*, and *C* were evidenced only in N5 line. The hairy root lines differed significantly in relative concentration of ajmalicine (0.007–0.08% dry wt.), catharanthine (0.01–0.04% dry wt.), and serpentine (0.01–0.08% dry wt.). Highest biomass accumulation was noted in line PG2, and highest catharanthine content

was recorded in line PI3 which were upscaled in bioreactor wherein a 9–11-fold augmentation in biomass was attained with twofold enhancement in ajmalicine (0.029% dry wt.).

5.8.3 Particle Bombardment

This method involves the delivery of microparticles, any of gold or tungsten in nature, layered with DNA which are then transmitted into the target cell or tissue at high velocity with the help of carrier gas like He or Ar, which can later be regenerated into well-developed transformed plants (Sanford et al. 1987). Earlier, tungsten-mediated particle bombardment was applied for transformation in suspension cultures with plasmid constructs containing two different gene promoters (CaMV 35S, FMV 34S) along with a fused Gus reporter gene to evaluate the gene expression. The exercise of 35S promoter together with a deletion derivative of 34S promoter was determined with particle bombardment on calli derived from leaves for the genetic engineering of this species (van der Fits and Memelink 1997; Hilliou et al. 1999). A rate of 60–80% transgenic calli was recovered out of total calli bombarded by means of a single plasmid. Conversely, 25–60% success rate was attained when bombarded with two separate plasmids, which increases to 90% when comprising both the plasmids. The gene for octadecanoid-responsive *Catharanthus* AP2/ERF-domain (*ORCA3*), a jasmonate-responsive APETALA2 (APs)-domain transcription factor from *C. roseus*, has been introduced by particle bombardment and over-expressed in *C. roseus* cell suspension cultures subsequent to elicitation with MJ (van der Fits and Memelink 2000; Memelink et al. 2001). The procedure resulted in improved expression of quite a few metabolite biosynthetic genes that, accordingly, enhanced the accumulation of TIAs. However, the biosynthetic genes, geraniol 10-hydroxylase (*G10h*) and acetyl-CoA 4-O-deacetylvindoline 4-O-acetyltransferase (*Dat*), were not induced, signifying that ORCA3 does not control these genes. A research was conducted by Collu et al. (2001) on molecular cloning and expression of G10h, a P450 enzyme responsible for biosynthesis of iridoid monoterpenoids and numerous types of monoterpenoid alkaloids. Transformation by particle bombardment was performed in *C. roseus* suspension cultures with a CaMV *35S-CYP76B6* gene sequence containing plasmid construct. CYP76B6 mRNA was detected in high levels and was not present in case of controls. Moreover, protein extract assays for G10 h activity in the transformed cell demonstrated the production of 10-hydroxygeraniol. Guirimand et al. (2009) reported the in situ localization of monoterpenoid indole alkaloid enzymes by means of particle bombardment-intervened GFP-fused cDNA transient expression in *C. roseus* cells. Standardization of transformation procedure was carried out in the initial phase to obtain the highest number of GFP-expressing cells. Afterward, the method was employed taking benefit of a variety of organelle markers as well as analyzing the subcellular localization of hydroxylmethyl butenyl 4-diphosphate (HDS) of MEP pathway. The environs of plastid-expressed HDS and ER-localized

G10h pointed to the possible role of inter-organelle exchange in preserving the metabolic flux in the TIA biosynthesis pathway. Most of the studies used CaMV 35S promoter to stimulate the over-expression of various transgenes. Two additional promoters were also investigated in *C. roseus*, the glucocorticoid-inducible promoter (GIP) (Hughes et al. 2002) and a deletion derivative of the 34S promoter (FMV 34S) (van der Fits and Memelink 1997).

5.8.4 Protoplast Transformation

Plant cells devoid of cell walls are the protoplasts that are extracted by mechanical or enzymatic methods. The cells of suspension culture can be genetically modified, just because of the totipotency of plant cells that can regenerate into a whole plant. Protoplast transformation has been conducted mediating bacteria and fungi (Katsumata et al. 1984; Ruiz-Díez 2002). The transmission of foreign genes into protoplasts can be carried out by various methods, i.e., direct transfer by treating the protoplast with chemical or mechanical techniques in which the DNA crosses the permeable cell membranes; distinctive protoplasts can be merged to share their genomes or by microinjecting the DNA into the protoplast or by means of *Agrobacterium* (Hasezawa et al. 1981; Okada et al. 1985). However, the strategy is extremely painstaking and lengthy, mainly the exclusion of cell walls and manipulation of protoplast as well as the following regeneration phase.

5.9 Short-Term Conservations by Synthetic Seed Production

In order to store and exchange the germplasms of several medicinal plants, synthetic seed production via alginate encapsulation of explants has been successively employed (Gantait et al. 2015a). Research in plant biotechnology has given an unconventional and greatly progressing gift in the form of artificial seed technology. In the current times, threatened and economically important plant species can be easily maintained, protected, and carried as well due to which this technique has gained much popularity (Gantait et al. 2015b). Cost involvement is less since any meristematic tissue like SEs, STs, and nodes can be employed for significant propagation and transport. The genetic modification of plants, their breeding and propagation for pharmaceutical purposes infer a vital scope for research; artificial seed program gathers importance regarding appreciable propagation of explants being exchanged between laboratories and nurseries (Gantait et al. 2017). Moreover, during ex vitro conservation, lethal cryoprotectants cannot harm the plant sample as it is encapsulated thus minimizing post-storage damages. But when it comes to *C. roseus*, this well-known technique has not been applied much, exception being a single report by Maqsood et al. (2012), who effectively encapsulated SEs obtained from hypocotyl region. This purpose was served using various combinations of calcium chloride and sodium alginate for encapsulation resulting in bead formation with 100 mM calcium chloride and 2.3% sodium alginate giving a perfect result.

MS medium supplemented with 1.1. μM BA and 1.3 μM NAA acid yielded higher seedling rate of 84% from encapsulated embryos and did not lose germination capabilities when stored efficiently at 4 °C for 10 weeks. Following a period of 30 days of storage, frequency became 81% that kept on decreasing with prolonged time period.

5.10 Long-Term Conservations by Cryopreservation

Cost-effective germplasm conservation over longer time period is possible only by the single technology available, known as cryopreservation, which involves storing of plant materials in liquid nitrogen at −196 °C, in a sustainable inactive condition, during which no physical modifications can occur as cell division and metabolism remain stagnant (Gantait et al. 2016b). The major benefit of this technique is decrease in contamination, space requirement, somaclonal variation, and *in vitro* culture costs. The freezing effect of dimethyl sulfoxide (DMSO) and sorbitol on cells of *C. roseus* was conducted by treating them singly or with combination of both sorbitol and DMSO using differential thermal analysis and nuclear magnetic resonance (Chen et al. 1984). When DMSO or sorbitol was added to the liquid growth medium, the resulting temperature range from initiation to completion process of ice crystal formation was significant. In case of DMSO-treated cells, water crystallization was low when compared with control, at under −30 °C. Sorbitol-treated cells also gave same results except that water crystallization rate was low at below −25 °C. Silent relationship was observed between percent cell survival upon 1 h of freezing in liquid nitrogen and percent of unfrozen water at −40 °C. Hence, it seems that for an effective cryopreservation of *C. roseus* suspension cultures, water at −40 °C was critical as both sorbitol and DMSO together prevented water from freezing. Fatima et al. (2009) established a protocol for efficient cryopreservation of *C. roseus* embryogenic cell suspension cultures involving method based on vitrification wherein, before their immersion in liquid nitrogen, embryogenic cells were exposed to a medium containing preculture/pretreatment. Upon comparing at different concentrations of sorbitol (0.2–0.6 μM) and sucrose (0.09–0.6 μM), 0.4 μM sucrose stimulated maximum cellular regrowth during preculture. Also, treatment using 5% DMSO and 0.4 M sucrose gave maximum cell colony number (10.06 ± 0.55) subsequent to preculture of cryopreserved cultures. Callus regrown in medium containing 5.37 μM NAA and 6.62 μM BA resumed usual growth producing SEs similar to those from non-frozen embryogenic cultures. All plantlets regenerated from such SEs displayed normal morphology.

5.11 Conclusions and Future Prospects

The biotechnological improvement of any pharmaceutically important plant species should be demarcated in terms of its enhanced regeneration rate and increased accumulation of particular pharmaceutically active metabolites. On this account, considerable advancement has taken place in the establishment of *in vitro* regeneration

systems of *C. roseus* in the earlier decades. Several successful protocols are at present obtainable for the development of *in vitro* cultures and subsequent regeneration into whole plants that can provide consistent amount of plant material for round-the-year mining of pharmaceuticals. Nonetheless, future research in several fundamental areas is needed to be addressed considering the prospect of this plant species. The upcoming study on *C. roseus* may comprise the selection of elite germplasm line and biochemical as well as molecular depiction of biosynthetic pathways of the compounds of interest. Further, research on the standardization of bioreactors has enormous utility in the large-scale production of secondary metabolites that are synthesized in low quantities. Additional research is also desirable to discover apposite elicitors to augment the bioactive ingredients in cell suspensions and revelation of the chief aspects accountable for their biosynthesis. Moreover, over-expressing the single gene to improve the production of secondary metabolite is another significant facet of metabolic engineering. This step may bring about boosting the enzymes involved in the process of metabolism and accretion of the target compound. In addition, cryopreservation technology has to be standardized in every possible manner so that the germplasm can be conserved. Even though numerous genetic transformation researches have been conducted, there is a prospect to develop transgenic *C. roseus* plants to increase the accumulation of vincristine and vinblastine. Lastly, the standardization of the available genetic modification procedures, or creation of new methods, will be a significant aspect for manipulating secondary metabolite pathways, which will help to develop genetically modified medicinal plants with superior yielding capacity for active metabolites.

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Medicinal Plants as a Potential Source of Chemopreventive Agents

6

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Abstract

Cancer is one of the most common deadly diseases and remains as one of the leading causes of death worldwide. High mortality and incidence make it an important public health and economic issue which requires an effective prevention. Radiotherapy, immunotherapy, and chemotherapy are the most common methods used for the treatment of cancer, but these techniques adversely affect the healthy cells. Thus, inhibition of damaging behavior to the healthy tissues with the use of commonly used therapies motivates to explore the new safe methods to treat cancer. Chemoprevention is a novel approach to control cancer; it involves utilization of specific natural or synthetic agents to suppress, prevent, or reverse the malignancy before development of invasive cancer. Products from natural sources such as medicinal plants and herbs and their phytocompounds including flavonoids, alkaloids, carotenoids, etc. show protective effects against various forms of cancers. Chemoprevention of cancer using phytocompounds may be one of the feasible approaches to control this disease. An herbal medicine which refers to the medicinal product of plant stem, roots, leaves, bark, seed, flower, and fruit is one of the best sources for extraction of chemopreventive agents as they are nontoxic in nature and cause less or no side effect to the patients. Consumption of chemopreventive agents containing phytocompounds shows an important insight to fight against cancer and yield promising outcomes. The aim of this chapter is to provide an overview of various scientific evidences that support the utilization of medicinal plants and chemopreventive agents for the treatment of cancer.

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6.1 Introduction

Human health is always a major area of concern for the society as it is well affected by the changing environment and food habit. Due to these changes, many new diseases as well as disorders are noticed from the past decades, and, as a consequence, concern over human health is increasing day by day. Cancer is one of the major diseases resulting from bad habits of human beings, especially the consumption of carcinogen-containing items such as tobacco and tobacco-derived products. Cancer development involves the alteration of epigenetic processes and their deregulation (Baxter et al. 2014). Control of hypermethylation of tumor suppressor genes on CpG islands is deregulated in cancer cells, due to which gene silencing and tumor suppressor gene inactivation occur. Based on the animal model studies, cancer is a multistep process, which includes origination, development, and advancement. In the first phase, the cancer-causing agent binds with DNA and produces a mutation. A molecular change depends upon the type of cancer-causing agent's metabolism and reaction of DNA repair activity. The proliferation of cancer-initiating cells or abnormal cells occurs during the promotion phase (Tsao et al. 2004). In progression phase, phenotypic and genetic changes occur, and it is the phase between premalignant lesion and invasive cancer development (Owuor and Kong 2002; Oshima 2002). In carcinogenesis, genetically distinct premalignant and malignant lesions occur within the whole region, which is exposed to carcinogens. One of the examples of this exposure is the carcinogenic effects of tobacco in the lungs and upper aerodigestive tract (Rabi and Gupta 2008).

In spite of worldwide efforts to combat cancer in the last few years by early diagnosis and prevention, cancer is still one of the leading causes of death. It is very difficult to figure out the specific cause for cancer. However, tobacco and liquor consumption, noxious waste of environment, contagious agents, traditional practices, and lifestyles are some commonly known reasons responsible for this disease (Sultana et al. 2014). High death rate associated with cancer requires better treatment for this deadly disease, which necessitates the search for novel factors to fight with this disease and it could be possible with the use of naturally occurring compounds. The high mortality rate due to cancer (one in six deaths) and incidences make it an important public health and economic issue, which requires effective prevention. Cancer has become the second principal cause of sacrificing human life globally.

According to World Health Organization, 8.8 million cancer cases were detected in 2015. This figure is expected to rise to 24 million by 2035. Cancer cases were noticed more in developed countries (1.8 times higher), compared to developing countries (Ferlay et al. 2013). According to the American Cancer Society and World Health Organization fact sheets, February 2015 (www.who.int), cancer has been

evidenced by 14 million new cases and 8.2 million deaths due to cancer in 2012. Earlier, cancer has been known to affect more in developed countries; the occurrence of numerous kinds of cancer is presently speedily escalating globally (Siegel et al. 2016). In 2017, almost 13% of all types of cancer were detected in US mature people (cancer-facts-figures 2017). Lung, prostate, colorectal, stomach, and liver cancer are the major five types of cancer in man, while in the female the major types of cancer are breast, colorectal, lung, cervix, and stomach cancer. Cervical cancer is a major type of malignancy since it is not diagnosed in early stage (Monsonego et al. 2004). Gastric adenocarcinoma is the second prominent reason of cancer mortality worldwide. Colon cancer and liver cancer are also the most important reasons of frequent mortality (Pool-Zobe 2005). In urban populations of developed nations, liver cancer, ovarian cancer, pancreatic cancer, prostate cancer, and skin cancer are consistently imperative or causative factors of recurrent death rate (Burges and Schmalfeldt 2011). Elevated ratio of body mass index, less fruit and vegetable consumption, not having physical exercise, and tobacco and liquor consumption have been found as the major lifestyle errors for the development of cancer. Among these errors, tobacco use has been found as the most deadly activity, which is responsible for 20% of worldwide cancer deaths and 70% of total lung cancer cases (WHO 2017). Radiotherapy, immunotherapy, and chemotherapy are the most common methods used for the treatment of cancer, but these techniques adversely affect the healthy cells (Kopeina et al. 2017).

Nature represents an attractive source of potential therapeutic agents derived from the tremendous diversity of chemicals found in millions of species of plants, animals, and microorganisms. The endless diversity of the plant and animal kingdom presents an extraordinary opportunity to develop novel anticancer drugs. There has been an unparalleled growth in the plant-derived products in healthcare. Isolation and identification of plant- or animal-derived compounds with pharmacological activities continue to expand, particularly in the discovery of cancer chemotherapy drugs (Gullett et al. 2010). In this scenario, development of novel anticancer drugs from natural resources may increase the efficacy of conventional chemotherapeutic drugs (Liberio et al. 2013). Plant extracts and animal toxins are known to be potent bio-resource and therapeutic agents against numerous kinds of cancer. Toxins of many animal species (scorpion, toad, snake, frog, etc.) and their active constituents like protein and nonprotein toxins, enzymes, and peptides have shown therapeutic potential in the treatment of cancer (Gomes et al. 2010). Currently, more than 50% of recent medications in clinical use are acquired from plant products (Newman and Cragg 2012). Plants provide food, shelter, clothing, and medicine. Herbal medicine provides opportunities for the treatment of cancer. The global market for herbal medicines currently stands at over US$ 60 billion annually, and it is growing steadily (Gunjan et al. 2015).

Herbs are a plant or part of the valuable plants and have potential medicinal values. Herbs contain various chemical compounds that act upon the body, and herbal preparations are called phytomedicine or phytopharmaceuticals. Herbal remedies are effective due to its easy availability and lower cost. Medicine from herbal origin is one of the oldest forms of healthcare known to mankind and was an integral part

of the development of modern civilization. Medicinal uses of plants have been developed through the observations of wild animals and by trial and error process. Many of the drugs that are commonly used today for the treatment of various diseases are of herbal origin. Some are made from the plant extracts, and others are synthesized to mimic the natural plant compound. Advantages of traditional medicine utilization are that it is more reasonable and reduces the fear about the adverse effects of synthetic medicines. The major utilization of herbal medicines is for the promotion of health and to treat chronic diseases. However, consumption of herbal medicine increases when conventional medicine is ineffective in the treatment of disease, such as in the case of advanced cancer (Cohen and Ernst 2010). Demand for plant-based products is increasing in both developed and developing countries due to their advantages over synthetic drugs. Medicinal plants play a key role not only as traditional medicines but also as trade commodities. Nowadays, there is an increased interest in herbal-based medicines due to the increasing realization of the potential health hazards associated with the indiscriminate use of modern chemically derived medicines.

Chemoprevention is a new move to defeat cancer, which demands utilization of precise natural or man-made agents to suppress the malignancy prior to an incurable form of cancer (Rabi and Gupta 2008). Consumption of chemopreventive agents containing phytocompounds shows an important insight to fight against cancer and yield promising outcomes. A number of anticancer compounds including Taxol, vincristine, vinblastine, camptothecin derivatives, irinotecan, topotecan, and etoposide obtained from epipodophyllotoxin are in experimental mode worldwide. Few potential agents like roscovitine, flavopiridol, betulinic acid, combretastatin A-4, and silvestrol are in developmental phase in laboratories (Shoeb 2006). Accurate dosage is a vital factor in cancer prevention and treatment. The aim of this chapter is to provide an overview of various scientific evidences that support the utilization of medicinal plants and chemopreventive agents for the treatment of cancer.

6.2 Medicinal Plants with Anticancer Potential

Plants have always been a basis for the traditional medicine systems, and they have provided continuous remedies to mankind for thousands of years. Therapeutic potential of plants is based on the findings of thousands of years of their use. Medicinal plants are appraised as a repository of various bioactive compounds and used for a long time for their therapeutic properties. Therapeutic potential of medicinal plants includes antitumor, antiviral, anti-inflammatory, and antimalarial activity. Knowledge of the medicinal plants for the preparation of various drugs has been of great significance. Recently, scientific interests in the study of plants as a source of new compounds for use in therapy against cancer have increased considerably. The National Cancer Institute, USA, has approximately screened 35,000 plant species for their anticancer activities, and about 3000 plant species have demonstrated reproducible anticancer activity (Desai et al. 2008).

Different plant extracts are proven for its biological activities which include antidiabetic, free radical scavenging, anti-inflammatory, and anticancer (David et al. 2016). Medicinal plants with their anticancer properties have been recognized for centuries. Medicinal plants such as *Combretum paniculatum*, *Pupalia lappacea*, *Petersianthus macrocarpus*, *Ruta graveolens*, *Cordyla madagascariensis*, *Withania somnifera*, *Centella asiatica*, *Picrorhiza kurroa*, and many more are known for their anticancer activities (Sowemimo et al. 2009; Hou et al. 2008; Fadlalla et al. 2011; Sehrawat et al. 2016; Roy et al. 2016; Roy and Bharadvaja 2017). They comprise a large collection of polyphenolic components that have shown to inhibit carcinogenesis. Numerous phytochemicals derived from plant resources interfere with specific stages of carcinogenesis.

Medicinal plants play a potential role in revealing the novel drug molecules, which can be utilized for the development of new therapeutic drugs targeting a particular disease. At present, most of the drugs are acquired from the medicinal plants, for example, morphine from the dried latex of unripe seedpods of *Papaver somniferum*, atropine from *Atropa belladonna*, and reserpine from *Rauvolfia serpentina* (Mondal et al. 2016). Likewise, many such plant secondary metabolites possess therapeutic potential, and, thus, medicinal plants serve as a reliable source of modern drugs. Generally, compounds derived from the plants are more tolerated and nontoxic to the normal human cells. Several studies have been done on naturally occurring compounds known to possess cytotoxicity effects, as they display a potential to destroy cancer cells. Anticancer activity of numerous plants is still being researched actively, and some of them showed promising results. Below we have discussed a list of plants showing the potential anticancer activity (Table 6.1).

6.3 Chemoprevention: A Measure to Cure Cancer

Various measures to cure cancer are being adopted based on its state such as surgery, radiation therapy, hormonal therapy, chemotherapy, and immunotherapy (Baskar et al. 2012). These therapies have adverse effects on the healthy cells. Therefore, an alternative measure for the prevention of cancer is either avoiding lifestyle-related risk factors such as high-fat-containing diet, smoking habit, and foods containing carcinogen or taking chemopreventive agents (Shukla and Gupta 2005; Gupta 2006). The latter alternative may be more practical as chemopreventive agents can be used as supplements (Rabi and Gupta 2008). Chemoprevention is a major therapy used in cancer treatment. It interferes with the carcinogenic process by hindering the neoplastic process induction or stopping transformed cells from developing to malignant phenotypes (Sporn et al. 1976; Sporn and Hong 1997). Sporn and Hong (1997) have coined the term chemoprevention to differentiate it from chemotherapy. Chemoprevention is mainly related to stopping the development of cancer, while chemotherapy deals with the management of already established cancer. Based on the idea of prevention is better than cure, major research is being conducted on the development of new chemoprevention agents. Chemoprevention involves the utilization of either natural or synthetic compounds

Table 6.1 Plant extracts showing anticancer activity

Cancer type	Scientific name	Plant family	Parts used	Solvent extract	Mechanism of action	References
Breast	*Trianthema portulacastrum*	Africa and North and South America	Leaves	Ethanol	Suppressed cell nuclear antigen proliferation and cyclin D1 expression, induced apoptosis, downregulated anti-apoptotic protein Bcl-2, upregulated pro-apoptotic protein Bax, and diminished nuclear and cytosolic β-catenin expression in mammary tumors	Bishayee and Mandal (2014)
	Eclipta alba	Asteraceae	–	Chloroform fraction	Extract activates the intrinsic apoptotic pathway and upregulated Hsp60	Arya et al. (2015)
	Perovskia abrotanoides	Lamiaceae	Flower	Hydro-alcoholic	Extract inhibited the growth of MCF-7 partly by inducing apoptosis	Geryani et al. (2016)
	Chamaemelum nobile	Asteraceae	Arial parts	Ethyl acetate fraction	Ethyl acetate fraction activated mitochondrial death pathway via the involvement of G2/M phase arrest and induces apoptosis in MCF-7 cells	Kandelous et al. (2016)
	Allium wallichii	Alliaceae	Whole plant	Aqueous ethanol	IC_{50} 55.29 μg/ml was effective for MCF-7 breast cancer cell lines	Bhandari et al. (2017)
	Thymus alternans	Lamiaceae	Arial parts	–	Essential oils exhibited antiproliferative activity against MDA-MB-231 cells	Dall'Acqua et al. (2017)
	Paramignya trimera	Rutaceae	–	Methanol	Exhibited antiproliferative activity against MCF-10A	Nguyen et al. (2017)
Cervical	*Petersianthus macrocarpus*	Lecydaceae	Leaves	Alcohol	It contains flavones, tannins, alkaloids, terpenoids, steroids, saponosides, and glycosides that show antiproliferative activity on colon cancer cells	Line-Edwige et al. (2009)
	Cissus debilis	Vitaceae	Stem	Alcohol		

	Celosia trigyna	Amaranthaceae	Whole plant	Ethanol	It shows cytotoxic activity toward cancer cells and also induces apoptosis	Sowemimo et al. (2009)
	Pupalia lappacea					
	Alternanthera sessilis					
	Justicia extensa	Acanthaceae	Whole plant	Ethanol		
	Hedranthera barteri	Apocynaceae	Leaves	Ethanol		
	Ethulia conyzoides	Asteraceae	Leaves	Ethanol		
	Ruta graveolens	Rutaceae	Leaves	Methanol	Extract dose dependently reduced the viability and clonogenicity of treated cells and induced G_2/M halt, abnormal mitoses, and caspase-3 stimulation. It also induced the p53 pathway	Fadlalla et al. (2011)
	Anthocephalus cadamba	Rubiaceae	Leaves	–	ACALK, the isolated fraction of the plant, effectively protected plasmid pBR322 DNA from damage caused by hydroxyl radicals and induced cell death in HeLa cells via apoptotic mode	Chandel et al. (2016)
	Phyllanthus glaucus	Euphorbiaceae	Leaf, flower	Alcohol fraction	Alkaloid fraction inhibited HeLa cell growth with IC_{50} value of 25.46 ± 1.79 µg/ml. Mitochondrial pathway is involved in the programmed cell death	Stefanowicz-Hajduk et al. (2016)
	Allium wallichii	Alliaceae	Whole plant	Aqueous ethanol	IC_{50} 46.51 µg/ml was effective for HeLa cervical cancer cell lines	Bhandari et al. (2017)
	Thymus alternans	Lamiaceae	Flowering aerial parts	–	Essential oils exhibited antiproliferative activity against HCT 116 cell lines	Dall'Acqua et al. (2017)
Liver	*Cannabis sativa*	Cannabaceae	Flowering tops of the plant	Methyl alcohol	Exert antitumor effects by the direct induction of apoptosis and can decrease telomerase activity by inhibiting the expression of the TERT gene	Hussein et al. (2014)

(continued)

Table 6.1 (continued)

Cancer type	Scientific name	Plant family	Parts used	Solvent extract	Mechanism of action	References
	Stemona collinsiae	Stemonaceae	Whole plant	Methanol	It shows antiproliferative activity on HepG2	Manosroi et al. (2015)
	Gloriosa superba	Colchicaceae	Whole plant	Methanol		
	Ventilago denticulata	Rhamnaceae	Whole plant	n-hexane fraction		
	Allium wallichii	Alliaceae	Whole plant	Aqueous ethanol	IC_{50} 69.69 µg/ml was effective for prostate cancer cell lines (PC-3)	Bhandari et al. (2017)
	Tripterygium wilfordii	Celastraceae	Roots	Dimethyl sulfoxide	Treatment failure of prostate cancer is sometimes due to bone metastasis. Pretreatment with celastrol (8 µmol/l) inhibits tumorigenicity of PC-3 cells and thus prevents bone invasion	Kuchta et al. (2017)
Lung	*Fritillaria ussuriensis*	Liliaceae	Bulbs	Chloroform and n-hexane	It shows cytotoxic activity toward cancer cells A549 and induces apoptosis	Wang et al. (2015)
	Chlorella sorokiniana	Chlorellaceae	Whole plant	Aqueous	Induces mitochondrial-mediated apoptosis in non-small cell lung cancer cells via downregulation of Bcl-2, XIAP, and survivin	Lin et al. (2017)
	Salvia miltiorrhiza	Lamiaceae	Roots	Methanol extract	Inhibits human non-small cell lung cancer proliferation and induced both early and late apoptosis. It induced a G2/M phase arrest, increased expression of p53 and p21, and activated caspase-3/9 and PARP1	Ye et al. (2017)

Prostate	*Ailanthus excelsa*	Simaroubaceae	Root barks	Chloroform	It shows anticancer property against wide range of cancer cell lines of diverse origin. It enhances tumor suppressor proteins p53 and p21 expression, decreases *c-Myc* (oncogene) expression, and downregulates cdk4 and cyclin d1. Furthermore, it may result to phosphorylation of p53 in B16F10 tumors	Lavhale et al. (2009)
	Paramignya trimera	Rutaceae	Leaves	Methanol	Exhibited antiproliferative activity against Du145	Nguyen et al. (2017)
Ovarian	*Paramignya trimera*	Rutaceae	Leaves	Methanol extract	It shows potent antioxidant activity, while the powdered PTL extract exhibited great antiproliferative capacity for A2780 (ovarian) cells	Nguyen et al. (2017)
	Fritillaria ussuriensis	Liliaceae	Bulbs	Chloroform and n-hexane	Purified total alkaloids of the plant induce cytotoxic activity against A2780 cell lines by inducing G0/G1phase arrest of A2780 cells	Wang et al. (2015)
	Emblica officinalis	Euphorbiaceae	Fruits	Powder of fruit dissolved in water	It significantly increases the autophagic proteins beclin1 and LC3B-II expression. It also reduced several angiogenic gene expression, which includes hypoxia-inducible factor 1α (HIF-1α) in OVCAR-3 cells	De et al. (2013)
	Rauwolfia vomitoria	Apocynaceae	Roots	Ethanol	Rau decreased growth of cell in OVCAR-5 and induces apoptosis in OVCAR-8 cell lines	Yu et al. (2013)
	Cordyla madagascariensis	Fabaceae	Fruits	Ethanol	Ethanol extract prevents the growth of protein kinase C through G_2 phase arrest and escalates the p21Waf1/Cip1, p27Kip1, and p15INK4B, a strong inhibitor of the cdk1	Hou et al. (2008)

(continued)

Table 6.1 (continued)

Cancer type	Scientific name	Plant family	Parts used	Solvent extract	Mechanism of action	References
	Elaeodendron sp.	Celastraceae	Wood	Ethanol	Extract (Mg 3232) exhibits significant antiproliferative property against the ovarian cancer cell (A2780). Mg 3232 is having cardenolides that can promote the apoptosis and G_1 level cell arrest	Cao et al. (2007c)
Pancreatic	*Mangifera indica*	Anacardiaceae	Leaves	Ethanol	It shows cytotoxic activity against pancreatic α-amylase	Ganogpichayagrai et al. (2017)
	Alternanthera sessilis	Amaranthaceae	Leaves	Methanol	It shows cytotoxic activity toward cancer cells and also induces apoptosis	George et al. (2010)
				Methanol		
	Amoora chittagonga	Meliaceae		Petroleum ether		
		Burseraceae				
	Bursera serrata					
Renal cell	*Soymida febrifuga, Tinospora cordifolia, Lavandula bipinnata, Helicteres isora*	Meliaceae, Menispermaceae, Lamiaceae, Malvaceae	Whole plant	Ethanol	It shows cytotoxic activity toward cancer cell line (PN-15)	Shaikh et al. (2014)
	Nigella sativa	Ranunculaceae	Seeds	Hydro-alcohol	It showed apoptosis of normal renal epithelial (GP-293) and human renal adenocarcinoma cell lines	Shahraki et al. (2016)
Skin	*Daucus carota*	Apiaceae	–	Oil extract	Significant reduction in tumor	Zeinab et al. (2011)
	Rosmarinus officinalis	Labiatae	–	Aqueous	Effective in preventing cutaneous photo damage induced by UV radiations	Martin et al. (2008)

Stomach	*Urtica dioica*	Urticaceae	Leaves	Hydro-alcohol	Plant extract inhibited proliferation of gastric cells (MKN45) and induced concomitant increase of apoptosis	Ghasemi et al. (2016)
	Astragalus membranaceus	Fabaceae	Roots	Methanol	It inhibited BGC-823 cell proliferation and human gastric cancer cell growth	Wang et al. (2013)
	Zingiber officinale	Zingiberaceae	Rhizome	Ethanol	*Zingiber officinale* prevents production of inflammatory cytokines, nitric oxide, prostaglandin synthase, and arachidonate 5-LOX in a dose-dependent manner. It also constrains the leukotriene production from COX-1 and COX-2 and LOX	Plengsuriyakarn et al. (2012)
	Atractylodes lancea	Compositae	Rhizome	Ethanol	The complex 2-[(2′E)-3′, 7′-dimethyl-2′, 6′-octadienyl]-4-methoxy-6-methylphenol extracted from the *Atractylodes lancea* twig displays potent inhibitory effects on 5-LOX and COX-1, responsible for arachidonic acid metabolism. It also decreases tumor size and also stops cancer metastasis	
	Angelica sinensis	Umbelliferae	Roots	Ethanol	Ethanol fractions show anti-inflammatory consequence by NF-κβ-dependent suppression	
	Cyathula uncinulata	Amaranthaceae	Leaves	Methanol	It shows cytotoxic activity toward cancer cells and also induces apoptosis	George et al. (2010)
	Hypoxis latifolia	Hypoxidaceae	Roots	Methanol		
	Lantana camara	Verbenaceae	Fruits and flowers	Ethyl acetate fraction		

to retard inhibition and reverse the progression of cancer in normal and preneoplastic conditions (Pezzuto 1997).

The aim of chemopreventive agents is to prevent DNA damage by free radicals, block mutagenic carcinogens, modulate specific steps in the carcinogenic process, suppress epithelial cell hyper-proliferation, and modulate epithelial cell differentiation and apoptosis. Developing a new class of chemopreventive agents from fruits, vegetables, teas, spices, herbs, and medicinal plants is an emerging concept. The most important requirement in the field of chemoprevention is to develop a novel agent to prevent cancer with fewer or negligible side effects. Although prior observations lead to several successful preclinical trials, only a restricted count of clinical trials have been able to fully implement dietary phytochemical for cancer prevention. Mechanisms of dietary agents to interact with cancer cells, the immune system, and oxidative stress pathways play an important role to discover safe and nontoxic anticancer therapeutics (Kotecha et al. 2016). The main goal of administering a chemopreventive agent is to prevent the development of cancers. An ideal chemopreventive agent should be safe, inexpensive, and effective in the prevention of cancer without compromising the life quality (Gupta 2006). A list of most archetypal cancers in human and their corresponding anticancerous compounds along with the proposed dosage is mentioned in Table 6.2. Also, the information related to the chemopreventive properties of plant phytochemicals is mentioned briefly.

6.4 Plant Sources as Anticancer Drugs in Clinical Study

Plants have been used as the main source of drug preparation for a long time to cure various human health problems. Plants play an important role in the discovery and development of novel drugs. Search for the plant-derived anticancer agents started in the 1950s with the discovery of vinblastine and vincristine. These two agents were discovered during the investigation of the plant as a potential source of oral hypoglycemic agents, and these two compounds were first to enter into clinical trials. In 2005, it was estimated that the worth of plant-derived drugs was $18 billion and the USA accounts for the 50% of the global plant-derived drug market (Saklani and Kutty 2008).

Clinical trials are important for the discovery of new treatment or new way to detect, diagnose, and reduce the disease risk. It helps the researchers to find out what does and does not work in a human being. On an average it takes 10–12 years for a drug to enter from design to clinical trials. Clinical trial phases include preclinical studies and phase I–IV trials. In the preclinical studies step, it is decided whether a drug is ready for the clinical trials or not, and its preliminary studies like toxicity, efficacy, safety, and pharmacokinetic information are evaluated. Further the drug undergoes various phases of clinical trials like phase I, phase II, phase III, and phase IV (https://www.profil.com/knowledge-center/trial-stages). Benefits of clinical trials include the access of new treatment or drug for the various diseases. Clinical trials help in the identification of possible risks and benefits of a treatment (https://www.nhlbi.nih.gov/studies/clinicaltrials/benefitsrisks). Clinical trials also have some drawbacks; for example, new treatments or strategies being studied are

Table 6.2 Chemopreventive agents from medicinal plants

Cancer type	Chemopreventive agents of plant origin	Plant	Parts used	Dosage	Mechanism of action	References
Breast cancer	Eudesmane	*Parasenecio roborowskii*	Aerial parts	303.2 μM	It possesses cytotoxic activity	Pang et al. (2017)
	Curcumin	Curcuma longa	Roots	–	It exhibits anti-breast cancer activities by regulation of matrix metalloproteinase (MMP)-2, B-cell lymphoma2 (Bcl-2), Bax, flap endonuclease 1 (Fen1), NF-E2-related factor 2 (Nrf-2) factors, and phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) signaling	Lv et al. (2014)
	Quercetin-3-O-glucuronide (Q3G)	*Nelumbo nucifera*	Leaves	0.1 μM	Q3G suppresses breast cancer cell invasion by controlling β2-adrenergic signaling and by suppressing the MDA-MB-231 breast cancer cell invasion and MMP-9 induction	Yamazaki et al. (2014)
	Sauchinone	*Saururus chinensis*	Roots	–	Controls VEGF, cyclin D1, Bcl-2, caspase-3, and the extracellular signal-regulated kinase (ERK) signaling pathway in breast cancer cells	Kim et al. (2011)
	Cucurbitacin B	*Trichosanthes cucumerina*	Fruit	–	It shows antiproliferative effects against SKBR3, MCF-7, T47D, and MDA-MB-435 cell lines	Kongtun et al. (2009)
	23,24-Dihydrocucurbitacin B	*Trichosanthes kirilowii*	Roots	–	Possesses anticancer activity against human breast cancer cell lines including Bcap37 and MCF-7	Yang et al. (2007)

(continued)

Table 6.2 (continued)

Cancer type	Chemopreventive agents of plant origin	Plant	Parts used	Dosage	Mechanism of action	References
Cervical cancer	Crocetin	*Crocus sativus*	Stigma	20 mg/Kg-40 mg/kg body wt.	Crocetin induces anti-inflammatory effect by suppressing the level of IL-1β and TNF-α as well as PMNs activity	Chen et al. (2015)
	Rhinacanthin-N	*Rhinacanthus nasutus*	Roots	17 μM	Rhinacanthin-N induces human cervical carcinoma (HeLaS3 cells) apoptosis. Rhinacanthin-treated cells partly arrest at G_2/M phase	Siripong et al. (2006)
Colon cancer	Isoliquiritigenin	*Licorice*	Roots	100 μg/ml	Isoliquiritigenin, a chalcone found in *Licorice* root, showed chemoprevention activity through phase II enzyme induction. It significantly induced quinone reductase-1 activity in the colon carcinogenesis	Cuendet et al. (2010)
	Falcarindiol	*Oplopanax horridus*	Roots barks	10 μM	Falcarindiol promotes cell arresting in G_2/M phase and prevents human colon cancer cell (HCT-116) proliferation by apoptosis induction at both earlier and later stage	Sun et al. (2010)
	Rotenolone	*Pongamiopsis pervilleana*	Roots barks	2.9 μM	Rotenolone demonstrates antiproliferative activity against colon cancer HCC-2998 cell lines. Rotenolone prevents proliferation of cell by apoptosis induction via p53 pathway	Harinantenaina et al. (2010)

	Ursolic acid and oleanolic acid	*Miconia fallax*	Aerial parts	–	Ursolic acid and oleanolic acid, extracted from *Miconia fallax*, are triterpenoid compounds which have antitumor activity against colon cancer cells, but its exact mechanism is not clearly understood	Furtado et al. (2008)
Liver cancer	Actein	*Actaea racemosa*	–	27 µg/ml	Actein reformed expression of fatty acid and cholesterol biosynthetic genes and p53 pathway genes. It moderates free fatty acid and cholesterol ingredients in the liver and prevents human HepG2 liver cancer cell growth	Einbond et al. (2009)
	Oridonin	*Rabdosia rubescens*	Flowers	10–50 µmol/l	Oridonin induces cytotoxic properties against cancer cells by an escalation in the apoptotic cell death and reactive oxygen species (ROS) generation. Cytotoxic effects of this compound include rise in the p53 as well as proteins associated with apoptotic expression	Huang et al. (2008)
	Ardisiacrispin (A + B)	*Ardisia crenata*	Fruits	1–10 µg/ml	Ardisiacrispin (A + B) is a combination of ardisiacrispins A and B and exerts cytotoxic potential against liver cancer cells (Bel-7402) via antiproliferative, pro-apoptotic, and microtubule disruptive actions	Li et al. (2008)

(continued)

Table 6.2 (continued)

Cancer type	Chemopreventive agents of plant origin	Plant	Parts used	Dosage	Mechanism of action	References
	Andrographolide	*Andrographis paniculata*	Leaves	–	Andrographolide inhibits IL-6 expression and IL-6-mediated signals. It causes cell apoptosis by the stimulation of mitogen-activated protein kinases including p38 kinase, c-Jun N-terminal kinase, and extracellular signal-related kinases	Ji et al. (2007)
	Furanodiene	*Curcuma wenyujin*	Oil	300 μM	Furanodiene obstructs HepG2 cell growth by triggering cell cycle halt at G_2 to M phase and encouraging apoptosis associated with mitochondrial transmembrane depolarization and stimulated the caspase-3 which is linked to the p38 activation and ERK1/2 MAPK signaling inactivation	Xiao et al. (2007)
	Kalopanaxsaponins A and I	*Nigella glandulifera*	Flowers	–	The cytotoxicity of two oleanane-triterpene saponins, kalopanaxsaponins A and I, was calculated against HepG2. They exert cytotoxic activity against liver cancer cells by pro-apoptotic and microtubule disruptive activities	Tian et al. (2006)
	Ganoderic acid	*Ganoderma lucidum*	Whole plant	50–500 mg/ml	Ganoderic acid shows effective in preventing the growth of BEL-7402 cells by preventing their transition from G_1 to S phase	Yang (2005)

	Lycopene	*Lycopersicon esculentum*	Fruits	–	Lycopene inhibits Hep3B cell growth in a dose-dependent fashion by mechanisms by comprising cell cycle arrest in the G_0/G_1 stage and DNA destruction. It also demonstrates anti-invasive and anti-migration activities against highly invasive SK-Hep-1 cells	Park et al. (2005)
	Cunabic acid	*Annona glabra*	Fruits	25 μmol/l	Cunabic acid stops human liver cancer (HLC) cell line SMMC-7721 development through S phase and affects arrest at the G_0/G_1 period. It also supports cell apoptosis by downregulating the *Bcl-2* gene expression and upregulating the *Bax* gene	Zhang et al. (2004)
	Asiatic acid	*Centella asiatica*	Leaves	–	Asiatic acid reduces the sustainability of HepG2 cells facilitated by an upsurge in intracellular calcium levels, which led to the increased tumor suppressor gene expression	Lee et al. (2002)
	Boswellic acids	*Boswellia serrata*	Leaves	–	Boswellic acids show antiproliferative activities against HepG2 cells, with apoptosis along with activation of caspase-3, caspase-8, and caspase-9	Liu et al. (2002)
Lung cancer	Paclitaxel	*Taxus brevifolia*	Bark	–	Microtubule disruptor; blocks mitosis; induces apoptosis; microtubules are polymerized and stabilized; disruption of spindle formation; inhibition of translational machinery	Che et al. (2015)

(continued)

Table 6.2 (continued)

Cancer type	Chemopreventive agents of plant origin	Plant	Parts used	Dosage	Mechanism of action	References
	Solamargine	*Solanum incanum*	Fruits	5.8 μM	Solamargine causes numerous apoptotic processes such as cytochrome c release and causes anti-apoptotic Bcl-xL and Bcl-2 downregulation, rise of caspase-3 activity, as well as DNA fragmentation. Thus, it can moderate the TNFRS and Bcl-2 expressions	Liu et al. (2004)
	Saikosaponin D	*Bupleurum umbellifera*	Roots	10 μM	Saikosaponin D withdrew A549 proliferation by inducing apoptosis as well as arresting cell cycle progress in the G_1 phase. It considerably amplified the p53 and p21/WAF1 expression, contributing to blocking of cell cycle and heightening in Fas/APO-1	Hsu et al. (2004)
	Hederacolchicoside A1	*Hedera colchica*	Leaves	4.5–12 μM	Hederacolchicoside A1 inhibits the cell proliferation by inhibiting the DNA synthesis of cancer cells	Barthomeuf et al. (2002)
	Lycobetaine	*Lycoris radiata*	Bulbs	1.3 μM	Lycobetaine inhibited topoisomerases I and II, stabilized DNA topoisomerase I intermediate, and induced apoptosis	
Prostate cancer	6-Acetyl-11-keto-β-boswellic acid (AKBA)	*Boswellia serrata*	Gum resin	1.68 μM/l	6-Acetyl-11-keto-β-boswellic acid (AKBA) suppresses tumor development in the human prostate tumor. It is an effective angiogenesis inhibitor and prevents numerous steps of VEGF (vascular endothelial growth factor), cell proliferation and also facilitates tumor angiogenesis	Pang et al. (2012)

	Neobavaisoflavone and psoralidin	*Psoralea corylifolia*	Seeds	50 μg	Neobavaisoflavone and psoralidin strikingly augment tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer cells	Szliszka et al. (2011)
	Phenols and terpenoids	*Cupressus lusitanica*	Root barks	>30 μg/ml	Extracted phenols and terpenoids are effective against DU145 prostate cells. They prevent cell explosion in dose-dependent manner via apoptosis initiation and cell halt at S phase	Mbaveng et al. (2011)
	Rotenolone	*Pongamiopsis pervilleana*	Roots	5.8 μg/ml	Rotenolone illustrates antiproliferative activity toward the prostate cancer DU 145 cell lines. It inhibits the cell proliferation by induction of apoptosis via p53 pathway and inhibiting 12-lipoxygenase	Harinantenaina et al. (2010)
	Ginsenosides and polyphenol compounds	*Panax ginseng*	Roots	–	Ginsenosides stimulated the different steroid hormone receptors. The antitumor properties of its ability to stimulate the cell death and effect against proliferation, invasion, and metastasis. The polyphenol prevents the growth and proliferation of tumors and also eliminates free radicals and active oxygen	Kim (2008)
	Plumbagin	*Plumbago zeylanica*	Roots	5 μM	Plumbagin promotes apoptosis, induces G_2-M arrest, produces reactive oxygen species (ROS), and also inhibits the PCA cell VEGF and MMP-9 expression	Aziz et al. (2008)

(continued)

Table 6.2 (continued)

Cancer type	Chemopreventive agents of plant origin	Plant	Parts used	Dosage	Mechanism of action	References
	Genistein	*Glycine max*	Seeds	11–54 mg/day	Genistein interrupts nutrient absorption of cancer cells inducing apoptosis which leads to a cancer cell proliferation reduction and angiogenesis	Kim (2008)
Ovarian cancer	Epipervilline	*Pongamiopsis pervilleana*	Roots	5.8 µg/ml	Epipervilline is a rich source of bioactive prenylated isoflavonoids and pterocarpans. It displayed an adequate antiproliferative action against human ovarian cancer cell line (A2780)	Harinantenaina et al. (2010)
	Genistein	*Glycine max*	Seeds	11–54 mg/day	Genistein restricts the nutrient absorption to cause apoptosis of cancer cells which leads to the decrease in the proliferation of ovarian cancer cell and angiogenesis	Kim (2008)
	Termicalcicolanones A and B	*Terminalia calcicola*	Leaves	14 µg/ml	Both of these compounds exhibited moderate action against the A2780 human ovarian cancer cell line and also promoted cell cycle arrest during S phase	Cao et al. (2007a)
	Guttiferones K and L	*Rheedia calcicola*	Fruits	15 µg/ml	Guttiferones K and L showed cytotoxicity toward the A2780 human ovarian cancer cell line and also stimulated antiproliferative activity by the induction of apoptosis	Cao et al. (2007b)

	Cassipourol and cassipouryl acetate	*Cassipourea madagascariensis*	Root and leaves	18.2 μg/ml	Cassipourol and cassipouryl acetate display moderate cytotoxic properties against the A2780 cell line. They induced apoptosis via caspase-8 and caspase-3 activation and reduction in expression of FAS-associated death motif and G_1 cell cycle arrest	Chaturvedula et al. (2006)
	Glaucolide M	*Vernonia pachyclada*	Leaves	3.3 μM	Glaucolide M reveals adequate cytotoxicity toward the A2780 cancer cell line and also stops tumor cell proliferation activity	Williams et al. (2005)
Pancreatic cancer	Xanthone V1	*Symphonia globulifera*	Leaves	20ug/ml	Xanthone V1 affects cell cycle distribution, caspase-3/7 activity, and apoptosis induction. It induces cell cycle arrest at higher concentration, and it also inhibits the growth of blood capillaries having negative effect on tumor promotion *in vivo*	Kuete et al. (2011)
	2-Acetylfuro-1,4-naphthoquinone	*Newbouldia laevis*	Roots	3.8ug/ml	2-Acetylfuro-1, 4-naphthoquinone induces apoptosis, without the activation of caspase-3/7. This also inhibits the blood capillary formation of cancer cells	

(continued)

Table 6.2 (continued)

Cancer type	Chemopreventive agents of plant origin	Plant	Parts used	Dosage	Mechanism of action	References
Skin cancer	Lupeol	*Mangifera indica*	Fruits	75 μg/ml	Lupeol can prompt apoptosis in a dose-dependent manner. Its activity is also related with caspase cell death pathway by the stimulation of Bax and Apaf1, reduction in Bcl-2 expression, and consequent cleavage of PARP. Lupeol usage is found to prevent cell existence by inactivation of NF-kβ through its inhibitor Ikβ upregulation	Prasad et al. (2009)
	6-O-(β-D-Glucopyranosyl)-1-O-octanoyl-β-D-glucopyranose and asperulosidic acid	*Morinda citrifolia*	Fruits	20 ng/ml	They are effective in EGF-stimulated cell transformation and related AP-1 activity suppression	Liu et al. (2001)
	Geraniin and corilagin	*Geranium thunbergii*	Leaves	43 mM for geraniin and 76 mM for corilagin	Geraniin and corilagin are investigated to stop the release of TNF-α	Okabe et al. (2001)
Stomach cancer	Curcumin compound	*Curcuma longa*	Rhizome	–	Curcumin has anti-cholangiocarcinoma properties. It causes the suppression of various stages of carcinogenesis (AP-1, transcription factor, and STAT3) and has capability to conquer pro-inflammatory pathways on COX-2	Plengsuriyakarn et al. (2012)

	Byakangelicin	*Angelica dahurica*	Flowers	–	Byakangelicin is purified from *Angelica dahurica* and causes the suppression of tumor necrosis factor-α, PGE2, and histamine release, via reduced COX-2	Plengsuriyakarn et al. (2012)
	Thymoquinone	*Nigella sativa*	Leaves	–	Thymoquinone promotes apoptosis in tumor cells by inhibiting the Akt activation, NF-κβ, and extracellular signal-regulated kinase signaling pathways and also obstructs tumor angiogenesis	

Table 6.3 Plant-derived anticancer drugs in clinical phases

Drug name	Activity	Phase	References
DHA-paclitaxel	Antimitotic agent: blocks cells in the metaphase	III	Newman et al. (2003)
Diflomotecan 100	Inhibitor of topoisomerase I	II	
Rubitecan		II	
Protopanaxadiol	Caspase-3, caspase-8, and caspase-9 stimulants	I	
Vinorelbine	Microtubule-destabilizing agents: bind to tubulin heterodimers	III	Anonymous (2004)
Roscovitine	CDK inhibitor	II	Butler (2005)
Exatecan mesylate	Inhibitor of topoisomerase I	III	Bradbury (2005)
Lurtotecan		II	
Meisoindigo	Induces apoptosis by locking STAT3 signaling	III	Letourneau et al. (2006)
Ortataxel	Antimitotic agent: blocks cells in the metaphase	II	
Homoharringtonine	Protein synthesis inhibition	II	Saklani and Kutty (2008)
Bruceantin	Inhibits peptidyl transferase elongation reaction	II	Georgaki et al. (2009)
Paclitaxel poliglumex	Antimitotic agent: blocks cells in the metaphase	III	

not always better than the current one, new treatments may have side effects that doctors don't know about, etc.

Kelloff et al. (1995) described the three broad approaches of clinical use of chemopreventive agents. This includes primary chemoprevention, secondary chemoprevention, and tertiary chemoprevention. Primary chemoprevention involves the administration of agents to the general healthy population with particular risk factor. Secondary chemoprevention involves the identification of individuals with premalignant lesions and administration of agents to prevent cancer progression. Tertiary chemoprevention involves the administration of agents to prevent the recurrence of cancers in individuals who have undergone successful early disease treatment (Steward and Brown 2013). Since the 1980s, several randomized clinical trials of chemoprevention have been undertaken. There have been some positive chemoprevention results in prostate and breast cancer and also some negative results in the case of colon and lung cancer. Plant-derived compounds which are in various phases of clinical trials are discussed in Table 6.3.

6.5 Conclusions and Future Prospects

Nowadays, there is an increased resistance of cancer to the current therapeutic agents which creates a problematic situation. Therefore, there is a need to discover new compounds that have potential to cure cancer and are safe. Conventional

therapies have several adverse effects on healthy cells. Varied biological and chemical diversity of the natural resources drives the interest of the scientific community for the development of novel anticancer drugs based on natural origin. Utilization of products that are derived from the medicinal plant for management of the carcinogenic process provides an alternative source for the treatment of cancer. Chemopreventive agents from natural source have gained importance in the field of drug development, because these compounds are often known to have less adverse effects. These agents directly modulate specific steps in the carcinogenic process, prevent DNA damage, block mutagenic carcinogens, or modulate epithelial cell differentiation and apoptosis. The evidence in this chapter suggests that plants can be used to lower cancer risk. Chemoprevention and treatment using natural phytochemicals have been an attractive approach. Natural dietary phytochemicals have been and will continue to be a promising and active research area in the near future. Natural compounds used in cancer chemoprevention are the natural analogs considered to enhance immune-modulatory as well as antioxidant activities and thereby have a tremendous potential to fight against various types of human cancers. These agents are relatively less toxic and have advantage over other synthetic agents. Clinical trials are important for the discovery and development of new treatment to detect, diagnose, and reduce the disease risk. Advantage of clinical trials includes the access of new treatment or drug for various diseases. Clinical trials help in the identification of possible risks and benefits of a treatment. These agents have emerged to be highly promising after preclinical safety and efficacy studies, but they have failed in human trials. Selection of specific chemopreventive agents as active compounds or whole plant extracts, dose, duration, and mode of application for particular cancers are very critical. Clinical trials also increase the risk of individuals included in the studies, and thus an alternative way could be the use of biomarkers. These are substances which indicate the presence of cancer in the body and are able to differentiate an affected person from an unaffected one. Biomarkers can be utilized for the assessment of patients in multiple clinical settings such as in screening primary cancers, estimating the disease risk, differentiating the benign from malignant, etc. Combinational therapy could be an alternative way to overcome this disease, and it combines two or more therapeutic agents to treat the cancer. It is the combination of anticancer drugs which enhance the efficacy as compared to the monotherapy method because it targets the key pathways. This approach reduces drug resistance and simultaneously provides the benefits of the anticancer agent such as reduction in tumor growth, arrest of mitotically active cells, reduction in cancer stem cells, etc. More research in the plant-derived compounds may result in the discovery of more potent chemopreventive agents. Chemoprevention could be a very effective way to reduce the risk of cancer in the future, the biomedical community needs to recognize and advocate approaches on clinical assessment of natural chemopreventive compounds for the prevention/treatment of different cancers in humans.

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Plant Metabolites as New Leads to Anticancer Drug Discovery: Approaches and Challenges

7

Sagar Satish Datir

Abstract

Cancer is one of the most life-threatening diseases widely affecting the mortality of the world's population. Anticancer therapies such as radiation treatment, chemotherapy, and surgery have gained limited success in cancer treatment, and therefore, there is a need to develop an alternative method to treat chronic diseases like cancer. Medicinal plants and plant foods such as vegetables and fruits are in use to treat human diseases since prehistoric times because of their health-promoting effects. Certainly, there is an increased public awareness regarding the use of medicinal plants in treating human diseases as they are promising source of drugs with minimal or no side effects. Plant metabolites from various parts of medicinal plants have attracted attention of researchers and pharmaceutical industries in treating cancer due to their indispensable use with reduced side effects. With the advent of techniques such as transgenics, bioinformatics, metabolomics, and nanotechnology, plant metabolites offer opportunities to anticancer drug discovery. The use of phytochemicals, computational approaches in drug design, the efficacy and efficiency, the mode of action, and further clinical studies might provide new avenues in cancer research. Plant-derived anticancer compounds are currently being used in clinical studies and are currently being investigated phytochemically to understand their antitumor actions against various cancers. The present chapter summarizes the information on anticancer medicinal plants and their derived phytochemicals or metabolites and also on the development of plant-derived substances to treat cancer.

Keywords

Alkaloids · Anticancer · Drug · Medicinal plants · Metabolites

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7.1 Introduction

Food and medicines are integral parts of human life. Besides their aesthetic and ornamental values, plants are an indispensable source of food and medicines. Plants have played a leading role not only in the development of traditional medicine systems but also in human civilization by providing food and shelter (Anand and Neetu 2011). Medicinal parts of plant used for development of drugs include flower, root/rhizome, stem, bark, leaf, fruit, seed, and whole plants. However, not all plants or parts are safe for health due to the presence of toxic compounds which show adverse effects in the body (Wink 2010). Therefore, the practical knowledge of identification of plants and proper understanding of the utilization of plant-derived products are essential. Plant-based food and pharmaceutical industries are gaining momentum in recent years with pharmaceutical industries occupying the prime position. On the other hand, due to changing lifestyle and unpredictable environmental conditions, the public health concerns about various health-related diseases become a major anxiety in both developing and developed countries. Both pharmaceutical industries and Ayurvedic practitioners are actively engaged to discover effective medicines to combat with various diseases. Plants are in use to treat human diseases since prehistoric times due to their health-promoting effects. The World Health Organization (WHO) estimates that approximately 80% of some Asian and African populations depend on traditional medicine in the form of herbals for primary health care. Herbal treatments are highly profitable in the international marketplace, and it has been estimated that the global market for herbal products is expected to reach $5 trillion by 2050 (Anand and Neetu 2011).

A number of infectious and parasitic diseases cause major threats to the health of millions of people throughout the world. Among them, cancer is one of the most life-threatening diseases widely affecting the mortality of the world's population and considered as the second leading cause of death after cardiovascular disease (Kaur et al. 2011). As the number of individuals living with cancer is expanding continuously, there is worldwide alarming rise in mortality rate which has geared up the discovery for effective anticancer agents (Khazir et al. 2014). There is major public health concern about cancer, and therefore, there is an increasing demand to develop alternative methods to treat this dreadful disease with no side effects. Although there is a constant demand for new therapies to treat and prevent cancer, due to their less toxic effects compared to current treatments such as chemotherapy, naturally derived compounds are gaining momentum in scientific research (Greenwell and Rahman 2015). One of the main disadvantages of synthetic drugs is associated side effects (Lahlou 2013). Such synthetic drugs are expensive and unaffordable to majority of people especially from developing countries. Moreover, due to the limited success of clinical therapies including radiation, chemotherapy, immunomodulation, and surgery in treatment of cancer (Dai and Mumper 2010), plant-derived natural products are becoming beneficial in combating cancer (Prakash et al. 2013). One of the major advantages of using plant-derived products over synthetic drugs is that natural products possess enormous structural and chemical diversity that cannot be matched by any synthetic libraries of small molecules and continue to inspire novel discoveries in chemistry, biology, and medicine (Shen

2015). Folashade et al. (2012) estimated about 25% of all modern medicines are directly or indirectly derived from higher plants demonstrating that plants have enormous medicinal potential in traditional medicine. Plant-derived products such as extracts, dry powders, and parts of plants, fungi, and algae have been used as complementary treatments alongside conventional drugs. Medicinal plants contain valuable substances such as secondary metabolites with therapeutic or beneficial effect in healing and prevention of various ailments in man and animals (Robinson and Zhang 2011). The present chapter summarizes the information on anticancer medicinal plants and their derived phytochemicals or metabolites and also on the development of plant-derived substances to treat cancer.

7.2 Plant-Derived Metabolites in Cancer Treatment

Bioactive compounds or plant-derived compounds can be defined as secondary metabolites causing pharmacological or toxicological effects in humans and animals. Plant-derived metabolites are organic compounds, classified as primary metabolites and secondary metabolites. Primary metabolites include glucose, starch, polysaccharide, protein, lipids, and nucleic acid which are beneficial for normal growth and development of the human body (Shakya 2016). Besides their primary biosynthetic and metabolite routes, these secondary metabolites are exclusively produced within the plants and are associated with plant growth and development and are regarded as products of biochemical "side tracks" in the plant cells and not needed for the daily functioning of the plant (Bernhoft 2010). Due to their antioxidant, anti-inflammatory, antitumor, antimutagenic, and anticarcinogenic activities, secondary compounds such as terpenoids, phenolic acids, lignans, tannins, flavonoids, quinones, coumarins, alkaloids, catechins, and isocatechins have been found to play an important role in the treatment of cancer (Nema et al. 2013; Kaur et al. 2011). These metabolites function in plants during various processes such as cellular signaling, photosynthesis, and biotic and abiotic stress conditions. While flavonoids protect against free radicals generated during photosynthesis (Kumar et al. 2013), Raghvendra et al. (2011) reported that anthocyanins are the potent antioxidants that inhibit chemically induced cancer.

Plant-derived products have a long history of use in the treatment of cancer that leads in the development of many anticancer drugs (Khazir et al. 2014). The application of plant-derived anticancer agents can be traced back to the 1950s (Prakash et al. 2013). The discovery and development of the very first anticancer agents are vinca alkaloids (vinblastine and vincristine) from *Catharanthus roseus* belonging to family Apocynaceae (Thingujam et al. 2015). Since then a number of plants were investigated for their potential anticancer activity, and more than 3000 plant species have been listed as anticancer plants (Kaur et al. 2011). The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity (Shoeb et al. 2006). Natural compounds isolated from medicinal plants are believed to be promising leads in the development of anticancer drugs (Rao et al. 2016). List of plants in treatment of various human diseases is huge; however, some of popularly used plants such as

Picrorhiza kurroa, *Cedrus deodara*, *Berberis aristata*, *Piper longum*, *Curcuma zedoaria*, *Cannabis sativa*, *Oroxylum indicum*, *Chelidonium majus*, *Taxus baccata*, *Curcuma longa*, *Typhonium flagelliforme*, *Phaleria macrocarpa*, *Catharanthus roseus*, *Selaginella corymbosa*, *Loathatreum gràcies*, *Taraxacum mongolicum*, *Brucea javanica*, *Allium sativum*, *Smilax china*, *Helianthus annuus*, *Solanum nigrum*, *Asparagus cochinchinensis*, *Glycyrrhiza glabra*, *Withania somnifera*, etc. have been used in the traditional medicine system for centuries for the treatment of various ailments including cancer (Kainsa et al. 2012; Singh et al. 2013). Due to their safety, low cost, and oral bioavailability, a great potential was found in plant-based compounds for the treatment and prevention of cancer (Alonso-Castro et al. 2011). Therefore, there is crucial demand of extensive scientific screening and clinical experimentations for the development of improved anticancer drugs based on plant-derived compounds. Although a vast number of medicinal plants are known to have biochemical constituents with anticancer properties, this association needs further research and experimentation in order to develop and design anticancer drugs (Raina et al. 2014). Hence, recent scientific developments and advances in clinical trials not only offer the opportunities to develop plant-based anticancer drugs but there is an ample scope to understand their mechanism of action and pharmacological and toxicological effects. Medicinal plants have historically proven their value as a source of molecules with therapeutic potential and nowadays still represent an important pool for the identification of novel drug leads (Song et al. 2014; Atanasov et al. 2015). Table 7.1 describes some medicinally important plant species with their anticancer properties.

Table 7.1 Some important medicinal plants with anticancer properties

Plant species	Compound	Pharmacological actions	References
Terminalia chebula	Tannins, shikimic acid compounds, triterpenoids, ellagic acid	Antioxidant, antidiabetic, renoprotective, hepatoprotective	Jena and Gupta (2012)
Catharanthus roseus	Vinblastine and vincristine	Anticancerous	Priyadarshini and Keerthi Aparajitha (2012)
Zingiber officinale	Mono- and sesquiterpenoids, zingerone and gingerols	Anticancerous, hepatoprotective, hypercholesterolemic, anti-atherosclerotic	Umadevi et al. (2012)
Camptotheca acuminata	Topotecan and irinotecan	Anticancer agents (ovarian and small cell lung cancers)	Gajalakshmi et al. (2013)
Curcuma longa	Curcumin	Anticancer, hepatoprotective	Sharma (2013)
Podophyllum peltatum	Etoposide and teniposide	Anticancer agents	Shiyou and Wanli (2014)
Withania somnifera	Steroidal lactones, withanolides, notably withaferin A	Chemopreventive, anticancerous, memory enhancer and immunomodulatory	Rathinamoorthy and Thilagavathi (2014)

7.3 Challenges and Opportunities in Cancer Drug Discovery from Plants

For successful development of plant-derived drugs, multidisciplinary approach is required. Expertise in the field of natural products chemistry, molecular and cellular biology, synthetic and analytical chemistry, biochemistry, biotechnology, and pharmacology is needed. However, the challenges associated with successful development of plant-derived compounds involve selection and identification of plant material, harvesting, isolation, identification, characterization, implementation of appropriate screening bioassays and synthesis, testing of their efficacy and toxicity, and finally clinical trials. Moreover, these processes can be tedious, time-consuming, and expensive. Not all natural products can be fully synthesized, and many natural products have very complex structures that limit their synthesis on an industrial scale. Moreover, in the past, drug discovery and the process of identifying the structures of bioactive compounds from plants were time-consuming (Lahlou 2013) mainly due to the lack of high-end equipment and automated high-throughput techniques. However, the recent advancements in computational biology and bioinformatics, metabolomics, biotechnology, and nanotechnology have not only offered opportunities for rapid screening of plant extracts but also geared up the process of drug development to overcome challenges. Instrumentations such as high-performance thin liquid chromatography (HPTLC-MS) coupled with mass spectrometry (MS), liquid chromatography coupled with mass spectrometry (LC-MSMS), higher magnetic field-strength nuclear magnetic resonance (NMR) instruments, 3D QSAR in modern drug design, computer-aided drug design, and robotics to automate high-throughput bioassays have minimized the time and efforts needed to identify a specific drug from plants (Lahlou 2013). The use of plant-derived metabolites as lead in cancer treatment is challenging, and therefore, proper scientific study is required. The challenges and opportunities in cancer drug discovery from plants using various fields are depicted in figure form (Fig. 7.1).

7.3.1 Biotechnological Approaches

Production of plant-derived anticancer compounds using biotechnological approaches such as genetic engineering and *in vitro* cell cultures has a major role to play in plant-based industries. It has been reported that biotechnological tools are important for the multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in vitro* regeneration and genetic transformation systems (Siahsar et al. 2011; Sheikhpour et al. 2014). Metabolomics approaches made it possible to identify pathways responsible for the production of important secondary metabolites, while genetic engineering technology offers enormous opportunities in modification of these pathways for the enhanced production of secondary metabolites by expressing certain gene/s. For example, engineering of plants to enhance anthocyanin content was a major attention. As anthocyanins are flavonoids associated with the protection against several human diseases, their natural levels in

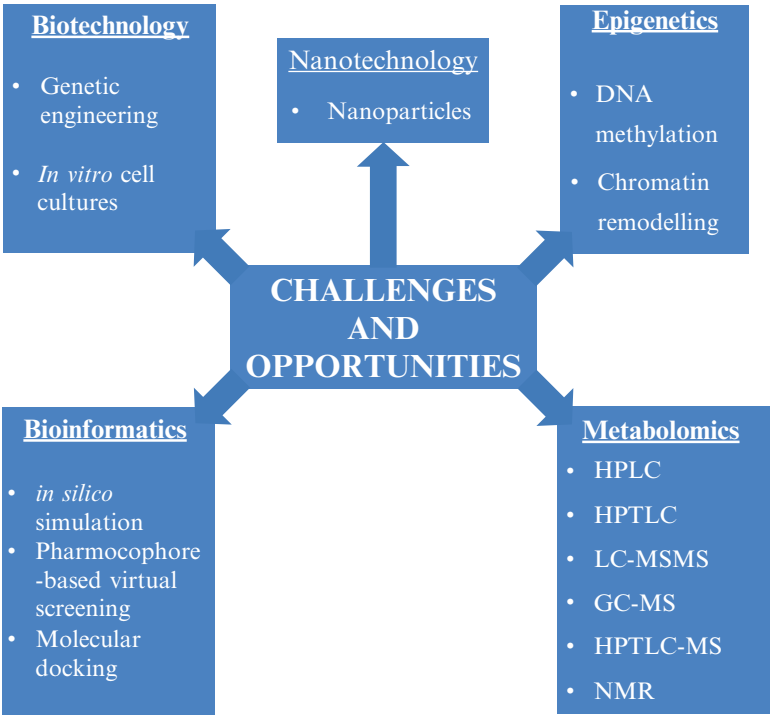

Fig. 7.1 Challenges and opportunities in cancer drug development in plants

plants are inadequate to confer optimal benefits. Therefore, the expression of two transcription factors in tomato led to the accumulation of higher quantities of anthocyanins at concentrations comparable to those found in high-anthocyanin-containing plants such as blackberries and blueberries (Diaconeasa et al. 2015). Moreover, it has been observed that the new variety has an intense purple coloration exhibiting a threefold enhanced antioxidant capacity, and it has been found to extend the life span of cancer-susceptible mice when these were fed with a diet supplemented with this tomato variety. Similarly, the overexpression of ORCA3 and G10H genes in *Catharanthus roseus* has been found to increase the production of anticancer indole alkaloid (Pan et al. 2012). Terpenoids that belong to the most diverse class of natural products (Misawa 2011) have been found to play an effective role on a variety of diseases, such as cancer, cardiovascular diseases, malaria, and Alzheimer's disease. Overexpressing terpenoid biosynthesis pathway genes using *Agrobacterium*-mediated genetic transformation in homologous and ectopic plants is an effective strategy to enhance the yield of pharmaceutical terpenoids. It has been suggested that *Agrobacterium*-mediated genetic transformation offers great opportunities to enhance the supply of scarce terpenoid drugs, reduce the price of expensive drugs,

and improve people's standard of living (Lu et al. 2016). Although there is a significant development in the production of protein and polypeptide drugs by means of genetic engineering technology, the production of secondary metabolites involves complex biosynthetic pathways utilizing multiple enzymes, many of which have yet to be isolated and characterized. Therefore, understanding the genes involved in biosynthetic pathway using metabolomics approach, appropriate promoters, and well-established transformation and regeneration tissue culture system may result in successful engineering of anticancer compound. Therefore, genetic engineering of the plant-derived secondary metabolites can be considered as a challenging and difficult task. Since the world demand for natural anticancer compounds is increasingly growing and the plant sources are limited, the application of genetic technology is going to be one of the most promising methods for industrial production of natural products.

Production and isolation of the plant-derived compounds by conventional techniques are a challenging task. Many natural products have very complex structures that are too difficult and expensive to synthesize on an industrial scale (Lahlou 2013). Moreover, most of the medicinal plants are collected from the wild, and because of their continuous harvesting, several medicinal plants have become endangered which ultimately leads to the loss of biodiversity. Novel biotechnological approaches such as cell-based bioprocess engineering confer cost-effective technology for the large-scale production of clinically/commercially important secondary metabolites such as paclitaxel. Of note, it is recognized as the best strategy since the extraction of natural products from plant sources may result in extinction of medicinal plant species (Khani et al. 2012). The production of secondary metabolites by growing undifferentiated tissues *in vitro* large amounts using plant cells has been the subject of extended research (Veeresham and Chitti 2013; Mohammed et al. 2015). A recent development to overcome the difficulties arising with cell suspension cultures is the production of hairy root cultures using genetic transformation of plants with *Agrobacterium rhizogenes*. Hairy roots have been found to be suitable for the production of secondary metabolites because of their stable and high productivity in hormone-free culture conditions (Veeresham and Chitti 2013). *Catharanthus roseus* (Apocynaceae) which is known as one of the most important anticancer medicinal plants produces dimeric indoles vinblastine and vincristine (Almagro et al. 2015). These anticancer alkaloids are present in very low amount in intact plant *Catharanthus roseus* (Apocynaceae), and organic synthesis of chemically complex molecules is not economical. Therefore, hairy root culture obtained from leaf explants showed an increased production of these alkaloids when treated with abiotic and biotic elicitors (Gaviraj and Veeresham 2011). *In vitro* production of anticancer drug produced from roots of various *Taxus* species in bioreactor has been reported (Filová and Rovna 2011). *Toddalia asiatica* belonging to family Rutaceae is well-known for its anticancer and anti-HIV activities. However, the yield of the nitidine (alkaloid) in this plant is too low, and plant tissue culture has been used as an alternative method for increasing the production of

secondary metabolites. For example, Murashige and Skoog medium supplemented with NAA (2 mg/l) and kinetin (1 mg/l) showed tenfold production of the nitidine alkaloid in this plant (Rajkumar et al. 2010). Therefore, bioreactors are the key step toward commercial production of anticancer compounds.

Another important challenge in plant-derived metabolites is limited by endangered, rare, and threatened status of plant species. Such plant species cannot be collected from the wild. Therefore, in such cases, plant tissue culture approaches have been used. *Gentiana kurroo* is a critically endangered medicinal herb belonging to the family Gentianaceae. The roots and rhizomes of the plant have been extensively used for anticancer activity. Therefore, a micropropagation of *Gentiana kurroo* resulting from apical meristem has been developed (Kaushal et al. 2014). Similarly, considering its endangered status, *in vitro* propagation protocol of *Zeyheria montana* has been reported. *In vitro* micropropagation from axillary bud and shoot tip explants of *Catharanthus roseus* has been reported (Mehta et al. 2013). *In vitro* propagation of *Acalypha indica* from nodal explants has also been reported (Saikia and Handique 2014). *In vitro* propagation of medicinal plants with enriched bioactive principles and advances in cell culture methodologies for secondary metabolite production could provide new means for the cost-effective and commercial production of rare or exotic plants. However, the knowledge of the biosynthetic pathways of desired compounds in plants as well as of cultures is often still rudimentary, and approaches are consequently needed to develop efficient and effective tissue culture-based system. The problems occurring in the production of secondary metabolites from plants stem from the complex and incompletely understood nature of plant cells in *in vitro* cultures. Therefore, continuation and intensification efforts in this field will lead to controllable and successful biotechnological production of specific, valuable, and as yet unknown plant chemicals (Veeresham and Chitti 2013). Biotechnological tools for multiplication of medicinally important anticancer plants using tissue culture and anticancer compounds by plant metabolic engineering strategies are presented in Table 7.2.

Table 7.2 Biotechnological tools for multiplication of medicinally important anticancer plants using tissue culture and anticancer compounds by plant metabolic engineering strategies

Metabolite	Plant Species	Strategy	References
Tanshinone	*Salvia miltiorrhiza*	Hairy root culture	Gupta et al. (2011)
Linalool	*L. latifolia*	Overexpression	Mendoza-Poudereux et al. (2014)
Alkaloids	*Catharanthus roseus*	Micropropagation	Pandey et al. (2014)
Geraniol	*Nicotiana tabacum*	Overexpression	Ritala et al. (2014)
Acalyphine	*Acalypha indica*	Micropropagation	Saikia and Handique (2014)
Camptothecin	*Ophiorrhiza pumila*	Overexpression	Cui et al. (2015)
Solanesol	*N. benthamiana*	Overexpression	Campbell et al. (2016)
Phenolic compounds	*Momordica charantia*	Hairy root culture	Chung et al. (2016)
Alkaloids	*C. roseus*	Overexpression	Sun et al. (2016)

7.3.2 Bioinformatics/Computational-Based Approaches

The traditional labor-intensive approaches toward discovery of plant-based drugs often involve expertise, time, and expenditure. However, the development of rapid high-throughput technologies across the biosciences, bioinformatics, plays a crucial role in modern drug design and discovery (Sharma and Sarkar 2012). Computational methods provide a very powerful knowledge-based approach that helps to select plant material or natural products with a high likelihood for biological activity (Atanasov et al. 2015). Computational-based approaches can be categorized as bioinformatics-based or in silico simulations and pharmacophore-based virtual screening and molecular docking (Atanasov et al. 2015; Hein et al. 2010). In silico simulations can be used to propose protein-ligand binding characteristics for molecular structures, e.g., known constituents of a plant material. Compounds that perform well in in silico predictions can be used as promising starting materials for experimental work (Rollinger et al. 2008). Moreover, bioinformatics approaches offer essential tools for the identification of genes and pathways that may be associated with important bioactive secondary metabolites from medicinal plants (Saito and Matsuda 2010). However, there has been limited attention to date to the potential application of bioinformatics approaches that can leverage plant-based knowledge (Sharma and Sarkar 2012).

Whereas pharmacophore-based virtual screening includes a 3D arrangement of physicochemical model (e.g., hydrogen bond donor/acceptor, hydrophobic area, aromatic ring) that represents the key interactions between a ligand molecule and its target protein, molecular docking is widely used to elucidate the mechanism of action and rationalize structure activity relationships of natural products (Hein et al. 2010). In pharmacophore-based virtual screening, 3D-multiconformational compound libraries can be screened against a pharmacophore model to retrieve molecules that map the pharmacophore features and consequently have a high likelihood of being active on the target (Hein et al. 2010). This method can be highly valuable for target identification if a general activity of an extract or pure compound is known. The structure can be screened against a set of models for multiple targets to reduce the experimental work in identifying the molecular target(s) related to the bioactivity (Duwensee et al. 2011; Steindl et al. 2007; Wolber and Rollinger 2013). In molecular docking, accurate prediction of the positioning of a ligand within a protein-binding pocket is essential, and it is important to estimate the strength of the binding with a docking score. Moreover, if the 3D structure of a protein is available, either from X-ray crystallography, NMR data, or homology modeling, then ligand molecules can be computationally positioned directly in the binding pocket to analyze their putative target-ligand interactions and thus identify the crucial binding features of the molecule (Hein et al. 2010; Waszkowycz et al. 2011).

Application of some bioinformatics software can highlight the mechanisms of action of molecules and can evaluate their properties based on their chemical structures. In view of this, flavonoids such as isoflavones, flavonols, morin, and flavone have been evaluated for their anticancer properties using PASS software. Furthermore, it has been concluded that consumption of vegetables and fruits

containing flavonoids can reduce the risk of cancers significantly (Hashemi et al. 2014). Recently, attempts have been made on in silico identification of anticancer compounds and plants from traditional Chinese medicine database. These studies predicted 5278 anticancer compounds from traditional Chinese medicine database, and it has been observed that the top 346 compounds were highly potent and active in the 60 cell line test. The further similarity analysis revealed that 75% of the 5278 compounds are highly similar to the approved anticancer drugs. Based on the predicted anticancer compounds, these studies identified 57 anticancer plants by activity enrichment and constructed a network of predicted anticancer plants for prediction of compounds and plants from TCM database. Such studies, however, offer an attractive starting point and provide a broader scope to mine for potential anticancer agents using in silico approach. Thus, bioinformatics methodologies offer enormous opportunities in drug design that can result in efficient, quicker, targeted searches and potentially cost-effective leads toward finding plant-based anticancer remedies. Taken together, in silico methodologies may be an important approach to reduce the cost and time involved by conventional screening strategies (Sharma and Sarkar 2012).

7.3.3 Metabolomics Approaches

Metabolomics is a branch of biology which offers comprehensive profiling of all cellular secondary metabolites and has become a powerful tool in drug discovery and development in medicinal plants (Rahman et al. 2011). Plant-derived secondary metabolites provide lead molecules for drug development. Platform of modern omics technique such as metabolomics not only provides a comprehensive analysis of phytochemicals but also offers a valuable tool for identifying potential biomolecules from medicinal plants. Moreover, recent advances in mass spectrometry (MS)-based platforms like GC-MS and LC-MS helped in separation and identification of several metabolites (Mukherjee et al. 2016). In recent years there has been significant increase in the use of metabolomics in prevention and diagnosis of human diseases such as cancer (Gomez-Casati et al. 2013). It has the potential to be useful in identifying novel diagnostic biomarkers and understanding cancer etiology (Verma and Banerjee 2015; Wojakowska et al. 2015). Carrots are known for their anticancer properties mainly due to the presence of bioactive compounds (Wassim et al. 2013). Juice from different carrot cultivars was analyzed using H-NMR approach and biochemical differences, mainly based on amino acids and organic acids (Tomassini et al. 2016).

There is a strong body of evidence which suggests that metabolic changes result in different metabolic landscapes in cancer cells versus normal cells (Blekherman et al. 2011). Metabolomics has been found to play an important role in the field of oncobiology. This is mainly due to the fact that tumor cells are highly proliferative and have a high transcription and translation rates, as well as a higher energy demand. Also, they have special metabolic requirements when compared to normal cells and frequently lose many regulatory functions (Gomez-Casati et al. 2013).

Moreover, different metabolites have been identified using metabolomics approaches and proposed that would serve as markers for several tumor processes. Thus, one of the greatest challenges in medicine is the use of metabolomics in predicting the appearance of tumor cells (Gomez-Casati et al. 2013). However, one of the challenges is the limited number of samples which were used and there might be sample-to-sample variation. Therefore, in order to overcome this weakness, the first requisite is that a sufficient number of samples are analyzed and that the techniques are fully standardized in order to improve the reproducibility of these technologies (Kim et al. 2010). Perhaps one of the major advantages of metabolomics is that it can be used to monitor early changes in metabolic pathways, and therefore, prediction of anticancer agent's toxicity at an early stage is feasible. The early diagnosis of toxicity is very necessary in cancer; however studies on this aspect are meager (Kasliwal et al. 2017).

Publicly available Medicinal Plant Metabolomics Resource (MPM) (http://metnetdb.org/mpmr_public/) provides a framework for generating experimentally testable hypotheses about the metabolic networks that lead to the generation of specialized compounds, identifying genes that control their biosynthesis, and establishing a basis for modeling metabolism in less studied species (Wurtele et al. 2012). Metabolomics require an interdisciplinary approach including comprehensive knowledge in many areas such as biochemistry, biology, physiology, and bioinformatics (Gomez-Casati et al. 2013). Therefore, the use of metabolomics is considered as one of the greatest challenges in predicting the appearance of tumor cells (Serkova and Glunde 2009). It is believed that metabolomics will certainly improve the efficiency of lead finding from plant-derived products and thus reinstate this prolific source of potential anticancer drugs (Kim et al. 2010). Metabolomics requires the development of sophisticated and powerful statistical software and methodologies to make clinical observations easy to follow. Furthermore, the deeper understanding of the metabolic basis of cancer has the potential to provide the foundation for the development of novel approaches targeting tumor metabolism (Verma and Banerjee 2015). Metabolomics uses high-throughput technologies such as mass spectrometry and magnetic resonance spectroscopy techniques which allow qualitative and quantitative profiling of small molecules present in biological systems. It has been revealed that this approach can be applied to study metabolic differences between different types of thyroid cancer metabolomics and to identify new potential candidates for molecular biomarkers as well as improvement of the tumor type classification and diagnosis (Wojakowska et al. 2015). Metabolomics-based investigations of plant-derived anticancer drugs, therefore, may be very crucial in development of novel therapeutics against various types of cancer.

7.3.4 Epigenetic Modifications

One of the major opportunities in the prevention and treatment of cancer involves epigenetic modifications. In recent years, targeting of aberrant epigenetic modifications has gained considerable attention in cancer chemoprevention research because,

unlike genetic changes, epigenetic alterations are reversible and occur during early carcinogenesis. Efforts were directed toward investigation of a number of naturally occurring phytochemicals in food and medicinal plants aiming to identify and develop anticancer agents which cause minimal harm to normal cells while effectively killing cancer cells (Thakur et al. 2014). Till date very little information is available on the role plant-derived compounds play in epigenetic modification. The research aiming at developing new therapeutic anticancer strategies against epigenetic targets has flourished in the last years (Andreoli et al. 2013). Both histone methyltransferases (HMT) and histone demethylases (HDM) have also been associated with cancer development (Huang et al. 2017). Although, in the last decade, several cancer pathologies have been associated to specific epigenetic changes, the way in which epigenetic modifications are regulated is still largely unknown (Andreoli et al. 2013). It has been documented that epigenetic alterations such as DNA methylation and chromatin remodeling play a significant role in breast cancer development and, although extensive research has been done, the causes, mechanisms, and therapies of breast cancer are still to be fully elucidated (Veeck and Esteller 2010; Lustberg and Ramaswamy 2010; Bombonati and Sgroi 2011). Several plant-derived compounds were identified for their anticancer properties with an emerging field regarding the modulation of epigenetic events. Studies suggested that most compounds were evaluated regarding histone modifications (mainly acetylation) and DNA methylation; however, evaluating the impact of these compounds on miRNA (Link et al. 2010; Huang et al. 2011; Karius et al. 2012; Schneider-Stock et al. 2012) and potentially other epigenetic targets is necessary to clarify the role of epigenetics on gene regulation for chemopreventive purposes and clinical applications (Schnekenburger et al. 2014).

Several phytochemicals from plants, particularly flavonoids, are suggested to be able to alter the epigenetic cellular mechanisms and act as epigenetic modulators (Thakur et al. 2014). Plant-derived epigenetic modulators with anticancer properties are presented in Table 7.3. Busch et al. (2015) reviewed the epigenetic activities of flavonoids such as flavanones, isoflavones, anthocyanidins, etc. isolated from various plant species in the prevention and treatment of cancer. They further claimed that a growing number of epigenetically active compounds such as flavonoids are currently being tested in clinical trials with their therapeutic potential (Busch et al. 2015). Therefore, a deeper understanding of the mechanisms of action of these flavonoids or other plant-derived metabolites needs to be obtained. These mechanisms include allosteric regulation, inhibition/activation, and enzymatic kinetics (e.g., reversible/irreversible, substrate and cofactor competition/noncompetition). These tasks may be challenging, especially when natural and/or dietary bioactive components are involved. Therefore, more studies on understanding of these mechanisms are needed to know how epigenetic framework will play a major role in the near future to develop new therapies against cancer (Andreoli et al. 2013). Ingestion of flavonoids has been suggested to reduce the risk of versatile cancer entities like pancreatic, prostate, lung, colon, breast, and prostate cancer even though results are sometimes inconclusive (Romagnolo and Selmin 2012). Root of *Scutellaria baicalensis* contains almost 70 flavonoids like anthocyanidins, chalcones,

Table 7.3 Plant-derived epigenetic modulators with anticancer properties

Chemical class	Compound	Plant source	Epigenetic target (s) and mechanism (s)	References
Betacyanin	Betanin	*Beta vulgaris* (beetroot)	*In vitro* DNMTi	Paluszczak et al. (2010)
Anthocyanidin	Cyanidin	Pigment – apple, plum, red cabbage, red onion and most red berries: grape, cherry, strawberry, blueberry…		
Polyphenol	Brazilin	*Caesalpinia sappan*	Downregulation of HDAC1 and HDAC2 expression, increases histone acetylation	Kim et al. (2012)
Alkaloid	Mahanine	*Murraya koenigii*	Inhibits DNMT activity	Agarwal et al. (2013)
Flavonol	Kaempferol	Found in common fruits and vegetables (tea, tomato, cruciferous vegetables, apple)	Decreases HDAC activity and increases acetylation	Berger et al. (2013)
Anthraquinone	Emodin	*Rheum emodi* (rhubarb), *Fallopia japonica*, buckthorns	Emodin decreases pH3Ser10 and increases H3K27me3 contributing to gene silencing in bladder cancer cells	Cha et al. (2013)

Source: Schnekenburger et al. (2014)

flavanonols, flavonols, flavanones, and flavones exhibiting the pharmacological effects such as antidiabetic, anti-inflammatory, antioxidative, hepatoprotective, antiviral, antianxiety, antitumor, and antihypertensive effects (Bhandari et al. 2010). Whereas curcumin (diferuloylmethane) is a naturally occurring flavonoid derived from the rhizome of *Curcuma longa*, genistein and daidzein are abundant polyphenols found in soybean. Both are known to possess anticancer and chemopreventive properties (Ahmad et al. 2012; Rietjens et al. 2013), and studies suggested that these anticancer properties might result from epigenetic changes triggered by curcumin, genistein, and daidzein (Rietjens et al. 2013; Teiten et al. 2013). Although plants are rich source of phytochemicals, further in-depth research and prospective as well as mechanistic studies are required to investigate the beneficial effects of the different flavonoids in detail thereby focusing on their distinct epigenetic activities (Busch et al. 2015). Several plant-derived compounds such as anthocyanidin, anthraquinone, betacyanin, etc. (Table 7.3) are reported to target and modulate epigenetic mechanisms showing promising potential for cancer prevention and therapy by modulating most hallmarks of cancer (Schnekenburger et al. 2014). The progress in epigenetic research opens new avenues for medicinal plants as resources for natural product-based drug development (Dawood and Efferth 2015).

7.3.5 Nanotechnological Tools for Cancer Therapy

Nanotechnology is the engineering and manufacturing of materials at the atomic and molecular scale. It is a tool to enhance therapeutic values of natural plant products (Kumari et al. 2012; Pandey and Pandey 2013). Plant-derived nanoparticles are potential remedy for various diseases such as malaria, cancer, HIV, hepatitis, and other acute diseases mainly due to the fact that secondary metabolites act as a precursor for the synthesis of nanomaterial in nonhazardous ways. Moreover, though several anticancer drugs are available, one of the major problems is their target specificity. Therefore, researchers are paying significant attention to the development of drugs at the nanoscale level to increase their target specificity and to reduce their concentrations (Rao et al. 2016).

Due to their minimal size, inexpensive, single-step, and eco-friendly nature (Kuppusamy et al. 2016), plant-derived secondary metabolites for synthesis of nanoparticles open up new avenues in anticancer research. Plant-derived nanoparticles are in high demand for cancer chemotherapy, and these nanoparticles have great effect against various cancer cell lines such as Hep 2, HCT 116, and HeLa cell lines (Raghunandan et al. 2011; Das et al. 2013). It has been reported that the plant-derived silver/gold nanoparticles have many applications in biomedical field such as control of free radical formation from the cell and induction of apoptosis mechanism in malignant cells (Dipankar and Murugan 2012; Rajeshkumar 2016). The green synthesis of silver nanoparticles exhibited a significant cytotoxic effect in HeLa cell lines compared to other chemical-based synthetic drugs (Suman et al. 2013). Using nanotechnology, the researchers found that a new herbal medicine compound was able to enter cancer cells without damaging the healthy cells of the human body. Several challenges are associated with the existing cancer treatments such as localization of the therapy to tumor sites, drug resistance by tumors, and short drug circulation times. Moreover, it has also been documented that the drug toxicity leads to major complications, such as heart problems and low white blood cell counts. However, such challenges are overcome by plant-derived nanoparticles (Hu et al. 2010). Recent advances in nanotechnology-based drug delivery systems of anticancer agent camptothecin showed improved efficiency of this drug due to development in nano-sized dosage forms of camptothecin-derived drugs (Pandey and Pandey 2013).

Presently, nanotechnology is considered as one of the most prominent and promising fields for the development of plant-derived anticancer drugs. Unfortunately, there are only a few plant-derived nano-based products that are currently used in cancer applications. Particle shape, size, and surface chemistry influence the cytotoxicity of AgNPs (Rao et al. 2016). Therefore, these challenges can be manipulated so as to reduce the effects of cytotoxicity of nanoparticles. Examples of plant-derived compounds use in nanotechnology in cancer treatment are presented in Table 7.4. More research is lacking on plant-derived anticancer drugs derived from AuNPs and iron oxide, titanium dioxide, cerium oxide, etc. nanoparticles. Although nanotechnology contributions are advantageous for several medicinal areas, clinical researchers have mentioned some negative factors, such as high cost, the difficulty

Table 7.4 Examples of plant-derived compounds use in nanotechnology in cancer treatment

Formulations	Active ingredient	Biological activity	Method of preparation	References
Artemisinin nanocapsules	Artemisinin	Anticancer	Self-assembly procedure	Chen et al. (2009)
Curcuminoid solid lipid nanoparticles	Curcuminoids	Anticancer and antioxidant	Microemulsion technique	Nayak et al. (2010)
Berberine-loaded nanoparticles	Berberine	Anticancer	Ionic gelation method	Chang et al. (2011)

of scaling up processes, and the easy incapability of nanoparticles, which can result in dangerous lung diseases and often lead to other diseases that can lead to changes in homeostasis or even death. Therefore, there is an urgent need to overcome these challenges using advances in developments in nanoscience and nanotechnology (Yadav et al. 2011; Singh et al. 2013). Although the problems associated with the lack of specificity of conventional chemotherapy can be overcome by targeted nanoparticles to deliver the drug/gene in cancer therapy (Ali et al. 2011; Heidel and Davis 2011), there are also potential risks and challenges associated with this novel strategy (Nguyen 2011). For instance, targeted nanoparticles might change the stability, solubility, and pharmacokinetic properties of the carried drugs. Apart from this, the use of targeted nanoparticles is limited by shelf life, aggregation, leakage, quick degradation, durability, and toxicity of materials used to make them (Jain et al. 2011). However, these aspects have not been extensively investigated, and therefore further investigation may shed light on this aspect. Despite these challenges, targeted nanoparticles have provided an effective platform for a better and more specific delivery of cancer therapeutics. Therefore, it has been believed that the role and scope of targeted nanoparticles for drug delivery in cancer therapy are increasing, and the development of effective multifunctional targeted nanoparticles will not be far in the near future (Nguyen 2011). Shabani (2015) suggested that investigating the use of gold nanoparticles with plant metabolites targeting to cancer cell can provide new avenues in cancer treatment.

7.4 Conclusions and Future Prospects

Plant-derived metabolites have tremendous potential to remedy dreadful diseases like cancer. Several medicinal plants are currently being investigated for their anticancer properties. Recent advances in biological sciences open up new avenues in cancer research. Biotechnological tools such as transgenics can be used to overexpress specific candidate gene/s that leads to the synthesis of desired anticancer metabolite. Anticancer drugs/metabolites can be produced or synthesized at larger scale using cell culture techniques and bioreactors. Discovery of metabolites using advanced techniques such as metabolomics and their targeting can also be studied using bioinformatics tools. The effective and efficient targeting to the cancer site can be ensured using nanotechnology-based particles. Therefore, advancement in

cancer research using modern tools can overcome the challenges faced by medical practitioners. Plant-derived metabolites not only provide an effective way to combat cancer but also offer sustainable approaches to fight this dreadful disease.

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Terpenes of the Genus *Salvia*: Cytotoxicity and Antitumoral Effects

8

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Abstract

Plants are an important source of natural compounds used in cancer chemotherapy. The *Salvia* genus includes around 900 species worldwide and is an important source of terpenes, a group of secondary metabolites. According to an estimate, 111 terpenes are isolated from 24 *Salvia* species till date, and only 48 terpenes showed the cytotoxic activity. Out of 24 species, 16 are the native of Asia and 7 of America and 1 is from Europe. On the basis of the chemical compounds, 82 correspond to diterpenes, 4 sesterpenoids, 16 sesquiterpenes, and 9 triterpenes. Out of total diterpenes, five (ferruginol, sclareol, cryptotanshinone, tanshinone I, and tanshinone IIA) have been tested for their antitumoral effect. The present chapter described detail account of terpenes isolated from plants of the *Salvia* genus with their cytotoxic and antitumor activity.

Keywords

Antitumor activity · Cancer · Cytotoxic · Diterpenes · Sesquiterpenes

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8.1 Introduction

Cancer, caused by changes to genes that control the cell function, is characterized by out-of-control cell growth, evasion of apoptosis, induction of angiogenesis, and the invasion to the other organs and tissues (Hanahan and Weinberg 2011). Most of cancers are formed by solid tumors, except leukemia. More than 100 different types of cancer have been described, the classification depends on the type of cell or organ that is initially affected, and it is known as primary cancer. When a cancer cell successfully spreads from the primary cancer to other parts of the body, they may invade and destroy healthy tissue forming a new cancer named secondary cancer or metastasis; this cancer is called according to the primary cancer. However, these cases are very difficult to treat (van Rensburg and Yang 2016).

In 2015, there were approximately 14 million new cases of cancer and 9 million deaths related with cancer (WHO 2017). Cancer treatment requires careful considerations, the types and stages of cancer. The most common treatments are surgery, chemotherapy, radiotherapy, and other therapies such as photodynamic, biological, and hormonal, among others. Conventional treatments include surgical intervention followed by chemotherapy and radiotherapy, which produce side effects as nausea, vomiting, alopecia, loss of appetite, digestive distress, etc. These side effects diminish the quality of life to patients and, in some cases, fail their efficacy (Siegel et al. 2016). Around 49% of the drugs used for cancer treatment have been obtained from natural sources (Newman and Cragg 2016). The discovery of paclitaxel from the bark of *Taxus brevifolia*; vinca alkaloids (vinblastine and vincristine), isolated from *Catharanthus roseus*; and podophyllotoxins, isolated from *Podophyllum peltatum*, is evidence that plant-derived compounds are a good source of anticancer agents (Newman and Cragg, 2016). The present chapter described detail account of terpenes isolated from plants of the *Salvia* genus with their cytotoxic and antitumor activity.

8.2 Generalities of the *Salvia* Genus

Approximately 900 species of the *Salvia* genus are found in tropical and temperate climates. Most of the members of the *Salvia* genus are shrubs, 30–150 cm in height, herbaceous, perennial or, occasionally, annual or biennial, with flowers of a great variety of colors (Cornejo-Tenorio and Ibarra-Manríquez 2011). *Salvia* genus has more than 700 species in the Lamiaceae family and is distributed worldwide as follows: 500 species in the American continent, 300 species in the Mediterranean region and Central Asia, and 100 species in the eastern portion of the Asian continent (Walker et al. 2004). In Mexico, there are approximately 300 species of *Salvia*, from which 85–88% are endemic. The *Salvia* genus in Mexico is the second genus with more species, followed by *Mammillaria* genus (Cornejo-Tenorio and Ibarra-Manríquez 2011). The plant name *Salvia* came from the Latin word salvare ("healer"). These plant species have been used as ornamentals, for culinary and aromatic purposes, as well as for the empirical treatment of different diseases. This name was changed to the French form Sauja and Sauge or "Sawge" in old English. Nowadays, the popular name is known as sage. In the Middle Ages, there was a

proverb: *Cur moriatur homo cui Salvia crescit in horto*? ("Why should a man die whilst sage grows in his garden?") (Dweck 2000). This clearly shows that plants from *Salvia* genus have been active in the treatments of numerous ailments during centuries. A great number of *Salvia* species are economically significant for their use in the perfumery and cosmetic industries. Some members of this genus are used to flavor pork, sausage, and poultry meats. Therefore, many of the members of *Salvia* genus, including *Salvia officinalis*, *S. verbenaca*, *S. fruticosa*, and *S. tomentosa*, among others, are cultivated for commercial purposes.

The isolation and identification of more than 700 compounds of *Salvia* genus has been reported. The terpenes and flavonoids are the main types of compounds found in this genus. Frequently, the branches and the leaves of these plants contain flavonoids, monoterpenes, and triterpenes, and the roots contain mainly diterpenes (Dziurzynski et al. 2013). Oleanolic acid, linolenic acid, and linoleic acid are some of the principal compounds found in *Salvia* species (Azcan et al. 2004). Linolenic acid is widely used as a remedy for cardiovascular diseases (Pan et al. 2012a), whereas oleanolic acid is used for treating inflammatory diseases (Kashyap et al. 2016a, 2016b; Baghi et al. 2016). These components corroborate the traditional practices of *Salvia* species. The content of these phytocompounds in *Salvia* species depends on genetic, climatic, seasonal, environmental, and culture site factors, among others (Maksimovic et al. 2007).

S. officinalis, a very well-known species in the *Salvia* genus, is used for aromatic and medicinal purposes, including inflammation and digestive disorders. Another most commonly used member of the *Salvia* genus is *S. miltiorrhiza* Bunge., which has been utilized in the traditional Chinese medications since ancient times. *S. miltiorrhiza* is known as Dan Shen and was documented in the ancient Chinese monographs *Shen Nong Ben Cao Jing* (Divine Farmer's Materia Medica) and Wu Pus Materia Medica (Du and Zhang 2015). *S. miltiorrhiza* is used in different formulations, and only in some cases this plant is given as a single herbal remedy. *Salvia* species are used for the empirical treatment of more than 60 ailments (Topcu 2006). Members of *Salvia* genus are included in the pharmacopeias of Germany, Austria, China, Poland, and Portugal, among others. In ancient times, different members of the *Salvia* genus were used in increasing the fertility of women, dispelling of evil spirits, and healing snake bite (Dweck 2000). Some of the pharmacological effects studied of plants from the *Salvia* genus include anti-inflammatory, antinociceptive, antibacterial, hypoglycemic, antihypertensive, and antihyperlipidemic properties, among others (Kianbakht et al. 2011; Rodrigues and Kanazawa 2012; Yang et al. 2012; Bisio et al. 2017). Therefore, this genus is an interesting option to find new compounds with pharmacological activities.

8.3 Terpenes of the *Salvia* Genus with Cytotoxic Effects

Terpenes are a family of chemical compounds composed of two or more isoprene units that can be found in animals, plants, and fungi. In plants, terpenes are involved in chemical defense (e.g., phytoalexins) and growth regulation (e.g., gibberellins). Among terpenes, diterpenes being the largest group are subclassified into abietane,

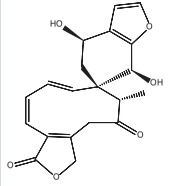
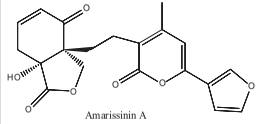
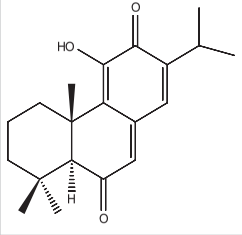
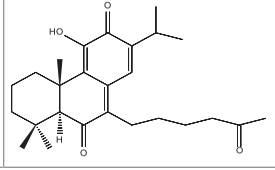
clerodane, labdane, and pimarane. Approximately, 111 terpenes have been isolated from 24 plants of the *Salvia* genus, and their cytotoxic activity was evaluated. These terpenes correspond to 82 diterpenes, 4 sesterpenoids, 16 sesquiterpenes, and 9 triterpenes. Many of these compounds have been obtained through bioassay-guided fractionation.

According to their origin, 16 of 24 plant species are a native from Asia (*S. austriaca*, *S. castanea*, *S. cavaleriei*, *S. chorassanica*, *S. eremophila*, *S. hypargeia*, *S. lachnocalyx*, *S. leriifolia*, *S. miltiorrhiza*, *S. przewalskii*, *S. plebeian*, *S. reuteriana*, *S. sahendica*, *S. scapiformis*, *S. urmiensis*, and *S. yunnanensis*), whereas 7 plants are native from America (*S. amarissima*, *S. ballotiflora*, *S. corrugate*, *S. herbacea*, *S. lachnostachys*, *S. leucantha*, and *S. pachyphylla*) and 1 is from Europe (*S. officinalis*). None of the plants listed in this book chapter are considered as endangered species by the International Union for Conservation of Nature and Natural Resources (IUCN 2017). The botanical names were verified using international databases (IPNI 2008; MBGT 2010).

According to the National Cancer Institute, pure compounds with inhibitory concentration of 50 (IC_{50}) values lower than 4 μg/ml are considered as cytotoxic (Suffness and Pezzuto 1990). Only 48 from 111 terpenes showed cytotoxic activity ($IC_{50} < 4$ μg/ml) (Table 8.1). The results showed that 53% (44 of 82) of the diterpenes were cytotoxic, whereas 33.3 and 6.2% of triterpenes and sesquiterpenes were active, respectively (Table 8.1). Nevertheless, none of the sesterpenes were cytotoxic. This clearly indicates that diterpenes are a good option to find new cytotoxic agents. The diterpenes 11α,12α-epoxyleukamenin E and 18-deacetyl-4-epihenryine A, among others, obtained from *S. cavaleriei* H. Lév, native from China, exerted cytotoxic effects with similar potency compared with reference drugs. The bibliographic search showed that 15 terpenes were isolated from *S. cavaleriei* and tested for their cytotoxic activity. From these, 13 showed cytotoxic activity. Similarly, four from five terpenes isolated from *S. przewalskii* and *S. yunnanensis*, each, showed cytotoxic activity. This suggests that *S. cavaleriei*, *S. przewalskii*, and *S. yunnanensis* are a good source of cytotoxic compounds.

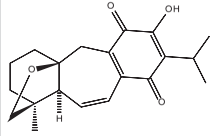
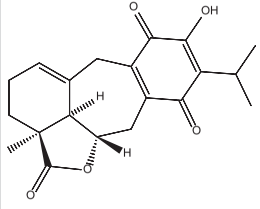
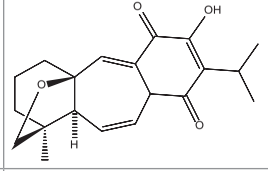
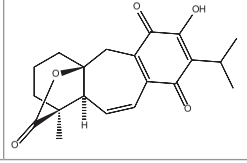
The use of non-tumorigenic cells such as keratinocytes (HaCaT), NIH 3T3 murine fibroblasts, human umbilical vein endothelial cells (HUVEC), and Chinese hamster lung fibroblasts (V79) has been reported in some studies. It is important to carry out cytotoxic studies with non-tumorigenic cells to study the selectivity of new cytotoxic agents on tumorigenic cells. These assays provide valuable information to perform *in vivo* studies. It is interesting to mention that most of *Salvia* species are not used as traditional remedies for cancer treatment. For instance, S. *miltiorrhiza* is commonly used as a folk therapy for cardiovascular diseases in China, and its use in cancer treatment has been increased during the last decade (Wang 2010). Only some species such as *Salvia virgata* (not mentioned in this book chapter), which is used as an empirical therapy for uterine cancer and leukemia (Tuzlaci and Aymaz 2001), showed cytotoxic effects (Topcu et al. 2008; Abu-Dahab et al. 2012; Firuzi et al. 2013). Therefore, the folk medicinal use of most *Salvia* species is not a factor for the selection of *Salvia* species for cancer treatment and the isolation of terpenes and their evaluation in cytotoxic studies.

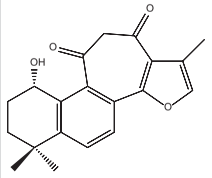
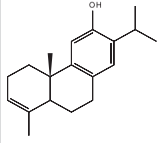
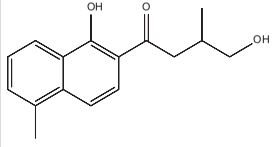
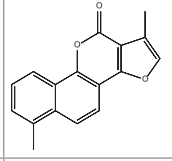
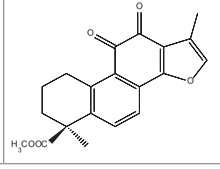
Table 8.1 Terpenes extracted from *Salvia* with cytotoxic activity

Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
S. amarissima Ortega The compounds were obtained from the acetone extract of the leaves and flowers (Bautista et al. 2015)	Teotihuacanin		IC_{50} (μg/mL): >20 (MCF-7), 12.3 (MDA-MB-231), 12.9 (HCT-15), 10.9 (HCT-116), and 13.7 (HeLa) (Bautista et al. 2015).
	Amarissinin A		IC_{50} (μg/mL): 18.2 (MCF-7), 19.3 (MDA-MB-231), NS (HCT-15), 10.9 (HCT-116), 14 (HeLa) (Bautista et al. 2016).
S. austriaca Jacq. The abietane terpenes were isolated from a root culture (Burmistrova et al. 2013	Taxodione[a] This compound was also isolated from the chloroform extract of the aerial parts of *S. xanthocheila* and *S. phlomoides* (Gandomkar et al. 2012*)* and the methanol extract of *S. chorassanica* (Tayarani-Najaran et al. 2013).		IC_{50} (μg/mL): 2.45 (WM-115), 2.21 (HL-60), 2.06 (NALM-6), 12.61 (HUVEC) (Kuźma et al. 2012). IC_{50} (μg/mL): 18.97 (K562), 17.79 (HL-60) (Tayarani-Najaran et al. 2013).
	7-(2-oxohexyl)-11-hydroxy-6, 12-dioxo-7,9(11),13-abietatriene[a]		IC_{50} (μg/mL): 0.30 (WM-115), 0.26 (HL-60), 0.27 (NALM-6), 2.18 (HUVEC) (Burmistrova et al.2013).

(continued)

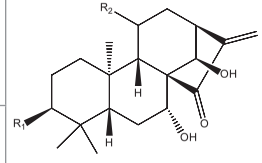
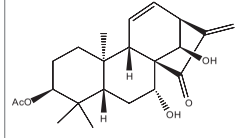
Table 8.1 (continued)

Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
S. ballotiflora Benth. These compounds were isolated from the chloroform and acetone extracts of the aerial parts (Esquivel et al. 1997; Campos-Xolalpa et al. 2017)	19-Deoxyicetexone		IC_{50} (μg/mL): 60 (A549), 45.29 (CT26), 69.3 (HeLa), 68.26 (MCF7), > 500 (J774A.1) (Campos-Xolalpa et al. 2017).
	7,20-Dihydroanastomosine		IC_{50} (μg/mL): 36.66 (A549), 39.13 (CT26), 96.02 (HeLa), 60.56 (MCF7), >500 (J774A.1) (Campos-Xolalpa, et al. 2017).
	19-Deoxyisoicetexone[a]		IC_{50} (μg/mL): 5.11 (A549), 6.17 (CT26), 3.2 (HeLa), 14.87 (MCF7), 8.81 (J774A.1) (Campos-Xolalpa et al. 2017).
	Icetexone		IC_{50} (μg/mL): 25.52 (A549), 29.20 (CT26), 129.15 (HeLa), 62.29 (MCF7), 48.48 (J774A.1) (Campos-Xolalpa et al. 2017).

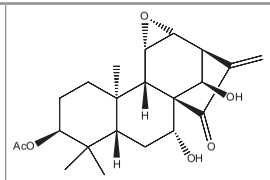
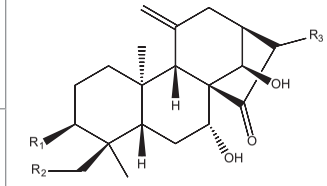
S. castanea Diels The norditerpenoids were isolated from the acetone extract of the whole plant (Pan et al. 2012a)	Castanol A		IC_{50} (μg/mL): >13.04 (HL-60, SMMC-7721, A549, MCF-7, SW480) (Pan et al. 2012a).
	Castanol B		IC_{50} (μg/mL): >10.81 (HL-60, SMMC-7721, A549, MCF-7, SW480) (Pan et al. 2012a).
	Castanol C This compound was also obtained from the acetone extract of *S. yunnanensis* root (Wu et al. 2014).		IC_{50} (μg/mL): >10.36 (HL-60, SMMC-7721, A549, MCF-7, SW480) (Pan et al. 2012a). IC_{50} (μg/mL), >6.76 (HeLa, KB-3-1, NCI-H460, PC3, MCF-7, K562) (Wu et al. 2014)
	Neo-tanshinlactone[a]		IC_{50} (μg/mL): >10.57 (HL-60), 5.29 (SMMC-7721), 7.07 (A549), 3.56 (MCF-7), 3.34 (SW480) (Pan et al. 2012).
	Methyltanshinoate[a]		IC_{50} (μg/mL): 5.49 (HL-60), 1.38 (SMMC-7721), 1.78 (A549), 1.16 (MCF-7), 2.15 (SW480) (Pan et al. 2012).

(continued)

Table 8.1 (continued)

Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
	(1) 11α-Hydroxyleukamenin E[a]		IC_{50} (μg/mL): 1.04 (HL-60), 0.46 (SMMC-7721), 1.41(MCF7), 0.58 (SW480), 1.45 (A549), 1.16 (BEAS-2B) (Zheng et al. 2013).
	(2) 11β-Hydroxyleukamenin E[a]		IC_{50} (μg/mL): 4.03 (HL-60), 1.58 (SMMC-7721), >4.15 (MCF-7), 2.28 (SW480), >4.15 (A549), >4.15 (BEAS-2B) (Zheng et al. 2013).
	(3) 11-Oxoleukamenin E[a]		IC_{50} (μg/mL): 1.16 (HL-60), 1.24 (SMMC-7721), 1.03 (MCF7), 0.66 (SW480), 1.12 (A549), 0.83 (BEAS-2B) (Zheng et al. 2013).
	(4) 3-Oxo-11α-hydroxyleukamenin E[a]		IC_{50} (μg/mL): 3.69 (SW480), >4.56 (HL-60, SMMC-7721, MCF7, A549, BEAS-2B) (Zheng et al. 2013).
	(5) 3β,7α,14β-Trihydroxy-ent-kaur-16-ene-11,15-dione[a]		IC_{50} (μg/mL): 2.82 (SW480), >3.71 (HL-60, SMMC-7721, MCF7, A549, BEAS-2B) (Zheng et al. 2013).
	(6) 3,11-Dioxoleukamenin E[a]		IC_{50} (μg/mL):1.22 (HL-60), 1.81(SMMC-7721) (Zheng et al. 2013).
S. cavaleriei H. Lév. The compounds were isolated from whole plant, using 95% aqueous ethanol extract (Zheng et al. 2013)	11,12-Didehydroleukamenin E[a]		IC_{50} (μg/mL):0.83 (HL-60), 1.11 (SMMC-7721), 0.68 (MCF7), 1.11 (SW480), 1.23 (A549), 0.35 (BEAS-2B) (Zheng et al. 2013).

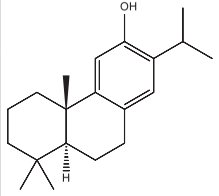
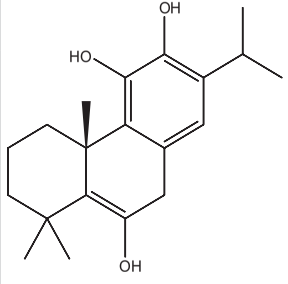
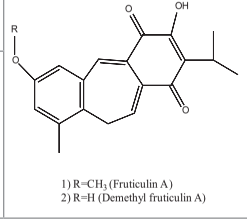
	R_1	R_2
1	OAc	α-OH
2	OAc	⇓-OH
3	OAc	=O
4	=O	α-OH
5	OH	=O
6	=O	=O

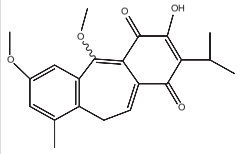
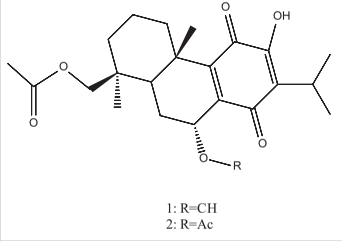
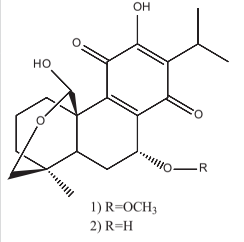
	11α,12α-Epoxyleukamenin E[a]		IC_{50} (μg/mL): 0.27 (HL-60), 0.54 (SMMC-7721), 1.24 (MCF-7), 0.74 (SW480), 0.45 (A549), 0.30 (BEAS-2B) (Zheng et al. 2013).
	(1) 18-Deacetyl-4-epi-henryine A[a] This compound was isolated from *Rabdosia henryi* (Bing-Nan et al. 1989).		IC_{50} (μg/mL): 0.29 (HL-60), 0.40 (SMMC-7721), 0.74 (MCF-7), 0.70 (SW480), 0.39 (A549), 0.36 (BEAS-2B) (Zheng et al. 2013).
	(2) 7α,14β-Dihydroxy-17β-methoxymethyl-3β-acetoxy-ent-kaur-11,15-dine[a]		IC_{50} (μg/mL): 1.65 (HL-60), 1.78 (SMMC-7721), 1.11 (MCF7), 0.89 (SW480), 1.29 (A549), 1.11 (BEAS-2B) (Zheng et al. 2013).
	(3) 7α,14β-Dihydroxy- 17α-ethoxymethyl-3β-acetoxy-ent-kaur-11,15-dione		IC_{50} (μg/mL): >4.59 (HL-60, SMMC-7721, MCF-7, SW480, A549, BEAS-2B) (Zheng et al. 2013).
	(4) 7α,14β-Dihydroxy-17β-ethyoxymethyl-3β-acetoxy-ent-kaur-11,15-dione[a]		IC_{50} (μg/mL): 1.44 (HL-60), 1.53 (SMMC-7721), 1.35 (MCF7), 1.31 (SW480), 1.53 (A549), 1.22 (BEAS-2B) (Zheng et al. 2013).
	(5) 7α,14β-Dihydroxy-17α-methoxymethyl-ent-kaur-3,11,15-trione		IC_{50} (μg/mL): >3.79 (HL-60, SMMC-7721, MCF-7, SW480, A549, BEAS-2B) (Zheng et al. 2013).
	(6) 7α,14β-Dihydroxy-17α-methoxymethyl-18β-acetoxy-ent-kaur-11,15-dione[a]		IC_{50} (μg/mL): 2.83 (SW480), >4.22 (HL-60, SMMC-7721, MCF7, A549, BEAS-2B) (Zheng et al. 2013).
	(7) 7α,14β-Dihydroxy-17β-methoxymethyl-18β-acetoxy-ent-kaur-11,15-dione[a]		IC_{50} (μg/mL): 1.18 (HL-60), 1.48 (SMMC-7721), 0.84 (MCF/), 1.10 (SW480), 127(A549), 1.27(BEAS-2B) (Zheng et al. 2013).

	R_1	R_2	R_3
1	H_2	a-OH	$=CH_2$
2	OAc	H	⇓-CH_2OCH_3
3	OAc	H	α–$CH_2OCH_2CH_3$
4	OAc	H	⇓-$CH_2OCH_2CH_3$
5	=O	H	α-CH_2OCH_3
6	H_2	OAc	α-CH_2OCH_3
7	H_2	OAc	⇓-CH_2OCH_3

(continued)

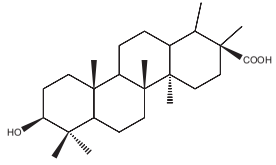
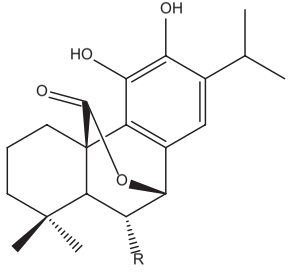
Table 8.1 (continued)

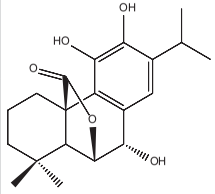
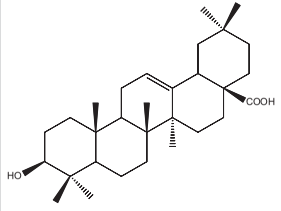
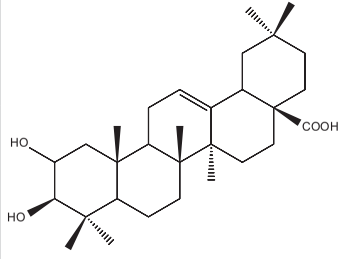
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
S. chorassanica Bunge The compounds were isolated from the root of methanolic extract (Tayarani-Najaran et al. 2013)	Ferruginol[a] This compound was also obtained from hexanic extract of *S. sahendica* Boiss. Root (Moghaddam et al. 1995).		IC_{50} (μg/mL): 5.59 (K562), 2.21 (HL-60) (Tayarani-Najaran et al. 2013). IC_{50} (μg/mL):25.9 (MIAPaCa-2) (Fronza et al. 2011) IC_{50} (μg/mL): <25 (HeLa, Jurkat and U937) (Tangarife-Castaño et al. 2004).
	6-Hydroxysalvinolone[a] This compound was also isolated from ethanol extract of *S. leriaefolia* Benth. whole plant (Choudhary et al. 2013) and acetone extract of *S. hypargeia* Fisch. Et. Mey aerial parts (Topcu et al. 2008).		IC_{50} (μg/mL): 10.53 (K562), 11.47 (HL-60) (Tayarani-Najaran et al. 2013). IC_{50} (μg/mL): 2.64 (HeLa), 1.29 (PC-3), 1.02 (3T3) (Choudhary et al. 2012).
	Also, it was isolated from *Salvia jaminiana* (Kabouche et al. 2005)		IC_{50} (μg/mL): 3.9 (A2780) (Topcu et al. 2008)
S. corrugata Vahl. The compounds were isolated from the aerial parts of dichlorometane extract (Giacomelli et al. 2013)	(1) Fruticulin A		IC_{50} (μg/mL): 12.07 (Hepa1c1c7), 13.14 (c3) (Giacomelli et al. 2013).
	(2) Demethyl-fruticulin A		IC_{50} (μg/mL): 5.46 (Hepa1c1c7), 7.69 (c3) (Giacomelli et al. 2013).

	Fruticulin C		IC_{50} (μg/mL): 5.77 (Hepa1c1c7), 7 (c3) (Giacomelli et al. 2013).
	(1) 7α-Methoxy-19-acetoxy-royleanone	 1: R=CH 2: R=Ac	IC_{50} (μg/mL): >19.92 (Hepa1c1c7), >19.92 (c3) (Giacomelli et al. 2013).
	(2) 7α,19-Diacetoxy-royleanon[a]		IC_{50} (μg/mL): 3.75 (Hepa1c1c7), 3.53 (c3) (Giacomelli et al. 2013).
	(1) 7α-O-methyl-conacytone	 1) R=OCH_3 2) R=H	IC_{50} (μg/mL): 18.80 (Hepa1c1c7), >19.92 (c3) (Giacomelli et al. 2013).
	(2) 7-Dehydroxy-conacytone		IC_{50} (μg/mL): 20.54 (Hepa1c1c7), >19.92 (c3) (Giacomelli et al. 2013).

(continued)

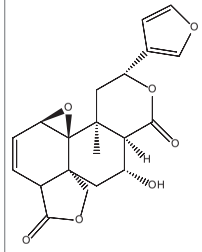
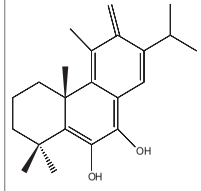
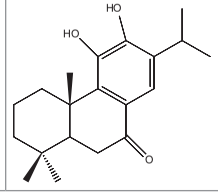
Table 8.1 (continued)

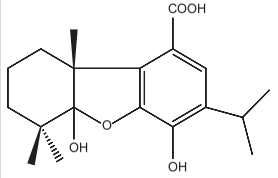
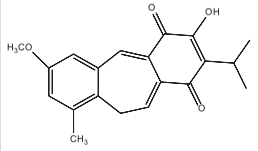
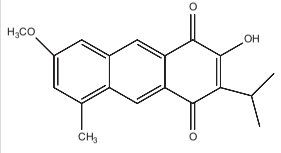
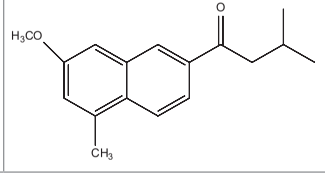
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
S. eremophila Boiss. The compounds were obtained from the acetone extract of the aerial parts (Farimani et al. 2012)	3β, 20-Dihydroxylupane-28-oic acid		IC_{50}: 72 (HL-60), 83 (K562), 97 (Hep G2), 66 (MCF-7), 53 (MOLT-4) (Farimani et al. 2012).
	Carnosol[a] This compound was also isolated from *S. pachyphylla* acetone extract (Guerrero et al. 2006).	 R= H (Carnosoll) R= OH (isorosmanol)	IC_{50} (μg/mL): 65 (HL-60), 58 (K562), 50 (Hep G2), 48 (MCF-7), 53 (MOLT-4) (Farimani et al. 2012)
			IC_{50} (μg/mL): 61.46 (AGS) (Areche et al. 2009)
			IC_{50} (μg/mL): 2 (P388) (Aoyagi et al. 2006)
			IC_{50} (μg/mL): 1.19 (A2780), 3.30 (SW1573), 8.59 (WiDr), 7.93 (T-47D), 1.29 (HBL-100) (Guerrero et al. 2006).
	Isorosmanol This compound was also obtained from *Perovskia abrotanoides and S. pachyphylla* acetone extract (Guerrero et al. 2006; Aoyagi et al. 2006).		IC_{50} (μg/mL): 98 (HL-60), 100 (K562), 47 (Hep G2), 58 (MCF-7), 121 (MOLT-4) (Farimani et al. 2012).
			IC_{50} (μg/mL): 6.58 (A2780), 7.97 (SW1573), >34.64 (WiDr), >34.64 (T-47D), 5.89 (HBL-100) (Guerrero et al. 2006), IC_{50} (μg/mL): 4 (P388) (Aoyagi et al. 2006)

	Rosmanol[a] This compound was also obtained from *Perovskia abrotanoides* (Aoyagi et al. 2006).		IC_{50} (μg/mL): 135 (HL-60), 110 (K562), 248 (Hep G2), 99 (MCF-7), 18 (MOLT-4) (Farimani et al. 2012) IC_{50} (μg/mL): 0.75 (P388) (Aoyagi et al. 2006).
	Ursolic acid		IC_{50} (μg/mL): 6.7 (518A2), 5.3 (A2780), 7 (A549), 6.4 (FaDu), 4.8 (HT29), 5.8 (MCF-7), 8.5 (NIH3T3) (Wiemann et al. 2016) IC_{50} (μg/mL): 12.38 (MGC-803), 17.12(HCT-116), 13.39 (T24), 13.81 (HepG2), 16.36 (A549), >45.7 (HL-7702) (Hua et al. 2015).
	Maslinic acid This compound has also been obtained from *Olea europea* L. (Reyes-Zurita et al. 2013).		IC_{50} (μg/mL): 6.48 (518A2), 13.61(HT29), 17.58 (MCF-7), 11.06 (A549), 9.22 (A2780), 8.04 (8505C) (Siewert et al. 2014) IC_{50} (μg/mL): 9.97 (NIH-3T3) (Sommerwerk et al. 2016).

(continued)

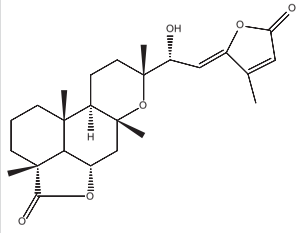
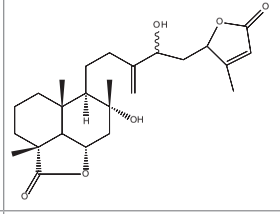
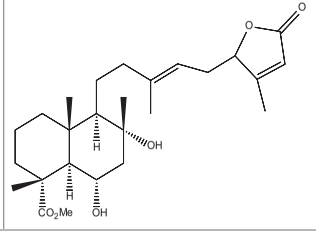
Table 8.1 (continued)

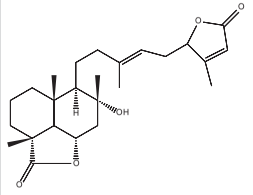
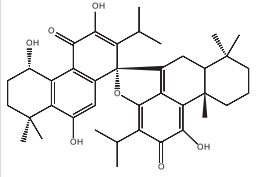
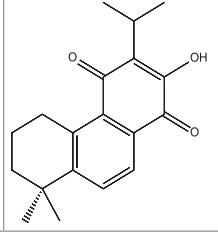
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
S. herbacea Benth. The compound was obtained from the acetone extract of the aerial parts (Bautista et al. 2012)	Tehuanine F		IC_{50} (μg/mL): 15.62 (U-251), 14.52 (SKLU-1) (Bautista et al. 2012)
S. hypargeia Fisch. & C.A. Mey. The compounds were isolated from the acetone extract of the aerial parts and roots (Topcu et al. 2008)	5,6-Didehydro-7-hydroxytaxodone		IC_{50} (μg/mL): 18.8 (A2780) (Topcu et al. 2008)
	Demethylcryptojaponol[a]		IC_{50} (μg/mL): 1.2 (A2780) (Topcu et al. 2008)

	Salvicanaric acid[a] This compound was also obtained from the ethanol extract of *S. leriifolia* Benth. whole plant (Choudhary et al. 2012), as well as *Salvia munzii* (Luis and Grillo 1993), and *Salvia texana* (González et al. 1989).		IC_{50} (μg/mL): 15 (A2780) (Topcu et al. 2008) IC_{50} (μg/mL): > 12.59 (PC-3), 6.87 (HeLa), >12.58 (3T3) (Choudhary et al. 2012) IC_{50} (μg/mL): 5.96 (PC-3),3.65 (HeLa), 2.11 (3T3) (Choudhary et al. 2013)
S. lachnostachys Benth The compounds were obtained from the ethanol extract of the leaves (Oliveira et al. 2016)	Fruticuline A		IC_{50} (μM): 5.5 (U251), 5.2 (MCF7),7.4 (NCI-ADR/Res), 5.2 (786.O), 7.4 (NCL-H460), 4.6 (PC-3), 4.6 (OVCAR), 380(K562), (HaCaT) (Oliveira et al. 2016)
	Fruticuline B		IC_{50} (μM): 32.8 (U251),19.9 (MCF7), 116.4 (NCI-ADR/Res), 36.3 (786.O), 22.6 (NCL-H460), 37.1 (PC-3), 16.4 (OVCAR), 28.5(K562), 29.3 (HaCaT) (Oliveira et al. 2016)
	Lachynostachyone (1-(7-methoxy-5-methylnaphthalen-2-yl)-3- methylbutan-1-one)[a]		IC_{50} (μM): 4.6 (U251), 3.7 (MCF7), 5.6 (NCI-ADR/Res), 4.6 (786.O), 5.2(NCL-H460), 3.7 (PC-3), 3.8 (OVCAR), 300 (K562), 6.5 (HaCaT) (Oliveira et al. 2016)

(continued)

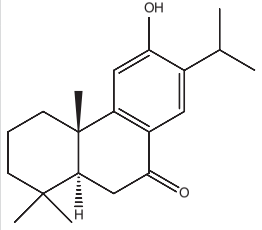
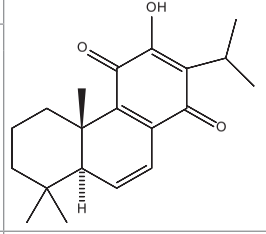
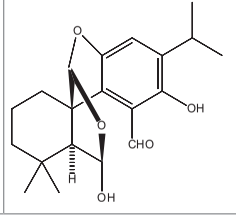
Table 8.1 (continued)

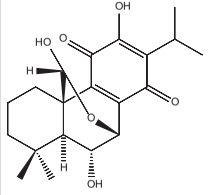
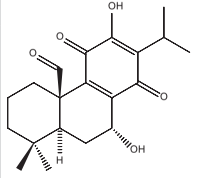
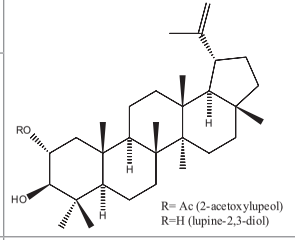
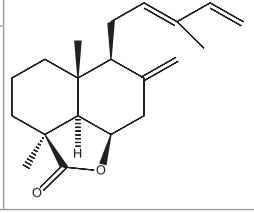
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
S. lachnocalyx Hedge. The compounds were obtained from the hexane and acetone extract of the aerial parts (Farimani et al. 2014)	Lachnocalyxolide A		IC_{50} (μg/mL): > 4.53 (HeLa), 41.91 (MCF7) (Farimani et al. 2014a)
	Lachnocalyxolide B		IC_{50} (μg/mL): >4.55 (HeLa), >4.55 (MCF7) (Farimani et al. 2014)
	Salvileucolide methylester This compound was also extracted from the aerial parts of *Salvia sahendica* Boiss & Buhe (Moghaddam et al. 1995), *Salvia hypoleuca* Benth. (Rustaiyan et al. 1988), and *Salvia syriaca* (Rustaiyan et al. 1987).		IC_{50} (μg/mL): > 4.49 (HeLa), 42.80 (MCF7) (Farimani et al. 2014)

	Salvileucolide-6,23-lactone This compound was also extracted from the aerial parts of *Salvia hypoleuca* Benth. (Rustaiyan et al. 1982).		IC_{50} (μg/mL): > 4.17 (HeLa), > 4.17 (MCF7) (Farimani et al. 2014)
S. leriifolia Benth. The compounds were obtained from the ethanol extract of the whole plant (Choudhary et al. 2012; Choudhary et al. 2013), and the aerial parts extracted with hexane and EtOAc (Farimani et al. 2016)	Salvialeriafone		IC_{50} (μg/mL): >12.59 (PC-3), 6.87 (HeLa), >12.58 (3T3) (Choudhary et al. 2012)
	Salvialerione[a]		IC_{50} (μg/mL): 4.77 (PC-3), 5.56 (HeLa), 1.43 (3T3) (Choudhary et al. 2012).

(continued)

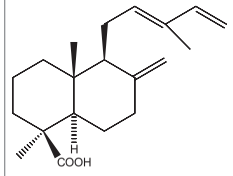
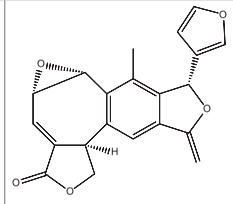
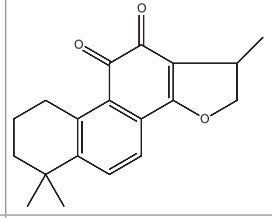
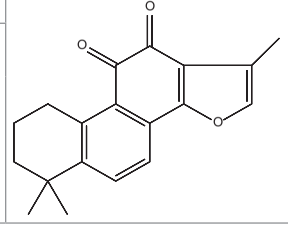
Table 8.1 (continued)

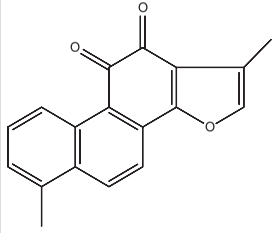
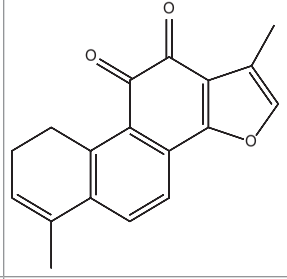
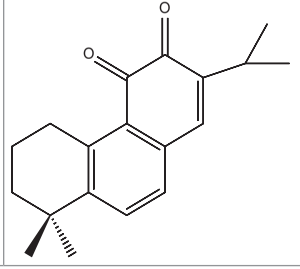
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
	Sugiol This compound was also obtained from the methanol extract of *Sequoia sempervirens* cones (Son et al. 2005), *Juniperus brevifolia* leaves (Moujir et al. 2011), roots of *Peltodon longipes* (Fronza et al. 2011), *Pygmacopremma herbacea* (Satish et al. 2011), cones *Metasequoia glyptostroboides* (Bajpai et al. 2014), *Salvinia molesta* (Li et al. 2013), and *Salvia miltiorrhiza* (Chun-Yan et al. 2015).		IC_{50} (μg/mL): <50 (SW620, HCT116, MDA-MB-231, NCI-H23, and A549) (Son et al. 2005) IC_{50} (μg/mL): >30.04 (MRC-5, PC-3), 23.98 (A549), 20.62 (Hl-60), 26.4 (PANC-1), 10.82 (BxPC-3) (Li et al. 2013).
	6, 7-Dehydroroyleanone[a] This compound was isolated from *Tetradenia riparia* (Demarchi et al. 2015), *Plectranthus forsteri* (Kubínová et al. 2013), *Tetradenia riparia* (de Oliveira et al. 2015), *Rabdoisia lophanthoides* (Lin et al. 2016), and *Sphacele Chamaedryoides* (Areche et al. 2009).		IC_{50} (μg/mL): 2.06 (PC-3), 2.96 (HeLa), 5.85 (3T3) (Choudhary et al. 2013) IC_{50} (μg/mL): 8.34 (HeLa), 3.7 (HCF-8), 2.87 (HepG2), and 19.1 (AGS) (Lin et al. 2016)
	Cariocal[a] This compound was also obtained from *Plectranthus barbatus* (Kelecom and Dos Santos 1985).		IC_{50} (μg/mL): > 6.93 (PC-3), 3.02 (HeLa), >6.92 (3T3) (Choudhary et al. 2013)

	Salvialeriol		IC_{50} (μg/mL): 16.48 (HeLa), 17.24 (PC-3) (Choudhary et al. 2013).
	Deacetylnemorone[a] This compound was also obtained from *Salvia nemorosa* (Takeda et al. 1997) and *Salvia pubescens* (Galicia et al. 1988).		IC_{50} (μg/mL): 0.90 (HeLa), 2.14 (PC-3) (Choudhary et al. 2013)
	2-Acetoxylupeol		IC_{50} (μg/mL): 8.18 (HeLa),10.41 (PC-3) (Choudhary et al. 2013)
	Lupine-2,3-diol[a]		IC_{50} (μg/mL): 1.05 (HeLa), 1.09 (PC-3) (Choudhary et al. 2013)
	8(17),12E,14-labdatrien-6,19-olide This compound was also obtained from the chloroform extract of the aerial parts of the *S. leriifolia* (Habibi et al. 2000).		IC_{50} (μg/mL): 21.03 (MD-MB231), 21.03 (DU-145) (Farimani et al. 2016)

(continued)

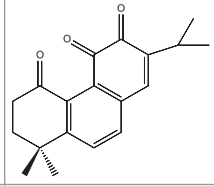
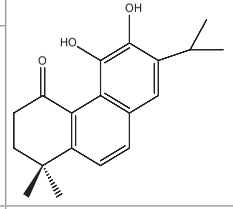
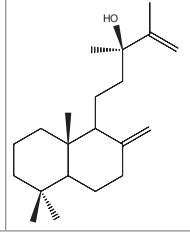
Table 8.1 (continued)

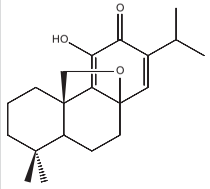
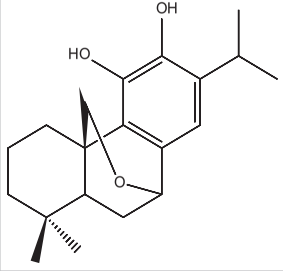
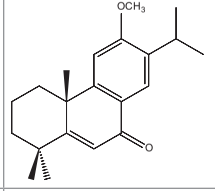
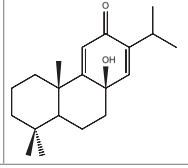
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
	(1S,4aR,8aR)-1,4a-dimethyl-6-methylene-5-((E)-3-methylpenta-2,4-dien-1-yl) decahydronaphthalene-1-carboxylic acid		IC_{50} (μg/mL): 7.55 (MCF-7), 15.10 (MD-MB231), 15.10 (DU-145) (Farimani et al. 2016)
S. leucantha Cav. The compound was obtained from the acetone extract of the aerial parts (Jiang et al. 2016)	Salvileucantholide		IC_{50} (μg/mL): 11.42 (HCT116), 8.77 (BT474), 13.08 (HepG2) (Jiang et al. 2016)
S. miltiorrhiza Bunge The compounds were obtained from the ethanol extract of the whole plant (Chang et al. 2013) and the hexane extract of the roots (Fronza et al. 2011)	Cryptotanshinone[a]		IC_{50} (μg/mL): 1.27 (A549), 1.39 (TOV-21G) (Chang et al. 2013)
	Tanshinone IIA[a]		IC_{50} (μg/mL): 0.59 (A549), 0.81 (TOV-21G) and 1.9 (MIAPaCa-2), NS (MV-3) (Chang et al. 2013; Fronza et al. 2011)
	This compound was also obtained from the acetone extract *S. yunnanensis* root (Wu et al. 2014)		IC_{50} (μg/mL): 2.97 (HeLa), 2.71 (KB-3-1), >2.94 (NCI-H460), 2.62 (PC3), 2.94 (MCF-7), 1.62 (K562) (Wu et al. 2014)

	Tanshinone I[a]		IC_{50} (μg/mL): 2.96 (A549), 2.14 (TOV-21G) (Chang et al. 2013) IC_{50} (μg/mL): 10.5 (MIAPaCa-2) (Fronza et al. 2011)
	1,2-Dihydrotanshinone		IC_{50} (μg/mL): 5.6 (MIAPaCa-2) (Fronza et al. 2011)
	Miltirone This compound was also obtained from *Salvia apiana* (González et al. 1992).		IC_{50} (μg/mL): 22.5 (MIAPaCa-2) (Fronza et al. 2011)

(continued)

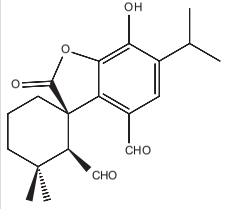
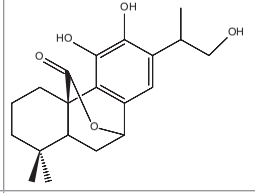
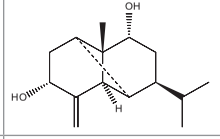
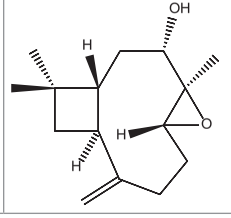
Table 8.1 (continued)

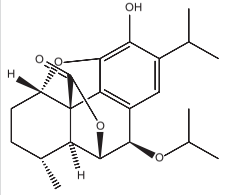
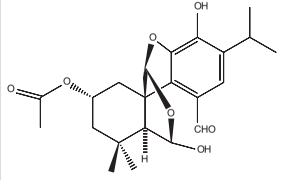
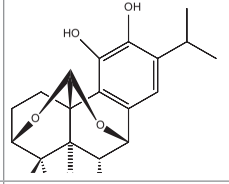
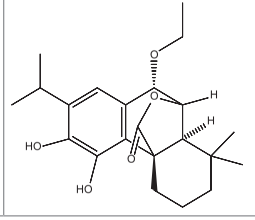
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
	1-Oxomiltirone It was also obtained from *Salvia molesta* (Li et al. 2013) and *Salvia glutinosa* (Senol et al. 2017).		IC_{50} (μg/mL): 29.9 (MIAPaCa-2) (Fronza et al. 2011), IC_{50} (μg/mL): 7.4 (A549), 5.62 (PC-3), 6.89 (HL-60), 9.5 (PANC-1), 6.09 (BxPC-3), 6.04 (MRC-5) (Li et al. 2013)
	Miltiodiol This compound was also obtained from *Salvia apiana* (González et al. 1992).		IC_{50} (μg/mL): 66.3 (MIAPaCa-2) (Fronza et al. 2011)
S. officinalis L. The compound was isolated from the dichloromethane extract of leaves (de Oliveira et al. 2016, Nicolella et al. 2014)	Manool This compound was also obtained from *Catharanthus roseus* L. (de Pinho et al. 2009).		IC_{50} (μg/mL): 49.3 (V79), 15.6 (B16F10), 17.1 (MCF-7), 6.7 (HeLa), 28.5 (HepG2), 9.6 (MO59 J), 6.7 (U343), 13.1 (U251) (de Oliveira et al. 2016)

Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
S. pachyphylla Epling ex Munz. The compounds were isolated from the acetone extract (Guerrero et al. 2006)	Pachyphyllone		IC_{50} (μg/mL): 4.43 (A2780), 7.28 (SW1573), 11.39 (WiDr), 10.13 (T-47D), 6.96 (HBL-100) (Guerrero et al. 2006)
	20-Deoxocarnosol[a] This compound was also obtained from *Coleus barbatus* (Andrews) Benth. (Kelecom 1984), *Sphacele chamaedryoides* (Balbis) Briq. (Areche et al. 2009), and ethanol extract of the whole *S. plebeia* (Zhang et al. 2017).		IC_{50} (μg/mL): 1.71 (A2780), 6.96 (SW1573), 10.13 (WiDr), 10.13 (T-47D), 1.46 (HBL-100) (Guerrero et al. 2006; Zhang et al. 2017)
	5,6–didehydro–*O*-methylsugiol		IC_{50} (μg/mL): 5.23 (A2780), 5.89 (SW1573), 9.48 (WiDr), 10.79 (T-47D), 5.23 (HBL-100) (Guerrero et al. 2006)
	8β-Hydroxy-9(11),13-abietadien-12-one		IC_{50} (μg/mL): 5.74 (A2780), 8.76 (SW1573), 25.07 (WiDr), 24.76 (T-47D), 25.07 (HBL-100) (Guerrero et al. 2006)

(continued)

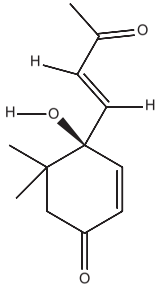
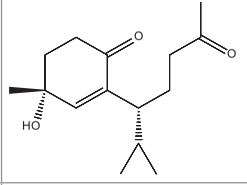
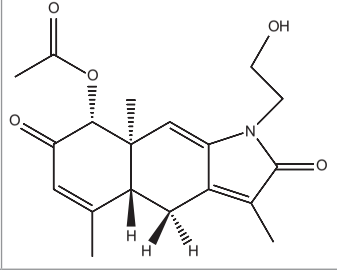
Table 8.1 (continued)

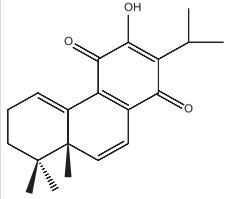
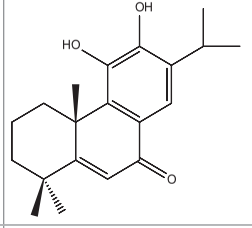
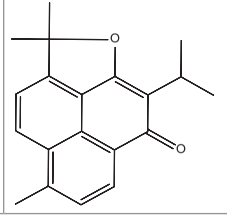
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
	Rosmadial		IC_{50} (μg/mL): 12.40 (A2780), 11.02 (SW1573), 20.32 (WiDr), 16.53 (T-47D), 8.95 (HBL-100), >20 (CaCo2), >20 (MCF-7) (Guerrero et al. 2006; Zhang et al. 2017)
	16-Hydroxycarnosol[a]		IC_{50} (μg/mL): 1.25 (A2780), 3.12 (SW1573), 9.70 (WiDr), 12.12 (T-47D), 1.25 (HBL-100) (Guerrero et al. 2006)
	Plebein B		IC_{50} (μg/mL): >20 (CaCo2), >20 (MCF-7) (Zhang et al. 2017)
S. plebeia R. Br. The compounds were obtained from the ethanol extract of the whole plant (Zhang et al. 2017; Ma et al. 2017)	Suberosols A[a]		IC_{50} (μg/mL): >20 (CaCo2), >20 (MCF-7) (Zhang et al. 2017)

	Plebein C[a]		IC_{50} (μg/mL): 3.81 (CaCo2),10.87 (MCF-7) (Zhang et al. 2017)
	Plebein E		IC_{50} (μg/mL): 7.20 (CaCo2), 9.91 (MCF-7) (Zhang et al. 2017)
	Plebein F		IC_{50} (μg/mL): 10.05 (CaCo2), >20 (MCF-7) (Zhang et al. 2017)
	7-Ethoxyrosmanol		IC_{50} (μg/mL): 4.6 (CaCo2),17.21 (MCF-7) and 25.52 (HeLa), 24.69 (HT29) (Álvarez et al. 2015)

(continued)

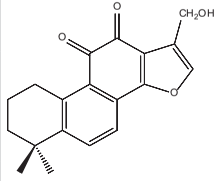
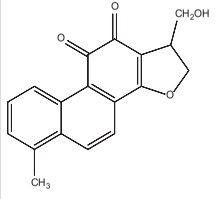
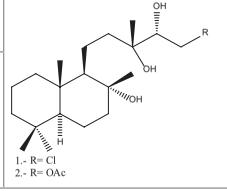
Table 8.1 (continued)

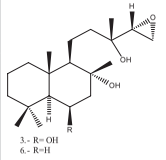
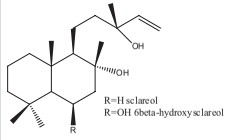
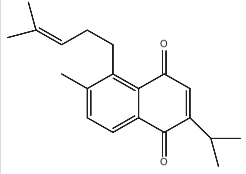
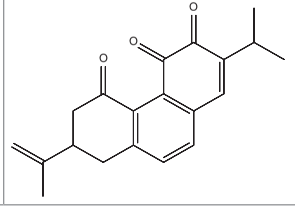
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
	4-Hydroxy-4-(3-oxo-1-butenyl)-3, 5, 5-trimethylcyclohex-2-en-1-one ((S)-(+)-dehydrovomifoliol)		IC_{50} (μg/mL): >20 (CaCo2), >20 (MCF-7) (Zhang et al. 2017)
	(4S)-4-hydroxy-1,10-seco-muurol-5-ene-1,10-dione		IC_{50} (μg/mL): >20 (CaCo2), >20 (MCF-7) (Zhang et al. 2017)
	Salplebeone B		IC_{50} (μg/mL): 4.98 (K562) (Zhang et al. 2017)

S. przewalskii Maxim. The compounds were isolated from the acetone extract of the whole plant (Ohsaki et al. 2011)	Salviskinone A[a]		IC_{50} (μg/mL):1.94 (HL-60) (Ohsaki et. al. 2011)
	Salvinolone[a]		IC_{50} (μg/mL): 0.97 (HL-60) (Ohsaki et. al. 2011)
	Salvilenone		IC_{50} (μg/mL): 9.06 (HL-60) (Ohsaki et al. 2011)

(continued)

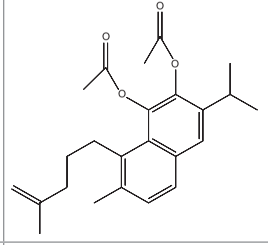
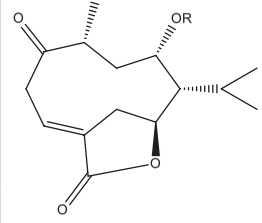
Table 8.1 (continued)

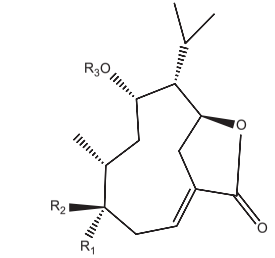
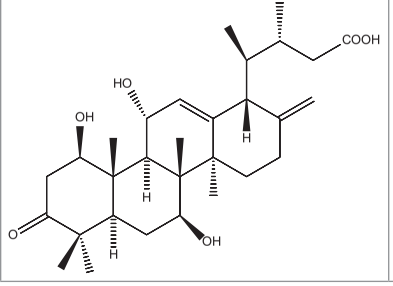
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
	Przewaquinone A[a]	 Przewaquinone A	IC_{50} (μg/mL): 0.77 (5637), 1.73 (A-427), 0.34 (MCF-7) (Mothana et al. 2009)
			IC_{50} (μg/mL): 2.3 (Hep-2), 1.7 (A549), 1.9 (SK-OV-3), 1.6 (SK-MEL-2) 0.8 (XF498) (Wang et al. 2007), IC_{50} (μg/mL): 23 (P-388) (Zhao et al. 2011)
			IC_{50} (μg/mL): 2.3 (A549), 1.7 (SK-OV-3), 1.9 (SK-MEL-2), 1.96 (XF498), 0.8 (HCT15) (Zhang et al. 2013)
	Przewaquinone B[a]	 Przewaquinone B	IC_{50} (μg/mL): 1.9(P-388) (Zhao et al. 2011)
S. reuteriana Boiss The compounds were obtained from the hexane extract of the aerial parts (Farimani et al. 2014)	(1) 14a-Hydroxy-15-chlorosclareol	 1.- R= Cl 2.- R= OAc	IC_{50} (μg/mL): 37.17 (HeLa), 42.16 (MCF7) (Farimani et al. 2014)
	(2) 14a-Hydroxy-15-acetoxysclareol		IC_{50} (μg/mL): 57.16 (HeLa), 46.74 (MCF7) (Farimani et al. 2014)

	6β-Hydroxy-14a-epoxysclareol		IC_{50} (μg/mL): 58.72 (HeLa), 37.29 (MCF7) (Farimani et al. 2014)
	14a-Epoxysclareol		IC_{50} (μg/mL): 59.41 (HeLa), 62.07 (MCF7) (Farimani et al. 2014)
	Sclareol		IC_{50} (μg/mL): 16.6 (HeLa), 32.65 (MCF7), 65.2 μM (MG63) (Farimani et al. 2014).
	6β-Hydroxysclareol		IC_{50} (μg/mL): 53.23 (HeLa), 36.98 (MCF7) (Farimani et al. 2014)
S. sahendica Boiss. & Buhse The compounds were obtained from the hexane extract of root (Fronza et al. 2011; Kafil et al. 2015)	Sahandinone		IC_{50} (μg/mL): 10.18 (MIAPaCa-2) (Fronza, et. al. 2011)
	Ketoethiopinone		IC_{50} (μg/mL): 8.6 (MCF7) (Kafil et al. 2015)

(continued)

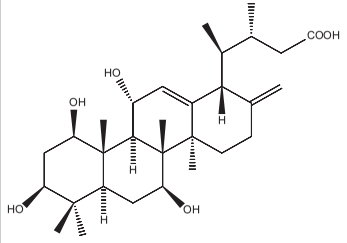
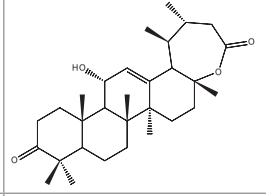
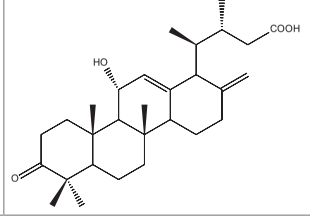
Table 8.1 (continued)

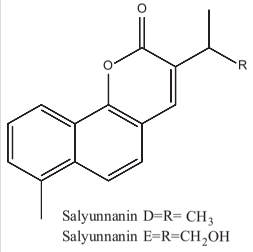
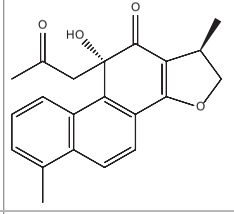
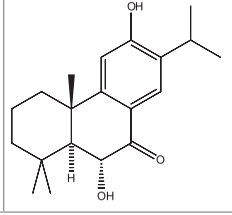
Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
	Ortho-diacetate aethiopinone		IC_{50} (μg/mL): 14.2 (MCF7) (Kafil et al. 2015)
S. scapiformis Hance. The compounds were obtained from acetone extract of the whole plant (Lai et al. 2013)	Scapiformolactone A	 1.- R=AMBT 2.- R=HMBT 3.- R=Tig 1.- scapiformolactone A 2.- scapiformolactone B 3.- scapiformolactone C	IC_{50} (μg/mL): 13.78 (HL-60), 8.72 (SMMC-7721), 13.50 (A549), 16.34 (MCF-7), >16.33 (SW480), 13.45 (BEAS) (Lai et al. 2013)
	Scapiformolactone B		IC_{50} (μg/mL): > 14.61 (HL-60), 8.43 (SMMC-7721), > 14.61 (A549, MCF-7, SW480, BEAS) (Lai et al. 2013)
	Scapiformolactone C		IC_{50} (μg/mL): 7.98 (HL-60), 6.38 (SMMC-7721), 9.10 (A549), 12.16 (MCF-7), > 13.93 (SW480), 5.38 (BEAS) (Lai et al. 2013)

	Scapiformolactone D	 scapiformolactone D =R_1=OH, R_2= H, R_3= AMBT scapiformolactone E =R_1=H, R_2=OH, R_3= AMBT scapiformolactone F =R_1=OH, R_2=H, R_3= HMBT scapiformolactone G=R_1=H, R_2= OH, R_3= HMBT scapiformolactone H=R_1=OH, R_2=H, R_3=Tig scapiformolactone I = R_1=H, R_2=H, R_3= Tig	IC_{50} (μg/mL): > 16.42 (HL-60, SMMC-7721, A549, MCF-7, SW480) (Lai et al. 2013)
	Scapiformolactone E		IC_{50} (μg/mL): > 16.45 (HL-60, SMMC-7721, A549, MCF-7, SW480) (Lai et al. 2013)
	Scapiformolactone F		IC_{50} (μg/mL): > 14.74 (HL-60, SMMC-7721, A549, MCF-7, SW480) (Lai et al. 2013)
	Scapiformolactone G		IC_{50} (μg/mL): > 14.74 (HL-60, SMMC-7721, A549, MCF-7, SW480) (Lai et al. 2013)
	Scapiformolactone H		IC_{50} (μg/mL): > 14.02 (HL-60, SMMC-7721, A549, MCF-7, SW480) (Lai et al. 2013)
	Scapiformolactone I		IC_{50} (μg/mL): > 14.02 (HL-60, SMMC-7721, A549, MCF-7, SW480) (Lai et al. 2013)
S. urmiensis Bunge The compounds were obtained from the acetone and hexane extract of the aerial parts of the plant (Farimani et al. 2015)	1β, 7β,11α-Trihydroxy-3-oxo-17,22-secours-12,17(28)-diene-22-oic acid		IC_{50} (μg/mL): 35.72 (HeLa), 44.65 (HepG2) (Farimani et. al. 2015)

(continued)

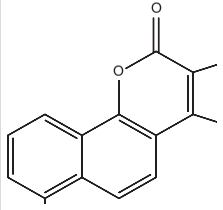
Table 8.1 (continued)

Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
	1β,3β,11α-Trihydroxy-17,22-secours-12,17(28)-diene-22-oic acid		IC_{50} (μg/mL): 12.45 (HeLa), 16.93 (HepG2) (Farimani et al. 2015a)
	Urmiensolide B[a]		IC_{50} (μg/mL): 7.01 (A549), 1.48 (MCF-7) (Farimani et al. 2015)
	Urmiensic acid[a]		IC_{50} (μg/mL): 6.56 (A549), 1.18 (MCF-7) (Farimani et al. 2015)

S. yunnanensis C.H. Wright The compounds were obtained from the acetone extract of the root (Wu et al. 2014)	Salyunnanin D[a]		IC_{50} (μg/mL): 2 (HeLa), >5.04 (KB-3-1, NCI-H460, PC3, MCF-7, K562) (Wu et al. 2014)
	Salyunnanin E[a]		IC_{50} (μg/mL): 0.22 (HeLa), 2.14 (KB-3-1), >5.08 (NCI-H460, PC3, MCF-7, K562) (Wu et al. 2014).
	Danshenol A[a]		IC_{50} (μg/mL): 1.93 (HeLa), 2.22 (KB-3-1), 3.97 (NCI-H460), 1.07 (PC3), 3.21 (MCF-7), 0.63 (K562) (Wu et al. 2014)
	6α-Hydroxysugiol[a]		IC_{50} (μg/mL): 2.45 (HeLa), 2.43 (KB-3-1), 2.36 (NCI-H460),1.90 (PC3),2.77 (MCF-7),1.59 (K562) (Wu et al. 2014)

(continued)

Table 8.1 (continued)

Scientific name/method or plant parts/references	Terpenes	Structure	Cytotoxic activity/references
	Dihydroneotanshinlactone		IC_{50} (μg/mL): >5.78 (HeLa, KB-3-1, NCI-H460, PC3, MCF-7, K562) (Wu et al. 2014)

MCF7 human cancer cell line of breast, *MDA-MB-231* human cancer cell line of breast, *HCT-15* human cancer cell lines of colon, *HCT-16* human cancer cell line of colon, *HeLa* human cervical cancer cell, *WM-115* human skin melanoma cells, *HL-60* human leukemia promyelocytic cells, *NALM-6* human leukemia lymphoblastic cells, *HUVEC* human umbilical vein endothelial cells, *CT26* murine colon cancer cell, *A549* human cancer cell line of lung, *K562* human erythromyeloblastoid leukemia, *Hep-G2* human hepato-carcinoma, *MOLT-4* human acute lymphoblastic leukemia, *PC-3* human prostate cancer cell, *3T3* mouse fibroblast normal cell, *TOV-21G* human ovarian cancer cell, *MIAPaCa-2* human pancreatic cancer cell line, *MV-3* human melanoma cancer cell line, *V79* Chinese hamster lung fibroblasts, *B16F10* murine melanoma, *MO59 J, U343*, and *U251* human glioblastoma, *HaCaT* human keratinocytes

[a]Refers to plant compounds with cytotoxic activity ($IC_{50} < 4$ μg/ml)

8.4 Mechanisms of Action of Terpenes

Taxodione promoted cancer cell death by apoptosis induced by the release of mitochondrial proteins, such as Smac/DIABLO, cytochrome C, and apoptosis-inducing factor, accompanied by the loss of the mitochondrial membrane potential (Burmistrova et al. 2013), whereas ortho-diacetate aethiopinone inhibited the proliferation of breast cancer cells by stimulating apoptosis via DNA and chromatin fragmentation (Kafil et al. 2015). Ferruginol activated the apoptosis via caspases 3, 8, and 9 in different cancer cells (Parsaee et al. 2013; Ho et al. 2015), whereas neotanshinlactone induced the apoptosis by the activation of the transcription factors ATM, Chk2, and p53 in breast cancer cells (Banerji et al. 2017). Likewise, rosmanol, a phenolic terpene, induced apoptosis through DNA fragmentation, cell shrinkage, and chromatin condensation and activation of procaspase 8 and Fas, as well as the release of cytochrome c and PARP cleavage in different cancer cells (Petiwala and Johnson 2015).

Tanshinone IIA promoted apoptosis and cell cycle arrest at the S phase and the downregulation in the expression of the vascular endothelial growth factor (VEGF) in lung cancer cells (Xie et al. 2015). Cryptotanshinone and sugiol, each, inhibited the activation of signal transducer and activator of transcription 3 (Stat3) pathways in colorectal cancer cells and prostate cancer cells, respectively (Li et al. 2015; Jung et al. 2015). In addition, sugiol inhibited topoisomerase I in the human pancreatic cancer cells (Fronza et al. 2012). Sclareol induced cell cycle arrest in G1 phase, apoptosis, and loss of mitochondrial membrane potential in osteosarcoma cells (Wang et al. 2015). Maslinic acid induced DNA fragmentation, activated apoptosis via caspase 3 and caspase 8, as well as reduced the expression of VEGF in pancreatic cancer cells (Lin et al. 2014). Maslinic acid and urosilic acid increased, each, the expression of pro-apoptotic factors such as Bax and the downregulation of anti-apoptotic factors such as Bcl-2 in cancer cells (Reyes-Zurita et al. 2009; Hassan et al. 2016). In ovarian cancer cells, maslinic acid arrested the cell cycle at G1/G0 phase (Siewert et al. 2013, 2014). Carnosol is an antagonist to androgen and estrogen receptors (Johnson et al. 2008; Akaberi et al. 2015) and downregulated proteins involved in proliferation such as AMPK, Akt, and PI3K and arrested prostate cancer in G2 phase of cell cycle (Johnson et al. 2008). Ursolic acid stimulated apoptosis by the release of cytochrome C and by the induction of caspase 3 (Kashyap et al. 2016a) and downregulated the expression of β-catenin/T-cell factor, which participates in cell proliferation (Kim et al. 2014).

8.5 Terpenes of the *Salvia* Genus with Antitumor Effects and Their Mechanisms of Action

Compounds that are active on *in vitro* studies might be assessed for their efficacy through *in vivo* xenograft studies. Five diterpenes obtained from *Salvia* species have been tested for their antitumoral effect. Ferruginol (100 μmol/kg i.p.) decreased tumor growth and tumor weight by approximately 70%, compared to the vehicle

group, in mice bearing CL1–5 tumor after 22 days of treatment. The induction of apoptosis in tumors was shown by immunohistochemistry (Ho et al. 2015). Sclareol (7.85 μg i.p.) decreased by approximately 90% the tumor volume in mice with spontaneous mammary tumor after 17 days of treatment. This antitumor activity was attributed to the activation of cellular immune response (production of IFN-γ and IL-4) by sclareol (Noori et al. 2010). Cryptotanshinone (100 mg/kg s.c.) decreased tumor volume by approximately 25% in nude mice bearing human lung tumor tissue by the induction of apoptosis evaluated by the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay and immunohistochemistry in the tumor samples (Chen et al. 2004). Tanshinone IIA (20 mg/kg i.p.) decreased tumor volume by approximately 25% in nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice bearing osteosarcoma tumor after 45 days of treatment. The induction of apoptosis in the tumor was shown by the induction of caspases 3, 8, and 9 and the upregulation of pro-apoptotic proteins such as Bax, Bad, and Bak (Huang et al. 2017). Tanshinone I (150 mg/kg p.o.) reduced the tumor weight by 67% in SCID mice bearing PC-3 tumor after 42 days, by the downregulation of proliferation elements such as ki67 (Gong et al. 2011). In another study, tanshinone I (80 and 200 mg/kg p.o.) decreased the tumor weight by 27.3% and 34%, respectively, in SCID mice bearing non-small cell lung tumor (H1299) after 42 days of treatments. The downregulation of anti-apoptotic proteins such as Aurora A and Bcl-2 was confirmed in tumors, which suggested apoptotic effects induced by tanshinone I (Li et al. 2013a). The terpenes isolated from plants of the *Salvia* genus could be promising compounds to carry out clinical trials. Nevertheless, it is necessary to perform dose-dependent experiments.

In the present scenario, multidrug resistance (MDR) is a health problem worldwide, with approximately 45% of all types of cancer presenting resistance to chemotherapy. This could be a serious problem in cancer treatment (Kapoor et al. 2013). In this regard, the terpenes from the *Salvia* genus such as teotihuacanin and amarissinin A were tested for their cytotoxic effect on MDR cancer cells. Only teotihuacanin reverted the MDR in these cells (Bautista et al. 2016). Further experiments are necessary to include MDR in cytotoxic experiments. Other plant terpenes have reverted MDR in cancer cells. For instance, the sesquiterpenoid β-elemene, isolated from *Rhizoma zedoariae*, induced high cytotoxicity in KB-C2 cells that overexpress the ABCB1 (P-glycoprotein) transporter, which is involved in MDR of paclitaxel and olaparib, among other cytotoxic agents (Guo et al. 2014). In most of the reports, only a single dose was used and no positive controls were used. In addition, more *in vivo* studies are necessary with terpenes obtained from *Salvia* species. Cancer cells are capable to adapt to toxic effects in monotherapy; when the chemotherapy is administered in combination of drugs with different mechanisms of action, the results in cancer treatment could be improved. This possibility could decrease the adverse effects of chemotherapy and enhanced the efficacy in cancer treatment. In some cases, the combination of plant-derived compounds such as withaferin A, a steroid lactone isolated from *Withania somnifera*, with cisplatin should be efficacious in the treatment of ovarian cancer cells under *in vitro* and *in vivo* assays, compared to the administration of cisplatin alone (Kakar et al. 2014).

In addition, the combination between terpenes, from *Salvia* genus, with different cytotoxic mechanisms should be assessed. For instance, the combination of sulforaphane, an isothiocyanate mainly found in cruciferous vegetables, with quercetin, a flavonoid firstly isolated from trees of *Quercus* genus, induced a synergistic activity decreasing the proliferation of pancreatic cancer cells, compared with either agent alone (Srivastava et al. 2011).

8.6 Conclusions and Future Prospects

Members of *Salvia* species are a good source of terpenes with cytotoxic and antitumor effects. Some terpenes have been studied for their cytotoxic mechanism of action. These terpenes could be promising compounds to carry out clinical trials. Although the various reports of the *Salvia* species are from the Asian continent, it is an utmost fact that the species of the *Salvia* genus are mostly distributed in the American continent. Thus, a huge effort should be needed to evaluate the pharmacological, phytochemical, and toxicological activities of species of *Salvia* genus, and their bioactive compounds should also be evaluated by future investigators for the finding of novel anticancer drugs.

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Essential Oil with Anticancer Activity: An Overview

9

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Abstract

An increased interest by consumers toward pharmacologically active plant-derived natural products as alternatives to synthetic drugs has increased the attention of global scientists. Among the various plant-derived natural products, essential oils have gained popularity because of its use in food, cosmetics, and pharmaceutical industries. Constituting an array of many lipophilic and highly volatile components, derived from a wide range of different chemical classes, essential oils are characterized by a wide range of biological activities, such as antiseptic, anti-inflammatory, spasmolytic, sedative, analgesic, and anesthetic. A growing interest has recently focused on the potential of essential oils as an anti-cancer treatment to overcome the development of multidrug resistance and important side effects associated with the currently used antitumor drugs. The anticancer potential of essential oils has been widely explored till date. A recent Medline survey on PubMed for "essential oil and cancer" retrieves 926 results with a remarkable surge in publications over the last 16 years (688 out of 926

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studies), while a search for "essential oil and cytotoxicity" has shown 434 results, of which 392 were published in the last 10 years. These numbers suggested that the studies in this field have been initiated rather lately, even though essential oils have been known since ancient times. The aim of the present chapter is to provide an overview on the scientific reports published for *in vivo* and *in vitro* studies in reference to essential oils of a wide variety of plants, viz., *Cymbopogon flexuosus*, *Eucalyptus benthamii*, *Laurus nobilis*, *Melissa officinalis*, *Myristica fragrans*, *Rosmarinus officinalis*, *Salvia miltiorrhiza*, *Satureja thymbra*, *Thymus broussonetii*, etc., or their main constituents. Moreover, the various mechanisms of action of different essential oils and their constituents having anticancer properties are also discussed.

Keywords
Antioxidants · Antiseptic property · Biological activity · Chemical constituents · Cytotoxicity

9.1 Introduction

Essential oils are defined by the International Organization for Standardization (ISO) as volatile products extracted from plants by hydrodistillation and steam distillation, by mechanical processes (e.g., *Citrus* spp. essential oils), or by "dry" distillation for some wood (Do et al. 2015). They are usually complex mixtures of lipophilic, volatile, and odoriferous secondary plant metabolites and are associated with an important role in plant protection such as insecticidal and antimicrobial properties (Lorenzi 2008; Radaelli et al. 2016). Various flowers, buds, seeds, leaves, twigs, barks, woods, and roots of different plant species are the source of essential oils. The term "essential" is derived from the word "essence," meaning smell or taste and is linked with specific flavors found in these substances (Calsamiglia et al. 2006). Essential oils are complex mixtures of different chemical compounds, which possess diverse biological activities (antioxidant, anti-inflammatory, antiviral, antimicrobial, stimulators of the central nervous system, etc.) (Do et al. 2015). They occur in a limited number of plant families such as Annonaceae, Apiaceae, Burseraceae, Compositae, Cupressaceae, Cyperaceae, Lamiaceae, Lauraceae, Leguminosae, Myrtaceae, Pinaceae, Poaceae, Rutaceae, and Zingiberaceae (Properzi et al. 2013). The constituents of essential oil may be terpenes, aromatic compounds, and some other compounds of various origins. Essential oils are known to be more powerful than their chemicals for their synergistic and more selective effect (Properzi et al. 2013). The different composition and various uses of essential oils from the same species (chemotypes) can be due to plants growing in varied environments (Do et al. 2015).

Most of the commercialized essential oils are chemotyped by gas chromatography and mass spectrometry analysis (Properzi et al. 2013). The composition, structure, and functional group of essential oils have an important role in determining their biological activities. Their versatile character of antibacterial, antifungal, antioxidant, and anticancer effects is well documented by many scientists (Properzi

et al. 2013; El-Kalamouni et al. 2017; Sharifi-Rad et al. 2017). Essential oils can be used as antimicrobial agents against a wide range of pathogenic microorganisms, including *Listeria monocytogenes*, *L. innocua*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Shigella dysenteriae*, *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella typhimurium* (Simic et al. 2004; Jirovetz et al. 2005; Mith et al. 2014; Batchu et al. 2017; De Santi et al. 2017; Mirzaei-Najafgholi et al. 2017).

Various essential oils have proven their antimicrobial activity against a wide spectrum of pathogenic bacterial and fungal strains. Therefore, they can be used for the purpose of food preservation, medicines, and cosmetics (Properzi et al. 2013). Angelini et al. (2006) showed the use of essential oils in the food industry as natural sanitizing agents. However, Tirillini et al. (2009) analyzed the antifungal activities of *Laserpitium garganicum* subsp. *garganicum* (Ten.) Bertol essential oil against some phytopathogens and opportunistic human fungi. Moreover, Pagiotti et al. (2011) performed the *in vitro* susceptibility test against various fungal isolates for two well-defined chemotypes of *Thymus schimperi* essential oil. They concluded that *T. schimperi* essential oil showed antifungal activity against all of the tested fungal isolates (*Penicillium chrysogenum*, *Verticillium* sp., *Aspergillus tubingensis*, *A. minutus*, *Beauveria bassiana*, and *Microsporum gypseum*). The minimal inhibitory concentration values were similar or lower than those of terbinafine (Pagiotti et al. 2011).

Essential oils are rich in phenolic compounds and for this reason have been researched extensively to evaluate their activity as antioxidants or free radical scavengers. Reactive oxygen species can react with lipids, proteins, and nucleic acids causing oxidative stress and molecular alterations related to cancer, cardiovascular disease, diabetes, aging, and neurodegenerative disorders (Properzi et al. 2013). Radical scavenging and antioxidant properties in the DPPH radical assay at room temperature have been proven for the essential oils of basil, cinnamon, clove, nutmeg, oregano, and thyme (Tomaino et al. 2005). The action of *Citrus limonum* Risso essential oil to control free radical-induced lipid peroxidation and preventing tissue damage to the skin was shown by Bertuzzi et al. (2006, 2012). Properties that could benefit the human skin have been found in lemon essential oil suggesting that its scavenging action could have a practical application for treating the human skin against oxidative damage as it undergoes environmental and chronological aging (Bertuzzi et al. 2012). To find out an overview of essential oil, an extensive Medline research of literature in the PubMed databases was carried out using the methodology of Liberati et al. (2009). The survey for "essential oil and cancer" retrieves 926 results with a remarkable surge in publications over the last 16 years (688 out of 926 studies), while a search for "essential oil and cytotoxicity" has shown 434 results, of which 392 were published in the last 10 years. The aim of the present chapter is to provide an overview on the scientific reports published for *in vivo* and *in vitro* studies in reference to essential oils on a wide variety of plants, viz., *Cymbopogon flexuosus*, *Eucalyptus benthamii*, *Laurus nobilis*, *Melissa officinalis*, *Myristica fragrans*, *Rosmarinus officinalis*, *Salvia miltiorrhiza*, *Satureja thymbra*, *Thymus broussonetii*, etc., or their main constituents. Moreover, the various mechanisms of action of different essential oils and their constituents having anticancer properties are also discussed.

9.2 Cytotoxicity and Cytoprotective Activities of Essential Oils

9.2.1 *In Vitro* Cytotoxicity and Cytoprotective Activities of Essential Oils

With regard to *in vitro* cell culture systems, a compound is considered to be cytotoxic if it interferes with cellular attachment to the surface and the rate of cell growth or if it is causing cell death (Horvath 1980). Different types of assays have been developed to measure cytotoxicity *in vitro* (Riss 2005). One of the mostly used cytotoxicity assays is the MTT, a colorimetric assay which has replaced traditional radioisotopic assays like (3H)-hypoxanthine incorporation (Riss 2005). The presence of numerous pharmacologically active compounds in essential oils makes it a nonspecific cell targeting cytotoxin. Essential oils are lipophilic. They pass through the cell wall and cytoplasmic membrane, which may disrupt the arrangement of the cellular layer comprising of polysaccharides, fatty acids, and phospholipids (Swamy et al. 2016). Altering membranes lead to reduced ATP pool, alteration of the pH gradient, and loss of mitochondrial transmembrane potential, thereby leading to cell death by apoptosis and necrosis. Furthermore, some essential oils can also act as prooxidants by affecting the cellular redox status and also compromise cellular survival (Sharifi-Rad et al. 2017). Observations by scanning and transmission electron microscopy (SEM and TEM) have revealed cell ultrastructural alterations in several compartments such as plasma membrane, cytoplasm, and nucleus (Bakkali et al. 2006). The *in vitro* cytotoxic and cytoprotective activities of some essential oils on various cancer cell lines are discussed below.

9.2.1.1 *Abies* spp. (Family: Pinaceae)

Legault et al. (2003) carried out an antitumor activity study regarding the cytotoxicity of the *A. balsamea* (L.) Mill. essential oil against tumor cell lines MCF-7, PC-3, A-549, DLD-1, M4BEU, and CT-26. *A. balsamea* essential oil was active against all tumor cell lines with GI50 values ranging from 0.76 to 1.7 mg/ml. The GC-MS revealed the presence of three majority constituents: β-pinene (30%), δ-3-carene (19.5%), and α-pinene (15.8%). Furthermore, the presence of α-humulene (0.16%) was found to be responsible for cytotoxicity (GI50 55 mM). The contact of L-929 cells with essential oil and α-humulene inhibits (in dose- and time-dependent manners) the activity of glutathione (GSH) content and the reactive oxygen species (ROS) production. In a recent study by Bonikowska et al. (2015), the cytotoxic activity of *Abies alba* and *A. koreana* (from crushed seeds) essential oil on two cancer cell lines (MCF-7 and MDA-MBA-231) has been highlighted. They concluded that the effect of the essential oils on cancer cells was however weak.

9.2.1.2 *Aristolochia mollissima* (Family: Aristolochiaceae)

Yu et al. (2007) tested the essential oil of the rhizome and the aerial part of the *A. mollissima* for their cytotoxicity against four human cancer cell lines (ACHN, Bel-7402, Hep G2, and HeLa). The rhizome oil possessed a significantly greater

cytotoxic effect on these cell lines than the essential oil from the aerial plant. Among the main compounds, (E)-β-santalol acetate (10.3%) and camphene (6.7%) were detected in the rhizome oil, while spathulenol (6.8%) was detected in the oil extracted from the aerial part of the plants.

9.2.1.3 *Cymbopogon* spp. (Family: Poaceae)

Cymbopogon, commonly known as lemongrass, is a genus of about 55 species, which are native of Asia, Southeast Asia, and Australia. Five species yield the three oils of main commercial importance (Ghosh 2013): lemongrass oil from *C. citratus* of Malaysian origin (West Indian lemongrass) and *C. flexuosus* (East Indian lemongrass) from India, Sri Lanka, Burma, and Thailand; palmarosa oil from *C. martinii*; and citronella oil from *C. nardus* (Sri Lanka) and *C. winterianus* (Java). *C. citratus* (DC.) Stapf is a widely used herb in tropical countries, especially in Southeast Asia. Some of the reported phytoconstituents are essential oils that contain citral α, citral β, nerol geraniol, citronellal, terpinolene, geranyl acetate, myrcene, and terpinol methylheptenone (Shah et al. 2011). Essential oils isolated from leaves have been tested for cytotoxicity on P388 mouse leukemia cells. The IC_{50} value was found to be 5.7 μg/ml (Dubey et al. 1997). *C. flexuosus*, also known as East India or Cochin lemongrass, is a perennial aromatic grass. Its essential oil is used in perfumery and pharmaceutical industries (Kumar et al. 2000). It has shown to have antifungal and antimicrobial activities (Chao et al. 2000). The chemical composition of the essential oil has been reported (Kumar et al. 2008), and various constituents (%) such as geraniol (20.08), geranyl acetate (12.20), α-bisabolol (8.42), and isointermedeol (24.97) have been reported for their cancer cell cytotoxicity (Cavalieri et al. 2004; Kumar et al. 2008).

The essential oil from *C. flexuosus* and sesquiterpene isointermedeol (ISO), which is its major component, was checked for their ability to induce apoptosis in human leukemia HL-60 cells, because deregulation of apoptosis is the hallmark of cancer cells. *C. flexuosus* essential oil and ISO inhibited cell proliferation for IC_{50} values of 30 and 20 μg/ml, respectively (Kumar et al. 2008). *C. flexuosus* essential oil induced has been shown to have *in vitro* cytotoxicity in colon (502713), neuroblastoma (IMR-32), liver (Hep-g-2), and cervix (SiHa) cell lines, with an IC_{50} value ranging from 4.2 to 6.5 μg/ml. The essential oil also induced dose-dependent growth inhibition and decreased the ascitic fluid volume and total ascites cell count in solid and ascitic Ehrlich and S-180 tumor models in mice (Sharma et al. 2009). In another experiment, Ghosh (2013) evaluated the anticancer effect of lemongrass essential oil and citral on cervical cancer cell lines (HeLa and ME-180) *in vitro*. The results have shown that lemongrass oil and citral emulsions initiate the cancer cell death by decreasing cell proliferation, increasing intracellular ROS, altering mitochondrial membrane potential, and initiating apoptosis in HeLa and ME-180 cell lines.

9.2.1.4 *Cyperus rotundus* (Family: Cyperaceae)

C. rotundus is a traditional herbal medicine with an almost global distribution. It's absent only in the northern region. Chemical composition of the rhizome essential oil had been extensively studied (Sonwa and König 2001; Jirovetz et al. 2004; Lawal

and Oyedeji 2009; Aghassi et al. 2013). Some studies also reported the antibacterial, antimutagenic, antiradical, antioxidant, and insecticidal activities of the rhizome essential oil (Kilani et al. 2005, 2007, 2008; Duarte et al. 2007; Essaidi et al. 2014; Liu et al. 2016). Different studies on the *C. rotundus* rhizome revealed the neuroprotective role, anti-apoptotic, and anxiolytic activity using SH-SY5Y human neurons (Lee et al. 2011; Sunil et al. 2011; Kumar et al. 2013, 2014). Based on this, SH-SY5Y cells were selected to investigate the cytotoxicity of essential oils from *C. rotundus* rhizomes. The relationship between the concentration of essential oils and their cytotoxic effect on SH-SY5Y cells was investigated by MTT and LDH release assays. Compared with untreated control cells, a significant decrease in cell viability and LDH leakage was observed above 150 μg/ml in *C. rotundus* essential oil treatment. The essential oil of *C. rotundus* rhizomes contains cyperene, α-cyperone, isolongifolen-5-one, rotundene, and cyperorotundene as principal chemical constituents (Kilani et al. 2005). An *in vitro* cytotoxicity assay suggested that *C. rotundus* essential oil from Tunisia was very effective against L1210 leukemia cells by MTT assay. It correlated with significantly increased apoptotic DNA fragmentation (Kilani et al. 2008). Furthermore, the presence of potential active compounds with antioxidant activity in the essential oil may also play a role in reducing cell numbers because reactive oxygen radicals play a relevant aspect in the carcinogenesis process (Abdelwahed et al. 2007).

9.2.1.5 *Laurus nobilis* (Family: Lauraceae)

L. nobilis is a culinary herb used either fresh or dried for both its flavor and fragrance (Kilic et al. 2004). Medicinally, it has been shown to have *in vitro* antibacterial effects (Dadalioglu and Evrendilek 2004; Marzouki et al. 2009) and wound healing properties on animal models (Nayak et al. 2006). The essential oil fraction of the fruit is also known for suppressing C32 amelanotic melanoma and ACHN renal cancer cell growth through apoptotic mechanisms *in vitro* (Loizzo et al. 2007). *in vitro* studies demonstrated inhibition of cancer cell growth by processed dried leaf of *L. nobilis* extracts in HT-29, HCT-116, Caco-2, and SW-480 human cancer cell lines, which were accompanied by variable levels of elevated apoptosis (Bennett et al. 2013). The leaf of *L. nobilis* also exerted moderate inhibition of cyclooxygenase 2 and 5-lipoxygenase enzymatic activity. In addition, these extracts significantly downregulated interferon-γ production in T helper type 1-stimulated whole blood from healthy donors. Furthermore, size fractionation of the extracts revealed that antiproliferative and proapoptotic activities were associated with low molecular weight, i.e., polyphenolics and essential oils.

9.2.1.6 *Lindera strychnifolia* (Family: Lauraceae)

It is widely used in traditional Chinese medicine. *In vitro* cytotoxic activity of the *L. strychnifolia* essential oil on three human cancer cell lines (A549, HeLa, and Hep G2) was examined using a modified MTT assay (Yan et al. 2009). The leaf essential oil showed the strongest cytotoxicity on the cancer cell lines tested with 50% inhibitory concentration (IC_{50}) values ranging from 22 to 24 g/ml after 24 h of treatment.

9.2.1.7 *Melaleuca alternifolia* (Family: Myrtaceae)

Tea tree oil (TTO) is an essential oil, steam-distilled from the Australian native plant (*M. alternifolia*). It is employed largely for its antimicrobial properties (Pazyar et al. 2013). Out of the over 100 components of the oil, the more represented are terpinen-4-ol, -terpinene, -terpinene, 1,8-cineole, and -cymene (Carson et al. 2006). Terpinen-4-ol, the most abundant component of the oil, is thought to be the main active constituent responsible for the several *in vitro* and *in vivo* activities reported for TTO (Mondello et al. 2006). TTO is known for its antibacterial (Carson et al. 2002, 2006), antifungal (Hammer et al. 2003; Homeyer et al. 2015), antiviral (Schnitzler et al. 2001), anti-inflammatory properties (Hart et al. 2000), and anticancer activities (Bozzuto et al. 2011; Pazyar et al. 2013).

In vitro TTO cytotoxic activity was tested on 14 cancer and 7 non-malignant cell lines, including cervical cancer (HeLa), acute lymphoblastic leukemia (MOLT-4), and erythromyeloblastoid leukemia (K562) and B cell derived from the bone marrow of a patient with acute myeloid leukemia (CTVR-1), fibroblast, and epithelial cells. In these studies, TTO showed an IC50 on cell growth ranging from 0.002% v/v to 0.27% v/v (Soderberg et al. 1996; Hayes et al. 1997; Mikus et al. 2000; Calcabrini et al. 2004; Loughlin et al. 2008; Liu et al. 2009; Greay et al. 2010a; Wu et al. 2012). Calcabrini et al. (2004) reported that TTO as well as terpinen-4-ol were able to impair the growth of human melanoma M14 wild-type cells, where upon the effect was stronger on their resistant variants (M14 adriamycin-resistant cells), which express high levels of P-glycoprotein in the plasma membrane, overcoming resistance to caspase-dependent apoptosis exerted by P-glycoprotein-positive tumor cells. The authors suggested that the greater sensitivity to the TTO treatment displayed by the drug-resistant cells could be ascribed to the different lipid composition of the plasma membrane since there is evidence indicating that multidrug resistance phenotype is also associated with changes in membrane lipid composition (Lavie et al. 1999; Santini et al. 2001). It is worth noting that an earlier study testing the cytotoxic effect of TTO on "normal" epithelial and fibroblast cells, having similar susceptibilities as basal keratinocytes to topical agents (Teepe et al. 1993), did not report toxic effects at concentrations that were shown to affect melanoma cell survival (Soderberg et al. 1996), thus confirming a higher sensitivity of tumor cells as compared to normal cells.

Greay et al. (2010b) has reported the cytotoxic effect of TTO in murine mesothelioma (AE17) and melanoma (B16) cell lines. In this case, TTO and terpinen-4-ol induced cancer cell cycle arrest and primary necrotic cell death with low levels of apoptosis. Recently, the potentiality of TTO and its dominant chemical component, terpinen-4-ol, has also been believed to interfere with the migration and invasion processes of drug-sensitive and drug-resistant melanoma cells (Giordani et al. 2006; Bozzuto et al. 2011; Ireland et al. 2012). Further investigation by Wu et al. (2012) determined terpinen-4-ol to mainly induce apoptosis through the intrinsic mitochondrial pathway in the non-small cell lung cancer (NSCLC) cell lines, A549 and CL1-0. Terpinen-4-ol decreased the levels of apoptosis inhibitor proteins (AIPs), activated caspase 3 and 9, and cleaved poly (ADP-ribose) polymerase (PARP). The combined observations that TTO and terpinen-4-ol not only induce cell death but

also inhibit the growth of aggressive tumor cells, highlighting the potential selective anticancer of these compounds.

9.2.1.8 *Melissa officinalis* (Family: Lamiaceae)

M. officinalis (lemon balm) is a traditional herbal medicine widely diffused in the European and Mediterranean region. *M. officinalis* essential oil has been shown to possess antibacterial (Friedman et al. 2004; Hancianu et al. 2008), antifungal (Mimica-Dukic et al. 2004; El Ouadi et al. 2017) and spasmolytic activities (Sadraei et al. 2003) and antitumoral effect (De Sousa et al. 2004; Queiroz et al. 2014). The anticancerogenic effect of the essential oil of the *M. officinalis* L. was investigated by De Sousa et al. (2004) by an *in vitro* MTT cytotoxicity assay. It showed that this oil was very effective against different human cancer cell lines (A549, MCF-7, Caco-2, HL-60, K562) and a mouse cell line (B16F10). As evidenced by the reduction of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), *M. officinalis* essential oil also possessed antioxidant activity. Queiroz et al. (2014) have studied the activity of the *M. officinalis* essential oil and its major component (citral) in glioblastoma multiforme (GBM) cell lines. GBM is the most common and aggressive form of glioma, and current therapies are not effective. Interestingly, both the essential oil and citral decreased the viability and induced apoptosis of GBM cells as demonstrated by DNA fragmentation and caspase-3 activation. Citral reduced the activity and downmodulated the expression of multidrug resistance-associated protein 1 (MRP1).

9.2.1.9 *Ocimum basilicum* (Family: Lamiaceae)

O. basilicum (basil) is native to Iran and India (Benzie and Wachtel-Galor 2011). In a study of Manosroi et al. (2006), antiproliferative activities of essential oil from *O. basilicum* and other 16 Thai medicinal plants on human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines by MTT assay were checked. In P388 cell line, the *O. basilicum* essential oil had the highest antiproliferative effect, which is 12.7 times less potent than the 5-FU (thymin antagonist 5-fluorouracil). An MTT assay was also conducted to find *in vitro* cytotoxicity of *O. basilucum* essential oil against the HeLa (human cervical cancer cell line), NIH 3 T3 mouse embryonic fibroblasts and Hep-2 (human laryngeal epithelial carcinoma cell line). The IC_{50} values obtained were 90.5, 120.7, and 96.3 mg/ml, respectively. The major constituents of basil essential oil from fresh leaves were investigated to be linalool (17.50%), β-elemene (2.60%), camphor (1.52%), and methyl cinnamate (70.10%) (Kathirvel and Ravi 2012). Thus, it is revealed from their study that basil oil can be used as a therapeutic drug against cancer (Kathirvel and Ravi 2012). The cytotoxic activities of *O. basilicum* essential oil were examined on nasopharyngeal cancer cell line (KB) and liver hepatocellular carcinoma cell line (HepG2) by Shirazi et al. (2014). IC_{50} for KB and HepG2 were 45 ± 4 and 40 ± 3 μg/ml, respectively. On the basis of these results, *O. basilicum* essential oil could be a promising candidate for antitumor drug design. However, the antiproliferative potential of *O. basilicum* and other Greek aromatic plant species essential oils was tested against a panel of human cancer cell lines and evaluated by using the sulforhodamine B (SRB) assay. All essential oil preparations exhibited a variable degree of antiproliferative activity,

depending on the cancer model used, with the most potent one being sweet basil against an *in vitro* model of human colon carcinoma (Fitsiou et al. 2016).

9.2.1.10 *Nigella sativa* (Family: Ranunculaceae)

N. sativa, black cumin, is a herbal plant that has been widely used for various medicinal and nutritional purposes. Human lung cancer cells were exposed to *N. sativa* essential oil and cell viability was measured by NRU assay, in a study conducted by Al-Sheddi et al. (2014). On exposure to *N. sativa* essential oil, cell viability decreased and the cell morphology of A-549 was altered, while essential oil concentrations of 0.1 mg/ml or more, for 24 h, were found to be cytotoxic. By using MTT assay, the decrease in cell viability at 1 mg/ml of NSO was found to be 13%. There was a loss of characteristic cell morphology as well as a decrease in size with A-549 human lung cancer cells exposed to 0.25, 0.5, and 1 mg/ml of *N. sativa* essential oil. Black cumin essential oil was shown to help in reducing human lung cancer cell viability (Ait Mbarek et al. 2007; Al-Sheddi et al. 2014).

Thymoquinone (TQ) is the main constituent of black cumin essential oil with promising *in vitro* antineoplastic activities in different tumor cell lines (Ichwan et al. 2014). Thymoquinone has been revealed in recent studies to give protection against doxorubicin-induced cardiotoxicity. In some cancer cell lines (Effenberger-Neidnicht and Schobert 2010), thymoquinone can act as a booster for the anticancer effect of doxorubicin (chemotherapy agent). Ichwan et al. (2014) report that thymoquinone stimulated distinct apoptotic pathways in two human cervical cell lines, Siha and C33A. Periasamy et al. (2016) used a *N. sativa* essential oil nanoemulsion (NSEO-NE) preparation on human cancer cells using a modified methyl-thiazolyl-diphenyl tetrazolium bromide (MTT) assay, as well as cellular uptake and nuclear morphological analyses. The NSEO-NE significantly reduced the viability of Michigan Cancer Foundation-7 (MCF-7) breast cancer cells. The nucleocytoplasmic morphological features of NSEO-NE-treated cells clearly indicate that NSEO-NE-induced apoptosis in MCF-7 cells.

9.2.1.11 Other Essential Oils

In addition to the essential oils discussed above, several others have shown anticancer activity *in vitro*. When treated with the essential oil of the conifer tree *Tetraclinis articulata* (Vahl) Mast., hallmarks of apoptosis were shown in a number of human cancer cell lines, including melanoma and breast and ovarian cancer (Buhagiar et al. 1999; Li et al. 2004). The essential oil of *Artemisia annua* (sweet wormwood) induced apoptosis in SMMC-7721 hepatocarcinoma cells (Li et al. 2004). Rezaie-Tavirani et al. (2013) investigated the effect of the essential oil of *Rosa damascena* Herrm. on human colon cancer cell line (SW742) and human fibroblast cells. The results have shown that the soluble part of *Rosa damascena* oil increases cell proliferation in high volumes and the non-soluble component decreases cell proliferation (Rezaie-Tavirani et al. 2013). In another study, the *in vitro* anticancer effect for the essential oil of *Salvia verbenaca* growing in natural sites in comparison with those of cultivated plants has been reported (Russo et al. 2015). Growth of the cancer cells (M14) examined was inhibited by both the essential oils, also inducing apoptotic

cell death; however, the most significant effects were demonstrated by the essential oil of cultivated samples.

Essential oils of the Greek aromatic plants *Satureja thymbra* L. and *S. parnassica* Heldr. & Sart. ex Boiss. were tested for cytotoxicity (using 2,2-diphenyl-1-picrylhydrazyl and sulforhodamine B assays) and antiproliferative potential against the MCF-7, A549, HepG2, and Hep3B cell lines (Fitsiou et al. 2016). *S. thymbra* and *S. parnassica* essential oils showed significant and diverse antiproliferative activities, mainly attributed to carvacrol and thymol, their main components (Fitsiou et al. 2016). Another study dealt with the antiproliferative effect of *Origanum vulgare* L. against human breast adenocarcinoma (MCF-7), and human colon adenocarcinoma (HT-29) was performed by sulforhodamine B assay. The results show that the essential oil, mostly composed of 4-terpineol, induces a high cytotoxicity effect in HT-29 (Begnini et al. 2014).

Essential oil from aerial parts of *Thymus carmanicus* Jalas (Hezar Mountain, Kerman Province, Southern Iran) was investigated for the cytotoxic property against human oral epidermoid carcinoma KB cells by MTT and neutral red assays (Fekrazad et al. 2017). The results showed that the essential oil (IC_{50} = 0.44 μl/ml) has potent cytotoxic property on KB cells and it could be used for oral cancer treatments (Fekrazad et al. 2017). The essential oil extracted from the leaves of *Vepris macrophylla* (Baker) I. Verd., a high evergreen tree endemic of Madagascar, was reported to have a cytotoxic effect (Maggi et al. 2013). On MDA-MB 231 human breast adenocarcinoma and HCT116 human colon carcinoma tumor cell lines, results showed that the essential oil exhibited strong inhibitory effects, with inhibition values comparable to those of the anticancer drug cisplatin (Maggi et al. 2013). In traditional medicine, the ginger plant (*Zingiber officinale*) is a very prominent species, probably native to Southeast Asia. Cytotoxicity of ginger essential oil against Dalton's lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cell lines were evaluated by the trypan blue exclusion method, while cytotoxicity to L929 cells was instead tested by MTT assay (Jeena et al. 2015). The IC_{50} value for DLA cell line was 11 μg/ml and for EAC cell lines 18 μg/ml. The IC_{50} of ginger essential oil was found to be 41 μg/ml against the L929 cell lines. This suggests the potential use of ginger essential oil as an anticancer agent. Also, another essential oil, this time from the fruits of the *Annonaceae*, *Xylopia parviflora* Spruce (from Chad and Cameroon), exerted more cytotoxic activity against MCF-7 cancer cell line than against normal cell line (ARPE-19), with IC_{50} values of 0.155 μl/ml and 0.166 μl/ml respectively (Bakarnga-Via et al. 2014).

9.2.2 *In Vivo* Anticancer Activity of Essential Oils

Some essential oils were tested against cancer *in vivo*. No test results were done on humans, only animal models were used, so it is rash to attribute anticancer properties to essential oils. The following results give more detail of the studies carried out with essential oils.

9.2.2.1 *Aquilaria crassna* (Family: Thymelaeaceae)

In vivo antitumor activity of *A. crassna* essential oil was tested on HCT116 colorectal carcinoma cells established in nude mice. Promising growth inhibition of the subcutaneous tumor was observed (Dahham et al. 2016).

9.2.2.2 *Boswellia sacra* (Family: Burseraceae)

Essential oil from gum resins was used to evaluate antitumor activity in a heterotopic human pancreatic cancer xenograft nude mouse model. The increased cell death caused by the interference in apoptotic pathway in which Akt and Erk1/2 were activated, while cyclin D1 cdk4 expression were suppressed (Ni et al. 2012).

9.2.2.3 *Brassica* spp. (Family: Brassicaceae)

Mustard essential oil containing allyl isothiocyanate (AITC) was studied on Swiss albino mice transplanted with Ehrlich ascites tumor (EAT) cells when compared with the normal HEK293 cells. AITC arrested the growth of EAT cells by inducing apoptosis. Antiangiogenic mechanisms were also observed (Kumar et al. 2009).

9.2.2.4 *Croton regelianus* (Family: Euphorbiaceae)

Antitumor effects of the essential oil from the leaves of *C. regelianus* and ascaridole was tested in sarcoma 180 murine model. Relatively low inhibition rates were observed, depending on the dose (Bezerra et al. 2009).

9.2.2.5 *Cupressus sempervirens* (Family: Cupressaceae)

The essential oil from *C. sempervirens* leaves was tested on experimental animals model cancer cell line (EACC). The modest results were augmented with a pre-initiation treatment (Fayed 2015).

9.2.2.6 *Curcuma aromatica* (Family: Zingiberaceae)

Spring *C. aromatica* essential oil (CSEO) and autumn *C. aromatica* essential oil (CAEO) were tested in nude mice bearing non-small cell lung cancer (NSCLC). CAEO is more active than CSEO (Ma et al. 2016).

9.2.2.7 *Curcuma longa* (Family: Zingiberaceae)

C. longa essential oil was tested on solid tumor development on mice induced with Dalton's lymphoma ascites cells. A reduction of cell proliferation was observed, but not a resolution of tumor cells (Liju et al. 2014).

9.2.2.8 *Curcuma wenyujin* (Family: Zingiberaceae)

The essential oil from the rhizome of *C. wenyujin* inhibited tumor growth of human cervical cancer HeLa in a xenograft mouse tumor model. The suggested mechanism of action of *C. wenyujin* essential oil is by inducing apoptosis (Lim et al. 2010). The antitumor activities were also evaluated in sarcoma 180 (S180) tumor-bearing mice by intraperitoneal administration. Between the essential oil main compound classes, the sesquiterpenoids seemed to be the more active (Song et al. 2014).

9.2.2.9 *Curcuma zedoaria* (Family: Zingiberaceae)

The essential oil from *C. zedoaria* was tested on mice inoculated with H1299 cells. A moderate activity was observed (Chen et al. 2013).

9.2.2.10 *Cymbopogon flexuosus* (Family: Poaceae)

The essential oil from *C. flexuosus* was tested on both solid and ascitic Ehrlich and Sarcoma-180 tumor models in mice. This study indicate that the oil act on the apoptotic pathway resulting in a moderate antitumoral activities (Sharma et al. 2009).

9.2.2.11 *Duguetia gardneriana* (Family: Annonaceae)

Essential oil from the leaves of *D. gardneriana* was tested on B16-F10-bearing mice. An antitumor activity *in vivo* was observed, but high doses produce relative low effects (Rodrigues et al. 2015).

9.2.2.12 *Guatteria friesiana* (Synonym *Guatteriopsis friesiana*) (Family: Annonaceae)

The antitumor activity of the essential oil from the leaves of *G. friesiana* and its main components (α-, β-, and γ-eudesmol) were tested in mice inoculated with sarcoma 180 tumor cells. The essential oil seems to not significantly affect body mass, macroscopy of the organs, or blood leukocyte counts, but the antitumoral effect dose-dependent requires too high a dosage for a nondefinitive effect (Britto et al. 2012).

9.2.2.13 *Guatteria pogonopus* (Family: Annonaceae)

The *in vivo* antitumor activity of the essential oil isolated from leaves of *G. pogonopus* was tested in mice bearing the sarcoma 180 tumor. Moderate tumor growth inhibition rates were observed at high dosage (Fontes et al. 2013).

9.2.2.14 *Kaempferia angustifolia* (Family: Zingiberaceae)

The effects of rhizoma essential oil of *K. angustifolia* on tumor growth and cell cycle were tested on MKN-45 human gastric cancer cells orthotopically transplanted in nude mice. The antitumoral effect acted via apoptosis arresting the cancer cells at G0/G1 phase (Xiao et al. 2006).

9.2.2.15 *Lippia gracilis* (Family: Verbenaceae)

In vivo antitumor study of leaf essential oil was tested on mice bearing sarcoma 180 tumor cells. Moderate tumor growth inhibition rates were observed even at high dosage (Ferraz et al. 2013).

9.2.2.16 *Mentha villosa* (Family: Lamiaceae)

In vivo antitumor activity of the *Mentha villosa* essential oil from the leaves was observed in sarcoma 180-bearing mice. The essential oil possesses low antitumoral activities and low systemic toxicity (Amaral et al. 2015).

9.2.2.17 *Nigella sativa* (Family: Ranunculaceae)

The *in vivo* antitumor activity of the essential oil of *N. sativa* L. seed was tested on DBA2/P815 (H2 d) mouse model. A reduction tumor volume was observed after injection of the essential oil into the tumor site after 30 days of treatment. This treatment also seemed to reduce the liver metastasis development (Ait Mbarek et al. 2007).

9.2.2.18 *Origanum vulgare* (Family: Lamiaceae)

The anticancer activity of *O. vulgare* essential oil was tested *in vivo* using F1 DBA C57 black hybrid mice with Lewis carcinoma. The essential oil was administered in drinking water for 3 months. The development of the tumor was moderately slowed down (Misharina et al. 2013). In other experiments, the essential oil modified the levels of tumor necrosis factor-α and viable activated macrophages and was capable to mitigate the effects of degradation of conjugated dienes (Grondona et al. 2014).

9.2.2.19 *Oxytropis falcata* (Family: Leguminosae)

The antitumor effects of the essential oil of *O. falcata* were tested in transplanted murine H22 solid tumors *in vivo*. Growth inhibition in H22 solid tumors was moderate (Yang et al. 2013).

9.2.2.20 *Pistacia lentiscus* (Family: Anacardiaceae)

Antitumor activities of mastic oil from *P. lentiscus* were studied in a xenograft mouse with Lewis lung carcinoma tumor model. The oil was administrated intraperitoneally. The growth inhibition tumor was moderate without toxicity. The mechanism of action is related to an increased apoptosis, a reduced neovascularization, and an inhibition of chemokine expression (Magkouta et al. 2009).

9.2.2.21 *Deverra tortuosa* (Synonym *Pituranthos tortuosus*) (Family: Apiaceae)

The essential oil of *P. tortuosus* was tested on mice bearing the B16F10 melanoma cancer cells. There was a high reduction of tumor weight but at very high doses (Krifa et al. 2016).

9.2.2.22 *Sphagneticola calendulacea* (Synonym *Wedelia chinensis*) (Family: Compositae)

The essential oil of *W. chinensis* was tested on male C57BL/6 mice injected with B16F-10 melanoma cells. There was an increase in the number of apoptotic cells in the essential oil treated group. The essential oil suppresses even the tumor-directed angiogenesis. It has shown an increase in the levels of p53 and caspase-3 (Manjamalai and Grace 2013).

9.2.2.23 *Xylopia frutescens* (Family: Annonaceae)

The leaf essential oil of *X. frutescens in vivo* antitumor activity was assessed in sarcoma 180-bearing mice. Moderate tumor growth inhibition rates were observed (Ferraz et al. 2013).

9.2.2.24 *Xylopia laevigata* (Family: Annonaceae)

The effect of the essential oil from leaves of *X. laevigata* was tested *in vivo* on mice bearing sarcoma 180 tumor cells. Moderate tumor growth inhibition rates were observed. The treatment with the essential oil did not significantly affect body weight, macroscopy of the organs, or blood leukocyte counts (Quintans et al. 2013).

9.2.2.25 *Zanthoxylum rhoifolium* (Family: Rutaceae)

The antitumor properties of the volatile oil from *Z. rhoifolium* leaves and some terpenes (α-humulene, β-caryophyllene, α-pinene, and β-pinene) were investigated *in vivo* using the Ehrlich ascites tumor model. Treatment of Ehrlich ascites tumor-bearing mice with the volatile oil and β-caryophyllene for 4 days significantly increased survival, whereas administration of α-humulene, α-pinene, and β-pinene were ineffective. α-Humulene, α-pinene, and β-pinene did not show antitumor activity (Da Silva et al. 2007).

9.2.2.26 *Zornia brasiliensis* (Family: Leguminosae)

The essential oil of the leaf of *Z. brasiliensis* was tested on mice inoculated with B16-F10 mouse melanoma. The antitumor activity *in vivo* was dose-dependent, but high doses produced relatively low effects (Costa et al. 2015).

9.2.3 *In Vivo* Anticancer Activity of Chemical Components

Some components of essential oils were tested *in vivo* and showed interesting antitumoral activity. However, some other components showed pro-tumoral activity such as methyleugenol and estragole (Ferraz et al. 2013; Liju et al. 2014).

9.2.3.1 1,8-Cineole

A statistically significant reduction of cell proliferation was observed compared to the control cells when tested on RKO cells and human colon cancer cell lines HCT116 injected into the SCID mice. The 1,8-cineole induced apoptosis by inactivating surviving and Akt, activating p38, inducing PARP, and cleaving caspase-3 (Rodd et al. 2015).

9.2.3.2 Allicin

For allicin, a reduction of tumoral cell proliferation was reported by Misharina et al. (2013).

9.2.3.3 Allyl Isothiocyanate

This compound was tested on Ehrlich ascites tumor cells transplanted in Swiss albino mice using HEK293 cells as control. There was a significantly reduced ascites secretion and tumor cell proliferation. Also the vascular endothelial growth factor expression was inhibited. The apoptosis was induced in tumor cells, and cell cycle was arrested at G1 phase (Ichwan et al. 2014).

9.2.3.4 β-Bisabolene

The first study seemed that β-bisabolene had promising activities against 4T1 mammary tumor cells *in vitro* and transplanted in mice (Krifa et al. 2016).

9.2.3.5 Camphene

This was tested against melanoma cells in a syngeneic model, and there was promising antitumor activity (Ma et al. 2016).

9.2.3.6 Cinnamaldehyde

This is promising in antitumor activity against NSCLC cells. The cells were induced in apoptosis and also the epithelial-mesenchymal transition was reversed affecting the Wnt/b-catenin pathway (Bouyahya et al. 2016).

9.2.3.7 Iso-egomaketone

This compound tested in *in vivo* models of B16 melanoma gave a significant cell growth inhibition. The tumor cells show an ROS-mediated mitochondria-dependent apoptotic-type death in sub-G1 cell cycle. Iso-egomaketone seemed to generate ROS in cells as well as upregulation of Bax and Bcl-2expression (Russo et al. 2015; Shen et al. 2012).

9.2.3.8 Elemene

For this compound, a reduction of tumoral cell proliferation in human and murine models was reported (Misharina et al. 2013).

9.2.3.9 Eugenol

This compound was tested on a model of skin tumor induced by DMBA croton oil in Swiss mice. The eugenol affects the cellular proliferation by increasing apoptosis cellular death. There is evidence for a downregulation of c-myc, H-ras, and Bcl-2 expression and an upregulation of p53, Bax, and active caspase-3 (Grondona et al. 2014).

9.2.3.10 Farnesol

A pharmacogenomic approach was used for the farnesol. Tests on many genes involved in apoptosis, regulation of transcription, and genes like INE1, CTRL, MRS2, NEB, LMO7, C9orf3, and EHBP1 are not conferred resistance to farnesol. The effects of farnesol on genes not related to the resistance to anticancer drugs may speculate the design of new drugs against tumor-resistant line (Manjamalai and Grace 2012a, 2012b; Ji et al. 2014).

9.2.3.11 Furanodiene

Tested on uterine cervical (UI4) and sarcoma 180 (S180) tumors in mice, the furanodiene has a significant tumor inhibition rate dose-dependent. The furanodiene was administered intraperitoneally as liposome. The effect of furanodiene on the inhibition of cell proliferation by activation of apoptosis was not hypothesized (Song et al. 2014, Zielińska and Matkowski 2014, Polo et al. 2013).

9.2.3.12 Geraniol

Starting from antitumor activity against several cell lines by an arrest occurring at the G0/G1 cell cycle and ultimately with an increase of apoptosis, this molecule was found to interfere with the mevalonic cycle enzyme. Suppression of prenylation of proteins leads to the inhibition of DNA synthesis, and the suppression of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) leads to a reduction of the mevalonate pool and thus limits protein isoprenylation. In the same way, a reduction of cholesterol biodisponibility was controlled (Pattanayak et al. 2009; Ni et al. 2012; Dahham et al. 2016).

9.2.3.13 Hinokitiol

Studied in xenograft tumors such lung adenocarcinoma cell, EGFR-TKI-resistant lines PC9-IR and H1975 in which the growth inhibition was observed, a novel antitumor mechanism was hypothesized. In summary, the hinokitiol can induce DNA damage and autophagy (Rodrigues et al. 2015).

9.2.3.14 D-Limonene

Tested as promising antitumor molecules in induced tumor on rat tissues, D-limonene was tested in preclinical studies in patients with advanced cancer. Limonene inhibits the activity of HMG-CoA reductase, subsequently reducing the possibility of cancer growth. The mechanism of action involves the inhibition of prenyltransferases with the activation of glutathione-S transferase and uridine diphosphoglucuronosyltransferase. More interest was pointed on the principal metabolite: perillyl alcohol which is more potent than limonene. The interest on perillyl alcohol is based on the necessity of a very high dosage of D-limonene in preclinical studies (about 1000 mg/kg/day in human mammary tumor) that can cause notably important side effects. The more active perillyl alcohol and the less low active doses hypothesized this molecule as a clinical candidate (Pattanayak et al. 2009; Chen et al. 2013; Fontes et al. 2013; Rani and Sharma 2013).

9.2.3.15 Linalool

Studies of antitumor activities and toxicity were done on solid S-180 tumor-bearing Swiss albino mice. It results in an induction of oxidative stress with an antitumor activities result. In comparison with cyclophosphamide, antioxidant effects were observed in the liver and modulation of proliferation of spleen cells in tumor-bearing mice challenged with lipopolysaccharides, while both were seriously affected by cyclophosphamide (Costa et al. 2015).

9.2.3.16 Myrtenal

Anticancer activity of myrtenal was tested against the diethylnitrosamine-induced hepatocellular carcinoma in Wistar albino rats. The apoptosis protein pattern was taken into account and resulted in upregulation of proteins anti-apoptotic (Ziech et al. 2012; Gautam et al. 2014).

9.2.3.17 Terpinen-4-Ol

Also this molecule exhibits antitumor effects by apoptotic mechanism. Studies were done in mice bearing A549 tumor xenografts (Quintans et al. 2013; Kiyan et al. 2014).

9.3 Conclusions and Future Prospects

Essential oils have been used in medicine since ancient times, and the present chapter is an attempt to highlight their *in vitro* and *in vivo* cytotoxic, chemopreventive, and therapeutic values. The main aim of summarizing the research in this area is to provide an overview of the existing knowledge regarding the pivotal role of essential oils as source of anticancer molecules and phytotherapeutics for humans. With more research, essential oils can efficiently be exploited in pharmaceutical preparations, some of which are already undergoing different phases of clinical trials. Compared with their individual constituents, essential oils are more effective in the preliminary studies. Essential oils and their constituents can be evaluated as therapeutic agents and can be used in addition to standard therapies. Due to the lack of target-specific release, the immense potential of essential oils needs to be explored further; in fact, research into essential oils as anticancer therapeutic agents is still in the early stages. Furthermore, studies including clinical trials are also required along with the use of advanced techniques for the targeted organ-specific release of the essential oils for making the treatment more effective.

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10

Fungal Endophytes from Anticancer Plants as Producers of the Antitumor Agent L-Asparaginase: A Look at Diversity, Ubiquity, and Enzyme Production

Yiing Yng Chow, Wei Shang Tan, and Adeline Su Yien Ting

Abstract

Endophytes are microorganisms inhabiting plants without causing any visible symptoms. In recent years, endophytes from medicinal or anticancer plants have demonstrated the ability to produce bioactive compounds similar to anticancer compounds derived from their host plant, i.e., taxol from *Taxomyces andreanae* found in the Pacific yew tree. In this study, we explored endophytes from several common medicinal plants for their potential to produce L-asparaginase, an anticancer agent that deprives tumor cells from L-asparagine. The plants selected have been documented for their ethnobotanical anticancer properties, which include *Cymbopogon citratus*, *Murraya koenigii*, *Oldenlandia diffusa*, *Pereskia bleo*, and *Andrographis paniculata.* The fungal endophytes were isolated and identified and their L-asparaginase production was determined. Results revealed that anticancer plants do harbor a diverse group of fungal endophytes, and their distribution and relative abundance vary according to plant parts (roots, stem, leaves). The number of endophytes isolated is highest from *A. paniculata* (50 isolates), followed by *P. bleo* (40 isolates), *O. diffusa* (25 isolates), *C. citratus* (14 isolates), and *M. koenigii* (10 isolates). Most of the fungal endophytes are species of *Colletotrichum*, *Fusarium*, *Penicillium*, *Phoma*, and *Aspergillus*. Comparison of the species diversity in each plant part showed that roots typically harbor the most number of endophytes isolated, followed by leaf or stem tissues. Nevertheless, the number of isolates with L-asparaginase production differs among plants. *A. paniculata* has the most number of endophytes with L-asparaginase activities (39 of the 50 isolates), compared to *P. bleo*, *O. diffusa*, *C. citratus*, and *M. koenigii* with 15, 7, 2, and 1 positive isolate(s), respectively. Their L-asparaginase activities quantified were between 0.009 and

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0.0246 M^{-1} mL^{-1} min^{-1} of mean L-asparaginase activity, with higher levels derived from endophytes from *O. diffusa*. Our study has shown that endophytes from anticancer plants have the potential as producers of L-asparaginase. They can be developed for upscale production under optimized conditions, to produce sufficient L-asparaginase for cancer treatment.

Keywords
L-Asparaginase · Anticancer plants · Endophytes

10.1 Introduction

Endophytes (Gr. *endon*, within; *phyton*, plant) are microorganisms found naturally inhabiting plants. They exist inside the internal tissues of living plants without causing any visible symptoms to the host plants (Nelson and Nelson 2012). Endophytes are ubiquitously found in many different plant species, colonizing almost every single plant organs (i.e., root, stem, leaf, flower). The number of publications on endophytes and plants increased throughout the years, attributed to the discovery of their various beneficial roles and applications (Fig. 10.1). These include their active role in promoting plant growth (Kang et al. 2007), in inducing plant defense mechanisms to tolerate pathogen infection (Bakker et al. 2007; Kang et al. 2007; Senthilkumar et al. 2007; Ting et al. 2009), and in producing valuable bioactive compounds (Newmann and Cragg 2007; Ting et al. 2011; Akinsanya et al. 2016; Hermanto and Ting 2016).

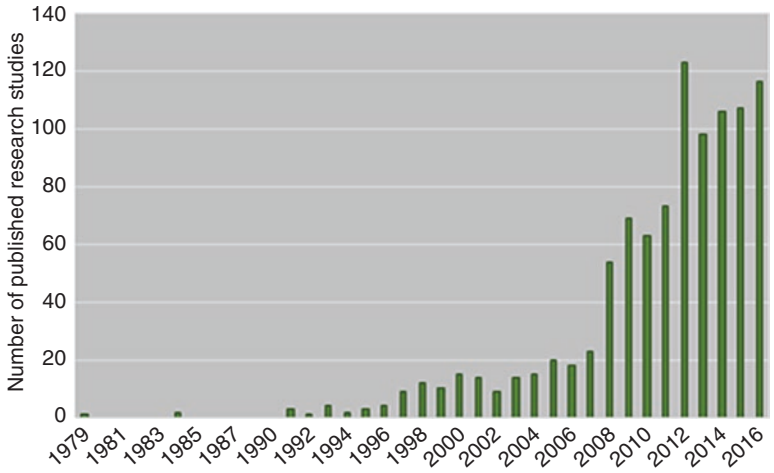

Fig. 10.1 A total of 988 research studies of endophytic fungi and plants that have been reported from 1979 to 2015. Data is sourced from ISI Web of Science using keywords "plants" in the topic field and "endophytic fungi" in the title field

It has been hypothesized that endophytes have the ability to produce compounds or metabolites similar to bioactive compounds found in the host plant, as a result of their close symbiotic relationship with the host plants (Theantana et al. 2009). The milestone to this hypothesis is the discovery of the anticancer agent taxol from the endophyte *Taxomyces andreanae.* Taxol was initially found only in the Pacific yew tree (Harper et al. 2003). With the discovery of taxol production by the endophyte *T. andreanae*, many researchers believe that isolation of these endophytic bioactive compounds is important as these compounds can be effective alternatives to modern medicine. Endophytes producing important bioactive compounds can be harnessed for large-scale production, without risking the biodiversity and resources for cultivating host plants such as medicinal plants (Mehanni and Safwat 2010). Furthermore, endophytes can produce a wide array of bioactive compounds with the following properties: antibiotic, anticancer, antioxidant, and anti-inflammatory (Newmann and Cragg 2007; Guo et al. 2008; Mehanni and Safwat 2010; Ting et al. 2011; Patil et al. 2012; Pandey et al. 2014; Akinsanya et al. 2016; Hermanto and Ting 2016). Production of bioactive compounds from microbial sources (such as endophytes) is also more economical and feasible in the long run (Zhao et al. 2011).

In this chapter, the anticancer agent of interest is L-asparaginase. This enzyme is clinically acceptable for the treatment of the disease acute lymphoblastic leukemia (ALL) (Verma et al. 2007). In healthy cells, L-asparagine is synthesized with the help of L-asparagine synthetase, which catalyzes the conversion of aspartate to asparagine by using adenosine triphosphate (ATP). In patients with ALL, the tumor cells are incapable of L-asparagine synthesis and must obtain L-asparagine from the serum for growth (Verma et al. 2007). Therefore, treatment with L-asparaginase aids in controlling tumor growth by catalyzing the hydrolysis of L-asparagine to L-aspartate and ammonia thereby restricts the amount of available L-asparagine in the serum for tumor cells. The other advantage of using L-asparaginase as an anticancer agent is that the enzyme is biodegradable and nontoxic and can be administered via localized administration quite easily and is also less painful and less costly compared to other chemotherapeutic agents (Kamble et al. 2012; Lopes et al. 2017). Extensive clinical studies have shown that administration of L-asparaginase increases survival rate (Pieters et al. 2011).

To develop and produce L-asparaginase for anticancer treatment, the enzyme is typically sourced from bacteria such as *Escherichia coli*, *Erwinia carotovora*, and *E. chrysanthemi* (Li et al. 2007; Theantana et al. 2009; Pieters et al. 2011). These bacterial-derived enzymes are commercially available in the USA as the following: *E.coli* native L-ASNase, erwinase, and pegasparaginase (PEG-ASNase) (Masetti and Pession 2009). However, the presence of L-glutaminase in this bacterial-derived asparaginase causes many side effects such as hypersensitivity (allergic reactions) and anaphylaxis (Kotzia and Labrou 2007; Li et al. 2007, Pieters et al. 2011). In addition, the half-life of L-asparaginase from bacteria is relatively short, deteriorating its pharmacokinetic profile as anticancer drug (El-Naggar et al. 2014). It is therefore of utmost importance that L-asparaginase is sourced from nonbacterial (eukaryotic) origin, to eliminate the side effects.

The first few attempts at eukaryotic sources for L-asparaginase are from plants, such as medicinal plants. Oza et al. (2009) extracted L-asparaginase from *Withania somnifera*, a traditionally used Indian ginseng (Oza et al. 2009). They found that L-asparaginase from plants is less harmful and available abundantly. Nevertheless, over the years, it was revealed that there are several serious limitations to sourcing for plant-derived asparaginase. Isolation of plant-derived asparaginase would increase the need to harvest slow-growing and possibly rare medicinal plants leading to diminishing biodiversity. In addition, the bioactivities of plant-derived L-asparaginase differed from bacterial-derived L-asparaginase, possibly attributed to the nonhomologous nature of their respective amino acid sequences. This prompted the biosourcing of fungal-type L-asparaginase, which does not cause allergic reaction and is safer to use (Patil et al. 2012). Several fungal isolates are known to produce L-asparaginase; examples are *Bipolaris* sp. from brown rice and *Aspergillus* sp. from soil (Lapmak et al. 2010; Rani et al. 2012). In recent years, endophytes are explored as producers of L-asparaginase. This approach is relatively new and innovative, with only few studies reporting such potential (Chow and Ting 2015). In their study, it was found that L-asparaginase producers were *Fusarium oxysporum* and *Penicillium simplicissimum* from *Murraya koenigii* and *Pereskia bleo*, respectively. These plants are lesser-studied medicinal plants rich in ethnobotanical value from Southeast Asia. L-asparaginase-producing endophytic fungi include *Alternaria alternata*, *Aspergillus aculeatus*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Petromyces flavus*, *and Pleospora allii*, which were successfully isolated from *Withania somnifera* (Moharram et al. 2016). Other reports have included *F. subglutinans* from *Thymus* sp., *F. solani* from *Tinospora cordifolia*, *A. niger* from *Datura innoxia*, *Talaromyces pinophilus* from *Curcuma amada*, and *Eurotium* sp. from *Curcuma longa* (Masumi et al. 2014; Jalgaonwala and Mahajan 2014; El-Said et al. 2016; Krishnapura and Belur 2016; Uzma et al. 2016). The aim of present chapter is to discuss the fungal endophytes from anticancer plants, the species discovered, their distribution, and their prevalence in the various anticancer plants. It will also describe the anticancer compounds they produce, specifically L-asparaginase (along with some other useful compounds), and the prospect of harnessing anticancer compounds from these fungal endophytes for future applications.

10.2 Anticancer Plants

Over 400,000 species of tropical flowering plants are known to have medicinal properties (Arehart and Rouleau 2013). Many medicinal plants all over the world have been examined for their anticancer activity and found to be effective in various experimental and clinical trials (Cragg and Newman 2003). Anticancer plants are simply defined as plants whose parts, extracts, and products can be used in the treatment of cancer. Plant-derived drugs are desired for anticancer treatment, as they are natural and readily available. They can be readily administered orally as part of patient's dietary intake. They are generally considered as nontoxic to normal human cells as the compounds are naturally derived from plants and are therefore safe to use

(Greenwell and Rahman 2015). The first two plant-derived anticancer agents for clinical use were vinblastine and vincristine. These agents were extracted from the Madagascar periwinkle (*Catharanthus roseus*) (Cragg and Newman 2003). Vinblastine and vincristine were found to be active against lymphocytic leukemia in mice. Other examples of plant-derived anticancer agents are taxol, which are isolated from the bark of *Taxus brevifolia* to treat breast cancer (Cragg and Newman 2003).

In recent years, these plants have been used for the isolation of endophytes they harbor. Endophytes are considered as attractive alternatives to plants as their microbial nature permits rapid isolation, purification, and fermentation to produce the anticancer compounds on a large-scale basis (Grothaus et al. 2010). In addition, endophytes have been found to produce the same anticancer compounds as their host plants. This includes recent reports of taxol production by *Phyllosticta spinarum* (Kumaran et al. 2008) and *Botryodiplodia theobromae* (Pandi et al. 2010) found in the needles of cypress (*Cupressus* sp.) and Indian mulberry (*Morinda citrifolia*), respectively. Another important anticancer compound, podophyllotoxin, is produced by *A. fumigatus* (Kusari et al. 2009) and *F. oxysporum* (Kour et al. 2008), which are endophytes of woody conifer (*Juniperus communis*) and Himalayan juniper (*Juniperus recurva*). The endophyte *Alternaria tenuissima* CH1307 from Hainan plum yew (*Cephalotaxus hainanensis*) produces the compound homoharringtonine (Hu et al. 2016). Endophytic *F. oxysporum* and *Talaromyces radicus*, residing in tissues of *C. roseus*, produce vincristine and vinblastine (Kumar et al. 2013; Palem et al. 2016). In addition to the major medicinal or anticancer plants mentioned in the earlier sections, several other lesser-known medicinal plants of ethnobotanical have also shown potential to harbor important endophytes. They include *Cymbopogon citratus*, *Murraya koenigii*, *Oldenlandia diffusa*, *Pereskia bleo*, and *Andrographis paniculata.* These plants are of interest as they are the lesser-studied medicinal plants from Southeast Asia but have shown anticancer properties. Nevertheless, they have many other uses and are popular in this region.

10.2.1 *Cymbopogon citratus*

C. citratus (DC.) Stapf, commonly known as lemongrass or oil grass, is a tropical plant from Southeast Asia valued for its oil (Sharma et al. 2008; Sousa et al. 2010). *C. citratus* is native to India, Sri Lanka, Malaysia, and most parts of Southeast Asia (Devi et al. 2011). This plant is from the family Poaceae and the order Poales (Lakshman et al. 2010). It is a tall aromatic coarse grass of 1.5 m high with long green leaves (Asaolu et al. 2009). The stem is reddish brown in color and it is attached to the bulb by stalk. It is a short-day plant and has profuse flowering. *C. citratus* is used for culinary and medicinal purposes. *C. citratus* produces essential oil which contains various chemical compounds used in perfumery, cooking, and pharmaceutical industries. The ethnobotanical uses of *C. citratus* include treatment of fever, infection, headaches, and cancer (Runnie et al. 2004). To date, studies on endophytes from *C. citratus* are limited to biodiversity studies. Krishnamurthy and Hemalatha (2008) revealed the following species that can be found in *C. citratus*:

Aspergillus sp., *Cladosporium* sp., *Gliocladium roseum*, *Macrophoma* sp., *Penicillium notatum*, and *Trichoderma viride*. Deshmukh et al. (2010) further identified *Cladosporium cladosporioides*, *Drechslera* sp., *Colletotrichum gloeosporioides*, and *Phyllosticta* sp. as endophytes from *C. citratus,* with higher endophyte abundance detected in leaf than the rhizome samples. In a recent study by Chow and Ting (2015), endophytic *Dothideomycetes* sp. P15E6, *F. proliferatum*, and *Phoma* sp. were found in *C. citratus.* Their study is among the first few to document the L-asparaginase production by these endophytes, revealing that endophytes from medicinal (anticancer) plants have the properties to produce anticancer compounds (L-asparaginase).

10.2.2 *Murraya koenigii*

M. koenigii (L.) Sprengel, also known as curry leaves, is used as a spice throughout India for its aromatic value. This plant is from the family Rutaceae and the order Sapindales (Shah et al. 2008). *M. koenigii* is an aromatic shrub growing up to 6 m in height (Muthumani et al. 2009). The leaves are pinnate, with each leaf measuring approximately 2–4 cm long and 1–2 cm broad (Patidar et al. 2010). This plant produce small, black shiny berries that are edible, but their seeds are poisonous. The traditional medical literature describes *M. koenigii* as a source for many vitamins and the domestic remedy for diabetes and cancer (Shah et al. 2008). Carbazole alkaloids present in *M. koenigii* leaves are used in traditional medicine for the treatment of piles, headache, stomachache, influenza, dysentery, skin eruptions, and poisonous bites (Patidar et al. 2010). To date, very few reports are available on endophytes in *M. koenigii*. One of the known endophyte species is *Colletotrichum gloeosporioides* MKL1, which has shown antimicrobial and antioxidant activities (Nath et al. 2014). Another endophyte, *Fusarium oxysporum* isolate h13, was found to produce L-asparaginase activity (Chow and Ting, 2015). These reports highlighted the potential of endophytes from *M. koenigii* as producers of valuable bioactive compounds.

10.2.3 *Oldenlandia diffusa*

O. diffusa is a well-known medicinal plant (Islam et al. 2009). This plant has one of the longest herb names in Chinese – *Bai Hua She She Cao* – which means "plant used as a treatment for snake bites" (Gupta et al. 2004). *O. diffusa* is from the family Rubiaceae and the order Gentianales (Islam et al. 2009). It is an annual plant, of 0.3 m in height, with small leaves. It has long been used in China for the treatment of hepatitis, sore throat, and tumors of the liver, lung, and stomach (Xu et al. 1997). Chemical compounds isolated from *O. diffusa* such as oleanolic and ursolic acid have anticancer, antibiosis, and transaminase-degrading properties (Rodriguez et al. 2003). The aqueous extract of this plant is effective in inhibiting the growth of cancer lines (Gupta et al. 2004). To date, endophytes isolated from *O. diffusa* have only been reported by Chow and Ting (2015), which are *Fusarium verticillioides* strain jb111, *As*comycota sp., and *Colletotrichum* sp., as producers of L-asparaginase.

10.2.4 *Pereskia bleo*

P. bleo (Kunth) DC., also known as "Seven Star Needle," is a plant from the family Cactaceae and the order Caryophyllales (Er et al. 2007). It is a spiny shrub of 2–8 m in height (Wahab et al. 2009) with orange-red flowers that bloom late in the afternoon and last only for 1 day (Sim et al. 2010). *P. bleo* originated from South America but is cultivated in many tropical countries (Wahab et al. 2009). *P. bleo* is usually consumed by eating the fresh leaves or drinking the soup boiled from the leaves (Er et al. 2007). The leaves of the plant are also used as remedy for the relief of headache, gastric pain, ulcers, hemorrhoids, and atopic dermatitis and the treatment of diabetes, hypertension, and also cancer (Tan et al. 2005). The pounded leaves of *P. bleo* paste could also be applied to wounds or cuts for pain relief (Sim et al. 2010). Endophytic communities in *P. bleo* are not widely understood. Chow and Ting (2015) conducted a simple study, which revealed 16 fungal endophytes detected in *P. bleo*, mainly *Colletotrichum gloeosporioides*, *Penicillium simplicissimum*, and *Fusarium proliferatum.* These endophytes were positive for L-asparaginase production, suggesting potential of endophytes from anticancer plant *P. bleo* in producing anticancer compounds (L-asparaginase).

10.2.5 *Andrographis paniculata*

A. paniculata (Burm.f.) Nees or commonly known as the "king of bitter," or *kariyat*, *kalmegh*, *hempedu bumi*, *pokok cerita*, is a member of the family Acanthaceae (Sanwal et al. 2016). It has lanceolate green leaves and grows to a height of 60–70 cm (Subramanian et al. 2008). *A. paniculata* have been used to treat fever, cold, inflammation, and diarrhea (Balap et al. 2017). Their leaves have the following properties: antithrombotic, anticancer, antioxidant, antihyperglycemic, and anti-inflammatory (Zhang and Tan 2000; Yu et al. 2003; Kumar et al. 2004; Subramanian et al. 2008). The endophytic communities in *A. paniculata* have been reported to include species of *Curvularia*, *Fusarium*, *Alternaria*, and *Penicillium*, which have been found to exhibit antimicrobial activities (Mohanta et al. 2007; Gajalakshmi et al. 2012). Other endophytic species such as *Aspergillus* sp. have also been isolated from *A. paniculata* with antimicrobial activities (Tan et al. 2016).

10.3 Endophytes from Anticancer Plants

Medicinal plants, especially those with anticancer properties, are highly sought as host plants for fungal endophytes. This is attributed to the hypothesis that endophytes in these plants may produce anticancer compounds similar to their host plants, as a result of their close symbiotic relationship with the host plants (Theantana et al. 2009; Lu et al. 2012). Endophytes are better alternatives than plants as their microbial nature permits upscaling and mass culturing to produce the anticancer compounds. This approach is far more sustainable and economically feasible in the long run. In recent years, there is a growing interest in exploring the potential of

fungal endophytes for the production of anticancer compounds (Mans et al. 1994; Kharwar et al. 2011). These compounds include L-asparaginase, piperine, podophyllotoxin, and lapachol, among others. Fungal endophytes producing these anticancer compounds include those from the genus of *Fusarium*, *Trichoderma*, *Aspergillus*, *Fomitopsis*, *Chaetomium*, *Penicillium*, *Colletotrichum*, *Guignardia*, and *Botryosphaeria*. Other than anticancer compounds, several other bioactive compounds belonging to the classes of alkaloids, phenols, quinones, peptides, pestacin, and polysaccharides have also been reportedly produced by fungal endophytes. These compounds are found to possess good antimicrobial and antioxidant activities and are discussed in the following sections.

10.3.1 Ubiquity of Endophytes Producing L-Asparaginase

Numerous fungal endophytes have been isolated successfully from different anticancer plants, each with varying degree of L-asparaginase production. In a study by Chow and Ting (2015), a total of 355 endophytic isolates were isolated from *Pereskia bleo*, *Murraya koenigii*, *Oldenlandia diffusa*, and *Cymbopogon citratus*. The endophytes were most prevalent in *P. bleo* as greater number of fungal endophytes was discovered (203 isolates), followed by *O. diffusa* (68 isolates), *C. citratus* (49 isolates), and *M. koenigii* (35 isolates). With greater abundance and prevalence in these plants, the numbers of L-asparaginase producers were also higher in these plants: 15 isolates from *P. bleo*, 17 isolates from *O. diffusa*, 2 isolates from *C. citratus*, and 1 isolate from *M. koenigii* (Chow and Ting 2015).

The prevalence of endophytes in anticancer plants was further evidenced by the endophytic infection rate (EIR), i.e., density of endophytes in each tissue. Tan et al. (2016) discovered 50 fungal endophytes from *A. paniculata*. The root tissues recorded the highest endophytic infection rate (EIR) (35.88%), followed by leaf (16.67%), stem (15.38%), and flower tissues (9.90%). It was also noted that EIR and number of endophytes isolated appeared to be higher in plant tissues (root and leaf tissues) that are commonly used for medicinal purposes (Behera et al. 2010, Xu et al. 2012). *A. paniculata* harbored more endophytes that are generally L-asparaginase producers (78% of isolates) compared to other anticancer plants (28%) such as reported by Chow and Ting (2015). Most of the endophytes with positive results were isolated from root tissues (19 isolates), leaf (13 isolates), and stem (7 isolates) tissues (Tan et al. 2016). Fungal endophytes from other anticancer plants were also found to produce L-asparaginase. Thirunavukkarasu et al. (2011) isolated 64 L-asparaginase producers (78%) from a pool of 82 endophytes from seaweed with cytotoxic activity in southern India. Manasa and Nalini (2014) discovered L-asparaginase producers from *Tabernaemontana*, a genus of flowering plants with cytotoxic activities. Clearly, endophytes with potential to produce anticancer compounds such as L-asparaginase are prevalent and ubiquitously harbored in various anticancer plants.

10.3.2 Diversity of Endophytes Producing L-Asparaginase

Both the commonly known and distinctive species have been reported to produce L-asparaginase. The common species of endophytes, which produce L-asparaginase, include *Fusarium* sp., *Penicillium* sp., and *Colletotrichum* sp. They are isolated as endophytes from a variety of medicinal plants (Audipudi et al. 2014; El-Said et al. 2016). *Fusarium* is a common genus of endophytes that can be found in many plants including medicinal plants. Many studies have shown that *Fusarium* sp. of endophytic origin have the potential of secreting alkaloids with anticancer properties such as podophyllotoxin (Kour et al. 2008) and rohitukine (Kumara et al. 2012). Manasa and Nalini (2014) identified *F. subglutinans* M24 from *Tabernaemontana heyneana* Wall as the isolate with the most potential for L-asparaginase activities. *Fusarium* sp. have also been isolated from several Malaysian anticancer plants (Chow and Ting 2015). *Fusarium proliferatum* was found in both *P. bleo* and *C. citratus,* while *F. verticillioides* and *F. oxysporum* were isolated from *O. diffusa* and *M. koenigii*, respectively. For endophytic *Penicillium* sp., they are known to produce bioactive compounds that have antimicrobial (Korejo et al. 2014) and antitumor properties (Lin et al. 2008). *P. simplicissimum* with L-asparaginase activities were isolated from both *O. diffusa* and *P. bleo* (Chow and Ting 2015). Anticancer plants in Thailand were also reportedly hosts of *Penicillium* sp., *Fusarium* sp., and *Eupenicillium* sp., which have L-asparaginase activities (Theantana et al. 2009).

Colletotrichum sp. is another common genus of endophyte with L-asparaginase activity. *Colletotrichum* sp. can be found in many medicinal plants with anticancer properties such as *Justicia gendarussa* and *Taxus mairei*. *Colletotrichum* sp. have also been found to produce taxol (Gangadevi and Muthumary 2008). In a study by Chow and Ting (2015) on endophytes from Malaysian anticancer plants, most of the endophytes with L-asparaginase production were identified as *Colletotrichum* sp. (Chow and Ting 2015). In their study, *Colletotrichum* sp. was found in *P. bleo* and *O. diffusa* (Chow and Ting 2015). Similar observation was also made by Tan et al. (2016) on the medicinal plant *Andrographis paniculata.* Ten of the 39 endophytic isolates (25.6%) that showed L-asparaginase production were of the genus *Colletotrichum*. In a separate study by (Theantana et al. 2009), 6 of the 15 selected isolates from Thai medicinal plants were identified to be *Colletotrichum* sp. (Theantana et al. 2009). In their study, *Colletotrichum* sp. E5T9 exhibited excellent L-asparaginase activity by inhibiting CaCo2 human Caucasian colon adenocarcinoma and HepG2 human Caucasian hepatocyte carcinoma cells. Other less common and distinctive L-asparaginase producers that have been reported include *Volutella* sp., *Verticillium lecanii, Alternaria* sp., *Chaetomium* sp., *Cladosporium* sp., *Petromyces* sp., and *Pleospora* sp. (Manasa and Nalini 2014; Moharram et al. 2016). These endophytes are sourced from various medicinal plants including the Indian ginseng plant *Withania somnifera* (Moharram et al. (2016). It is therefore evident that endophytes producing L-asparaginase can be isolated from various anticancer plants.

10.4 Anticancer Compounds Produced by Endophytes from Anticancer Plants

The discovery of anticancer compounds produced by endophytes is important, as these compounds are alternative novel lead molecules for chemical synthesis to produce anticancer agents (Narang and Desai 2009; Kumar and Sobha 2012; Kumar et al. 2015). According to the US Food and Drug Administration (FDA), anticancer compounds may originate from the following class of compounds: the alkylating agents, antimetabolites, inhibitors, natural products (from plants or microorganisms), or hormones (Feng and Zhao 2017). A summary of the various anticancer agents produced by endophytes from anticancer plants is shown in Table 10.1. Anticancer compounds derived from endophytes are preferred because they are microbial based and are therefore amenable to upscaling and are relatively renewable compared to plant-derived compounds, without risking the biodiversity and resources for cultivating host plants such as medicinal plants (Mehanni and Safwat 2010). These compounds are also organic and have less harmful side effects than synthetically derived anticancer compounds. Of the many anticancer compounds produced by endophytes, L-asparaginase is the least studied. This is because bacteria have produced the enzyme over the years. The exploration of fungal isolates producing L-asparaginase is still in its infancy. To date, only 13 studies on screening and isolation and 1 study on optimization of L-asparaginase production from fungal endophytes were performed in the last two decades. Chow and Ting (2015) studied L-asparaginase production from fungal endophytes isolated from anticancer plants in Malaysia. They found *Fusarium oxysporum* and *Penicillium simplicissimum* from *Murraya koenigii* and *Pereskia bleo,* respectively, as effective producers of L-asparaginase. In addition to L-asparaginase, endophytes from anticancer plants have also been established as producers of other valuable anticancer, antimicrobial, and antioxidant compounds. This is further supported by many reports on discovery of these anticancer agents in different species of endophytic fungi either from same or different host plants (Table 10.1).

10.5 Other Bioactive Compounds by Fungal Endophytes from Anticancer Plants

Other than L-asparaginase and various anticancer compounds, fungal endophytes are also rich sources of bioactive compounds with antifungal activities. The active compounds responsible for these beneficial properties include alkaloids, phenolics, quinones, and antioxidants, and they are produced by a variety of endophytes. Pyrrocidines A and B isolated from the endophyte *Acremonium zeae* in maize demonstrated effective inhibition toward pathogens *Aspergillus flavus* and *Fusarium verticillioides* (Yu et al. 2010). Strobel et al. (1997) reported that *Acremonium* sp. from *Taxus baccata* produces leuesnostatin A, which inhibited the growth of the plant pathogen *Pythium ultimum.* Another bioactive compound with antimicrobial properties, chaetoglobosins (A and C), was also effective toward the pathogenic

Table 10.1 Examples of anticancer compounds produced by endophytic fungi isolated from anticancer plants

Bioactive compounds	Cancer diseases	Endophytes	Host plant	References
Camptothecin	Ovarian, small lung and refractory ovarian cancers	*Fomitopsis* sp.	*Miquelia dentate*	Shweta et al. (2013)
		Trichoderma atroviride	*Camptotheca acuminata*	Pu et al. (2013)
Indole diketopiperazines	Breast cancer	*Chaetomium* sp.	*Cymbidium goeringii*	Wang et al. (2015a)
L-Asparaginase	Leukemia	*Penicillium simplicissimum*	*Pereskia bleo*	Chow and Ting (2015)
		Aspergillus niger	*Datura innoxia*	El-Said et al. (2016)
Lapachol	Anti-inflammatory, antimitotic, antiproliferative	*A. niger*	*Tabebuia argentea*	Channabasava and Govindappa (2014)
Piperine	Prostate cancer	*Colletotrichum gloeosporioides*	*Piper nigrum*	Chithra et al. (2014)
Podophyllotoxin	Antineoplastic	*Fusarium solani*	*Podophyllum hexandrum*	Nadeem et al. (2012)
Stemphyperylenol	Active against HCT116 cancer line	*Botryosphaeria dothidea*	*Melia azedarach*	Xiao et al. (2014)
Taxol	Breast, ovarian, lung, head, and neck cancers	*Guignardia mangiferae*	*Taxus media*	Xiong et al. (2013)
Vinblastine and vincristine	Leukemia, lymphoma, breast and lung cancers	*F. oxysporum*	*Catharanthus roseus*	Kumar et al. (2013)

Mucor miehei. Chaetoglobosins are produced by *Colletotrichum globosum,* an endophyte from the leaves of *Ginkgo biloba* (Qin et al. 2009). In addition, endophytic *Pestalotiopsis adusta* produces Pestalochloride A and B, which were antifungal in nature, towards several other fungal species such as *Fusarium culmorum*, *Gibberella zeae*, and *Verticillium alboatrum* (Yu et al. 2010).

Endophytes also produce antibacterial compounds, which are able to inhibit the growth of bacterial pathogens. *Ampelomyces* sp., an endophyte from the medicinal plant *Urospermum picroides*, produces 3-O-methylalaternin and altersolanol A. These quinones were inhibitory toward *Staphylococcus aureus*, *Streptococcus epidermidis*, as well as *Enterococcus faecalis.* Inhibition was achieved via alteration to electron-acceptor sites in the membrane of the pathogen, subsequently limiting their growth (Yu et al. 2010). In addition, endophytes have also been reported to synthesize peptides demonstrating antimicrobial activities. Cyclo (Pro-Thr) and cyclo (Pro-Tyr) produced by *Penicillium* sp. from mangrove plants have strong antibacterial activities against various bacterial pathogens (Strobel et al. 1997). In addition to antifungal and antibacterial activities, the bioactive compounds produced

by endophytes were also rich in antioxidants, phenol and phenolic acids, and various polysaccharides (Xu et al. 2008). Pestacin and isopestacin are bioactive compounds produced by the endophytic *Pestalotiopsis microspora* (from *Terminalia morobensis*). These compounds demonstrated strong antioxidant and antimicrobial potential (Harper et al. 2003).A study by Huang et al. (2007) revealed that the antioxidant properties of endophytic fungal cultures of medicinal plants are correlated to their total phenolic contents. Liu et al. (2009) discovered polysaccharides with antioxidant activities (strong scavenging activities on superoxide and hydroxyl radicals) from endophytes isolated from the root of *Stemona japonica* Miquel, a traditional Chinese medicine. Evidently, endophytes from anticancer and medicinal plants are effective producers of antimicrobial, antioxidant, and phenolic compounds (in addition to L-asparaginase), with potential for various applications.

10.6 Upscaling, Commercialization Potential, and Innovations

There is tremendous potential in upscaling the production of anticancer agents, particularly microbial-derived compounds. Nevertheless, industrial production of an anticancer agent is a tedious and time-consuming process. The process in discovering new anticancer agents encompasses the initial discovery of bioactive compounds and screening tests, followed by the final optimization for large-scale production. Cachumba et al. (2016) and Lopes et al. (2017) explained the industrial production of L-asparaginase in two separate phases. Phase 1 involves the fundamental research in sourcing of microbial L-asparaginase followed by growth of microbial culture and fermentation conditions. Phase 2 includes further works on the bioactive compound, which usually include isolation, purification, verification, formulation, and commercialization. For L-asparaginase, the following factors are important for consideration in implementing Phase 1 and 2 so as to produce abundant L-asparaginase for commercial use. The factors to be considered are the source of L-asparaginase, carbon and nitrogen source requirements, fermentation conditions (e.g., pH, temperature, duration, submerged-state fermentation (SMF) or solid-state fermentation (SSF)), and enzyme stability (Krishnapura and Belur 2016). These factors vary depending on the different isolates used, whether bacteria or fungal species. For nutrients, carbon and nitrogen sources are important components as they are the primary sources of nutrients in the medium. Studies have reported that glucose, starch, and maltose are desirable carbon sources, whereas L-asparagine, L-glutamine, and L-proline are effective nitrogen sources (Cachumba et al. 2016). It is proven that fungal L-asparaginase production can be enhanced using the solid-state fermentation (SSF) with agro-industrial residues as substrates. Dias et al. (2015) successfully increased L-asparaginase yield by *Aspergillus niger* up to 94.21 U/g using a mixture of agro-industrial residues (wheat bran, soybean meal, and cotton seed meal).

The upscaling of L-asparaginase from fungal endophytic sources has not been fully explored. To date, research on L-asparaginase production by fungal endophytes is mostly on verification of their enzyme-producing nature but has yet to fully

progress into optimization, fermentation, large-scale production, and commercialization. Nevertheless, production of L-asparaginase from bacteria is relatively well established. Bacteria such as *E. coli* (currently marketed as Elspar®) (Ratych et al. 1970) and *E. chrysanthemi* (currently marketed as Erwinaze®) (Abribat 2011) are known to produce effective L-asparaginase enzymes. Oncaspar® is another L-asparaginase agent produced by *E. coli,* which is much more stable and more cost efficient than Elspar® as only a single dose is required to obtain a similar antileukemic activity compared to 6–9 doses with the administration of Elspar® (Samudio and Konopleva 2013; Fernandes et al. 2017). Nevertheless, *E. coli* and *E. chrysanthemi* are the only two current main microorganisms for industrial L-asparaginase production. There is no fungal-based L-asparaginase that is commercialized for pharmaceutical purpose. To date, the only reported fungal L-asparaginase is derived from *Aspergillus oryzae*, used commercially for reduction of acrylamide formation in food industry (Hendriksen et al. 2009). Hence, there is much to be explored on fungal endophytes from medicinal plants and their production of L-asparaginase.

Optimization for L-asparaginase production has been attempted, mostly at the laboratory stage. The optimization trials adopt protocols used for non-endophytic microorganisms. Kumar et al. (2012) has a comprehensive protocol that is transferable to endophytes, whereby production of microbial L-asparaginase was achieved via submerged fermentation (SMF) and solid-state fermentation (SSF), followed by gradual purification and biochemical characterization. Key parameters optimized were carbon and nitrogen concentrations, pH, temperature, duration for fermentation, and oxygen transfer rate. In their study, they found that maximum L-asparaginase production can be achieved by culturing L-asparaginase-producing microbes in medium consisting of nutrient broth, brain heart infusion, peptone, and yeast extract, with a pH range of 5.5–pH 7.0 and at temperature range of 25–37 °C. L-asparaginase production is also enhanced via cell-immobilization, which has thus far revealed to be useful to optimize production of L-asparaginase by marine *Aspergillus terreus* (Farag et al. 2015). In their study, L-asparaginase production by immobilization using *A. terreus* immobilized on sponge increased by 1.33-fold compared to production by free cells. Immobilized cells on a supporting matrix (sponge) enabled better uptake of nutrients, leading to better production of L-asparaginase. Immobilized cells also allowed recycling and reuse of fungal isolates for continuous production of the enzyme. Vimal and Kumar (2017) further developed the use of in silico validation to confirm the production of L-asparaginase enzymes under various optimization procedures and comparing the quality with commercially available L-asparaginase. This ensures the quality of the L-asparaginase produced. In their study, they found that the L-asparaginase produced by *Penicillium lilacinum* is similar to L-asparaginase derived from *E. chrysanthemi* by comparing the docking scores.

There are several approaches to conducting optimization studies, particularly related to the nutrient requirements. The Plackett-Burman experimental design, response surface methodology (RSM), and artificial neural network (ANN) are among the many approaches to statistically predict the yield of the enzyme in response to the parameters and the number of experiments conducted. The

Plackett-Burman experimental design allows for the determination of useful nutrients to enhance L-asparaginase production. El-Naggar (2015) used the design and discovered that L-asparagine and yeast extract had the most significant impact on L-asparaginase production of *Streptomyces parvus* NEAE-95. The RSM on the other hand is useful to investigate the interactive effects of factors such as the interaction between growth and metabolite formation during fermentation process (Hymavathi et al. 2009). Erva et al. (2017) also used RSM to increase L-asparaginase activity of *Enterobacter aerogenes* KCTC2190/MTCC111 by 2.4 times higher, as the RSM enabled optimization of parameters (concentration, inoculum size, pH, temperature, incubation time, carbon and nitrogen sources). The ANN approach is a new method to optimize enzyme production. In this method, different aspect of biological information processing is mimicked for data modeling (Sivapathasekaran et al. 2010). Baskar et al. (2011) have adopted the ANN approach and successfully obtained maximum L-asparaginase activity of 18.59 IU/mL, which was closed to the experimental value of 18.72 IU/mL. This value was achieved using the optimized medium of 2.09% sodium citrate, 0.25% DAHP, and 0.92% L-asparagine, predicted by the ANN method. With factors optimized, the fermentation procedure can be carried out efficiently to yield high production of L-asparaginase (Lopes et al. 2017).

In recent years, L-asparaginase production responded effectively to modern biotechnological techniques. These techniques are introduced to innovate and improve the L-asparaginase production by fungal species. The primary approach is the use of genetic engineering by transferring, overexpressing, or modifying genes encoding the rate-limiting enzymes of secondary metabolites biosynthetic pathways (Aly et al. 2013). Wang et al. (2007) have successfully improved taxol production in endophytic fungus, *Ozonium* sp. with the use of restriction enzyme-mediated integration (REMI) transformation. REMI is commonly used to transfer nonhomologous linearized DNA into host chromosome via restriction enzymes (Wang et al. 2007). Comparatively, REMI is able to increase transformation rate and gene tagging and yield desired anticancer compounds at a much higher rate than the conventional fungi transformation method using $CaCl_2$/polyethylene glycol (PEG). Another innovative biotechnological technique, genome shuffling, is a new, powerful technique to allow genetic breeding of microorganisms using repetitive multi-parental protoplast fusions to achieve phenotypes of interest (Biot-Pelletier and Martin 2014). This is achieved by subjecting protoplasts to an electric pulse or incubated in the presence of PEG that alters membrane fluidity (protoplast fusion) (Biot-Pelletier and Martin 2014). Conjugational transfers of genetic materials from different organisms then take place. The resulting genome recombination alters the metabolic network in the whole genome to increase yield of desired compounds progressively (Wang et al. 2016). Genome shuffling has been attempted on several microorganisms and has successfully improved the yield of antibiotic tylosin in *Streptomyces fradiae* (Zhang et al. 2002), production of antibiotic pristinamycin in *Streptomyces pristinaespiralis* (Xu et al. 2008), and production of the antitumor deacetylmycoepoxydiene in *Phomopsis* sp. A123 (Wang et al. 2016). Researchers are looking forward to adopting this technique to improve yield of anticancer compounds by endophytes, especially with the success in genome shuffling of

taxol-producing *Nodulisporium sylviforme* (Zhao et al. 2008). This genome shuffling approach has no implications on the bioactivities of the antitumor activities by *N. sylviforme*, as demonstrated by Wang et al. (2015b). This interesting new approach therefore has tremendous potential for exploration as a technique to improve L-asparaginase production in endophytes.

10.7 Conclusions and Future Prospects

This chapter has shown that fungal endophytes from medicinal plants with anticancer properties (including *C. citratus*, *M. koenigii*, *O. diffusa*, *P. bleo*, and *A. paniculata*) have the potential as producers of L-asparaginase. Although studies on L-asparaginase-producing fungal endophytes from medicinal plants are paltry, these studies have shown that fungal endophytes can be a potential alternative source of L-asparaginase. In addition, our study has demonstrated that host plant species and tissue specificity are the factors influencing diversity of endophytes. Common and potent anticancer plant-derived endophytic L-asparaginase producers include *Penicillium* sp., *Fusarium* sp., *Aspergillus* sp., and *Colletotrichum* sp. However, optimization studies for upscaling and commercialization purpose are still in their infancy. Therefore, research efforts should be addressed to these areas, as well as enhancement of L-asparaginase production under optimized condition or using modern biotechnological techniques, to produce safer L-asparaginase with reduced side effect for cancer treatment.

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Xylooligosaccharides and Their Anticancer Potential: An Update

11

Praveen Kumar Gupta, Pushpa Agrawal, Prabha Hedge, and Mohd Sayeed Akhtar

Abstract

Xylooligosaccharides (XOS), which are sugar oligomers that consist of 2–10 units of xylose, are non-digestible food ingredients produced mainly by the hydrolysis of xylan. The production of XOS from agricultural residues serves as a good source of products for the nutraceutical and pharmaceutical industries. XOS have a characteristic prebiotic effect, promoting the growth of probiotic organisms. XOS affect various physiological functions, such as reducing cholesterol levels, maintaining gastrointestinal health, and improving immunity. XOS are also used as potential anticancer agents, mainly for breast cancer and colon cancer. In this chapter we highlight the role of XOS as prebiotics, as well as their role in the suppression of carcinoma cells.

Keywords

XOS · HCT-116 cells · IC_{50} · MCF-7 · MTT assay · Nutraceuticals · Prebiotics · Xylan

11.1 Introduction

During the period 36–400 BC, there was a famous hypocrite opinion in the form of a Chinese proverb "Let food be thy medicine and medicine be thy food" which means, "Whatsoever was the father of disease, an ill diet was the mother"

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(Blackmores 2015; Food that act as natural medicine 2015). Therefore, the significance of eating a good diet with a variability of nutrient rich unprocessed materials is acknowledged for its benefit on our health and well-being (Graf et al. 2015). Whole foods can be defined as those that are essentially untreated and untouched and are rich in important nutrients such as minerals and vitamins; examples are fruits and green vegetables, pulses, dry fruits and beverages.

In this context, prebiotics have attracted growing interest, and with intensive research and development, it has been proposed to revisit the original definition of 'prebiotic'; suggestions have been made to exclude the descriptor 'non-digestible oligosaccharides'. The updated definition of the term prebiotic encompasses the concept of a selectively fermented element that allows unambiguous alterations in the structure and/or activity of the gastrointestinal (GI) microflora, which may confer benefits, in terms of good health, to the host (Roberfroid 2002; Gibson et al. 2004; Leach 2007). However, Chandrasekhariah et al. (2007) have stated that prebiotics are non-viable food constituents, components, or any supplements that can selectively modulate the growth of the microbiota in the digestive ecosystem and facilitate improvements in health and wellbeing. Furthermore, in addition to the fundamental action of prebiotics on the GI tract, attempts have been made to broaden the action sites of prebiotics, to areas such as the skin, oral cavity, and female genital tract (Samanta et al. 2010). However, the original proposers of the prebiotic concept have opposed the philosophy of broadening the action sites to areas such as the vagina and skin, and they wish to again limit the action sites to the GI microflora. The accepted definition of prebiotic in 1995 was a selectively fermented element that produces specified changes in the composition and activity of the GI microbiota that result in benefits to the host health (Leach 2007). Studies conducted by other researchers (Hsu et al. 2004; Crittenden 2006; Samanta et al. 2011) have suggested the following criteria for a prebiotic, in relation to the digestive systems of animals:

1. Selective fermentability
2. Causes alterations in the composition of the gut microflora
3. Ensures host health and wellbeing
4. Originates from plants or is synthesized by microorganisms or enzymes
5. Remains intact while passing through different parts of the GI tract
6. No residue problems
7. Compatibility with other feed ingredients or premix
8. Has no adverse effects on productivity or product quality
9. Increases nutrient digestibility or reduces enteric methane emission in ruminants

Recently, the potential utility of non-digestible oligosaccharides has been realized, owing to their low degree of polymerization, their capacity as sweetening agents, their water-binding capacity, fat replacement value, possibility of fermentation, and action on digestion in higher regions of the GI tract. Non-digestible oligosaccharides are also incorporated in processed food and are promising functional

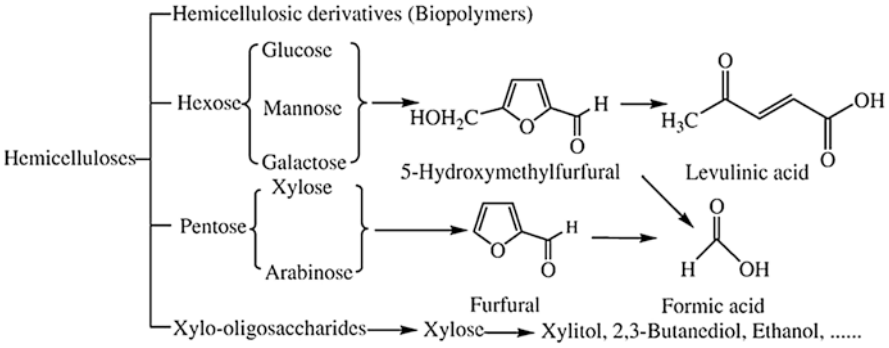

Fig. 11.1 Various derivatives produced from hemicelluloses (Peng et al. 2012)

ingredients in nutraceutical products (Rossi et al. 2011; Nyangale et al. 2012). The utility of non-digestible oligosaccharides in nutraceutical products is not their only value; these agents also show economic and environmental feasibility, offering an opportunity for the agriculture and food industries to produce value-added products from agriculture or fruit wastes. Xylooligosaccharides (XOS), a sub-category of non-digestible oligosaccharides, are regarded as emerging prebiotics; there are also several other common prebiotics that have different origins and chemical properties, e.g., inulin, fructooligosaccharides, galactooligosaccharides, lactulose, and polydextrose (Stowell 2007). XOS are extracted from hemicelluloses, the second most abundant biomaterial after lignin. The hemicellulose content of various organic wastes, such as maize stems (28.0%), barley straw (34.9%), wheat straw (38.8%), and rye straw (36.9%) varies widely (Fang et al. 2000). In the variety of applications, hemicelluloses are utilized to produce both pentose and hexose sugar. On hydrolysis the hemicelluloses produce xylose or arabinose as pentose sugars and glucose or galactose is produced as hexose sugars. Both pentose and hexose sugars can be transformed into other value-added products such as bioethanol, xylitol, and XOS (Canilha 2004; Fig. 11.1). In humans, XOS are used to treat cough and stomach ulcers, and they increase immunity (Kitamura et al. 1994; Kulicke et al. 1997; Kardosova et al. 2002; Cipriani et al. 2006). XOS are also used for the treatment of viral diseases and cancer, as well as being used as tablet binders (Damonte et al. 1996; Stone et al. 1998; Watson et al. 1999). In this chapter we highlight the role of XOS as prebiotics, as well as their role in the suppression of carcinoma cells.

11.2 Xylooligosaccharides (XOS)

Xylooligosaccharides (XOS), which are sugar oligomers that consist of 2–10 xylose units, are considered as non-digestible food ingredients. XOS are naturally available in various fruits, bamboos, green vegetables, honey, and milk. They are produced on an industrial scale from xylan-rich materials (Ebringerova and Hromadkova 1999).

Xylooligosaccharides have attracted the attention of scientists because of their important actions in maintaining health, such as their prebiotic activity, anticancer activity, and antioxidant activity (Stone et al. 1998; Gibson et al. 2004; Chandrasekhariah et al. 2007).

11.2.1 Chemistry of Xylooligosaccharides

Depending upon the various xylan sources used for XOS production, the structures of XOS vary in their degree of polymerization (DP), monomeric units, and types of linkages. Generally, XOS are mixtures of oligosaccharides formed by xylose residues linked through β-(1 → 4)-linkages. The number of xylose residues involved in their formation can vary from 2 to 10, and XOS are thus known as xylobiose, xylotriose, etc. For food applications, xylobiose (DP = 2) is considered to be an XOS. In addition to being found in combination with xylose residues, xylan is usually found in combination with other side groups, such as α-D-glucopyranosyluronic acid or its 4-O-methyl derivative, acetyl groups, or arabinofuranosyl residues (Figs. 11.2 and 11.3). The presence of these side groups results in branched XOS with diverse chemical properties (Gupta et al. 2012; Fig. 11.4).

11.2.2 Biological Properties of Xylooligosaccharides

XOS are virtually not digested or absorbed, but, by utilizing *Bifidobacterium*, they may directly enter the intestine, where they inhibit the growth and proliferation of detrimental bacteria. XOS have been reported to be selectively utilized by beneficial bacteria; of note, the low DP range of XOS such xylobiose and xylotriose is known to facilitate the growth of *Bifidobacterium* numbers, overwhelming the growth of pathogenic bacteria such as Clostridia. The esterified feruloyl group of

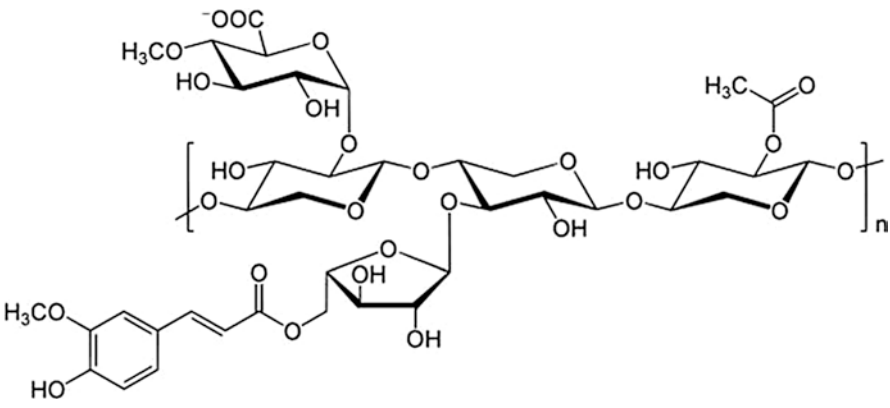

Fig. 11.2 Xylan polysaccharides associated with side chains from lignin (Sixta et al. 2009)

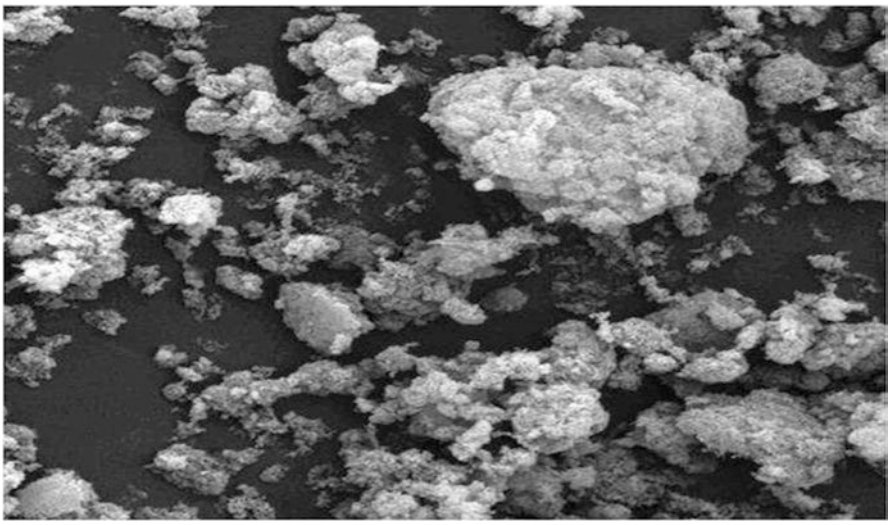

Fig. 11.3 Scanning electron microscope image of xylan powder (da Silva et al. 2012)

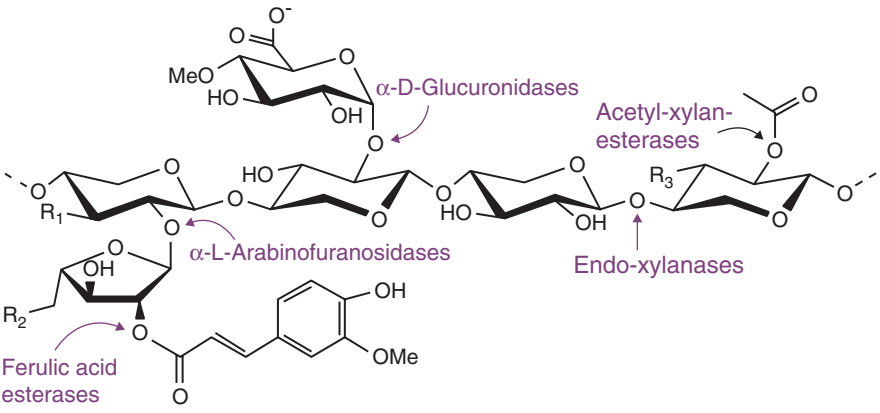

Fig. 11.4 Chemical structure of xylan (Shallom and Shoham 2003)

XOS encourages the growth of *Bifidobacterium bifidum* (Hsu et al. 2004; Gupta et al. 2015). It has been reported, by Okazaki et al. (1990), that XOS increased the proliferative activity of *Bifidobacteria* by up to 20-fold as compared with the effect of other polysaccharides. It is not easy to ferment XOS. Thus, they cannot be used for metabolizing various GI enzymes. Hence, with the use of XOS, there is a chance of diarrhea owing to substantial retention of water. This very common type of GI distress is usually associated with a high intake of dietary fiber. However, XOS are not toxic in humans (Okazaki et al. 1990). In another study, it was shown that XOS did not affect the number of *Escherichia coli* colonies, but that XOS

improved the growth of *Lactobacillus*. Under in-vivo conditions, as the growth of probiotic bacteria increases, the growth of pathogenic bacteria will be reduced (Samanta et al. 2015).

In food processing industries, XOS has proven benefits over other oligosaccharides, especially in terms of overcoming bacterial resistance at low pH and at very high temperatures.

XOS can be used in a variety of carbonated drinks and low-pH fruit juices (Modler 1994). XOS have various applications in food industries, e.g., as low-calorie sweeteners (Moure et al. 2006). XOS can also be used as flavor enhancers in certain kinds of beverages; the addition of XOS significantly increased the change in pH, chemical constituents, and color of beverage without any change in flavor perception (Ando et al. 2004; Manisseri and Gudipati 2010).

The National Centre for Health Statistics (NCHS) 2007–2008 for Food codes representative National Health and Nutrition Examination Survey (NHANES) (CDC, 2010; USDA, 2010) as per Title 21 and Section 9 for XOS GRAS under the Code of Federal Regulations (U.S. FDA, 2008) examined a variety of food categories in which XOS could be used (Table 11.1). For the premarket approval requirements of the United States Federal Food, Drug, and Cosmetic Act XOS ingredients were not recommended for use with meat- or poultry-containing products.

In regard to other properties of XOS, Ando et al. (2004) reported the cytotoxic effects of XOS and their capacity to reduce the growth of leukemia cell lines (obtained from acute lymphoblastic leukemia) under in-vitro conditions. The immunomodulatory, anticancer, antimicrobial, anti-inflammatory, anti-hyperlipidemic, anti-allergic, and antioxidant activities of XOS have also been explored. Xylooligosaccharides are also used in the preparation of nanoparticles and hydrogels for drug delivery and for the treatment of GI disorders (Manisseri and Gudipati 2010; Shimoda et al. 2011; Gupta et al. 2015). Oku and Nakamura (2002) reported that XOS reduced insulin secretion from the pancreas by increasing intestinal mineral absorption, while Cummings et al. (2001) reported that bowel action is altered by XOS owing to their laxative nature. The maximum permissible dose of XOS varies from person to person, and is calculated as 0.12 g/kg body weight for male adults. The details of XOS on human clinical study shown (Table 11.2). XOS intake has been reported as being highly effective for reducing severe constipation in pregnant women, without any adverse effects (Oku and Nakamura 2002). Nutritional formulas for infants that include XOS have been claimed to improve gut barrier maturation and to provide synergistic effects with with substituent-cleaving enzymes such as xylanase along the entire intestinal tract (Table 11.2). However, Samanta et al. (2010) concluded that dietary supplements that included XOS suppressed the production of secondary bile acids and physiologically active fatty acids during the digestive process. Red seaweed *Nothogenia fastigiata*-derived sulfated xylogalactans and an algal xylomannan were found to exhibit antiviral activity against herpes simplex virus types 1 and 2 (Candurra et al. 1996). A mixture of polysaccharides (derived from xylose and glucose) obtained from marine algae was reported to have antiviral activity and to increase the immune response (Seidner et al. 2005).

Table 11.1 Proposed xylooligosaccharides (XOS) intakes for different age groups

Food category	Proposed food use	Serving size (g)	Shandong Longlive XOS concentration					XOS usage level (g)/serving
			95P	70P	35P	20P	70 L	
Baby and toddler foods	RTE cereals for toddlers	20	0.40	0.54	1.08	1.90	0.77	0.38
	Cookies, crackers, 'puffs' and baby food	7	0.25	0.34	0.68	1.20	0.49	0.24
	RTS fruit-based baby/toddler food	60 (Strained)	0.25	0.34	0.68	1.20	0.49	0.24
		110 (Junior)	0.25	0.34	0.68	1.20	0.49	0.24
		125 (Toddler)	0.40	0.54	1.08	1.90	0.77	0.38
	Fruit juices, baby food	125	0.25	0.34	0.68	1.20	0.49	0.24
	RTS fruit-based baby/ toddler food	60 (Strained)	0.25	0.34	0.68	1.20	0.49	0.24
		110 (Junior)	0.25	0.34	0.68	1.20	0.49	0.24
		17 (Toddler)	0.40	0.54	1.08	1.90	0.77	0.38
Beverages and beverage bases	RTS energy, sport, and isotonic beverages	225	0.50	0.68	1.37	2.40	0.98	0.48
	Carbonated and non-carbonated beverages, water, and beer	225	0.50	0.68	1.37	2.40	0.98	0.48
	Processed fruits, juices, drinks, and punch	244	0.30	0.41	0.83	1.45	0.59	0.29
	RTD non-milk-based meal replacement and protein beverages	266	0.30	0.41	0.83	1.45	0.59	0.29
Dairy product analogs	RTD soy beverages, chocolate milk, and flavored milk	225	0.50	0.68	1.37	2.40	0.98	0.48
	Frozen dairy desserts and milk	68	0.30	0.41	0.83	1.45	0.59	0.29
Milk products	RTD flavored milk and milk drinks	250	0.30	0.41	0.83	1.45	0.59	0.29
	RTD milk-based meal replacements	266	0.30	0.41	0.83	1.45	0.59	0.29
	Yogurt, pudding, and jellies	225	0.50	0.68	1.37	2.40	0.98	0.48
Health foods	Medicinal foods	40	1.20	1.63	3.25	5.70	2.33	1.14
	Chewable tablets and capsules	2	1.20	1.63	3.25	5.70	2.33	1.14

(continued)

Table 11.1 (continued)

Food category	Proposed food use	Serving size (g)	Shandong Longlive XOS concentration					XOS usage level (g)/serving
			95P	70P	35P	20P	70 L	
General foods	RTE cereals and cereal bars, granola bars, protein bars, and 'power' bars	40	0.30	0.41	0.83	1.45	0.59	0.29
	Cookies, crackers, and 'puffs'	40	0.30	0.41	0.83	1.45	0.59	0.29
	Chocolate and other confectionery	40	0.30	0.41	0.83	1.45	0.59	0.29
	Chewing gum	1 stick	0.10	0.14	0.27	0.48	0.19	0.09

95P = 95% XOS Powder; 70P = 70% XOS Powder; 35P = 35% XOS Powder; 20P = 20% XOS Powder; 70 L = Liquid form of 49% XOS; RTE = Ready-to-eat; RTD = Ready-to-drink; RTS = Ready-to-serve

Table 11.2 XOS Prerequisites for clinical trials and premarket approval

Subjects	Daily dosage (g)	Duration	Measurement endpoint
Elderly men and women	4 g	3 Weeks	Serum hematological and biochemical variables; fecal microflora, including *bifidobacteria*; fecal pH/moisture; and stool consistency.
Men	0.4 g	2–4 Weeks	Stool consistency
Healthy women	2–10 g	Single dose	Fecal microflora, including *bifidobacteria*; fecal pH/moisture; and stool consistency
Healthy men	1–2 g	3 Weeks	Fecal microflora, including *bifidobacteria*
Healthy men	0.12 g/kg body weight	Single dose	Gastrointestinal tolerance
Type 2 diabetics	4 g	8 Weeks	Blood sugar and lipid profiles
Constipated pregnant women	4.2 g	4 Weeks	Stool consistency

Table 11.3 Growth of *Lactobacillus acidophilus* at different concentrations and time intervals compared with standard sample

Time (h)	Sample 2%	Sample 3%	Sample 4%	Xylooligosaccharides
0	1.2×10^7	1.2×10^7	1.2×10^7	1.2×10^7
6	3.6×10^7	3.9×10^7	3.2×10^7	4.1×10^7
12	4.4×10^8	5.2×10^8	4.0×10^8	5.4×10^8
24	5.1×10^9	5.6×10^9	5.1×10^8	6.2×10^9

11.2.2.1 Prebiotic Activity

The prebiotic activity of XOS was determined by the protocol of Huebner et al. (2007, 2008). This prebiotic analysis is based the determination of a prebiotic score that effectively portrays the functionality of several prebiotics and allows comparison of samples in previous studies. To determine a quantitative score for XOS activity in a given sample, a prebiotic activity score equation is used. This equation was chosen for its simplicity, as well as being based on the standardization of XOS prebiotic activity against that of a non-prebiotic sugar (in this case glucose) and the standardization of prebiotic activity against a selected non-fermenting organism. For prebiotic analysis, the growth of bacteria on 2%, 3%, and 4% XOS samples was observed and compared with growth on the standard media (Table 11.3). The growth curves for prebiotic media and media without carbohydrates were determined by plating growth with the aid of a hemocytometer. The criteria for a probiotic bacterium to be selected were growth on the prebiotic medium that resembled growth on standard XOS medium. The given sample was taken anaerobically in liquid minimal medium in order to assess the utilization of the selected *Lactobacillus acidophilus* and compare the utilization patterns of the prebiotics. For this, liquid

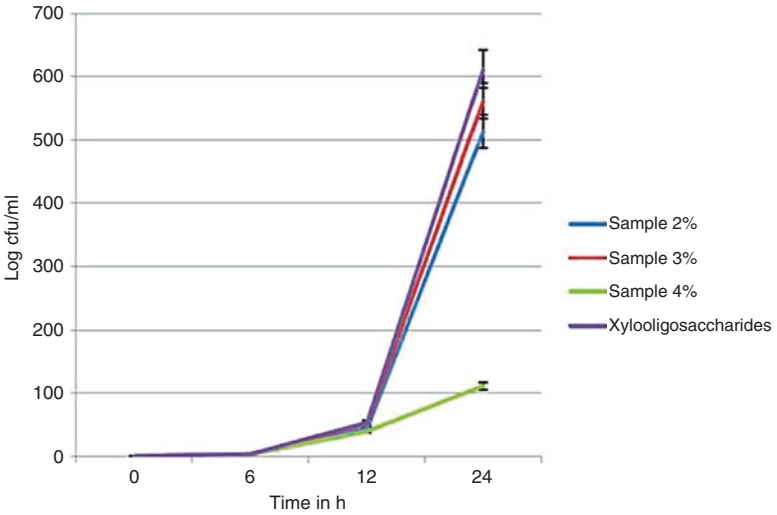

Fig. 11.5 Comparative prebiotic studies of xylooligosaccharides (XOS) samples with respect to XOS standard

Table 11.4 Growth of *Escherichia coli* in 2% samples at different time intervals compared with standard sample

Time (h)	Sample 2%	Xylooligosaccharides
0	0.8×10^7	0.8×10^7
6	2.1×10^7	2.6×10^7
12	2.8×10^8	2.8×10^8
24	3.5×10^8	3.8×10^8

minimal media containing prebiotics (2%, 3%, and 4%) were used, and pH was set at 6.5. At the end of 6, 12, and 24 h viable cells were enumerated at 0 and 24 h (Table 11.3; Fig. 11.5). The culture was inoculated to give an initial cell count of about 7 log cfu/ml and incubated at 37 °C. Similarly, *E. coli* (MTCC 443) cells were grown in two MRS agar plates containing 2% of sample product and standard XOS, respectively. The pH in both plates was set at 7.2 and the plates were incubated aerobically at 37 °C. Growth was observed at regular intervals, at 0, 6, 12, and 24 h, and cells in both plates were enumerated as colony-forming units (CFU) using a hemocytometer (Table 11.4). Probiotics are live bacteria and yeasts that are good for the human health, especially the digestive system (Table 11.4).

11.2.2.2 Anticancer Activity

Determination of the anticancer activity of XOS was based upon the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay. The basic principle of this assay is the reduction of the yellow water-soluble MTT dye to insoluble formazan crystals, which, upon dissolution, produce a purple color.

The intensity of the produced purple color is directly proportional to the number of viable cells and can be determined at 570 nm by spectroscopic techniques. Complete analysis was performed on MCF-7 and HCT 116 cancer cell lines. Three assay controls were used: a control (medium without cells), a negative control (medium with cells but without the experimental drug/compound), and a positive control (medium with cells treated with a known drug, metformin; 5μM).

11.2.2.2.1 Procedure for Determining Cell Cytotoxicity

Cell seeding of a 200-μl cell suspension was performed in a 96-well plate at the required cell density (25,000 cells per well), without the test agent. The cells were allowed to adhere to the culture plate for about 24 h, and then XOS were added (50, 150, 250, 350, and 450 μg/ml). The fixed conditions are mentioned such as 3 h at 37 °C in a 5% CO_2 atmosphere. After the incubation period, the plates were removed from the incubator and MTT reagent was added, to a final concentration of 10% of total volume. This volume was the same as the volume used for determining optimum cell density. To avoid exposure to light, the plate was wrapped with aluminum foil. The plate was again placed in an incubator for 3 h. For adherent cells, the culture medium was aspirated without disturbing the monolayer. Then solubilization solution (dimethylsulfoxide) was added in an amount equal to the culture volume. This was followed by gentle stirring, done in a gyratory shaker, which aimed to enhance dissolution. However, occasional pipetting up and down was required to completely dissolve the MTT formazan crystals, especially in dense cultures. Finally, the absorbance was recorded on a spectrophotometer at 570 nm. The IC_{50} value, which is a measure of the effectiveness of the substance in inhibiting a specific biological or biochemical function, was determined by using a linear regression equation (Gonzalez and Tarloff 2001; Hattori et al. 2003) (Figs. 11.6 and 11.7).

Other physiological properties of XOS, in addition to their effect on the intestinal microflora, include the following:

- Improving immune function in animals
- Preventing constipation, by fermenting the carbohydrate in food
- Potential to synthesize vitamins (B_1, B_2, B_6, B_{12}, nicotinic acid, and folic acid) owing to their activity on *Bifidobacterium*.

11.2.2.3 Pharmaceutical Effects

XOS had good antioxidant activity, but it is varied due to the presence of phenolic content in different organic waste materials (Rashad et al. 2016). XOS also possess anti-allergy, antimicrobial, anti-infective, and anti-inflammatory activities. In addition, XOS have blood-related effects and show activity against skin disorders, as well as showing cytotoxic activity and immunomodulatory activity. Therefore, XOS are potentially useful for skin treatment and in the cosmetic industry (Aachary and Prapulla 2011; Maeda et al. 2012). Owing to their good physicochemical and physiological properties, XOS are widely used in layer production (Zhou et al. 2009). On the basis of its prebiotic attributes, synbiotic food products that contain a prebiotic (XOS) and a live microbial food ingredient (probiotic) having health-promoting effects have been developed (Vazquez et al. 2000).

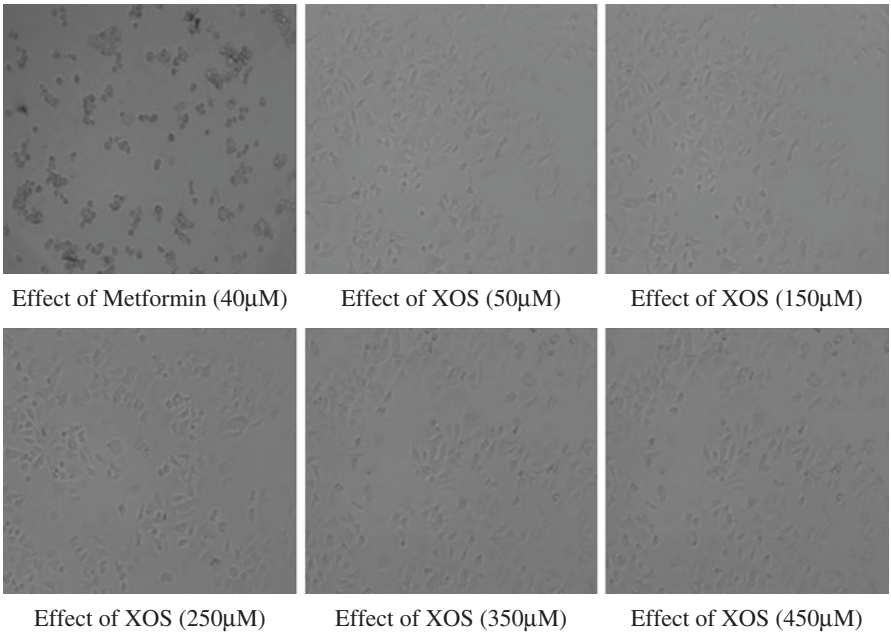

Fig. 11.6 Comparative effect of XOS on MCF-7 cell lines

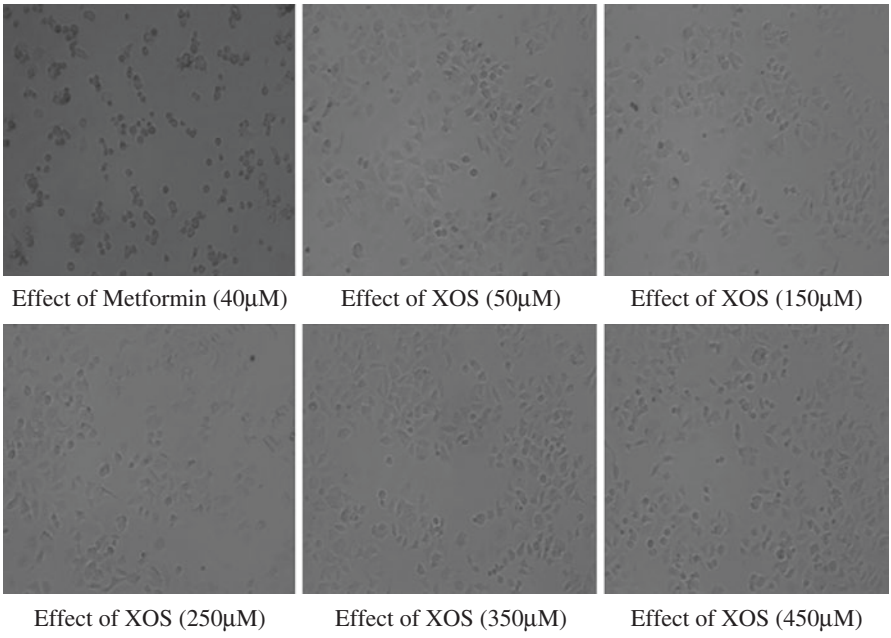

Fig. 11.7 Comparative effect of XOS on HCT-116 cell lines

In a survey conducted in 1000 randomly selected consumers by the International Food Information Council (1998), it was found that more than 95% of the survey respondents believed that certain foods, depending on the quality of nutrition, have various properties that can improve health, and by eating such foods they could reduce the risk of disease; 92% of the respondents believed that they could control their own health by changing their life style; and 78% of the respondents were able to name a particular food or component that was closely associated with various health benefits. Various food materials; green vegetables such as broccoli; carrots; fish and fish oil; oranges and orange juice; garlic; ginger; cucumber; and milk were mostly mentioned by the respondents, in that order (Gupta et al. 2017). In this regard, oranges are an example of a good food, as they have a variety of phytochemicals, such as carotenoids, including β-carotene, lutein, and β-cryptoxanthin; and flavonoids, in the form of abundant volatile organic compounds producing an orange aroma, mainly aldehydes, esters, terpenes, alcohols, and ketones (Ensminger et al. 1983). Orange peel mostly contains soluble sugars and pectin. According to Rivas et al. (2008), the component of orange peel that has the highest percentage is soluble sugars (16.9%), followed by starch (3.75%); fiber, which includes cellulose (9.21%); hemicelluloses (10.5%); lignin (0.84%); pectins (42.5%); ash (3.50%); proteins (6.5%); and fats (1.95%). The total cellulose content in orange peel ranged from 12.7 to 13.6% and hemicellulose content ranged from 5.3 to 6.1%. Taking these findings into consideration, the cost of materials in orange peel is about 45% lower than that of the conventional pulp that other than peels full fruit materials. Moreover, dried orange peel showed a lower concentration of fat than the pulp of other fruits, which may further increase prebiotic components (Rivas et al. 2008). In addition, other studies showed that, because of the presence of hemicellulose and pectins, orange peels can be utilized to produce prebiotic materials such as XOS, fructooligosaccharides, and pectin (Ma et al. 1993; Grohmann et al. 1995). Although orange peels contain high amounts of pectic oligosaccharides, these oligosaccharides have low prebiotic potential as compared with XOS and even as compared with fructooligosaccharides. The pretreatment of hemicellulose is one of the most important steps in the process of recovering xylan from dried orange peel powder. Studies have proven that XOS can be produced from various types of organic wastes such as cotton stalks, rice husk, and sugarcane bagasses by using enzymatic treatment methods (Samanta et al. 2012).

11.3 Production of XOS by Enzymatic Treatment Methods

Pretreatment of hemicellulose with alkaline solution (NaOH or KOH), followed by steam extraction, produces the maximum possible xylan extraction. A study in which similar approaches were used was conducted by the present authors; different concentrations (2, 4, 8, and 12%) of NaOH were used, followed by steam treatment (120 °C, 15 lb = 103421.355 pascal pressure for 45 min) of dried orange peel powder with a solid-to-liquid ratio of 1:10. Then alkali-solubilized xylan was centrifuged at 5000 rpm for 20 min and filtered first with zero filter paper, followed by whatman filter paper grade 40. By using glacial acetic acid at pH 5.0, the acidified

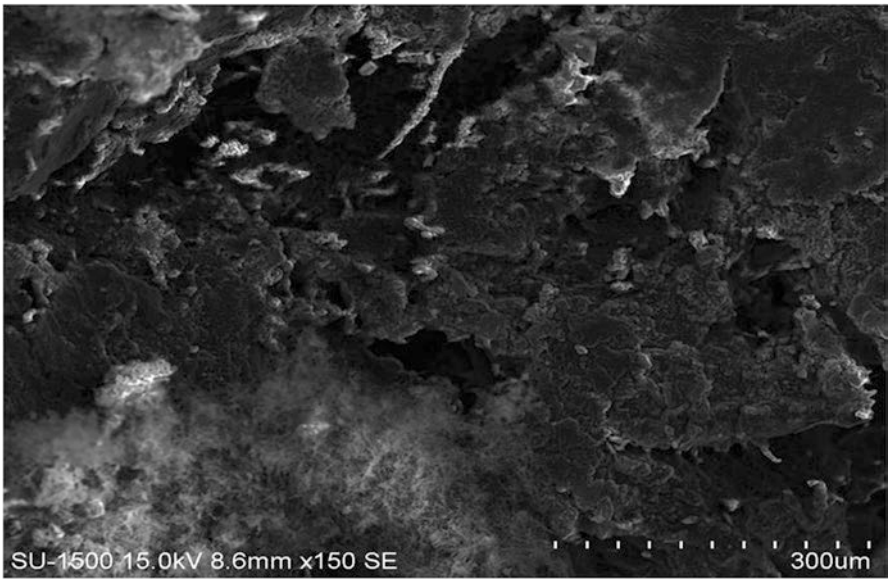

Fig. 11.8 Electron microscope image of enzyme-hydrolyzed xylan

condition in the supernatant was maintained. On the addition of 3 volumes of ice-cold 70% ethanol, xylan was extracted. Later the solution was centrifuged at 8000 rpm for 10 min at room temperature and xylan was precipitated; the xylan was dried in a hot air oven at 60 °C until it reached a constant weight. For the continuation of XOS production, the extracted xylan has to be submitted to an enzyme hydrolysis process. A similar study was carried out using the enzyme endoxylanase extracted from *Trichoderma viride*, procured from Sigma India Bangalore. To start the enzymatic hydrolysis process, an enzyme buffer solution was first prepared. Based upon previous study findings, sodium citrate buffer was chosen, and it was used at various temperature and pH conditions (Akpinar et al. 2009; Hendriks and Zeeman 2009). In order to maximize XOS production five parameters were optimized such as pH, temperature, enzyme concentration, alkaline concentration, and incubation period. All these parameters were identified based upon the previous findings (Samanta et al. 2015) (Fig. 11.8).

11.4 Conclusions and Future Prospects

It is evident from experimental reports that XOS can offer opportunities to prevent or mitigate GI disorders and the development of cancer cells. The experimental data obtained for XOS is limited and more studies are needed to extend the encouraging preliminary results to the clinical trial stage. The study conducted by authors of this chapter has proven that the XOS has got potential effect over cancer cells; however, this still has to be proven by conducting more studies in similar areas. The

recommendation of XOS as nontoxic and anticancerous agent will be completely based upon after the conduction of few clinical trials in future. In addition, the XOS effects need to be assessed more by further studies. However, in future, research on the combined effects of prebiotics and probiotics is desired to explore our knowledge of the symbiotic relationships between colonic microbiota, XOS, and the whole body.

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Phytochemicals Targeting Endoplasmic Reticulum Stress to Inhibit Cancer Cell Proliferation

12

Sukriti Srivastava, Gauransh Jain, Shweta Dang, Sanjay Gupta, and Reema Gabrani

Abstract

Cancer is marked by uncontrolled cell proliferation avoiding the programmed cell death. Rapid cell division demands increased protein synthesis, overloading endoplasmic reticulum (ER) machinery with enhanced folding, packaging and transportation of proteins causing ER stress. Unfolded protein response (UPR), the adaptive pathway activated by the cell in response to this stress, promotes survival of the cancer cells and hence tumorigenesis. The duration and severity of stress determine the fate of the cell, that is, whether the cell will survive and follow adaptive response or it will lead to apoptosis. Targeting the ER chaperones, UPR machinery and elements that assist in protein folding in the ER lumen might provide a significant therapeutic target against cancer. Phytochemicals can modulate various cellular functions and have been promising agents against cancer. The aim of this chapter is to summarize the possible impact of various phytochemicals like carnosic acid, diallyl sulphur compounds, gambogic acid, galangin, pachymic acid, pomegranate fruit extract, proanthocyanidins and resveratrol, *Saccharina japonica* n-hexane fraction, 6-shogaol and sulphureuin B, which has the ability to alter ER stress. The anti-proliferative mechanisms of these phytochemicals and their impact on ER stress and cancer progression have also been discussed.

Keywords

Apoptosis · Cancer · ER chaperones · ER stress · Phytochemicals · UPR

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12.1 Introduction

The endoplasmic reticulum (ER) contains different domains which accomplish diverse tasks (Wang et al. 2014) including modification and folding of proteins in the lumen of ER, protein incorporation in the cell membrane, secretory protein transport, detoxification and calcium ion storage in the lumen and synthesis of steroids and their discharge in the cytosol (Schwarz and Blower 2016). ER plays an important role in cell survival, and any shift from its regular role leads to cellular damage and programmed cell death. Several conditions that perturb the ER functions are known as ER stress. It may encompass alterations in calcium levels, protein glycosylation and disulphide bond pattern. Redox imbalance in the cell may lead to accumulation of wrongly folded proteins and decreased protein translocation to the Golgi bodies. Cells trigger unfolded protein response (UPR) in response to ER stress (Corazzari et al. 2017). The role of various phytochemicals like carnosic acid, diallyl sulphur compounds, gambogic acid, galangin, pachymic acid, pomegranate fruit extract, proanthocyanidins and resveratrol, *Saccharina japonica* n-hexane fraction, 6-shogaol and sulphureuin B in preventing cancer progression is well described in the chapter.

12.2 Unfolded Protein Responses

UPR comprises of a cascade of signalling events that are activated due to accrual of misfolded proteins in the ER. The UPR initially aims to maintain the homeostasis of cell by retarding the process of protein translation, degrading wrongly folded proteins and increasing the expression of molecular chaperones assisting protein folding. Under certain conditions when UPR is not able to counter ER stress, cellular apoptosis may occur (Fig. 12.1). To summarize, unfolded proteins initially will favour UPR and cell survival but under extreme conditions can trigger programmed cell death (Kato and Nishitoh 2015). UPR signalling is enabled by three main transmembrane transducers: inositol-requiring enzyme 1-alpha (IRE1 alpha), activating transcription factor 6 (ATF6) and RNA-dependent protein kinase-like ER kinase (PERK). These sensors normally in an inactive state remain bound to the ER chaperones, mainly glucose-regulated protein of 78 kDa (GRP78). Nonetheless, GRP78 dissociates in response to stress conditions in the ER, rendering UPR sensors active. Many disturbances such as nutrient exhaustion, redox imbalance, hypoxia and disturbed calcium ion homeostasis contribute significantly in triggering stress to tumour. This mainly leads to build-up of misfolded proteins and its subsequent UPR activation in the cell. Various triggers of stresses, which lead to tumour formation, are represented in figure form (Fig. 12.2).

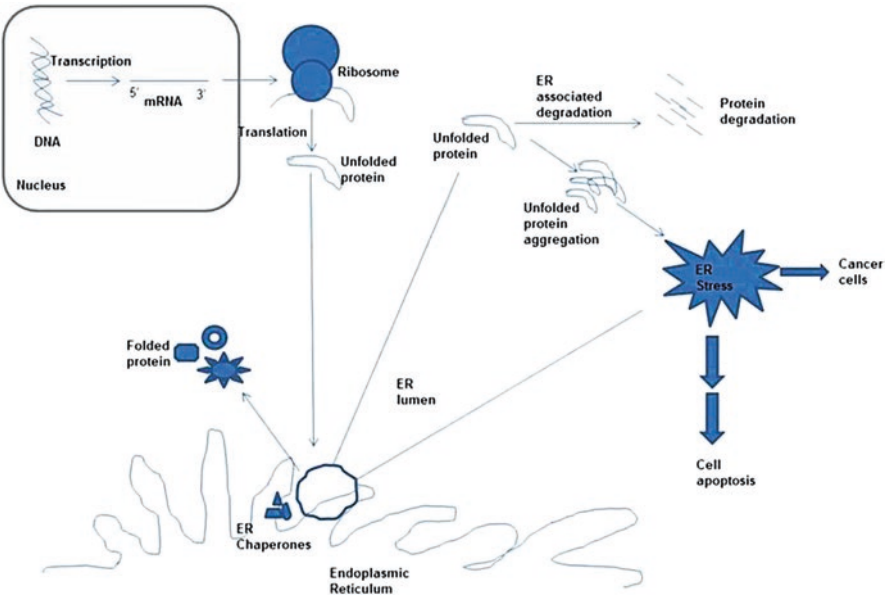

Fig. 12.1 Unfolded protein response governs the fate of the cell

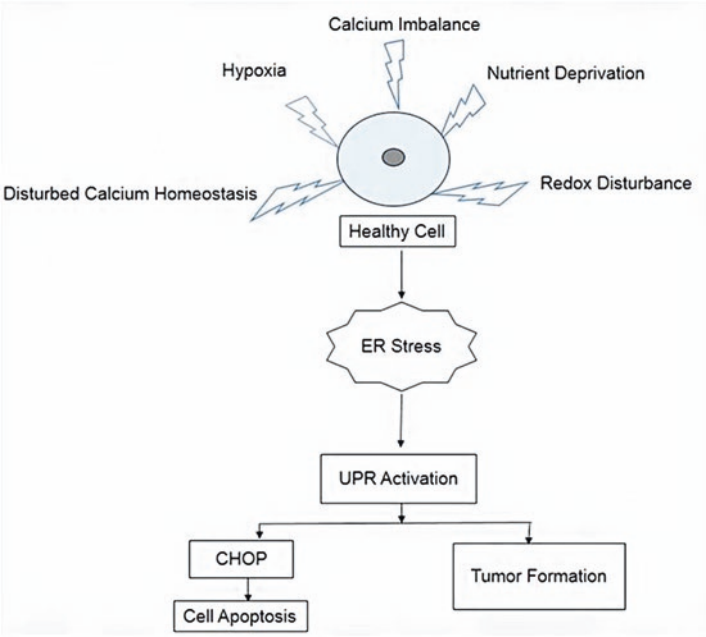

Fig. 12.2 Triggers of endoplasmic reticulum stress leading to tumour formation

12.2.1 The PERK Branch

PERK, a transmembrane protein, phosphorylates eIF2-alpha which inhibits the synthesis of new proteins and folding of the existing proteins. In addition, the activating transcription factor 4 (ATF4) expression is selectively induced by eIF2α phosphorylation, which induces upregulation of UPR genes and growth arrest, including ER oxidoreductase1α, CCAAT/enhancer-binding protein-homologous protein (CHOP) and various pro-apoptotic factors (Quick and Faison 2012; Carreras et al. 2017). Haem-regulated eIF2α kinase and multiple kinases including protein kinase R also induce eIF2α phosphorylation. The integrated stress response (ISR) is referred to as signalling related to eIF2α phosphorylation. Nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) is another substrate which upon phosphorylation dissociates from its cytoskeletal anchor and goes and binds to antioxidant redox element (ARE) in the nucleus. It has twofold part to play in cancer, oncogenic function and tumour suppressor function depending upon the ER stress in the cell. In normal circumstances, Nrf-2 interacts with kelch-like ECH-associated protein I to preserve the inactive state in the cytoplasm. PERK dissociates phosphorylated Nrf-2 from kelch-like ECH-associated protein I, which results in its translocation from membrane to the nucleus and antioxidant genes getting expressed promoting cell survival.

For survival of the tumour during nutrient starvation and hypoxia, it is considered necessary for the ISR and PERK signalling to get activated. Reactive oxygen species (ROS) concentration is increased as a result of oxidative stress and hypoxia. Additionally, PERK signalling upregulates ER oxidoreductase1α that controls ER redox status. As notified in different kinds of cancers, due to upsurge in the levels of ROS in tumours, ER oxidoreductase 1α is overexpressed (Wang et al. 2014). Inhibition of ISR or PERK signalling causes production of ROS that restricts growth of tumour by means of oxidative DNA damage. Retorting to prolonged and intense ER stress, cell death is induced by CHOP, a transcription factor and a downstream target of ATF4 (Hiramatsu et al. 2014). Hence there are possibilities of ISR and PERK being the therapeutic targets for cancer on account of their dual role in signalling pathways (Farooqi et al. 2015).

12.2.2 The IRE-1α Branch

IRE1-α is an ER type 1 transmembrane protein with both a kinase and an endoribonuclease domain. Activated IRE1 acts on mRNA of X-box-binding protein 1 (XBP1) and cleaves a 26-nucleotide intron from it, which promotes ER-associated degradation (ERAD). Regulated IRE1α-dependent mRNA decay (RIDD) is a process through which non-specific mRNAs are cleaved by IRE1α. XBPs help in marking up of wrongly folded proteins, so they are easily degraded via proteasome on their consequent translocation to the cytosol. XBP1s (spliced XBP) expression is necessary for independent tumour angiogenesis in the absence of vascular endothelial growth factor (VEGF). Hypoxia-inducible factor-1α pathway constitutively activates XBP1s retorting to hypoxia that leads to development of

tumour (Ojha and Amaravadi 2017). Interaction of IRE1-α with apoptosis signalling-regulating kinase 1 and tumour necrosis-associated factor 2 also activates c-Jun N-terminal kinase (JNK). Contribution of IRE1α-JNK pathway to both apoptotic cell death and cell survival via c-Jun activation portrays its dual role in cell fate. Thus, in some circumstances of chronic stress, cell death and tumour survival both are contributed by IRE1α signalling. Since discrete classes of adenosine triphosphate (ATP) competitive inhibitors can selectively control activity of endoribonuclease and kinase of IRE1α, it is believed that modulation of pathway can provide therapeutic approach for cancer (Kato and Nishitoh 2015).

12.2.3 The ATF 6 Branch

ATF 6 is a transmembrane protein of ER type 2 class. ATF 6 upon dissociation from the GRP-78 is translocated to Golgi apparatus from ER membrane to be cleaved via proteases. Cleaved ATF 6 translocates to nucleus and binds the UPR elements, which leads to the ER chaperones' expression in the cell that enhances protein folding capability of the cell. ATF 6-induced signals are purely pro-survival since no incident has been reported of ER stress-mediated apoptosis due to signalling from ATF 6 (Farooqi et al. 2015). Apoptosis modulators induced by the ER stress comprise of DNA damage-inducible gene 153 (CHOP/GADD153), C/EBP homologous protein, Bcl-2 family genes and caspase-12. ER stress induces the major pro-apoptotic transcription factor CHOP, and p38 mitogen-associated protein kinase further enhances its expression (Zorzi and Bonvini 2011). The drugs aim at increasing the ER stress by different processes leading to cellular apoptosis which can mitigate the uncontrolled growth of cancer cells. The role of UPR and its branches leading to cell apoptosis is represented in Fig. 12.3.

12.3 Therapeutics of Phytochemical Origin

Phytochemicals are the chemical compounds naturally present in plants and have protective or disease-preventive properties. On account of their antioxidant properties, some phytochemicals guard the cells against oxidative damage and reduce the risk of developing several cancer types, whereas others induce reactive oxygen species (ROS) to increase ER stress level to an extent that it leads to cellular apoptosis. The intracellular concentration of ROS is generally raised in the tumour cells, exposing a key role of ROS in the course of carcinogenesis and cancer advancement. Raised levels of ROS induce mutations and thus promote cancer (Yang et al. 2013). Under mild oxidative stress environment, ER behaves as a cellular stress sensor, seeking to restore cellular homeostasis. If the stress-induced damage is widespread, cellular apoptosis is initiated in ER by means of the unfolded protein response (Hetz 2012). Phytochemicals restrict DNA replication leading to inhibition of cancer cell proliferation.

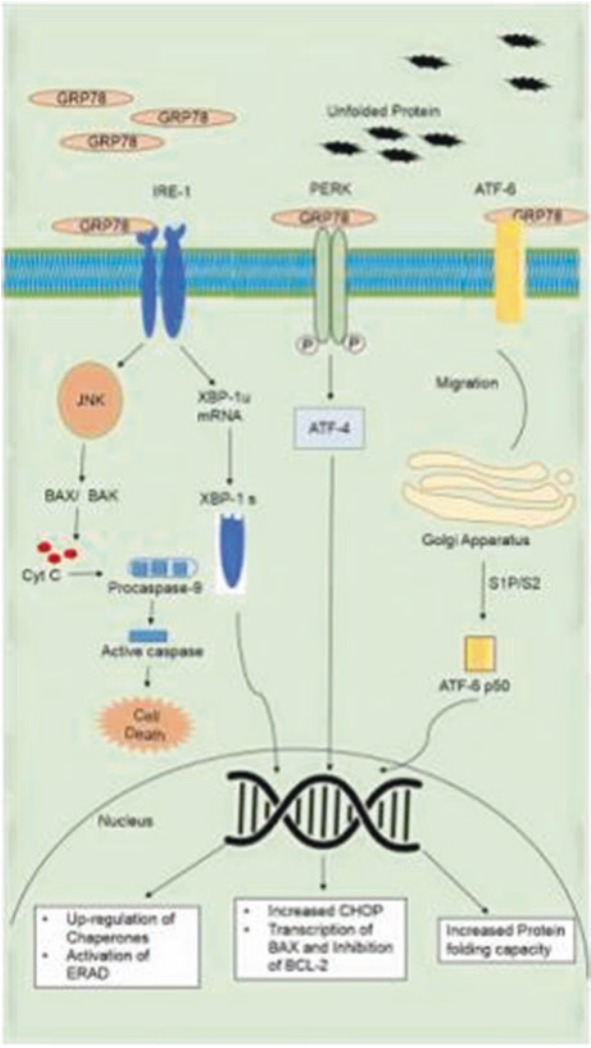

Fig. 12.3 Role of UPR branches in cell death and cancer inhibition. XBP1u, XBP1 unspliced; XBP1s, XBP1 spliced

12.3.1 Carnosic Acid

Carnosic acid is a diterpene found in rosemary (*Rosmarinus officinalis*), exhibiting anti-proliferative effects when observed *in vitro* in human renal carcinoma cells and cervical carcinoma cells (Su et al. 2016) including inhibition of cell proliferation, angiogenesis and cell migration. ER stress is induced by the carnosic acid present in the extract from rosemary, inducing cancer cell apoptosis (Cao and Kaufman 2014; Petiwala et al. 2014). Carnosic acid induced dose-dependent expression of ATF4 and CHOP and knockdown of CHOP and ATF4 by siRNA-suppressed apoptosis.

The role of ROS was investigated in the induction of ER stress by carnosic acid. ATF4 and CHOP expression induced by carnosic acid was markedly inhibited by ROS scavengers. Hence, this information implies that ATF4 and CHOP expression mediated by ER stress plays a significant part in apoptosis of human kidney Caki cells induced by carnosic acid (Min et al. 2014). Treatment of 22Rv1 and LNCaP, human prostate cancer cell lines, by carnosic acid degraded the androgen receptor (AR), and antigen specific to the prostate also showed reduced expression. Proteasomal degradation pathway dependent on ER stress mediates AR degradation. Induction of CHOP protein, degradation of AR and reduction in the growth of xenograft prostate cancer tumours by 53% were also detected *in vivo*. Additionally, carnosic acid induces ER stress only in prostate cancer cells and not in normal cells (Petiwala et al. 2016).

12.3.2 Diallyl Sulphur Compounds

Garlic compounds diallyl trisulphide, diallyl disulphide and diallyl sulphide produce ROS that decreases Ca^{2+} concentration in ER while increasing its cytosolic concentration, which leads to apoptosis of cancer cell. Calcium depletion in the ER interrupts the normal function of ER chaperones, and hence ER stress is induced (Wu et al. 2005). On the other hand, cytotoxicity is triggered under certain circumstances like enhanced generation of ROS, when intracellular calcium concentration is increased, inducing very high levels of ER stress to an extent that a cell could not sustain and dies. Experiments conducted on glioblastoma cells (Wu et al. 2017) and skin cancer cell line (Wang et al. 2012a) demonstrate a direct relation between the cell apoptosis and increase in activities and expression of cysteine proteases and stress kinases. Moreover, increase in levels of calreticulin (another ER chaperone required for the synthesis of proteins) and caspase-4 further ensures the role of increased ER stress in apoptosis (Wu et al. 2017). In adenocarcinoma COLO 205 cells, garlic compounds induce apoptosis by caspase activation, enhanced concentration of Bax and reduced concentration of Bcl2 proteins (Nicastro et al. 2015; Tung et al. 2015).

12.3.3 Gambogic Acid (GA)

GA, extract from *Garcinia hanburyi*, the most important active constituent of gamboge, inhibits the heat shock protein (Hsp90) (Zhang 2010). Hsp90 is the major protein in tumour regeneration as well as cell signalling, and its inhibition results in induction of ER stress, thereby activating UPR target genes and ER stress sensors which further cause cell death (Lamoureux et al. 2014). Severe or sustained ER stress increases CHOP expression and stimulates IRE1α apoptotic signalling, ATF6 and PERK. CHOP being the major regulator of Bcl2, Bim and DR5 leads to cellular apoptosis preventing tumour progression (Krajarng et al. 2015).

12.3.4 Galangin

Galangin is a flavonol that is derived from *Alpinia officinarum*, a plant from the ginger family, grown in Southeast Asia. The extract from the rhizome suppresses cell proliferation of hepatocellular carcinoma cells (Su et al. 2013). ER stress is induced by galangin as is evident by rise in concentration of cytosolic Ca^{2+} and other UPR target genes like CHOP, GRP 78 and GRP 94. The ER is a major storage site for intracellular calcium. Normal functioning of ER chaperones is disrupted by calcium exhaustion in ER which produces ER stress and hence activation of UPR (Hotamisligil 2010; Mekahli et al. 2011). CHOP and 4-polybutyric acid siRNA, well-known ER stress inhibitor, substantially blocked stress induced by galangin in the HCC cell line. To sum up, ER stress is upregulated by galangin, inhibiting cancer cell proliferation, and galangin can prove to be an effective anticancer agent (Su et al. 2013).

12.3.5 Pachymic Acid

Extracted from *Poria cocos*, pachymic acid (PA) is a tri-terpenoid (a lanostane type), which is reported to have anticancerous properties. NCI-H460 and NCI-H23 cells, the lung cancer cell lines, were studied for the effect of pachymic acid. Expression of a major UPR target gene-regulated protein, CHOP, was upregulated resulting in cell apoptosis after PA treatment. It was discovered that ROS inhibitors blocked the ER stress activation induced by PA. The results indicated that ROS production is also regulated by PA in lung cancer cells (Cheng et al. 2015; Jun et al. 2015). As per the studies conducted on MIA PaCa-2 and PANC-1 cell lines, PA-induced apoptosis in pancreatic cancer cell resistant to gemcitabine was dependent on *in vivo* and *in vitro* activation of ER stress. PA can be exploited for its anticancer effects for treatment of pancreatic cancer resistant to chemotherapy (Cheng et al. 2015).

12.3.6 Pomegranate Fruit Extract

Pomegranate fruit extract (PFE) downregulated the proteins involved in cell cycle regulation during G1 phase (Gullett et al. 2010). Moreover it inhibited growth, progression and angiogenesis of tumour by suppressing MAP kinase pathway, mTOR and NF-κB signalling pathway in the animal model for primary lung tumour (Khan and Mukhtar 2015) and urothelial carcinoma (Song et al. 2013). The MAPK/ERK pathway conducts a signal for sustained growth of tumour. Unrestrained growth is an essential step for the advancement of all cancers. In many cancers (e.g. melanoma), a flaw in the MAP/ERK pathway heads to the uncontrolled growth. Many compounds can obstruct steps in the MAP/ERK pathway and therefore are possible drugs for the treatment of cancer (De Luca et al. 2012; Vicinanza et al. 2013). Pro-apoptotic Bak and Bax are overexpressed, while anti-apoptotic Bcl-XL and Bcl2 are downregulated by administration of PFE, triggering apoptosis

(Hausmann et al. 2014). Furthermore, apoptosis might be induced by activation of pro-caspase 12 by dysregulated valosin-containing protein triggered by PFE (Derry et al. 2014).

12.3.7 Proanthocyanidins and Resveratrol

Grape seed extract (GSE) has high quantities of proanthocyanidins and resveratrol. Human colorectal cancer cell (CRC) exposure to GSE (Schonthal 2012) resulted in modified ER chaperones glucose-related protein-78 (GRP78) and protein disulphide isomerase (PDI). GRP78 is needed for integrity of ER as well as stress-induced autophagy (Flaumenhaft et al. 2015). PDI, a thiol-disulphide oxidoreductase, catalyses the disulphide bonds in newly synthesized proteins in the ER lumen (Han et al. 2013; Wang and Kaufman 2014; Perri et al. 2016), and PDIA3 is localized to the ER and is involved in protein folding (Wang et al. 2012b). Studies on the role of PDI in cancer have demonstrated a pro-oncogenic, pro-survival role for PDI in cancer and therapeutic resistance. PDI aids in ameliorating stress-induced apoptosis and causes degradation of wrongly folded proteins (Dinicola et al. 2010; Xu et al. 2014). Hence, any obstruction caused in the activity of PDI will induce stress in the cell and lead to cell apoptosis. Fructose bisphosphate aldolase, a key enzyme involved in glycolysis, is also a potential target of GSE (Vandewynckel et al. 2016). The ER stress leads the cell to undergo apoptotic pathways in multiple ways and hence inhibits the proliferation of the targeted cells (Farooqi et al. 2015).

12.3.8 *Saccharina japonica*

As per the studies conducted on SK-Hep1 cell line, n-hexane fraction of *S. japonica* of Phaeophyceae family activates caspase-dependent as well as caspase-independent cell apoptosis in hepatocellular cancer cell and also disturbs the calcium homeostasis that produces ER stress (Jo et al. 2012). ER stress is produced by the disruption of calcium homeostasis and therefore upregulates ER stress-related genes, TRAF2, ATF6α, Bip, CHOP and phospho-JNK, that lead to cell apoptosis. In human prostate cancer cells (267B1/K-ras), *S. japonica* extract induces cell cycle arrest and apoptosis and has shown to have potential for preventing cancer (Jung et al. 2014).

12.3.9 6-Shogaol

6-Shogaol is the dehydrated product of 6-gingerol, extracted from the rhizome of ginger. Treatment of HCC cell line with 6-shogaol resulted in cells with apoptotic phenotypes, which showed signs of cell and nuclear shrinkage as well as substantial chromatin condensation. De-phosphorylation of PERK and activation of the expression of CHOP initiate caspase cascade reaction inducing apoptosis in HCC. Two-dimensional gel electrophoretic analysis of proteome revealed that in

response to the treatment with 6-shogaol, a significant stimulation was observed in proteins related to the ER stress, signifying that apoptosis induced by 6-shogoal did involve ER stress. Cells showed marked rise in the UPR target expression, HSP70, Grp94, Grp78/Bip and the other ER chaperones on exposure to 6-shogoal in a time-dependent manner, which elicited activation of caspase-3 and degradation of poly ADP ribose polymerase (PARP). Various ER chaperone proteins improve adaptation of cancer cells to hypoxic environment and aid in developing resistance against anticancer therapy (Zorzi and Bonvini 2011; Urra et al. 2016). Screening of specific inhibitors of Grp78 as antitumour agents (Hu et al. 2012; Liu et al. 2013; Venkatesan et al. 2015) implies that inhibition of Grp78/Bip is a very promising anticancer strategy. HCC cells are selectively killed by 6-shogaol in the absence of any noticeable toxic consequence on normal healthy cells and very little toxicity as studied on SMMC7721 xenograft mice. Administration of 6-shogaol and salubrinal together for distinct time intervals resulted in significant increase in ER stress in the cell. It appears that 6-shogaol in combination with salubrinal has great therapeutic value against various malignancies including HCC (Hu et al. 2012).

12.3.10 Sulphureuine B

Laetiporus sulphureus is the edible mushroom from which sulphureuine B was extracted, and glioma cells were used to examine its anti-proliferative properties. Sulphureuine B elevates the expression levels of CHOP, caspase-12 and GRP8 leading to the generation of ER stress. ER stress can initiate UPR via triggering PERK, ATF6 and IRE1 signalling proteins. CHOP inhibition by CHOP siRNA also obstructed PARP cleavage giving an idea that PARP cleavage is brought about by sulphureuine B (Biswas et al. 2015; Zhang et al. 2015). We have summarized some of the phytochemicals that modulate ER stress to confine carcinogenic development of cell in tabular form (Table 12.1).

12.4 Conclusions and Future Prospects

Contribution of ER stress in cancer cell apoptosis has been examined very clearly, and the anticancer therapeutics can be designed using phytochemicals to target ER stress. Under extreme cases, high level of ER stress induced is not enough to induce cell death. Thus, a specific apoptotic pathway is required to activate the controlled cell death through the production of ROS, the mitochondrial-mediated pathway and the arrest of cell cycle. The results of various experimental findings disclosed that the essential function of CHOP in apoptosis is regulated by ER stress. The phytochemicals like carnosic acid, gambogic acid, galangin, pachymic acid, S*accharina japonica* and 6-shogoal mediate ER stress regulated UPR signaling. In contrast, diallyl sulphur compounds, pomegranate fruit extract, proanthocyanidins and resveratrol and sulphureuin B may increase the oxidative stress in the cell for its survival in stress conditions. Though these phytochemicals appear promising and act via

Table 12.1 Some of the phytochemicals that modulate endoplasmic reticulum (ER) stress to restrict cancerous growth of cell

Phytochemicals	Sources	Targets	Cancer types	References
Carnosic acid	*Rosmarinus officinalis*	ROS, ER stress, ATF4, CHOP	Cervical carcinoma, renal carcinoma, prostate cancer	Berrington and Lall (2012), Park et al. (2016) and Petiwala et al. (2013)
Diallyl sulphur compounds	*Allium sativum*	ROS, MAPK, cytosolic Ca^{2+}concentration↑, caspase 4	Glioblastoma, skin cancer, adenocarcinoma	Omar and Al-Wabel (2010)
Gambogic acid	*Garcinia hanburyi*	Integrin β1, VGEF, Hsp90, ER stress, UPR sensors, CHOP	Cervical cancer	Tang et al. (2017) and Wen et al. (2015)
Galangin	*Alpinia officinarum*	Cytosolic Ca^{2+} concentration, ER chaperones, ROS, ER stress	Hepatocellular carcinoma	Zhang et al. (2012)
Pachymic acid	*Wolfiporia extensa*	ER stress, CHOP, XBP1α, ATF4, ATF6	Lung cancer, pancreatic cancer	Cheng et al. (2015) and Ling et al. (2010)
Pomegranate extract	*Punica granatum*	NF-κB, MAP kinase pathway, mTOR signalling Bak and Bax, BCl2	Lung tumour; urothelial carcinoma; prostate cancer	Gullett et al. (2010), Sharma et al. (2017) and Vicinanza et al. (2013)
Proanthocyanidins and resveratrol	*Vitis vinifera*	Protein modification in ER chaperones and PDI, ALDOA	Colorectal cancer	Chen et al. (2014) and Derry et al. (2014)
n-Hexane fraction	*Saccharina japonica*, member of family Phaeophyceae	Cytosolic Ca^{2+} concentration↑	Hepatocellular carcinoma, prostate cancer	Jo et al. (2012), Jung et al. (2014), Song et al. (2017)
6-Shogaol	*Zingiber officinale*	ER stress, CHOP, ER chaperones	Hepatocellular carcinoma	Weng et al. (2012) and Wu et al. (2015)
Sulphureuine B	*Laetiporus sulphureus*	Caspase-12, GRP78, CHOP ER stress, UPR activation	Glioma	Jing et al. (2015)

regulating ER stress, still a detailed study for toxicity evaluation is required to consider these as anticancer agents. Currently, targeting of therapeutics using UPR pathways and ER chaperones is in progress. If the direct assessment of the above mentioned phytochemicals will be proven successful, then it may revert the progression of cancer and its effect on the physiology of the normal cells either alone or together with existing therapy. Furthermore, their efficacy can be enhanced by using them in combination with other phytochemicals on existing anticancer drugs.

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Remedy of Targeting Cancer and Cancer Stem Cells with Botanicals

13

Rajanna Ajumeera, Ganapathi Thipparapu, and Suresh Challa

Abstract

The cancer disease burden is increasing regularly due to various factors like mutation occurrence in genes, environmental changes, adopting a modern lifestyle, less physical exercises, and modern food habits. To cure this disease, chemotherapy is the major option including surgery for solid tumors followed by radiation therapy. Chemotherapy is associated with lots of side effects with synthetic compounds, whereas natural source of plant extracts and products is reliable and has less side effects of treatment. In line with this, plant extracts with active ingredients having anticancer potential are becoming popular for cancer therapy. These plant extracts are associated with more than one active compound. But, extraction procedures to convert them into final drug products are associated with different hurdles. However, recent research advocates that the real culprit of the disease is cancer stem cells present in tumor. There is a need to target cancer stem cells to bring novel strategies for the therapy. The present chapter summarizes the anticancer activities of plant active components targeting cancer as well as cancer stem cells for therapeutics.

Keywords

Cancer stems cells · MDR · Phytochemicals · Signaling · Therapy

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13.1 Introduction

Cancer disease is the second largest cause of morbidity and mortality worldwide (Antoni et al. 2016). The World Health Organization (WHO) report says 14.1 million new cases, 8.2 million deaths, and 32.6 million cancer patients were in the year 2012 globally, and the new registries count was estimated to rise by 70% in the next 20 years (Antoni et al. 2016). The rapid growth of cancer burden is drastically affecting the economy of all countries. Among all the cancer types, lung, colon, breast, pancreatic, prostate, leukemia, liver, ovarian, esophageal, and lymphomas are the most commonly occurring cancers worldwide (Ferlay et al. 2010). The incidence and mortality of cancer are increasing constantly with mutations in genes, change in lifestyle and environment, stress, obesity, less physical exercise, exposure to carcinogens and radiation, alcohol, viral or bacterial infections, hormonal changes, immune dysfunction, and genetic or hereditary transfer of cancer (Hussain et al. 2003; Danaei et al. 2005).

Chemotherapy is the major treatment option including surgery followed by radiation. Hormone therapy and immune therapy are also employed as an adjuvant therapy in cancer. Recently, personalized and targeted therapies are employed to cure patients of certain cancers specifically in order to reduce side effects. Combinations of one or more of these strategies can be applied for complete remission of the disease (Sawyers 2004; Couzin-Frankel 2013). However, the challenge of complete remission still remains elusive due to failures of cancer therapies like development of drug resistance and lack of specificity in targeting cancer cells. Further, side effects of chemo- and radiotherapy are increasing the complexity of disease and leading to rise in mortality rates worldwide. A rigorous and continuous research is going on around the globe to answer these challenges, and a number of drug candidates are being synthesized and evaluated for their safety and efficacy. Actually, drug design and development of new compounds in new drug discovery process take a long time and are expensive too, and finally, a limited number of lead molecules enter the clinical trials where most of them fail to be successful drug candidates (Turkson 2008; Schwartsmann et al. 2002). At this juncture, researchers are looking back to the alternate and primary sources of lead molecules from phytochemicals or plant extracts, as they are the richest source of leads for many diseases including cancer (Balunas and Kinghorn 2005). Earlier, many phytochemicals were scientifically evaluated as anticancer agents, and some of them are in clinical use. Phytochemicals and their analogues or derivatives are being investigated against different cancer targets. The mechanism of action includes alteration of cell signaling pathways, induction of apoptosis, inhibition of angiogenetic and metastatic progression, regulation of cell cycle and uncontrolled proliferation, inhibition of DNA damage or promoting DNA repair in non-cancer cells, alterations in gene expressions, reducing oxidative stress, metabolism of carcinogens, and epigenetic modifications (Aggarwal and Shishodia 2006).

The advancement in oncological research has enabled the identification as well as isolation of CSCs, a subset in cancer cell pool. These CSCs have been identified as the real culprits for the tumorigenesis, metastasis, drug resistance, and relapse of

the disease. Many research groups are trying to target these elusive CSCs by various approaches like targeting cell signaling, self-renewal, and differentiation of CSCs (Reya et al. 2001). One of the most important approaches is the use of phytochemicals. In recent years, a paradigm shift in cancer research opens a new window of opportunity to explore phytochemicals to cure cancer by targeting CSCs (Wicha et al. 2006). The present chapter summarizes the anticancer activities of plant active components targeting cancer as well as cancer stem cells for therapeutics.

13.2 Natural Compounds as Anticancer Agents

Natural compounds are being used for cancer prevention and treatment since many years. These are the primary source of medication in ancient days of anticancer therapy. The identification and clinical use of some of the important anticancer phytochemicals like vinca alkaloids (vincristine, vinblastine), podophyllotoxins (etoposide, teniposide), taxanes (paclitaxel, docetaxel), camptothecins (camptothecin, irinotecan), etc. started the new era of exploring phytochemicals against cancer (Shoeb 2006). Some phytochemicals and their semisynthetic compounds like roscovitine, flavopiridol, betulinic acid, silvestrol, combretastatin A-4, schischkinnin, montamine, betulinic acid, pervilleine A, curcumin, genistein, soy isoflavones, indole-3-carbinol, etc. are undergoing preclinical or clinical trials for future development.

The sources of the anticancer phytochemicals are either dietary components or medicinal plants. β-Carotene, fucoxanthin, halocynthiaxanthin, β-cryptoxanthin, lutein, lactuca xanthin, ellagic acid, epigallocatechin gallate, curcumin, hentriacontane, 1-acetoxychavicol acetate, etc. are obtained from food sources (Murakami et al. 1996). Some compounds are obtained from spices including curcumin, capsaicin, eugenol, gingerol, cumin, anise, fennel, ursolic acid, diallyl sulfide, S-allylmercaptocysteine, ajoene, ellagic acid, zerumbone, and many more (Aggarwal et al. 2008). Whatever the source of the anticancer phytochemicals, basically they all belonged to the phytochemical classes of alkaloids, lignans, coumarins, naphthoquinones, cucurbitacins, diarylheptanoids, phenanthrenes, fatty acids, polyacetylenes, flavonoids, stilbenoids, iridoids, polyphenols, limonoids, oligorhamnosides, terpenoids, physalins, glucosinolates, and organosulfur compounds (Balunas and Kinghorn 2005; Greenwell and Rahman 2015). The structures of some anticancer compounds are shown in Fig. 13.1a, b.

13.3 Different Chemical Classes of Anticancer Phytochemicals

Phytocompounds are classified based on various criteria. Recently, many novel bioactive plant products with pharmacological significance have been identified and isolated by various scientific groups involving both chemists and biologists. Some of these phytocompounds exhibit anticancer properties, and their chemical classes

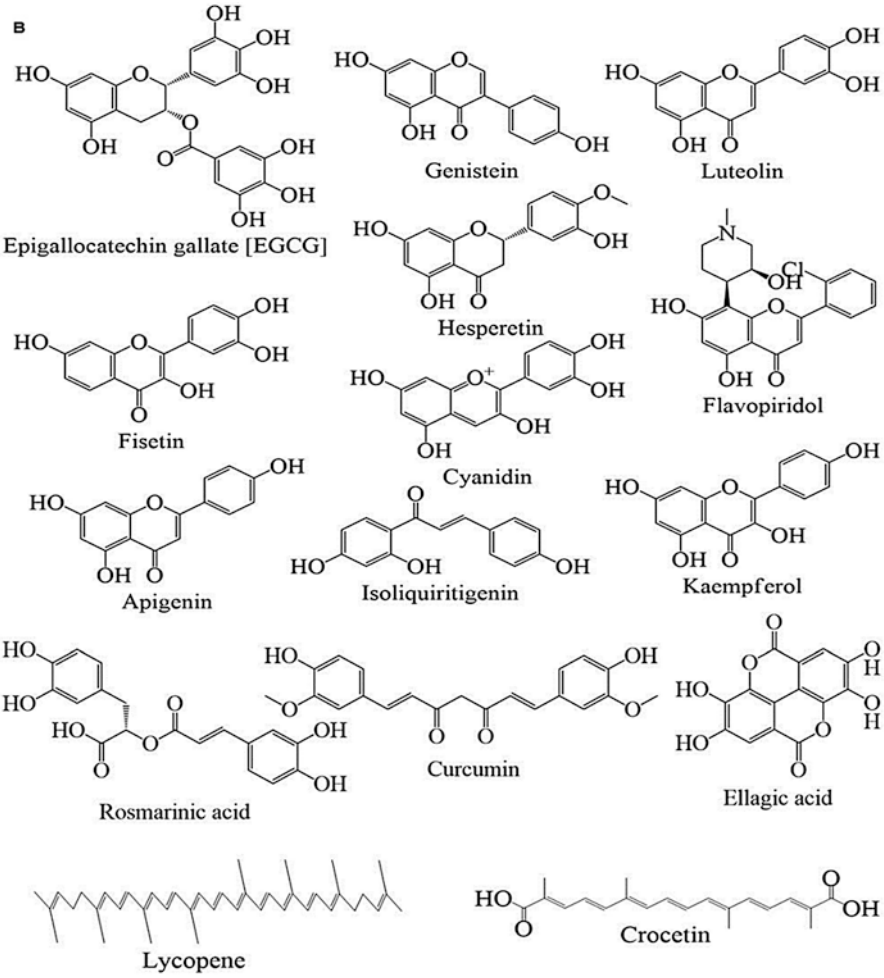


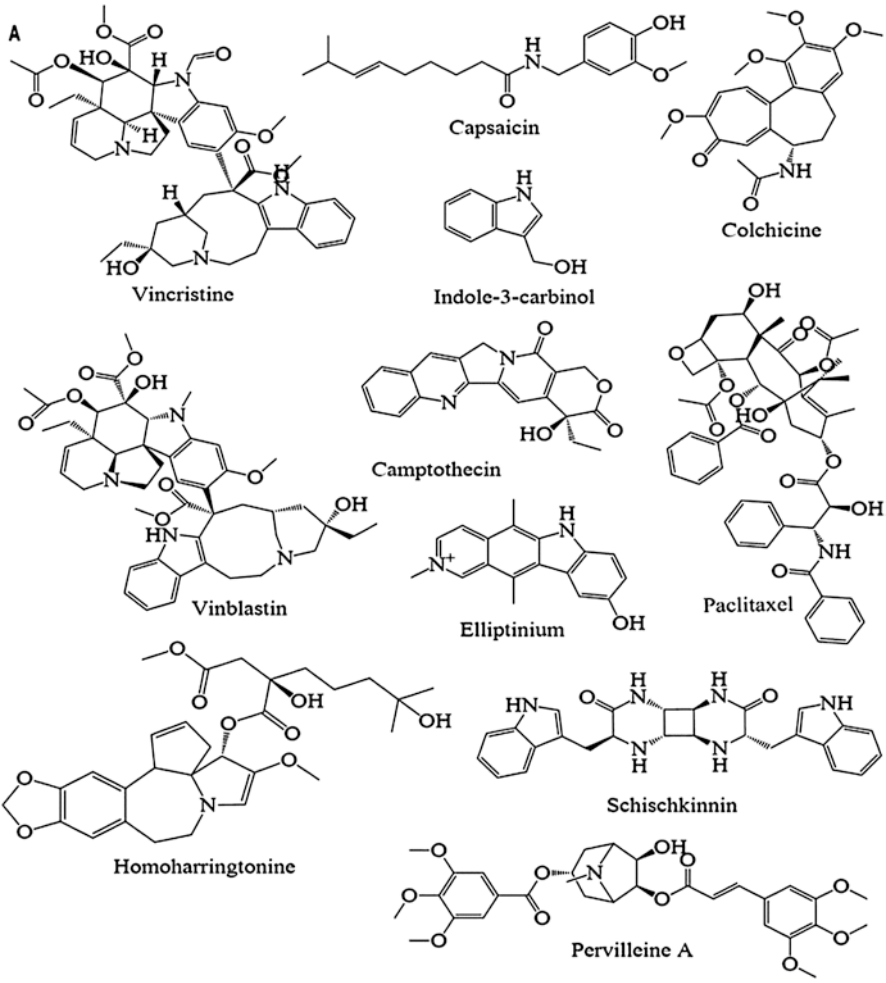
Fig. 13.1 (**a**, **b**) Structures of major anticancer phytocompounds

are mentioned in tabular form (Table 13.1), and structures of some anticancer compounds are shown in figure (Figs. 13.1), and their details are discussed in the following sections.

13.3.1 Flavonoid Compounds

13.3.1.1 Epigallocatechin Gallate (EGCG)

EGCG is an ester of gallic acid and epigallocatechin and is a catechin compound (Murakami et al. 1996). It is found most abundantly in green tea. It can be used to



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treat brain, prostate, cervical, and bladder cancers (Wang et al. 2012). It suppresses the ornithine decarboxylase action, an enzyme that leads to rapid proliferation and furthermore circumvents apoptosis (Singh et al. 2016a). It suppresses nuclear factor (NF-κB) activation and expression of Bcl-2 (B-cell lymphoma 2) as well as COX-2 (cyclooxygenase-2) in prostate cancer cells and causes induction of apoptosis. It hamper the matrix metallopeptidase-9 (MMP-9) activation in bladder and lung cancer cells and suppresses the synthesis of VGEF (vascular endothelial growth factor) in head and neck cancers. It prevents ERK (extracellular signal-regulated kinase) phosphorylation and MMP-2 and MMP-9 activation and suppresses ERK, c-Jun N-terminal kinase (JNK), and MMP-9 expressions in gastric carcinoma cells

Table 13.1 Classification of phytochemicals as anticancer agents

Classes of phytochemicals	Phytocompounds
Flavonoid	EGCG, fisetin, genistein, kaempferol, luteolin, apigenin, cyanidin, flavopiridol, isoliquiritigenin
Alkaloid	Vincristine, vinblastine, capsaicin, colchicine, indole-3-carbinol, camptothecin, paclitaxel, homoharringtonine, elliptinium, pervilleine A, schischkinnin, montamine
Terpenoid	Zerumbone, ursolic acid, betulinic acid, andrographolide, ixocarpalactone, β-boswellic acid
Carotenoid	β-carotene, lycopene, fucoxanthin, halocynthiaxanthin, lutein, lactucaxanthin, crocetin, α-carotene, β-cryptoxanthin
Isothiocyanate	Phenethyl isothiocyanate, sulforaphane
Polyphenolic	Rosmarinic acid, ellagic acid, curcumin
Others	Anethole, eugenol, isoeugenol, gingerol, diosgenin, gambogic acid, thymoquinone, diallyl sulfide, ajoene, hentriacontane, 1'-acetoxychavicol acetate, epipodophyllotoxin, roscovitine, combretastatin A-4, bullatacin, silvestrol, resveratrol, withaferin A, (−)-wikstromal, (−)-matairesinol, dibenzylbutyrolactol

(Singh et al. 2016a). It is binding and inhibits the antiapoptotic protein Bcl-xL, interferes with EGFR (epidermal growth factor receptor) signaling, and inhibits hepatocyte growth factor-induced cell proliferation and MAPK (mitogen-activated protein kinase), CDK (cyclin-dependent kinase), and cell signaling linked to growth factors (Wang et al. 2012; Du et al. 2012).

13.3.1.2 Fisetin (7,3′,4′-Flavon-3-Ol)

Fisetin is a plant polyphenol from the flavonoid group. It occurs in fruits and vegetables including persimmons, strawberries, onions, cucumbers, and apples. It is an antioxidant, exerts anticarcinogenic effects in HCT-116 (human colon carcinoma) cells, and modulates protein kinase and lipid kinase pathways (Wang et al. 2012). Fisetin alter signaling pathway like MAPK, NF-κB, activators of transcription (JAK/STAT), Janus kinase/signal transducers, phosphoinositide-3-kinase-protein kinase (PI3K/Akt), Wnt, and mammalian target of rapamycin (mTOR), thereby leading to cell cycle arrest in HL-60 cells (human acute promyelocytic leukemia cells) (Singh et al. 2016b). Thus, it exhibits inhibitory effects on adhesion, migration, invasion, and multidrug resistance (Suh et al. 2009).

13.3.1.3 Genistein

It is an isoflavone and is obtained from a variety of plants like psoralea (*Psoralea corylifolia*), kudzu (*Pueraria lobata*), faba beans (*Vicia faba*), and soybeans (*Glycine max*). It exhibits anticancer effect by inhibiting NF-κB and protein kinase B (Akt) signaling pathways (Singh et al. 2016b). It blocks the proliferation of cancer cells via the inhibition of cell growth enzymes and survival like tyrosine kinase and topoisomerase II; hence it is used to treat leukemia. Genistein increases the growth rate of some estrogen receptors in breast cancer cells and the rate of proliferation of

estrogen-dependent breast cancer by competitive binding to the estrogen-β receptors. It may be involved in JNK pathway in inducing activator protein-1(AP-1) activity (Wang et al. 2012; Dixon and Ferreira 2002).

13.3.1.4 Kaempferol

Kaempferol is one of the secondary metabolites found in some plants, plant-derived foods, and traditional medicines. It is a flavonoid compound obtained from some edible plants including grapes, tea, strawberries, broccoli, tomato, cabbage, leek, kale, endive, and beans. It inhibits growth and migration of pancreatic cancer cells by acting on proto-oncogene tyrosine kinase (Src), ERK1/2, and AKT pathways (Singh et al. 2016a). It is being investigated in pancreatic and lung cancers to evaluate its antiangiogenic, anticancer, and radical scavenging activities. It shows moderate cytostatic activity in PC3, HeLa, and K562 human cancer cells. It is identified as aryl hydrocarbon receptor antagonist and acts against ABCG2 (ATP-binding cassette subfamily G member 2)-mediated multidrug resistance by preventing the ABCG2 upregulation in esophageal carcinoma. It induces the apoptosis of ovarian cancer cell by activating p53 in intrinsic pathway mechanism. It is an inhibitor of breast cancer resistance protein (BCRP), quinine reductase-2, and a substrate of BCRP (Calderon-Montano et al. 2011; Wang et al. 2012).

13.3.1.5 Luteolin

It is a flavone with yellow crystalline appearance. Dietary sources of luteolin include oregano, celery, orange, broccoli, rosemary, green pepper, peppermint, parsley, olive oil, thyme, carrot, dandelion, chamomile tea, and perilla. It is found to obstruct epithelial-mesenchymal transition (Singh et al. 2016b). It is inhibiting the cancer cell proliferation, angiogenesis, and metastasis. In addition, it suppresses the pathways like PI3K/AKT, NF-κB, and X-linked inhibitor of apoptosis protein (XIAP) which enhances the cell growth and function. It also induces apoptosis and tumor suppressor p53. Hence, luteolin can be used as a potential antineoplastic agent in different cancers (Lin et al. 2008).

13.3.1.6 Apigenin

It is a flavone compound found in many fruits and vegetables and abundant in chamomile tea, parsley, celeriac, and celery. It induces apoptosis by targeting leptin/leptin receptor pathway and by targeting caspase-dependent extrinsic pathway as well as STAT3 signaling pathway in lung adenocarcinoma and BT-474 breast cancer cells, respectively (Singh et al. 2016b). It shows antitumor activity against breast cancer MCF-7 cells and colon cancer HCT 116 cells and is a mediator of cancer chemoprevention and an inducer of autophagy. It can be used to treat colon cancer as it induces apoptosis in colon cancer cells. It also increase melanogenesis in B16 cells by activating the p38 MAPK pathway (Wang et al. 2012).

13.3.1.7 Cyanidin

It is a kind of anthocyanidin and a common pigment found in many red berries including raspberry, grapes, acai berry, bilberry, loganberry, blackberry, hawthorn,

blueberry, elderberry, cherry, and cranberry. It is an antioxidant and free radical scavenging agent. It affects the colon cancer cell growth by inhibiting nitric oxide synthase and COX-2 gene expression. The derivative, cyanidin-3-glucoside (C3G), plays a role in the reduction of AP-1 activation and NF-κB and phosphorylation of MEK, MKK4, Akt, and MAPKs and blocks the activation of Fyn kinase signaling pathway and ErbB2/cSrc/FAK pathway. It is inhibits the UVB-induced COX2 expression and PGE2 secretion by suppressing AP-1, NF-κB, MKK4, MEK1, and Raf-1. Cyanidin-3-galactoside and cyanidin-3-glucoside are found to be BCRP substrates, and cyanidin, cyanidin-3,5-diglucoside, and cyanidin-3-rutinoside are found to be BCRP inhibitors (Wang et al. 2012).

13.3.1.8 Flavopiridol

It is a synthetic derivative of rohitukine plant alkaloid which is obtained from *Dysoxylum binectariferum*, which is active against leukemia, lymphomas and solid tumors (Shoeb 2006).

13.3.1.9 Isoliquiritigenin

It is extracted from *Dipteryx odorata* seeds, found to be active in induction of quinine reductase, and thus prevents chemical carcinogenesis. In a dose- and time-dependent manner, it significantly inhibits the proliferation of prostate cancer cells, and this isoliquiritigenin-induced cell cycle arrest and antiproliferative effects may be manifested by growth arrest- and DNA damage-inducible gene 153 (GADD153). It induces apoptosis in prostate cancer cells through mitochondrial apoptosis pathway in which mitochondrial membrane potential is disrupted, cytochrome-c and Smac/Diablo are released, and caspase-9 is activated. It is reported to enhance the expression of universal inhibitor of cyclin-dependent kinases, $p21^{CIP1/WAF1}$, in a dose- and time-dependent manner in A549 human lung cancer cells (Kanazawa et al. 2003; Ii et al. 2004; Balunas and Kinghorn 2005; Jung et al. 2006).

13.3.2 Alkaloid Compounds

13.3.2.1 Vinca Alkaloids

Vincristine and vinblastine are extracted from Madagascar periwinkle, *Catharanthus roseus.* These are primarily used in combination with other chemotherapy drugs in treating various cancers such as lung cancer, leukemia, testicular cancer, breast cancer, and lymphomas (Shoeb 2006). Vinca alkaloids and their semisynthetic derivatives cause depolymerization of tubulin by specific binding to it, leading to the arrest of metaphase of mitosis (Balunas and Kinghorn 2005). Vinblastine targets activator protein-1 (AP-1) signal pathways (Singh et al. 2016b).

13.3.2.2 Capsaicin

Capsaicin is the major pungent ingredient in red and green chili pepper. It is reported to induce apoptosis selectively in cancer cells and can suppress the activation of NF-κB through suppression of NF-κB inhibitor IκBα (Aggarwal and Shishodia

2004). It shows anticancer effects in animal models and suppresses carcinogenesis in colon, skin, lung, tongue, and prostate cancers by altering the metabolism of carcinogens. It selectively suppresses the human cancer cell growth of prostate, leukemic, glioma, gastric, and hepatic cancers. It inhibited the tumorigenesis linked and IL-6-induced activation of STAT-3 and STAT3-regulated gene products like cyclin D1, Bcl-2, Bcl-xL, survivin, and VGEF. It arrests cells in G1 phase and induces apoptosis (Aggarwal et al. 2008; Clark and Lee 2016).

13.3.2.3 Colchicine

It is a natural toxic secondary metabolite, extracted from *Colchicum* genus plants. It prevents gastric cancer by upregulating the dual specificity phosphatase 1 (DUSP1) gene. It is also reported to upregulate transforming growth factor beta 2 (TGF-β2) and A-kinase anchoring protein 12 (AKAP12) in hepatocellular carcinoma (Singh et al. 2016b).

13.3.2.4 Indole-3-Carbinol (I3C)

I3C is a bioactive compound majorly found in *Brassica* vegetables including broccoli, cauliflower, and collard greens. I3C and its derivative diindolylmethane (DIM) have been investigated for cancer prevention and treatment of breast, prostate, and ovarian cancers. I3C partially modulates the tyrosine kinase/PI3K/Akt signaling pathway which leads to the prevention of lung adenocarcinoma which is induced by using tobacco carcinogen in A/J mice. DIM transduces signaling via aryl hydrocarbon (Ah) receptor, NF-κB/Wnt/Akt/mTOR signaling pathways, cell cycle arrest, modulated cytochrome P450 enzymes, and altered angiogenetic and invasive, metastatic, and epigenetic behavior of cancer cells. Combination of DIM and I3C induces Nrf2-mediated phase II drug metabolizing genes (GSTm2, UGT1A1, and NQO1) and antioxidant genes (HO-1, SOD-1) (Weng et al. 2008; 2012).

13.3.2.5 Camptothecin(CPT)

CPT is extracted from *Camptotheca acuminata*, also called Chinese ornamental tree. Irinotecan and topotecan are semisynthetic derivatives of camptothecin, which can be used for the therapy of colorectal and ovarian and small cell lung carcinoma, respectively (Shoeb 2006). Camptothecin is a potent antitumor agent that targets topoisomerase I (Desai et al. 2008). The synthetic derivatives of camptothecin [20-*(S)*-9-nitrocamptothecin and 20-*(S)*-camptothecin] have the antitumor effects in breast, prostate, and melanoma cancers. CPT-11 is a new derivative that shows antitumor effects against metastatic colorectal cancer (Hosseini and Ghorbani 2015). It selectively inhibits topoisomerase I which is involved in cleavage and reassembly of DNA (Balunas and Kinghorn 2005). Camptothecin inhibits the synthesis of nucleic acid in L-120 cells and HeLa cells (Desai et al. 2008).

13.3.2.6 Paclitaxel

It is isolated from the bark of *Taxus brevifolia* generally known as pacific yew. It is primarily used in ovarian, small, and non-small cell lung cancers and advanced breast cancer (Shoeb 2006). It binds to tubulin but neither depolymerizes it nor

interferes with its assembly (Balunas and Kinghorn 2005). Taxol targets activator protein 1 signaling pathways (Singh et al. 2016b).

13.3.2.7 Homoharringtonine

This compound is isolated from *Cephalotaxus harringtonia*. A racemic mixture of harringtonine and homoharringtonine is used for acute and chronic myelogenous leukemia (Shoeb 2006).

13.3.2.8 Elliptinium

It is an ellipticine derivative and is obtained from *Bleekeria vitensis*, a Fijian medicinal plant. It is used to treat breast cancer (Shoeb 2006).

13.3.2.9 Pervilleine A

This phytocompound is extracted from the roots of *Erythroxylum pervillei*. It selectively kills the multidrug-resistant KB-V1 (oral epidermoid) cancer cells in the presence of chemotherapeutic agent vinblastine (Shoeb 2006). It could restore the sensitivity of vinblastine in cultured multidrug-resistant KB-V1 and CEM/VLB cells, at a half maximal inhibitory concentrations of 0.36 μM and 0.02 μM, respectively (Mi et al. 2001). It is more effective than verapamil in reversing multidrug resistance in KB-8-5 cells when tested in the *in vivo* hollow fiber model (Balunas and Kinghorn 2005).

13.3.2.10 Other Alkaloids

Montamine and schischkinnin are alkaloid compounds, isolated from the seeds of *Centaurea montana* and *Centaurea schischkinii*. These phytochemicals showed considerable anticancer activity against human colon cancer (Shoeb 2006; Singla et al. 2014).

13.3.3 Terpenoid Compounds

13.3.3.1 Zerumbone

Zerumbone is isolated from the rhizomes of the wild ginger *Zingiber zerumbet*. It is found to suppress the proliferation of breast cancer and colon cancer cells and also to prevent initiation and promotion of skin cancer in mice. It also suppresses the NF-κB activation and its sequential suppression of IκBα kinase activity, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, nuclear translocation, and acylation. It downregulates the NF-κB-regulated gene products like FLIP, cyclin D1, TRAF1, COX2, Bfl-1/A1, MMP-9, Bcl-xL, ICAM-1, Bcl-2, *c-myc*, XIAP, survivin, IAP1, and IAP2, and thus, it potentiates apoptosis (Murakami et al. 2002; Sakinah et al. 2007).

13.3.3.2 Ursolic Acid

It is a triterpenoid, derived from basil, and suppresses the activation of NF-κB by inhibiting a kinase that activates NF-κB(IKK) (Aggarwal and Shishodia 2004). It

suppresses tumorigenesis, tumor promotion, and angiogenesis by downregulation of the expression of lipoxygenases (LOX), MMP-9, and COX-2. It is reported to induce apoptosis in breast cancer, melanoma, hepatoma, prostate cancer, and acute myelogenous leukemia by preventing replication of DNA, inhibition of protein tyrosine kinases, activation of caspases, induction of calcium release, and downregulating apoptosis gene cellular inhibitor. Ursolic acid inhibits IκBα kinase activity, degradation of IκBα, phosphorylation, NF-κB-dependent reporter gene expression, p65 nuclear translocation, and p65 phosphorylation. Inhibition of NF-κB leads to the reduction of cyclin D1, COX-2, and MMP9 expression. This downregulates STAT-3 activation and its regulated gene products such as cyclin D1, Mcl-1, Bcl-xL, survivin, Bcl-2, and VEGF. It also induces the expression of tyrosine phosphatase SHP-1 protein and of mRNA (Aggarwal et al. 2008; Hsu et al. 2004).

13.3.3.3 Betulinic Acid

This compound is a pentacyclic triterpene obtained from *Betula* and *Zizyphus* species, which shows selective cytotoxicity against human melanoma cells (Shoeb 2006). It generates reactive oxygen species, activates MAPK cascade, inhibits topoisomerase I, inhibits angiogenesis, modulates pro-growth transcriptional activators, modulates the activity of aminopeptidase-N, and thereby induces apoptosis in cancer cells (Desai et al. 2008; Fulda 2008).

13.3.3.4 Andrographolide

It is a labdane diterpenoid, which shows cytotoxic effect against different cancer cell lines like human epidermoid cancer cells (KB), lymphocytic leukemia cells (P388), breast cancer cells (MCF-7), and colon cancer cells (HCT-116). It inhibits the proliferation of colon cancer cells (HT-29) and exerts pro-differentiative effect on mouse myeloid leukemia M1 cell line (Desai et al. 2008).

13.3.3.5 Ixocarpalactone

This terpenoid compound is isolated from the edible plant *Physalis philadelphica*. The leaf and stem extract induces quinine reductase, a phase II enzyme, needed for metabolism of chemical carcinogens. The anticancer activity of ixocarpalactone is exerted through G2/M phase arrest in cell cycle, downregulation of E2F-1, DP-1, upregulation of hyper-phosphorylated retinoblastoma, and induction of apoptosis in SW480 human colon cancer cells (Balunas and Kinghorn 2005; Choi et al. 2006).

13.3.3.6 β-Boswellic Acid

It is a natural pentacyclic triterpenediol and occurs in *Boswellia serrata* plants as an isomeric blend of 3α, 24-dihydroxyolean-12-ene and 3α, 24-dihydroxyurs-12-ene. It induces apoptosis in various cancer cells. The events involved in this process are excessive reactive oxygen species (ROS), nitric oxide (NO) formation, DNA ladder formation, and increased sub-G0 phase in cell cycle. This ultimately results in cleavage of Bcl-2 and translocation of Bax to mitochondria, which lead to the loss of mitochondrial potential and release of cyt-c and other factors into cytosol. All

these events are linked with reduced expression of survivin and inhibitor of caspase-activated DNases (ICAD) that leads to the activation of poly(ADP-ribose) polymerase (PARP). The triterpene upregulates the expression of cell death receptor-4 (DR-4) and tumor necrosis factor receptor-1 (TNF-R1), where both lead to activation of caspase-8 (Huang et al. 2000; Desai et al. 2008; Lu et al. 2008).

13.3.4 Carotenoid Compounds

13.3.4.1 Carotenoids

These are one of the major plant pigments in green-yellow vegetables. These including α-carotene, β-carotene, halocynthiaxanthin, fucoxanthin, β-cryptoxanthin, lactucaxanthin, and lutein are being explored as cancer-preventive agents (Murakami et al. 1996).

13.3.4.2 Lycopene

It is a red-colored pigment found in fruits and vegetables like carrot, watermelon, gac, and papaya, which shows anticancer activity against prostate, endometrial, breast, and colon carcinomas. It inhibits human cancer cell proliferation by activation of cancer-preventive enzymes like phase II detoxification enzymes, by suppression of insulin-like growth factor-I-stimulated growth (Wang et al. 2012). It also activates antioxidant enzymes like GST, GSH, and GPx and protects from oxidative stress caused by carcinogens. It alters PI3K/AKT pathway and ERK and Bcl-2 signaling in pancreatic and gastric carcinoma cells, respectively (Singh et al. 2016b).

13.3.4.3 Crocetin

It is a natural apocarotenoid dicarboxylic acid obtained from saffron, and saffron is a spice from the flower of the *Saffron crocus* and present in the dry stigmas of the plant *Crocus sativus* which can be used as a food colorant. It is reported to be a novel anticancer agent against hepatocellular carcinoma, pancreatic cancer, lung cancer, skin carcinoma, breast cancer, and colorectal cancer. It inhibits the nucleic acid synthesis, enhances antioxidant system, induces apoptosis, hinders growth factor signaling pathways, and thus affects the growth of cancer cells. It shows effects in the reduction of LPS-induced nitric oxide release; in the reduction of produced IL-1β, TNF-α, and intracellular ROS; in the activation of NF-κB; and in the blockade of LPS (Wang et al. 2012; Gutheil et al. 2012).

13.3.5 Isothiocyanate Compounds

13.3.5.1 Phenethyl Isothiocyanate (PEITC)

PEITC is a naturally occurring isothiocyanate, found mostly in cruciferous vegetables including cauliflower, watercress, cabbage, Brussels sprouts, garden cress, broccoli, and bok choy. It shows chemopreventive and anticancer activity against melanoma, breast cancer, myeloma, human liver hepatoma, non-small cell lung cancer, cervical cancer, prostate cancer, and osteogenic sarcoma. It induces apoptosis in

some drug resistance cell lines. Its mechanism of action is much diversified in various cancer cells. It induces apoptosis in highly metastatic human non-small cell lung cancer, L9981 cells via caspase-3 activation, and cell cycle arrest in G2/M phase by modulation of cyclin B1 expression while targeting MAPK/AP-1 pathway. In cervical cancer cells, it increases the death receptor (DR4, DR5) expression, cleaves caspase-3, induces caspase-8, truncates BID, and downregulates ERK1/2 and MEK phosphorylation while maintaining the expression of JNK and phospho-p38 MAPK. In human prostate cancer (DU 145) cell line, PEITC induces apoptosis by activating caspase cascade pathway (Wang et al. 2012; Cheung and Kong 2010).

13.3.5.2 Sulforaphane

It is an isothiocyanate compound found in cruciferous vegetables like broccoli, Brussels sprouts, and cabbages. It induces phase II drug metabolism enzymes of xenobiotic transformation and enhances the transcription of tumor suppression proteins. It promotes cytotoxicity in p53-deleted colon cancer cells by mitochondria- and lysosome-dependent cell death. Due to the effect of sulforaphane, Bax is also being increased in the presence of inhibition of JNK-induced Bcl-2 followed by mitochondrial cytochrome-C release and activation of apoptosis. Self-renewal Wnt/β-catenin signaling pathway is downregulated by sulforaphane in breast cancer stem cells. It has been reported to inhibit the activity of histone deacetylase (HDAC) and to reduce the number of polyps in $Apc^{min/+}$ mouse by inhibiting AKT and ERK signaling and protein expression of COX-2 and cyclin-D1. Sulforaphane also inhibits the growth of SW620 cells by inducing apoptosis (Clarke et al. 2008). In human colon cancer cells (HT-29), sulforaphane showed increased dose-dependent luciferase activity of AP-1, induced JAK activity, and inhibited NF-κB luciferase activation induced by LPS. It is also reported to inhibit cellular proliferation and to induce apoptosis. In HepG2 human hepatoma cells, sulforaphane significantly induces the expression of the Nrf-2 protein and activation of ARE-mediated transcription, delays Nrf-2 degradation by inhibition of Keap1, and activates the expression of transcription of the antioxidant HO-1 enzyme. This activation of the transcription is partially modulated by the signaling pathway of p38 MAPK, while p38 MAPK phosphorylates Nrf-2 and improves the binding between the proteins Nrf-2 and Keap1. In the PC-3 cells of human prostate cancer, sulforaphane suppresses the expression regulated by NF-κB and NF-κB signaling pathway by the IκBα and IKK pathways (Wang et al. 2012).

Sulforaphane strongly inhibits the expression of TNFα, IL-1β, COX-2, and iNOS and the expression of mRNA stimulated by LPS in the primary peritoneal macrophages of wild-type mouse. The expression of HO-1 is also increased significantly. The anti-inflammatory effects have been mitigated in the primary peritoneal macrophages of Nrf2 (−/−), and therefore the anti-inflammatory activity is mainly exerted by the Nrf2 pathway in mouse peritoneal macrophages. Sulforaphane induces detoxification enzymes metabolizing drugs via phase I (modification), phase II (conjugation), and phase III (further modification and excretion) and also increases the expression of genes that detoxify directly the exogenous carcinogenic toxins, endogenous ROS, and the genes which can recognize and repair damaged proteins (Wang et al. 2012).

13.3.6 Polyphenolic Compounds

13.3.6.1 Rosmarinic Acid

This is a polyphenol and an ester of caffeic acid and 3,4-dihydroxyphylllique acid. This is commonly found in Boraginaceae species and Nepettoideae, a Lamiaceae subfamily. It plays an important role in anti-inflammatory, antitumor, and antiproliferation activities. It shows a dose-dependent inhibition of invasion, migration, and adhesion of LS-174-T human colon carcinoma cells. It inhibits the bone metastasis from breast cancer via NF-κB and its downward IL-8 pathway (Wang et al. 2012). It reduces ERK and COX-2 phosphorylation in colon carcinoma. It reduces the activity of DNA methyltransferase and interferes with RANKL/RANK/OPG networks in breast cancer cells. It targets NF-kB pathway in U938 cells and PKA/CREB/MITF pathways in melanoma cells (Singh et al. 2016b). In U938 human leukemic cells, it sensitizes TNF-α-induced apoptosis by affecting NF-kB and ROS and inhibits the activation of NF-kB by inhibiting IκBα phosphorylation and degradation. It also reduces the levels of promoter activity of COX-2 protein induced by TPA in HT-29 colon cancer cells and antagonizes TPA stimulating effects on COX-2 expression (Petersen and Simmonds 2003).

13.3.6.2 Ellagic Acid

It is a phenolic compound extracted from pomegranate. It is an antiproliferative and antioxidant compound (Murakami et al. 1996). It induces apoptosis in cancer cells of the prostate and breast and prevents the process of metastasis in different cancers (Singh et al. 2016b).

13.3.6.3 Curcumin

It is a yellow-colored polyphenolic compound found in turmeric and used as a food additive. It has antitumor effects involved in mutagenesis, cell cycle regulation, apoptosis, oncogene expression, and metastasis. Thus it regulates the initiation, promotion, and progression of disease (Hosseini and Ghorbani 2015). Its mechanism of action is diversified and convoluted. 10 μM curcumin suppresses binding of the TPA response element (TRE) by c-Jun/activator protein-1 in NIH 3 T3 cells of mouse fibroblasts. Both protein kinase C and ornithine decarboxylase are also inhibited by curcumin. Inhibition of cyclooxygenase and lipoxygenase leads to suppression of arachidonic acid cascade (Murakami et al. 1996). Curcumin is an impressive blocker of the activation of NF-κB by inhibiting IκB kinase (IKK). Curcumin also downregulates cyclin D1, suppresses the cell growth, and induces apoptosis in prostate, breast, acute myelogenous leukemia, and multiple myeloma cancer cells. It may act against psoriasis by inhibition of phosphorylase kinase enzyme (Aggarwal and Shishodia 2004). Curcumin downregulates the TNF-induced NF-κB-regulated gene products involved in cellular proliferation (cyclin D1, COX-2, c-myc), antiapoptosis (IAP2, IAP1, Bcl-2, XIAP, Bcl-xL, TRAF1, Bf1–1/A1, Cflip), and metastasis (MMP-9, VEGF, ICAM-1). It also suppresses the activity of IκBα kinase, κBα degradation, IκBα phosphorylation, p65 nuclear translocation, p65 phosphorylation, and p65 acetylation (Aggarwal et al. 2008). It upregulates the

expression of p53, p16, p21, EGR1 (early growth response protein1), ERK (extracellular signal-regulated kinase), JNK(c-Jun-N-terminal kinase), ElK1, Bax, and caspase 3, caspase8, and caspase9 proteins and downregulates Bcl2, mTOR, p65, Bcl-xL, AKT, EGFR, cdc2, retinoblastoma protein (Prb), c-myc, and cyclin D1 proteins (Singh et al. 2016b). It can dissociate raptor from mTOR and inhibit mTOR complex1. The inhibition of the Akt/mTOR signaling results from the dephosphorylation dependent on the calyculin A-sensitive protein phosphatase. Further, it modulating effect on AP-1 in HT-29 human colon cancer cells was found to be a dose-dependent increase of AP-1 luciferase activity (Ravindran et al. 2009).

13.3.7 Other Phytocompounds

13.3.7.1 Anethole

It is one of the major constituents of essential oil of fennel and anise and belongs to the class of phenylpropenes. It has the capacity to block both inflammation and carcinogenesis. It is an antioxidant and also a suppressor of NF-κB activation by IκBα degradation (Aggarwal and Shishodia 2004).

13.3.7.2 Phenylpropenes

The compounds eugenol and isoeugenol are the active compounds of *Syzygium aromaticum*. These are reported to reduce the NF-κB activation by IκBα degradation (Aggarwal and Shishodia 2004).

13.3.7.3 Gingerol

It is a plant polyphenol and an active constituent of *Zingiber officinale*, which showed antioxidant, anti-inflammation, and antitumor properties. It has the capacity to inhibit NOS, TNF-α, and COX-2 enzymes which are regulated by NF-κB (Aggarwal and Shishodia 2004; Wang et al. 2012). It hinders the cell growth of prostate, gastric, and breast cancer cells and suppresses the lung metastasis of B16F10 melanoma. It exhibits antitumorigenic effect in human colorectal cancer cells via upregulating NSAID-activated gene-1 (NAG-1) (Aggarwal et al. 2008). It alters ERK1/2/JNK/AP1 pathway and induces apoptosis in colon cancer cells in a caspase-dependent manner (Singh et al. 2016b). ROS levels were significantly increased in K562 and MOLT4 cells treated with gingerol, and apoptosis was induced in leukemia cells by mitochondrial pathway (Wang et al. 2012).

13.3.7.4 Diosgenin

It is a steroidal saponin and legumes and yams are the rich sources of it. It is a notorious precursor of several synthetic steroidal drugs. It suppresses growth of cells and induces apoptosis in human osteosarcoma, colon cancer, and leukemia. Its anticancer mechanism is by arresting the cell cycle, disrupting the calcium homeostasis, activating p53, releasing apoptosis inducing factors, and modulating caspase-3 activity. It suppresses NF-κB activation induced by TNF as a result of DNA binding, IκBα kinase activation, degradation, phosphorylation, p65 nuclear translocation,

and phosphorylation. It suppresses proliferation and invasion and induces apoptosis by downregulation of cFLIP, cyclin-D1, TRAF1, COX-2, c-myc, Bfl-1/A1, Bcl-xL,IAP1, Bcl-2, and MMP-9 (Aggarwal et al. 2008; Raju and Mehta 2008).

13.3.7.5 Gambogic Acid

Gambogic acid is a xanthonoid, also known as kokum that is a resin from *Garcinia indica*, that can inhibit the proliferation of human breast, hepatoma, lung, and gastric carcinoma cells. It is also an apoptotic inducer and inhibits telomerase and the expression of telomerase reverse transcriptase mRNA and its promoter. It also suppresses CDK7-mediated CDC2/p34 phosphorylation, downregulates Bcl-2, and interacts with c-myc. By interacting with transferrin receptors, it mediates apoptosis of cancer cells. It inhibits NF-κB pathway. *In vivo* and *in vitro* experiments determined its potential to inhibit angiogenesis and VGEF-2 receptor, and thus it inhibits human endothelial cell growth, invasion, and migration, microvessel growth, and tube formation (Zhang et al. 2004; Pandey et al. 2007; Aggarwal et al. 2008).

13.3.7.6 Thymoquinone

It is the primary bioactive constituent of seed oil of *Nigella sativa*. It is a chemopreventive agent and suppresses the proliferation of breast, colorectal, leukemia, osteosarcoma, ovarian, and pancreatic cancer cells. It suppresses TNF-induced activation of NF-κB in a time- and dose-dependent manner and the subsequent inhibition of IκBα kinase activation, IκBα degradation, phosphorylation, p65 nuclear translocation, and phosphorylation. It specifically suppresses the nuclear p65 and recombinant p65 direct binding to DNA. It also downregulates the expression of NF-κB-regulated antiapoptotic (survivin, IAP1, Bcl-xL, XIAP IAP2, Bcl-2), proliferative (cyclin D1, c-myc, and COX-2), and angiogenic (VEGF and MMP-9) gene products. It suppresses the activation of ERK and AKT pathways and blocks *in vitro* and *in vivo* angiogenesis (Aggarwal et al. 2008).

13.3.7.7 Diallyl Sulfide

It is a thioether, found in garlic, inhibits cytochrome P450 IIE1 isoform, and thereby suppresses carcinogenesis (Aggarwal and Shishodia 2004). The consumption of garlic provides protection from gastrointestinal cancers and also suppresses the progression of colorectal adenomas (Hosseini and Ghorbani 2015).

13.3.7.8 Ajoene

It is an organosulfur compound obtained from garlic and induces apoptosis in leukemia cells (Aggarwal and Shishodia 2004). Ajoene demonstrated a significant cytotoxic activity in different cancer cell lines including FS4 (human primary fibroblast), BHK21 (a permanent and non-tumorigenic cell line derived from kidney cells of baby hamster), and BJA-B (tumorigenic lymphoid cell line derived from Burkitt lymphoma) (Scharfenberg et al. 1990).

13.3.7.9 Hentriacontane

It is also called as untriacontane, a solid, long-chain alkane hydrocarbon. It is obtained from different plants like peas (*Pisum sativum*), *Acacia senegal*, and *Gymnema sylvestris*. A kind of pleiotrophic effect induced by the tumor promoter is the inhibition of gap junctional intracellular communication, and this cell-cell communication is restored by low concentrations of hentriacontane (Murakami et al. 1996).

13.3.7.10 1′-Acetoxychavicol Acetate (ACA)

The anticancer activity of ACA involves the prevention of free radical generating systems like xanthin oxidase and NADPH oxidase systems (Murakami et al. 1996).

13.3.7.11 Epipodophyllotoxin

It is a lignan compound; podophyllotoxin is isolated from the roots of *Podophyllum peltatum* and *P. emodi*. Teniposide and etoposide are the semisynthetic derivatives of epipodophyllotoxin, and they are used for lymphomas and bronchial and testicular cancers (Shoeb 2006). These compounds break DNA molecules during the G2/M phase in cell cycle by binding to tubulin and irreversibly inhibiting DNA topoisomerase II (Balunas and Kinghorn 2005). Podophyllotoxin prevents the breast cancer cell growth by modifying checkpoint kinase 2 (Chk2) signaling pathway and also induces apoptosis in non-small cell lung cancer by cell cycle arrest, autophagy, and ER stress (Singh et al. 2016b).

13.3.7.12 Roscovitine

Roscovitine a derivative of olomoucine which is isolated from *Raphanus sativus* is under clinical trials (Shoeb 2006).

13.3.7.13 Combretastatin A-4

It is a stilbenoid compound and the active among all combretastatins isolated from the bark of *Combretum caffrum* and is used for colon, lung, and leukemia cancer therapy (Shoeb 2006).

13.3.7.14 Bullatacin

Bullatacin is a bis(tetrahydrofuranoid) fatty acid lactone found in some fruits from Annonaceae family. It is a member of acetogenin class of phytochemicals. It fights against the MDR phenotype of human mammary adenocarcinoma. It has shows antitumor properties by induction of chromatin migration and tumor cell condensation followed by apoptosis (Oberlies et al. 1997; Desai et al. 2008).

13.3.7.15 Silvestrol

It is a natural flavogline compound from the group of cyclopenta[b]benzofurans, extracted from the fruits of *Aglaila sylvestre*, which shows anticancer activity against lung and breast cancers (Shoeb 2006). The plant extract is reported to be potent against P-388 cells in *in vivo* experiments. In the hollow fiber *in vivo* assay, it shows cytotoxicity in a dose-dependent way. It works by altering the cell signal

pathways that related to the cell survival as well as angiogenesis and exerts its effects by inhibiting the translation of mRNA preferentially associated with malignancy (Balunas and Kinghorn 2005; Cencic et al. 2009).

13.3.7.16 Resveratrol

Resveratrol is a stilbinoid, found in the skin of grapes, peanuts, berries, and other fruits. The cytotoxic effect of resveratrol is mediated via the inhibition of several transcription factors; upregulation of caspases, Bax, and p53; and downregulation of survivin, cyclins, and Bcl-2. Increase in Bax/Bcl-2 ratio and upregulation of caspases lead to apoptosis. The beneficial effects of resveratrol against cancer have been shown in all the stages of cancer including carcinogenesis, initiation, promotion, and progression. It could inhibit Wnt target gene expression in normal colonic mucosa of the colorectal cancer patients. It increases the caspase-3 in malignant hepatic tissue and induces anticarcinogenic effects in human gastrointestinal tract (Hosseini and Ghorbani 2015). It has the ability to inhibit the development of DMBA-induced phenoblastic lesions in the mammary gland organ culture (MMOC) model of carcinogenesis and in two-stage full-term mouse model (Balunas and Kinghorn 2005). It prevents carcinogenesis by upregulating Bax and p53 proteins and downregulating NF-κB, COX-2, AP-1, cyclin-dependent kinases, hypoxia-induced factor 1α (HIF-1α), cyclins, MMPs, cytokines, and Bcl-2 proteins (Singh et al. 2016b). It plays a pivotal role in preventing the initiation, promotion, and progression of cancer by inducing phase II drug metabolizing enzymes, by mediating anti-inflammatory effects and inhibiting COX and hydroperoxidase functions, and by inducing cell differentiation, respectively, in human promyelocytic leukemic cells (Jang et al. 1997; Aggarwal et al. 2004).

13.3.7.17 Steroidal Lactones

Withanolide A and withaferin A are the main chemical components of *Withania somnifera* that contribute to the anticancer activity of the plant extract. The leaf extract is very toxic to the tumor models of mouse sarcoma-180, Ehrlich, and ascites. It is observed that the plant extract increases the levels of Th-1 cytokines, IL-2, and INF-γ in association with the $CD4^+$, $CD8^+$, and $CD3^+$ T-cell population and activates peritoneal macrophage functions after detection of the antigen. This remarkably increases the $CD40L^+$ and $CD40^+$in Ehrlich ascites tumor-bearing mouse. Withaferin A binds to Hsp90, inhibits Hsp90 chaperone activity by an ATP-independent mechanism, causes degradation of the client Hsp90 protein, and has *in vivo* anticancer activity against pancreatic cancer. Withanolide A is reported to induce apoptosis in Par-4 and, FOXO3a, Bim-dependent ways, respectively, in prostate and breast cancer cells (Desai et al. 2008; Yu et al. 2010).

13.3.7.18 (−)-Wikstromal, (−)-Matairesinol, and Dibenzylbutyrolactol

These are the chemical constituents of lignin mixture of *Cedrus deodara*, a medicinal plant. This inhibits Molt-4 cell proliferation with increased sub-G0 phase, produces apoptotic bodies, and induces DNA ladder formation. It shows time-related

apoptosis and pro-apoptosis necrosis. It decreases the mitochondrial membrane potential as a result of increased nitric oxide and peroxide formation in Molt-4 cells. The mixture causes twofold activation of caspase-3 in Molt-4 cells and fivefold activation in HL-60 cells where caspase-9 and caspase-8 are also activated in HL-60 cells (Desai et al. 2008; Sharma et al. 2008).

13.4 Phytochemicals Targeting Cancer Stem Cells (CSCs): A Paradigm Shift

An accumulated amount of research is reporting the identification of a subset of cancer stem cells within the tumor mass. CSCs have been identified and isolated based on various biomarkers like CD44, CD24, CD133, CD29, and aldehyde dehydrogenase (ALDH1A) from different cancers (Klonisch et al. 2008). These cancer stem cells are responsible for aggressiveness and poor prognosis of cancer (Manson et al. 2007). These CSCs are the major culprits for the initiation, progression, and metastasis of tumor. These forms heterogeneous population cells that constitute tumor. These cells are quiescence in nature and are resistant to most of the chemotherapeutic drugs. Their self-renewal and differentiation lead to the elusiveness of disease (Kawasaki et al. 2008).

The very crucial steps in cancer disease management are prevention of primary tumor formation and prevention of recurrence after therapy. As mentioned above phytochemicals can inhibit tumor formation and its progression by means of diversified mechanisms like antioxidant, antiproliferative, and pro-apoptotic effects. As these are abundantly available in food sources and are safer to consume on a long-term basis, it is advisable to employ phytochemicals in the prevention and therapy of disease. An extensive research is being conducted on phytochemicals in targeting cancer stem cells in recent years, and a paradigm shift has been made toward this direction. The key mechanisms involved are targeting multidrug resistance pumps and cancer stem cell signaling pathways like Wnt, Hedgehog, and Notch and also differentiation of cancer stem cells and altering the CSC niche. Table 13.2 describes few active phytocompounds targeted against cancer stem cells involved in different pathways.

13.4.1 Targeting Self-Renewal Pathways of CSCs

Many of the genes and signaling pathways showed important regulatory functions in normal and cancerous stem cells. Anyhow, misregulated signal pathways lead to cancer causing abnormal processing and eventually tumorigenesis (Singh et al. 2016a). The self-renewal property of stem cells is crucial in survival and propagation of tumor, and the underlying molecular mechanisms that control this property are a little known. Based on gene expression studies, specific pathways found to play a crucial role include Wnt, Hedgehog, and Notch signaling pathways (Kawasaki et al. 2008; Subramaniam et al. 2010; Takebe et al. 2011).

Table 13.2 Some of active components of phytochemicals targeting cancer stem cells involved in different pathways

Targets	Compounds	References
Wnt/β-catenin pathway	Selenium, EGCG, vitamin D, genistein, sulforaphane, curcumin	Kawasaki et al. (2008), Kim et al. (2012), andDandawate et al. (2013)
Notch signaling	Resveratrol, curcumin, piperine, genistein, EGCG	Kim et al. (2012) andDandawate et al. (2013)
Hedgehog signaling	Cyclopamine, cholesterol, cholecalciferol, curcumin	Kawasaki et al. (2008), and Kim et al. (2012)
Multidrug resistance	EGCG and EGC, curcumin	Pistollato et al. (2015) andDandawate et al. (2013)
Differentiation of CSCs	Retinoic acid, choline, fat, ceramide	Kawasaki et al. (2008) and Dandawate et al. (2013)
Epithelial-mesenchymal transition	Sulforaphane, genistein, isothiocyanates, quercetin	Kim et al. (2012)
CSC niche	Targeting hypoxia: brucine, licorice phenol licochalcone E, mulberry leaf extract, pterostilbene, isoflavones, *Semecarpus anacardium* nut extract	Pistollato et al. (2015)
	Targeting inflammation: lupeol, oleanolic acid, flavanones, flavonols, isoflavones, anthocyanins, chalcones, terpenoids, carotenoids, glucosinolates, ellagitannins, anthocyanins	Pistollato et al. (2015)
	Targeting low pH: genistein, silibinin, EGCG, resveratrol, benzylvincoside, abietic acid, cephaeline, minimiflorin, mundulin, mammea A/BA and mammea C/OA	Pistollato et al. (2015)

13.4.1.1 Wnt/β-Catenin Signaling

It is a conserved pathway and is associated with a variety of developmental and malignancy mechanisms including maintenance of stem cells and their compartments (Kawasaki et al. 2008). Wnt signaling cascade includes three main routes (Takebe et al. 2015):

- Canonical pathway – implication of tumorigenesis
- Noncanonical planar cell polarity pathway – regulation of the cytoskeleton
- Noncanonical Wnt calcium pathway – regulation of intracellular calcium levels

Frizzled protein receptors and low-density lipoprotein receptor-related proteins are activated by the Wnt ligand, a secretory glycoprotein attached to the extracellular matrix. Many developmental processes are activated by the transcriptional

coactivator β-catenin. A three-protein complex (glycogen synthase kinase 3β, Axin, adenomatous polyposis coli) destabilizes β-catenin and causes its destruction in the absence of Wnt, while activation of Wnt restores its stabilization (Kawasaki et al. 2008; Takahashi-Yanaga and Kahn 2010). The sensitivity of cells to the Wnt ligand is potentiated by the R-spondins attached to LGR5/6. When Wnt ligand binds to the frizzled receptor, the co-receptor LRP5/6 forms a cell surface complex with Wnt-bound frizzled. This complex activates the cytoplasmic phosphoprotein that is hevelled (Dvl) which in turn displaces the GSK-3β from the Axin/APC/GSK-3β complex. The stabilized β-catenin translocated to the nucleus by Rac1 binds to the LEF/TCF transcription factors, displaces corepressors, and recruits coactivators for Wnt target genes (c-myc, cyclin D1, TCF-1, PPAR-δ, MMP-7, Axin-2, CD44 etc.). In the absence of Wnt ligand, the Axin/APC/GSK-3β complex and CK1 phosphorylate the β-catenin, which further leads to the ubiquitination and proteosomal degradation of β-catenin by β-TrCP/Skp pathway (Takebe et al. 2015).

13.4.1.1.1 Phytochemicals Targeting Wnt/β-Catenin Pathway

Selenium, an essential micronutrient, exhibits anticancer effects. Combination of 1,4-phenylene bis(methylene)selenocyanate (p-XSC), an organoselenium compound, with docosahexaenoic acid (DHA) and omega-3 fatty acid reduces synergistically the viability of colon carcinoma cells with a concomitant reduction of β-catenin expression. Treatment with p-XSC also leads to the reduced expression of β-catenin in the adenomas (Kawasaki et al. 2008). EGCG is reported to modify the Wnt/β-catenin signaling in breast cancer cells. It also inhibits Wnt-activated gene responses like reduced TCF/LEF binding and c-myc expression, which is mediated by stabilization of HBP-1, a Wnt transcriptional repressor of and oncogenesis suppressor (Kawasaki et al. 2008).Wnt signaling is suppressed in MDAMB-231 cell line by a dose-dependent usage of EGCG. This reduction involves the induction of a Wnt suppressor called HMG box-containing protein-1 (HBP-1) transcriptional factor. The biological consequences include suppression of c-myc, a Wnt signaling target gene. The proliferation of leukemic blast cells and the ability of attachment and neural stem cell differentiation have been significantly suppressed with the use of 100 μM and 20 μg/ml of EGCG, respectively (Kim et al. 2012; Dandawate et al. 2013). EGCG promotes APT-competitive inhibition of PI3K and mTOR as well as Akt phosphorylation and inhibition of cell growth in MDAMB-231 and A549 cell lines, respectively. EGCG directly antagonizes the androgen activity. It inhibits nuclear translocation of AR and expression of proteins in a xenograft model. A dramatic reduction of androgen-regulated miR-21 and increase of a tumor suppressor miRNA, miR-330, in EGCG treated mouse tumors. Thus it can be used against prostate cancer (Dandawate et al. 2013).

Vitamin D can also mediate some of the antineoplastic effects via inhibition of Wnt signaling. In colon cancer, vitamin D reduces the expression of β-catenin-responsive genes, thereby reducing c-myc levels, peroxisome proliferator-activated receptor, CD44, and TCF-1. It alters β-catenin subcellular localization from predominantly nuclear to almost exclusively cytoplasmic. In addition, it induces expression of E-cadherin, scaffolding, and tumor-suppressing protein involved in

sequestering and inhibiting the activity of β-catenin. Additionally, overexpression of the vitamin D receptors counteracted the inhibitory actions of vitamin D upon Wnt/β-catenin signaling (Kawasaki et al. 2008). The oncogenic effects of β-catenin are decreased by 1,25-dihydroxyvitamin D3 which interferes with the activation of Wnt signaling. It modulates the Wnt signaling inhibitor (DKK-1 and DKK-4) expression and also translocates β-catenin to the plasma membrane from the nucleus in order to inhibit β-catenin-responsive gene expression in colon cancer cells at nanomolar range. Vitamin D promotes the formation of vitamin D receptor-β-catenin complex in the nucleus and leads to the competitive repression of β-catenin-/TCF4-mediated gene transcription (Kim et al. 2012).

Genistein can increase the expression of sFRP-2 (secreted frizzled-related protein-2), a Wnt antagonist involved in stem cell self-renewal process. However, the estrogen activity-dependent implications of genistein in noncanonical Wnt pathway need further attention (Kim et al. 2012). Sulforaphane generally targets Nrf-2 and can suppress the formation of CSC (isolated from MCF-7 and SUM159 breast cancer cells) mammospheres. Supplementation with sulforaphane significantly reduced the tumor size with a decreased stem cell population *in vivo* (Kim et al. 2012), while *in vitro* assays report that it could downregulate the Wnt/β-catenin self-renewal pathway (Dandawate et al. 2013).

Curcumin exhibits cytotoxic effects by altering the expression of GSK-3β, E-cadherin, and Wnt signaling integral proteins, in MCF-7 and MDA MB231 cells. Curcumin modulates the Wnt signaling through decreasing the p300, CBP, and Tcf-4 protein levels which further decrease the transcriptional activity of β-catenin/TCF-4 and β-catenin target gene expressions in androgen-dependent prostate cancer. Curcumin represses the downstream effects of β-catenin such as regulation of c-myc and E-cadherin that are mediated by HBx. Curcumin causes p53- and p21-independent cell cycle arrest at G2/M phase and apoptosis in HCT-116 (p53+/+, p53−/−, p21−/−) cell lines. The leading causes behind these effects are caspase-3-stimulated β-catenin cleavage and reduction in the β-catenin/TCF-LEF transactivation, in the β-catenin/TCF-LEF promoter DNA-binding activity and in the c-Myc protein levels. Curcumin treatment impairs Wnt/β-catenin pathway by inducing caspase-3-mediated degradation of E-cadherin, APC, and β-catenin proteins. Curcumin and its analogues demethoxycurcumin, bisdemethoxycurcumin, and tetrahydrocurcumin inhibit β-catenin-responsive transcription (CRT) and also downregulate a positive regulator protein p300 and thus inhibit the Wnt/β-catenin pathway (Dandawate et al. 2013).

13.4.1.2 Notch Signaling

Notch signaling is an evolutionarily conserved, primordial ligand-receptor cell fate determining pathway that plays significant role in cancer biology, CSCs, angiogenesis, survival, apoptosis, and differentiation (Dandawate et al. 2013; Takebe et al. 2015). A direct contact between cells is essential for the activation of the Notch signaling (juxtacrine cellular signaling). The interaction between the ligand and receptor of signal-sending and signal-receiving cells, respectively, leads to a two-step proteolytic cleavage of intracellular domain of Notch (ICN). Disintegrin

and metalloproteinase enzymes mediate the initial cleavage, while the latter cleavage is mediated by γ-secretase by releasing an intracellular fragment which interacts with nuclear factors that regulate target gene expression (Takebe et al. 2015). There are four isoforms of Notch receptors (Notch 4) and five canonical Notch ligands (Delta like ligand 1, DLL3, DLL4, Jagged 1, and Jagged 2). Various cell types present within a tumorlike vascular endothelial cells, immune cells, CSCs, and tumor cells are affected simultaneously by targeting Notch signaling. Different tumors and tumor subtypes express different Notch ligands and receptors. The mechanistic understanding of the role of Notch in specific cancers is needed to develop mechanism-based combination regimens to target Notch pathway (Takebe et al. 2015). Notch signaling promotes the expansion of neural, breast, and brain cancer stem cells (Kawasaki et al. 2008). Notch signaling is implicated in the CSC regulation and in the epithelial-mesenchymal transition (EMT) phenotype acquisition as EMT is associated with drug resistance, and hence targeting Notch pathway can eliminate these phenotypes. Hence, γ-secretase activates the Notch signaling; the γ-secretase inhibitors are used to inhibit cancer cell proliferation and induce apoptosis (Dandawate et al. 2013; Espinoza et al. 2013).

13.4.1.2.1 Phytochemicals Targeting Notch Pathway

Resveratrol is reported to decrease the Notch protein expression in acute lymphoblastic leukemia cells. As Notch mRNA levels are not affected with resveratrol, it may affect the posttranslational Notch signaling. In addition, resveratrol can decrease the mRNA levels of Notch downstream effectors like pre-TCRα and hairy and enhancer of split (HES1) (Kawasaki et al. 2008). Combination treatment of curcumin and piperine inhibits the formation of breast cancer cell mammospheres by half and by total at 5 μM and 10 μM, respectively, but it is unclear that the mechanism through which this inhibition is achieved may be either by alterations in the Notch pathway or by the modifications in the Wnt pathway (Kim et al. 2012). Genistein downregulates the Notch-1, Akt, and FoxM1 and suppresses cell viability and induces apoptosis in PCa cells (Dandawate et al. 2013). EGCG majorly affects the Notch signaling pathway. It may inhibit cancer cell growth by affecting all cell cycle-related networks (Dandawate et al. 2013).

13.4.1.3 Hedgehog Signaling (HH)

HH signaling pathway is involved in normal tissue repair, EMT, and tissue patterning in embryonic development, right-left asymmetry, central nervous system polarity, organogenesis, and spermatogenesis (Takebe et al. 2015). HH also contributes to the temporal-, spatial-, and concentration-dependent regulation of differentiation, migration, and proliferation of target cells, as well as regulation of proliferation, maintenance, and differentiation of stem cells (Dandawate et al. 2013; Singh et al. 2016a). HH signaling progresses via two transmembrane proteins called patched (PTCH1) and smoothened (SMO). In the absence of HH ligands (Indian, Sonic, and Desert), PTCH1 represses the SMO and subsequently leads to the phosphorylation of HH signal transcription factor GLI protein to a transcriptional repressor by PKA, CK1, and GSK-3β. While in the presence of HH ligands that bind to PTCH1, the

activated SMO dissociates GLI proteins from the key intracellular HH regulatory complex. Phosphorylation of GLI proteins to HH transcriptional activators and subsequent translocation into the nucleus result in the transcription of HH target genes like cyclin D, cyclin E, myc, Gli1, patched, and HIP. Modifications of any crucial component of this pathway lead to tumorigenesis and tumor growth (Clement et al. 2007; Kawasaki et al. 2008; Merchant and Matsui 2010).

13.4.1.3.1 Phytochemicals Targeting Hedgehog Pathway

Cyclopamine is a natural compound that inhibits the Hedgehog signaling pathway. Cyclopamine targets Hedgehog by specifically hindering SMO activation. Cyclopamine therapy of murine medulloblastoma resulted in the inhibition of proliferation, induction of neuronal differentiation, effective depletion of CSCs, and reduction of tumor burden in a mouse tumor allograft. Cyclopamine is effective in killing of pancreatic, breast, and multiple myeloma CSCs. Cyclopamine in combination with gemcitabine inhibits metastatic spread and reduces primary tumor burden in pancreatic orthotopic xenografts. Mammosphere formation in breast carcinoma and SC proliferation in multiple myeloma can be reduced by cyclopamine (Kawasaki et al. 2008). The HH ligand activation requires cholesterol at their carboxyl ends, and 22-OH-cholesterol and 20-OH-cholesterol are reported to increase the HH target gene expression, and this hypothesis of cholesterol-dependent HH signal transduction is investigated in M2-10B4 pluripotent mesenchymal stem cells. The mechanism underlying the positive regulation of HH signaling by these oxidative species of cholesterol is not clear. However, this oxidative status of cholesterol is altered by the endogenous ROS. Hence, the bioactive food components that control the ROS levels can be important in regulating self-renewal and HH pathways (Kim et al. 2012). Cholecalciferol (vitamin D3, an isoform of vitamin D) is reported to be HH antagonist *in vitro* but not *in vivo*. Binding of cholecalciferol to SMO receptors results in the reduction of HH signaling in MDAMB231 and C3H/10 T1/2 fibroblast cells. 1 μM vitamin D3 shows more potent SMO inhibitory action than 10 μM cyclopamine in PTCH1-transfected C3H/10 T1/2 cells (Kim et al. 2012). Curcumin interferes with the Gli1 mRNA or Gli reporter activity and inhibits HH signaling in transgenic mouse prostate adenocarcinoma cells (Kim et al. 2012).

13.4.1.4 Epithelial-Mesenchymal Transition (EMT)

Enhanced motility and invasion are the characteristic properties of EMT phenotype cells, and these cells can migrate to various parts of the body and cause metastatic spread of cancer in the progressive stages of malignancy. Many studies reported that EMT is involved in cancer progression besides being involved in metastasis (Dandawate et al. 2013). The process of EMT (significant morphological change in epithelial to fibroblastic mesenchymal phenotype) involves disturbance of cell-to-cell junctions, actin cytoskeleton reconstitution, increased invasion, and cell motility. The other cellular features of EMT include relocation and downregulation of zonulaoccludence-1 and E-cadherin; upregulation of vimentin, fibronectin, and N-cadherin; and nuclear translocation of β-catenin from plasma membrane. EMT accompanies with multidrug resistance and stemness or cancer stem cell phenotype.

These cancer stem cells or cancer initiating cells are involved in the recurrence of cancer (Dandawate et al. 2013). Targeting of EMT process is a way to inhibit CSC phenotype and prevent invasion and metastasis (Singh and Settleman 2010; Scheel and Weinberg 2012).

13.4.1.4.1 Phytochemicals Targeting EMT

Sulforaphane inhibits the protein expression that is involved in EMT such as β-catenin, vimentin, twist1, and ZEB1, thus preventing the early metastasis signaling. Combination therapy of sulforaphane with sorafenib induces apoptosis, inhibits proliferation as well as angiogenesis, and downregulates the sorafenib-induced protein expression which is involved in EMT (Dandawate et al. 2013). FoxM1 overexpression gives rise to the acquisition of EMT via activation of mesenchymal stem cell markers like ZEB2, ZEB1, E-cadherin, vimentin, and Snail2 as well as enhanced sphere-forming efficiency and expression of CSC markers such as CD44 and EpCAM and, at last, decreased miRNA expression of let-7a, let-7b, let-7c, miR-200b, and miR-200c.

Genistein decreases the protein expression of cancer stem cell markers like CD44 and EpCAM and inhibits proliferation, invasion, and migration and the acquisition of EMT phenotype. It also reduces the capacity of clonogenesis and formation of pancreatospheres (Dandawate et al. 2013). Isothiocyanates are reported to inhibit neoangiogenesis, cancer cell growth, EMT phenotype, and self-renewal of CSCs. Quercetin reduces the *in vitro* expression of proteins that are involved in EMT and suppresses *in vivo* xenografts derived from cancer stem cells. It also decreases the proliferative, angiogenetic, and stemness-associated gene expressions. Quercetin in combination with sulforaphane blocks the pancreatic CSC self-renewal capacity and induces apoptosis by reducing the expression of XIAP, Bcl-2, vimentin, β-catenin, Zeb1, and twist-1 and activating caspase-3 and FOXO1 (Pistollato et al. 2015).

13.4.2 Targeting Multidrug Resistance (MDR)

Cancer stem cells show quiescence and are resistant to toxins and drugs by expressing ABC transporters and have the DNA repairing ability and show evasion from apoptosis. The multidrug resistance of CSCs is due to mutations, overexpression of the target receptors, chemical transformation of drug, or its elimination from the cell. Through the multidrug resistance and quiescence, the cancer stem cells are left behind untreated in chemotherapy or radiotherapy and cause relapse of cancer (Gottesman et al. 2002; Dean et al. 2005b; Szakacs et al. 2006; Moitra et al. 2011). ATP-binding cassette (ABC) transporters play a crucial role in the drug efflux process in MDR cells, and mitoxantrone resistance protein (MXR or ABCG2), multidrug-resistant-associated protein (MRPs), and P-glycoprotein (P-gp) are the important transporter proteins of ABC family (Kawasaki et al. 2008). Cancer stem cells may promote the cancer cell proliferation by lowering the accumulation of drugs in cells through MDR drug efflux mechanisms. This correlation between multidrug resistance and cancer stem cells gives rise to a lead in targeting CSCs (Dean et al. 2005; Donnenberg and Donnenberg 2005; Kawasaki et al. 2008).

13.4.2.1 Phytochemicals Targeting MDR

EGCG and EGC are the active polyphenol compounds found in green tea, found to inhibit p-glycoprotein transport activities in Chinese hamster ovary (p-gp^{+}) cells. EGCG facilitates the retraction of MDR phenotype by reducing cellular drug efflux when given in combination with vinblastine or doxorubicin. Hesperetin, quercetin, daidzein, silymarin, naringenin, and resveratrol also inhibit the MRP1, MRP4, and MRP5 (Kawasaki et al. 2008). Curcumin increases the cellular accumulation of anticancer agents like cisplatin, tamoxifen, daunorubicin, vincristine, and doxorubicin and thereby effectively sensitizes the drug-resistant cancer cells. A reduction in MDR1B expression in L1210/Adr cells (mouse leukemic MDR cells) by curcumin is mediated by PI3K, Akt, and NF-κB pathways. It also inhibits the ABCG2 transporter activity. In addition curcumin facilitates the accumulation of mitoxantrone and doxorubicin in ABCG2-expressing HEK cells and hence reverses MDR (Kawasaki et al. 2008; Dandawate et al. 2013).

13.4.3 Differentiation of CSCs

The basic and primary properties of CSCs are the differentiation into heterogeneous populations. Most of the chemotherapy drugs target different checkpoints or phases of cell division cycle and thus inhibit the cancer cell proliferation. The complete eradication of cancer cells that are at different phases of cell cycle needs a number of repeated treatments. As these CSCs are quiescent in nature, the drugs cannot affect them, because they will not enter the active cell cycle stages. Therefore, induction of CSC differentiation may bring CSC liable to chemotherapy that is an effective way to fight cancer, and this strategy emerged as differentiation therapy (Kawasaki et al. 2008).

13.4.3.1 Phytochemicals Targeting CSC Differentiation

Retinoic acid (RA) is the active form of vitamin A, while β-carotene is the precursor to RA. RAs play a key role in embryogenesis and hematopoiesis, and RA has thus been seen as a potential mediator of differentiation therapy, which is evidenced from the treatment of APL (acute promyelocytic leukemia). APL is caused by translocation of chromosome that produces a chimeric protein between the retinoic acid receptor-alpha (RARα) and promyelocyte leukemia protein. Addition of retinoic acid to promyelocytic leukemic cells leads to their differentiation into mature neutrophils (Kawasaki et al. 2008). Retinoic acids upregulate the expression of SOX3 (a transcriptional factor involved in the developmental processes) by directly interacting with it, and this upregulation is an important determinant for the differentiation of neuronal cells. Vitamin A induces differentiation of embryonal NT2/D1 cancer cells and reduces the self-renewal capacity which is associated with the Nanog (a lineage-specific transcriptional factor) on chromosome 12p (Kim et al. 2012).

Choline is another dietary component which can differentiate the hepatic oval stem cells into mesenchymal cells. This trans-differentiated mesenchymal tumor

formation from the transplantation of LE/6 hepatic stem cells *in vivo* is inhibited by choline. Choline is an important element during the early fetal brain cell differentiation process. Comprehensive understanding of these studies warrants the effect of choline on brain cancer stem cells (Kim et al. 2012). Resident preadipocytes and mesenchymal stem cells undergo differentiation to yield adipocytes in adipocyte tissue, and this differentiation is altered by ceramide and fat. For instance, increased circulatory adipocyte progenitor cells and their differentiation into adipocytes are achieved by the 3–5-week high fat treatment in C57BL/6 mice. Ceramide is believed to suppress the mesenchymal stem cell differentiation into adipocytes. Conclusively, fat promotes the differentiation of stem cells to adipocytes, while ceramide opposes it (Kim et al. 2012).

13.4.4 Targeting CSC Niche

Niche is the specialized microenvironment around the stem cells that plays a crucial role in maintenance of the tissue physiology and integration of stem cell signals which are involved in tumorigenesis, proliferation, self-renewal, differentiation, invasion, and metastasis (Sneddon and Werb 2007). This niche protects the stem cell depletion by controlling excessive differentiation. Niche consists of all necessary elements required for the maintenance of stem cell. Niche is crucial in reducing cell-to-cell contacts and migration of stem cells to the distant parts of the body via the process of metastasis (Li and Neaves 2006; Scadden 2006; Yang and Wechsler-Reya 2007). The CSCs are being protected by niche from apoptosis induced by chemotherapeutic drugs. So, ways to target niche is an emerging point of research in fighting CSCs (Singh et al. 2016a). Niche is characterized by inflammation, hypoxia, low pH, and oxidative stress. In addition, there are many other cells associated with CSC niche that may activate the signaling pathways which promote the migration, EMT, and proliferation of CSCs (Pistollato et al. 2015). HIF-1 regulates the oxygen homeostasis and maintenance of tumor microenvironment and is also associated with CSC drug resistance. Hence, targeting niche is one of the important therapeutic strategies to eliminate CSCs (Li and Neaves 2006; Sneddon and Werb 2007; Pistollato et al. 2015).

13.4.4.1 Phytochemicals Targeting CSC Niche

Hypoxia and HIF-1 signaling are crucial in maintaining the CSC niche. Treatment of hepatocellular carcinoma using brucine is reported to suppress the expression of four target genes of HIF-1 (MMP2, cathepsin D, fibronectin, and LOX) which are involved in metastasis. Licorice phenol licochalcone E (LicE) downregulates the expression of CDKs, cyclins, and Ki67 and induces apoptosis by enhanced expression of caspase-3 and Bax and reduced expression of Bcl-2. Lic E also suppresses the expression of HIF-1α, CD31, iNOS, VEGF-A, COX-2,VEGF-C, CD45, lymphatic vessel endothelial receptor-1, and VEGF-receptor 2. Mulberry leaf (ML) extract is reported to downregulate HIF-1α along with the downward GLUT-1and VEGF gene expression, and under hypoxia conditions, it reduces the

expression of MMP2. Pterostilbene is also found to downregulate the HIF-1α expression. Isoflavones block the activation of Src/STAT3/HIF-1α pathway and hinder the transcription of HIF-1α. *Semecarpus anacardium* nut extract is involved in the blockade of HIF-1 and inhibits CA-IX, iNOS, VEGF, and GLUT1 genes (Pistollato et al. 2015).

The CSC niche consists of a continuous chronic inflammation, and the following compounds can target the pro-inflammatory signaling pathways including lupeol, oleanolic acid, flavonols, flavanones, anthocyanins, chalcones, isoflavones, carotenoids, terpenoids, glucosinolates, ellagitannins, and anthocyanins (Pistollato et al. 2015). A physiological low pH is favorable for the CSC extracellular microenvironment. A potassium-rich natural diet and lower consumption of proteins are an alternate to increase the extracellular pH. Many plant compounds like genistein, resveratrol, EGCG, and silibinin are used to bring the acidic pH of niche to neutral by inhibiting aerobic glycolysis. Proton pump inhibitors like benzylvincoside, abietic acid, cephaeline, mundulin, minimiflorin, mammea C/OA, and mammea A/BA can raise the peri-tumoral pH to neutral by inhibiting gastric H^+, K^+-ATPase (Pistollato et al. 2015).

13.5 Conclusions and Future Prospects

Even though an enormous research has been progressed in the field of oncology, the complete remission of disease is not achieved yet. Synthetic chemotherapeutic drugs synthetic chemotherapeutic drugs are associated with adverse side effects, and the process of their discovery and development to the point of clinical usage faces a plenty of hurdles and also requires a huge amount of money and time. Hence, naturally occurring phytochemicals identified and isolated from plant sources serve as the best anticancer drug candidates with a safe and effective targeting of cancer and cancer stem cells as explained in the above sections. The current research focus is on the extraction, isolation, and evaluation of these anticancer phytochemicals. But, in reality, a considerable amount of inhibitions come into the picture of complete profiling of all the potent plant compounds. Many numbers of papers nominate new anticancer plant extracts. But these studies cannot be reproduced by others or are simply not robust enough to justify their effectiveness alone, because most of the plant products are limited to a preliminary evaluation studies and this under appreciation is leading to a potential missing of valuable plant products. Continuous and dedicated efforts in identification and isolation of active compounds from botanical sources and in their preclinical and/or clinical trials are needed to bring the phytochemicals into a better anticancer therapeutics.

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Therapeutic Strategies of Natural Agents on Triple-Negative Breast Cancer

14

Ashok Kumar Pandurangan and Mohd Rais Mustafa

Abstract

Triple-negative breast cancers (TNBC) are described as a human breast cancer subtype that does not express estrogen receptor (ER) and progesterone receptor (PR), and they lack overexpression and/or lack gene amplification of human epidermal growth factor receptor 2 (HER2). TNBC are highly aggressive, and they are present among 10–15% of all breast cancer subtypes which constitute about 80% of all basal-like tumours. Plants produce diverse number of organic compounds. Traditionally these natural products are known as secondary metabolites or phytometabolites. Bioactive natural products' chemical structure and function have been studied since the 1850s. Comprehending these chemical structure and functions led to recognition of their biological properties as anticancer drugs. Among phytometabolites, phytoestrogens have been widely studied as a chemopreventive and chemotherapeutic agent due to their high affinity to interact with estrogen receptors. However, TNBC are indifferent to hormonal therapy, they became a therapeutic hurdle. The only systemic therapy currently left is chemotherapy which often fails due to ability of TNBC to develop resistance to chemotherapeutic drugs. Therefore, different theories exist, which suggest focusing on drugs that target TNBC survival-dependent molecular pathways instead. For instance, several studies reported TNBC depend on NF-κB to survive and avoid apoptosis. The aim of this chapter is to discuss the various bioactive natural products extracted from various parts of plants and mushrooms showing potential inhibitory effect against TNBC.

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Chemotherapy · Hormonal receptors · Natural products · Molecular pathway · Triple-negative breast cancer

14.1 Introduction

Breast cancer (BC) is the leading cause of cancer-related deaths among women worldwide with an estimated 1.7 million newly diagnosed cases in 2015 (Siegel et al. 2015, 2016). BC in India is on the rise, in line with most developing and medium-income countries in Asia (Sandhu et al. 2016). It was estimated that approximately 145,000 new patients were diagnosed with BC in India, and nearly 70,000 women died of the disease in the year 2012. The 5-year BC survival for Indian women diagnosed with BC was 60% compared with >80% in Western countries especially in the USA (Khokhar 2012; Sandhu et al. 2016). BC in Malaysia is also on the rise, when compared to other developing countries. In Malaysia, less than 50% occur in the age 50, and 60% of patients are premenopausal, while in the Western countries, majority of BCs occur in postmenopausal women (Yip et al. 2006, 2009). There are also differences in the mean age of occurrence between the three main ethnic groups in Malaysia: in Malays it is 48.1 years, Chinese 51.4 years and Indians 52.3 years (Yip et al. 2006). Without any doubt BC is an alarming threat to women population in the world. The major receptors such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) were absent in the triple-negative breast cancers (TNBC) and represent the focus of increasing interest of intense investigation by the researchers (Irvin and Carey 2008; Reis-Filho and Tutt 2008; Stockmans et al. 2008). TNBC is aggressive in nature due to absence of receptors and leads to the poor prognosis and lack of targeted therapies (Mersin et al. 2008; Tan and Swain 2008; Kaplan et al. 2009). The reason behind the failure of chemotherapeutic agents to TNBC cells when compared with non-TNBC breast cancer cells is illustrated in Fig. 14.1. Approximately 15–20% of all BC represents TNBC. TNBC characteristically has a higher recurrence rate, and about 34% of patients experience a distant recurrence with the average time of relapse being 2.6 years. Also the 5-year survival rates tend to be shorter for TNBC than other subtypes (Pan et al. 2015). The classification of TNBC in earlier system was into subgroups, viz., molecular apocrine, claudin low and basal like, which relied purely on their stages of differentiation and expression of specific cell surface markers alone (Ma et al. 2010).The molecular apocrine subtype of TNBC is unique, the most differentiated among all other subtypes of TNBC and found mostly in progressive ages, accounting for 0.5–4% of TNBC with characteristic ER-, PR-, HER2- and AR+ signatures (Ma et al. 2010). On the other side, the basal-like subgroups manifest basal or myoepithelial cell-like properties, and it was estimated for 75% of all TNBC types (Khan and Bui 2010). Besides having the molecular signatures of cytokeratin 5/6, 14 and 17, basal-like subgroups lack DNA repair and tumour suppressor genes attributed to locus 5q11 and have

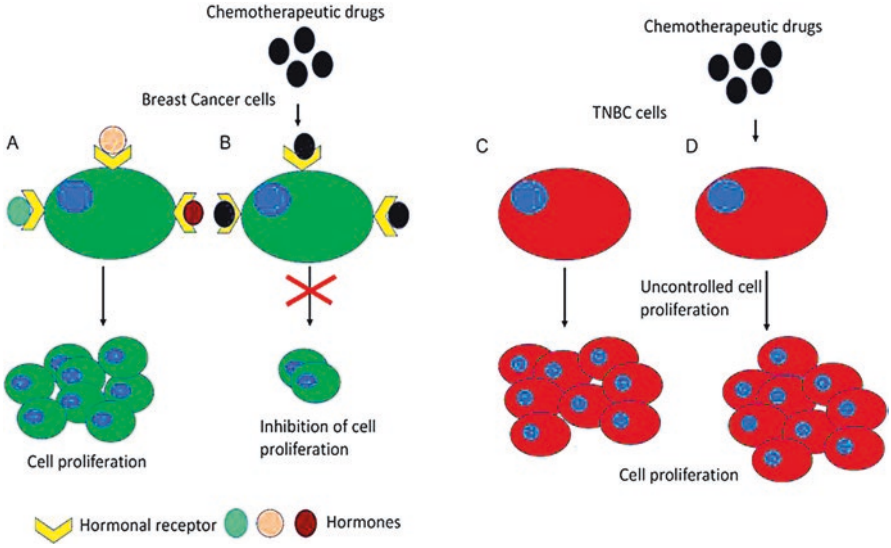

Fig. 14.1 The fundamental difference of TNBC cells and normal cancer cells. (**a**) Normal breast cancer cells (MCF-7, T47D) possess receptors like estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) in their cell surface; when hormones bind to their respective receptors, the cells start growing abundantly. (**b**) TNBC cells exposed to the conventional chemotherapeutic drugs (tamoxifen, doxorubicin and Taxol), it occupies the hormonal receptor and blocking its growth, hence the cell proliferation will be halted; (**c**) TNBC cells lack hormonal receptors. (**d**) Due to the lack of hormonal receptors, exposure to conventional chemotherapeutic drugs has no effect on TNBC cells, and the cells start to grow abundantly without any regulation

overexpression of EGFR (Ma et al. 2010). The other subgroup, claudin, was highly comprised of poorly differentiated proliferating mesenchymal stem cells (MSCs) (Perou 2010) that had a characteristic stem cell signature of $CD44^{+}/CD24^{-}$ and upregulated signature of mesenchymal markers like Slug, Twist and Snail genes with potential role in tumour recurrence associated with worst patient outcome (Ma et al. 2010; Kwon 2013). Based on the gene expression profile of 587 TNBC patients, it was identified that six different subtypes of TNBC, namely, BL1 and BL2, an immunomodulatory (IM) subtype, a mesenchymal subtype, a mesenchymal stem-like (MSL) subtype and a luminal androgen receptor (LAR) subtype, are present (Lehmann et al. 2011). Further, identification of molecular drives in corresponding cell line models will provide the preclinical base to develop effective therapies for the alleviation of TNBC. For example, Montagna et al. (2013) showed that BL1 lines were the most chemosensitive to cisplatin and that the mesenchymal and MSL lines were most chemosensitive to the Abl/Src inhibitor dasatinib. The same group used the intrinsic subtype tool to examine the composition of each TNBC subtype. Montagna et al. (2013) showed that all TNBC subtypes except MSL and LAR were composed primarily of the BL intrinsic subtype (BL1 99%, BL2 95%, IM 84% and mesenchymal 97%). The LAR subtype is classified as

HER2 (74%) and luminal B (14%), and the MSL subtype includes BL (50%), normal-like (28%) and luminal B (14%) (Montagna et al. 2013; Yam et al. 2017). Other gene expression analyses have also defined a claudin-low tumour subtype (Herschkowitz et al. 2007). Molecular characterization of TNBC showed that these tumours are enriched in epithelial-to-mesenchymal transition (EMT), stem-like features and immune system response but show low expression of luminal and proliferation-associated genes (Prat et al. 2010).

Surgery is the primary treatment coupled with chemotherapy and radiotherapy for TNBC patients due to the resistance to HER2-targeted therapies (trastuzumab) and hormonal therapies. The currently used chemotherapeutic drugs are 5-fluorouracil, epirubicin, cyclophosphamide, adriamycin and cyclophosphamide which causes severe side effects. Hypercalcemia, neutropenia and preservation of fertility are considered as the three major side effects which are main concerns to the patients undergoing chemotherapy (Yip et al. 2014). The lack of targeted therapies for TNBC intensified the interest for improving the overall survival of patients diagnosed with TNBC. Therefore, there is a dire need for the discovery of agents to kill TNBC cells and also for overcoming therapeutic resistance so that conventional therapeutics could be safely used with lower dosage without losing their activity (Kalimutho et al. 2015; O'Reilly et al. 2015). To that end, search is continuing, and recent focus has been targeted to natural agents because of their attributes as non-toxic or less toxic to normal cells and for their low cost. Previously, in our lab, we showed the killing of TNBC cells by both synthetic and natural products through apoptosis, and we showed their molecular mechanisms (Ananda Sadagopan et al. 2015; Hasanpourghadi et al. 2017). The aim of this chapter is to discuss the various bioactive natural products extracted from various parts of plants and mushrooms showing potential inhibitory effect against TNBC.

14.2 Natural Products on TNBC Therapy

Natural products are considered as an excellent and abundant source of therapeutics for many decades. They are classified into flavonoids (oxygenated derivatives of the aromatic ring structure) and alkaloids (having an indole ring). Natural products are used as traditional medicine for many decades (Laraia and Waldmann 2017). They are already proven to have beneficial effects including antidiabetic (Aziz et al. 2017; Ganesan et al. 2017), anti-inflammatory (Pandurangan et al. 2014a, b, 2015a, b; Arulselvan et al. 2016), antimicrobial, anti-colitis (Arulselvan et al. 2012; Huang et al. 2013; Pandurangan et al. 2015a, b, 2016), hepatoprotective (Kuriakose et al. 2017; Tzankova et al. 2017), cardioprotective (Giribabu et al. 2016; Hemmati et al. 2017), neuroprotective (Gopinath et al. 2011; Prakash and Sudhandiran 2015), nephroprotective (Karthivashan et al. 2016, Giribabu et al. 2017), wound healing (Muhammad et al. 2016) and antitumour activities (Pandurangan et al. 2014b, c; Saadatdoust et al. 2015; Tan et al. 2016). Some of the natural agents treated against TNBC with proper mechanistic actions (Fig. 14.2) are listed in tabular form (Table 14.1).

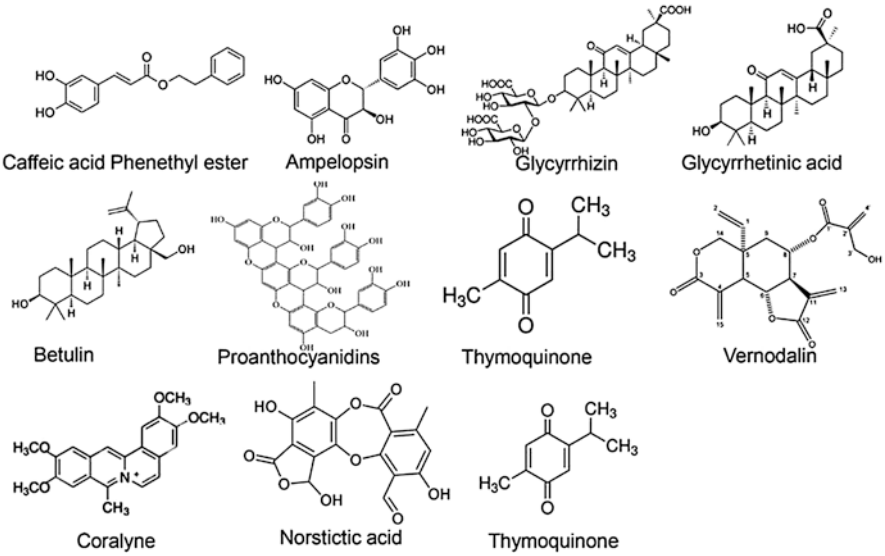

Fig. 14.2 Structures of some bioactive natural agents

14.2.1 Caffeic Acid Phenethyl Ester (CAPE)

Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE) is a natural phenol compound and an active component of propolis from honeybee hives. Torki et al. (2017) investigated the effect of phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway dual inhibitor, NVP-BEZ235, and CAPE on TNBC cell line (MDA-MB-231), stimulated with transforming growth factor (TGF)-β1 for 14 days. CAPE in combination with NVP-BEZ235 synergistically inhibited cell growth and reduced CXCR4 expression. Also, treatment of MDA-MB-231 cells with CAPE and NVP-BEZ235 time-dependently inhibited the expression of p-Forkhead Box O3a (FOXO3a). In a nutshell, these results indicate that tumour metastasis and progression in TNBC cells can be effectively reduced through the concurrent use of NVP-BEZ235 and CAPE (Torki et al. 2017).

14.2.2 Glycyrrhizin and Glycyrrhetinic Acid

Glycyrrhizin (GL) is a saponin-like compound that provides the main sweet flavour for *G. glabra* (licorice), and it is commonly used in clinical treatment for its outstanding pharmacological action such as antitumour, anti-inflammatory and anti-oxidative actions (Rebhun et al. 2015; Wu et al. 2015). Cai et al. (2017) tried to evaluate the anticancer properties of GA and GL against TNBC in combination with etoposide. GA is transformed from GL through specific bacteria in the human

Table 14.1 The list of natural products and its effect on TNBC

Natural agent	Model	Mechanism of action	References
CAPE	MDA-MB-231 cells	CAPE in combination with NVP-BEZ235 is cytotoxic to MDA-MB-231 cells and time-dependently inhibits the expression of p-FOXO3a	Torki et al. (2017)
Glycyrrhizin or glycyrrhetinic acid	MDA-MB-231 cells	Glycyrrhetinic acid in combination with etoposide inhibits the topoisomerase 2α and induces apoptosis	Cai et al. (2017)
Ixeris dentata	MDA-MB-231	It induces apoptosis via inhibiting p-Akt and p-NF-κB signaling pathway and reduces the volumes and weights of tumours in MDA-MB-231-bearing mice	Shin et al. (2017)
Ampelopsin E	MDA-MB-231	Induces apoptosis and G2/M phase cell cycle arrest	Rahman et al. (2016)
Vernonia amygdalina extract	MDA-MB-231, HCC1806 and MDA-MB-468	VA extracts reduce viability of TNBC cells	Howard et al. (2016)
		VA extracts inhibit DNA synthesis and induce DNA damage	
		VA extracts reduce pERK1/2 and increase caspase-3 activation	
Coralyne	MDA-MB-231	Coralyne inhibits cell proliferation in combination with paclitaxel	Kumari et al. (2017)
		The combination inhibits cancer cell migration and induces apoptosis	
SH003	MDA-MB-231	SH003 inhibits cell proliferation and induces apoptosis combined with doxorubicin by modulating the expressions of Bax, Bcl-2 and cleaved caspases 3, 8 and 9. SH003 and doxorubicin reduce the tumour volume and ki67 expression in MDA-MB-231 cell-induced xenograft mice	Woo et al. (2016)
Maximiscin	MDA-MB-468	MACN induces G1 phase cell cycle arrest. MACN caused DNA double-strand breaks with concomitant activation of the DNA damage response pathways, indicated by phosphorylation of p53, Chk1 and Chk2	Robles et al. (2016)
Ganoderma lipsiense extract	MDA-MB-231-HM in a mouse model	GLE reduced the tumour weight and microvessel density and cyclin D1 expression	Qi et al. (2016)

(continued)

Table 14.1 (continued)

Natural agent	Model	Mechanism of action	References
Dillenia suffruticosa dichloromethane root extract	MDA-MB-231	DCM-DS induced apoptosis, G2/M phase cell cycle arrest and oxidative stress in MDA-MB-231 cells. The reduction in apoptosis was possibly due to the activation of proapoptotic JNK1 and downregulation of anti-apoptotic ERK1, which may initiate the mitochondrial apoptotic pathway	Foo et al. (2016)
Aqueous *Pimenta dioica* extract or allspice fruit extract	MDA-MB-231	AAE induces cytotoxicity and apoptosis due to activating LC3B. It also inhibits Akt and mTOR signaling and tumour formation in MDA-MB-231 cell-inoculated athymic nude mice	Zhang et al. (2015)
Hibiscus syriacus extract and betulin derivatives	MDA-MB-231	HSSE and botulin derivatives induce cytotoxicity and apoptosis in MDA-MB-231 cells. HSSE activated the signaling pathway regulated by p53 family genes, leading to the inhibition of breast cancer cell viability	Hsu et al. (2015)
Grape seed Proanthocyanidins	HCC1937	GSP inhibits the viability of TNBC cells. The number of vascular channels was significantly reduced when cells were exposed in GSPs (100 μg/ml) and GSPs (200 μg/ml) groups. GSP promotes the transformation of mesenchymal-to-epithelial transition by modulating Twist1 protein	Luan et al. (2015)
Polysaccharide of Korean citrus hallabong peels (*Citrus sphaerocarpa*)	MDA-MB-231	HBE-II inhibited MDA-MB-231 cell migration, through downregulation of matrix metalloproteinase-9	Park et al. (2016)
Thymoquinone	MDA-MB-231	Thymoquinone acts on cell cycle and inhibits progression from G1 to S phase by targeting the cell cycle-related proteins such as cyclin D1, cyclin E and p27	Barkat et al. (2017)
		It also exhibits histone deacetylase (HDAC) inhibitory effects and targets p21 and Maspin	
		Induces pro-apoptotic gene, Bax, and downregulates anti-apoptotic gene Bcl-2	

(continued)

Table 14.1 (continued)

Natural agent	Model	Mechanism of action	References
Vernodalin	MDA-MB-231 LA7 cells induced tumour model in rats	Vernodalin activates the expression of FOXO3a and its downstream targets such as Bim, p27Kip1, p21Waf1/cip1, cyclin D1 and cyclin E. It also inhibits the tumour cell proliferation in the LA7-induced mammary gland tumour model in rats	Ananda Sadagopan et al. (2015)
Norstictic acid	MDA-MB-231	Norstictic acid significantly suppressed the proliferation, migration and invasion. It also significantly suppressed MDA-MB-231/GFP tumour growth of a breast cancer xenograft model in athymic nude mice	Ebrahim et al. (2016)

intestine (Taiko 2000). It was reported that GA at noncytotoxic concentration showed synergistic effect in combination with anticancer drug, etoposide (VP-16). Specifically, GA enhanced cytotoxicity through regulating topoisomerase II-α targeted by etoposide. Also, GA sensitized the cells to etoposide through elevating topoisomerase II-α with a 2.4-fold rate at 12 h time point. From 12 to 48 h, GA halved the expression of topoisomerase II-α and stimulated apoptosis, which exhibited its antineoplastic effect. They reported that GA was more potentially eliminating the TNBC cells when compared with GL (Cai et al. 2017).

14.2.3 Ixeris Dentate

Ixeris dentata NAKAI is commonly available in Korea, Japan and China, and it was a traditional herbal medicine used in Korea to treat indigestion, pneumonia, hepatitis and tumours (Ahn et al. 2006). *Ixeris dentata* is well known for its beneficial effects including neuroprotective effects (Oh et al. 2003), antidiabetic effects (Lee et al. 2013), protection effects against colitis and skin inflammation (Kim et al. 2013a, b) and anti-allergic effects (Yi et al. 2002). Shin et al. (2017) investigated the anticancer effects of *Ixeris dentata* extract (IDE) and studied the molecular mechanism of inhibiting breast cancer cells such as T47D, MCF-7 (ER and PR positive, HER2 negative), SK-BR-3 (ER- and PR negative, HER2 positive) and MDA-MB-231 (triple-negative) through *in vitro* studies. In addition, they also checked the antitumour effects of IDE through *in vivo* studies. The results indicated that IDE-induced apoptosis was mediated via various cell survival pathways on four breast cancer cells by identifying the factors including Bcl-2 family, p-Akt and p-NF-κB. With the promising *in vitro* findings of IDE-induced apoptosis via Akt-NF-κB signaling, they further investigated the effects of IDE on MDA-MB-231 cell-inoculated mice. The results showed that IDE significantly decreased MDA-MB-231 tumour volume and weight via inducing apoptosis by suppressing p-Akt. They concluded that IDE

induces apoptosis through the Akt-NF-κB signaling pathway in MDA-MB-231 breast cancer cells and tumours, and it may serve as a therapeutic agent for TNBC (Shin et al. 2017).

14.2.4 Ampelopsin E

Oligostilbenoid constituents of natural products have considerable attention for the multiple beneficial effects, namely, ampelopsin E, flexuosol A and Malaysianol D, found in *Dryobalanops beccarii*. *Dryobalanops beccarii* is widely distributed in the tropical forest of Malesia such as Peninsular Malaysia, Sumatra and Borneo (Oshima and Ueno 1993; Li et al. 1998; Wibowo et al. 2014). Wibowo et al. (2014) reported that ampelopsin E was the most cytotoxic towards breast adenocarcinoma cells (MCF-7). Further, the anti-tumorigenic effect of ampelopsin E was tested against the TNBC MDA-MB-231 cells. The results of the study showed that ampelopsin E was potentially cytotoxic towards MDA-MB-231 with the IC_{50} (50% inhibition of cell viability compared to control) of 14.5 ± 0.71 μM at 72 h time point. The hallmark of apoptotic characteristics such as cell shrinkage, membrane blebbing and formation of apoptotic bodies characteristic of apoptosis was observed when the MDA-MB-231 cells were exposed to ampelopsin E. The annexin V/PI flow cytometric analysis further confirmed that ampelopsin E induced apoptosis in MDA-MB-231 cells. Flow cytometric analysis of cell cycle disbursement revealed that ampelopsin E induced G2/M phase cell cycle arrest in the MDA-MB-231 cells (Rahman et al. 2016). They also revealed more experiments are necessary to study the complete underlying mechanism of action of ampelopsin E on TNBC cells.

14.2.5 Vernonia Amygdalina

Vernonia amygdalina (VA) (also called as African tea) is a small shrub from the member of the Asteraceae family that is widely available in the regions of tropical Africa. The aqueous extracts of VA, a bitter leaf plant, have been shown to be more than 1400 times more efficacious as an anticancer agent than some other plant extracts reported (Izevbigie 2003; Izevbigie et al. 2004). Aqueous extracts of VA have many known beneficial activities including antioxidant, hypoglycemic/antidiabetic, antibacterial, hepatoprotective, analgesic, gastric secretory, amoebicidal and phytotoxic actions (Ijeh and Ejike 2011). It was already shown that aqueous extract of VA inhibits the proliferation of tamoxifen-resistant PC3 prostate cancer cells by modifying the expressions of MAPK, c-Myc, AKT and Pgp (Cameron et al. 2013). The aqueous extract of VA leaf reduces cell proliferation of estrogen receptor-positive (ER+), estrogen receptor-negative (ER−) and triple-negative human breast carcinoma cells, as well as PC-3 androgen-independent prostate adenocarcinoma cells, a broader range than has been described with other anticancer agents, promoting apoptosis in BC and prostate cancer cells with no effect on normal human peripheral blood mononuclear cells (Howard et al. 2003, 2011). Recently, they reported aqueous extract of VA showed a significant reduction in tumour volume in

the MDA-MB-468 cell-induced tumours. They also showed aqueous extract of VA induced intrinsic mode of apoptosis in MDA-MB-468, MDA-MB-231 and HCC-1806 cells (Howard et al. 2016). They concluded that aqueous VA extracts induce apoptosis, exhibit additive effects, inhibit tumour growth and display chemopreventive actions against TNBC.

14.2.6 Coralyne

Alkaloids are a class of nitrogenous organic compounds of plant origin that have distinct physiological actions on humans. Coralyne [($C_{22}H_{22}O_4N^+$) 5, 6, 7, 8, 13, 13a hexadehydro-8-methyl-2, 3, 10, 11-tetramethoxy berberinium] is a heterocyclic analog of protoberberine alkaloid with antileukaemic activity and relatively high DNA/RNA-binding and intercalative potential compared to other isoquinoline compounds. Kumari et al. (2017) reported that treatment of coralyne in combination with paclitaxel (conventional chemotherapeutic drug) may exhibit synergistic effect on inhibition of proliferation, migration and induction of apoptosis in MDA-MB-231 TNBC cells. They reported that coralyne (10 μM) in combination with paclitaxel (0.1 μM) effectively inhibits MDA-MB-231 cell proliferation and migration at 48 h time point. Further, the combination altered the gene expression of the apoptotic mediators Bax and Bcl-2 (Kumari et al. 2017). Their study revealed that low doses of coralyne and paclitaxel showed promising therapeutic potential against TNBC cells.

14.2.7 SH003

Choi et al. (2014) previously developed SH003, a new herbal extract of three components, namely, *Astragalus membranaceus* (Am), *Angelica gigas* (Ag) and *Trichosanthes kirilowii Maximowicz* (Tk), and found that it suppressed breast tumour growth and metastasis (Choi et al. 2014). Accordingly, they aimed that a combination of SH003 with conventional chemotherapeutic agents might have a synergism in breast cancer treatment. Hence, they treated the SH003 and doxorubicin to TNBC MDA-MB-231cells *in vitro* and *in vivo*. SH003 alone and in combination with doxorubicin effectively induces apoptosis by modulating the apoptotic mediators such as Bax, Bcl-2 and cleaved caspases 3, 8 and 9. In addition, it controls the tumour volume and cancer cell proliferation in MDA-MB-231 cell-inoculated xenograft BALB/c nude mice (Woo et al. 2016). Woo and his colleagues finally conclude that the combinational treatment of SH003 and doxorubicin may improve TNBC treatment with less or no adverse effects.

14.2.8 Maximiscin

Maximiscin (MACN) is a secondary metabolite, and it was a by-product of the fungal isolate *Tolypocladium* sp. *Salcha MEA-2* and purified by column chromatography and

HPLC (purity >99%). The fungus was cultured in 20 L of modified potato dextrose broth containing 10 g/l of mashed potatoes and 5 g/l of glucose. Robles et al. (2016) reported that treatment of MACN has selective cytotoxic efficacy against basal-like 1 MDA-MB-468 cells compared to cell lines modelling other TNBC molecular subtypes. This compound also exhibited antitumour efficacy in a xenograft mouse model. Further, MACN treatment caused accumulation of cells in the G1 phase of the cell cycle, suggesting induction of DNA damage. Indeed, treatment with MACN caused DNA double-strand breaks with concomitant activation of the DNA damage response pathways, indicated by phosphorylation of p53, Chk1 and Chk2. Collectively, these results suggest basal-like TNBC may be inherently sensitive to DNA-damaging agents relative to other TNBC subtypes (Robles et al. 2016).

14.2.9 *Ganoderma lipsiense*

Ganoderma lipsiense is a commonly known mushroom. To study the inhibitory effect and mechanism of *Ganoderma lipsiense* extract (GLE) on the growth of TNBC cell line MDA-MB-231-HM inoculated in a mouse model. GLE treatment reduced the average tumour weight about 51.4%. In addition, the GLE treatment also reduced the microvessel density and cyclin D1 expression (Qi et al. 2016). They concluded that GLE could inhibit malignant proliferation of tumour cells by suppressing angiogenesis of blood vessels in tumour tissues and regulating cell cycles, thereby inhibiting TNBC.

14.2.10 *Dillenia suffruticosa*

Dillenia suffruticosa (Griffith ex Hook. F. and Thomson) Martelli (family, Dilleniaceae) is a medicinal plant commonly available in Malaysia and used for the treatment of cancerous growth (Ahmad and Holdsworth 1995). It was already reported that the root dichloromethane and ethyl acetate extract of *Dillenia suffruticosa* from sequential solvent extraction induced G0/G1 phase cell cycle arrest and apoptosis towards human caspase-3 deficient MCF-7 BC cells by modulating the expression of mitogen-activated protein kinases (MAPKs) (Foo et al. 2014; Tor et al. 2014). Foo et al. (2016) reported that treatment with DCM-DS induced apoptosis, G2/M phase cell cycle arrest and oxidative stress in MDA-MB-231 cells. The induction of apoptosis in MDA-MB-231 cells by DCM-DS is possibly due to the activation of proapoptotic c-Jun N-terminal kinases (JNK)1 and downregulation of anti-apoptotic extracellular signal-regulated kinases (ERK)1, which in turn downregulates anti-apoptotic Bcl-2 to increase the Bax/Bcl-2 ratio to initiate the mitochondrial apoptotic pathway. The cell cycle arrest in DCM-DS-treated MDA-MB-231 cells is possibly via p53-independent but p21-dependent pathway. A total of three triterpene compounds were isolated from DCM-DS. They identified that betulinic acid appears to be the most major and most cytotoxic compound in DCM-DS. And they assume that it could be the reason for the antiproliferative action of DCM-DS against TNBC cells (Foo et al. 2016).

14.2.11 *Pimenta dioica*

Pimenta dioica [(L)Merr] is termed allspice fruit, and they are in the form of dried unripe berries. Allspice fruit is also called Jamaican pepper, pimenta, or Newspice. It was a native plant from the Caribbean island Jamaica; *P. dioica* belongs to the family Myrtaceae (Zhang and Lokeshwar 2012). Scientific research on *Pimenta dioica* revealed that it has aromatic constituents *of Pimenta* leaves, and its unripe berries, allspice, paved the way for the discovery of many and novel aromatic compounds, mostly glycosides and polyphenols, that show antibacterial, hypotensive, anti-neuralgic and analgesic properties (Zhang and Lokeshwar 2012). It was reported that the identification of the aqueous allspice extract (AAE) as an anticancer formulation against prostate cancer and also identified an active ingredient namely Ericifolin (Eugenol 5-O-β-galloylglucopyranoside) from AAE that inhibits transcription of androgen receptor mRNA (Shamaladevi et al. 2013). Zhang et al. (2015) tested the effect of AAE against TNBC *in vitro* and *in vivo*. It was reported that AAE reduced the MDA-MB-231 cell viability and clonogenic growth of several types of BrCa cells ($IC_{50} \leq 100$ μg/ml) with minimal toxicity in non-tumorigenic cells. AAE-induced cytotoxicity in MDA-MB-231 cells was inconsistent with apoptosis but was associated with increased levels of autophagy markers light chain (LC3B) and LC3B-positive puncta. They also showed silencing the expression of autophagy-related genes (ATGs) prevented AAE-induced apoptosis. Further, AAE caused inhibition of Akt/mammalian target of rapamycin (mTOR) signaling and showed enhanced cytotoxicity when combined with rapamycin, a chemotherapy drug and an inhibitor of mTOR signaling. Oral administration of AAE into athymic nude mice inoculated with MDA-MB231 tumours inhibited tumour growth slightly but not significantly, when mice were gavagedAAE after the MAD-MB-231 cells injectedabrupt ending. Administration of AAE for 2 weeks showed delay in tumour palpability (about 38%) and growth rate (time to reach tumour volume $\geq 1000\ mm^3$) (Zhang et al. 2015). They concluded the antitumour and chemopreventive activity of AAE against TNBC cancer *in vivo* and *in vitro* and potential use as adjuvant for mTOR inhibition.

14.2.12 *Hibiscus syriacus* and Betulin Derivatives

Hibiscus syriacus was widely found in eastern and southern Asia, and it is an ornamental shrub. The plant is cultivated for decorative purposes because its flower is the national flower of Korea. Extracts of several parts of *H. syriacus* have been used as a prescription and effective alternative medicine in Asia (Yoon et al. 2017). Hsu et al. (2015) reported that *H. syriacus* skin extracts (HSSE) of 15 varieties were screened, including seven crude extracts and eight pure compounds. After treating estrogen receptor (ER)-negative and TNBC cell line (MDA-MB-231) with the extracts, functional assays were performed, which showed cell viability-inhibitory effects. In addition, triterpenoids (betulin and its derivatives) isolated from HSSE activated the signaling pathway regulated by p53 family genes, leading to the

inhibition of BC cell viability or even the induction of apoptosis. And also, these researchers found that all the triterpenoids had no effect on normal breast cells. These findings provide an important basis for the use of those triterpenoids in the development of alternative therapies for breast cancer treatment (Hsu et al. 2015).

14.2.13 Grape Seed Proanthocyanidins

Grape seed proanthocyanidins (GSPs) are considered as a group of proanthocyanidins that primarily contain dimers, trimers and other oligomers of catechin and epicatechin, and their gallic acid esters have been reported to protect against oxidation injury and to scavenge oxygen-free radicals (Yang et al. 2017). It also protects against diabetes and ischemia. GSP was reported to have antitumour activity against variety of cancer types (Yao et al. 2016). And dietary GSPs significantly inhibited UVB-induced skin tumour development in wild-type mice but not in XPA-deficient mice (Katiyar et al. 2017). Luan et al. (2015) examined the effect of GSPs on vasculogenic mimicry information in HCC1937 TNBC cell model. In this study, they identified the vasculogenic mimicry structure via the three-dimensional (3D) matrix *in vitro*. The effects of GSPs on human TNBC HCC1937 in terms of related proteins of vasculogenic mimicry information were determined using western blot analysis. *In vitro*, the tubular networks were found in highly invasive HCC1937 cells but not in the non-invasive MCF-7 cells when plated on matrigel. The number of vascular channels was significantly reduced when cells were exposed in GSPs (100 μg/ml) and GSPs (200 μg/ml) groups (all $p < 0.001$). Furthermore, they found that treatment with GSPs promoted transition of the mesenchymal state to the epithelial state in HCC1937 cells as well as reducing the expression of Twist1 protein, a master EMT regulator. GSPs can inhibit vasculogenic mimicry information by the suppression of Twist1 protein that could be related to the reversal of epithelial-to-mesenchymal (EMT) process. It is firstly concluded that GSPs may be a potential anti-vasculogenic mimicry natural agent for human TNBC (Luan et al. 2015).

14.2.14 Citrus Hallabong Peels (*Citrus sphaerocarpa*)

The fruit hallabong is the hybrid of mandarin and orange in the South Korean region. It was mainly grown in Halla mountain in Jeju Island and was thus named after the place. It was used as traditional medicine for a variety of diseases including treatment for stomach upsets, coughs, skin inflammation, muscle pain and ringworm infections, as well as to lower blood pressure (Li et al. 2009; Han et al. 2010). The hallabong fruit is used for the manufacture of juice, and its peel which is normally wasted during the process of making juice is rich in coumarins, carotenoids, pectin, limonoids and flavonoids that are valuable and functional dietary ingredients for human health (Tatum and Berry 1979; Horie et al. 1986). Park et al. (2016) reported that they aimed to utilize the hallbong fruit peel waste and identify properties it may have against breast cancer metastasis. Hallabong peel extract containing

crude polysaccharides was fractionated by gel permeation chromatography to produce four different polysaccharide fractions (HBE-I, HBE-II, HBE-III and HBE-IV). Treatment with HBE polysaccharides to human umbilical vein vascular endothelial cells (HUVECs) significantly blocked tube formation at a concentration of 12.5 or 25 μg/ml. The fraction of HBE-II disturbs the tube formation potentially than to other HBE polysaccharides. Further, HBE-II also inhibited MDA-MB-231 cell migration, through downregulation of matrix metalloproteinase-9 (MMP-9). Therefore, inhibition of tube formation and MMP-9-mediated migration observed in HUVEC and MDA-MB-231 cells, respectively, is likely to be an important therapeutic target in TNBC metastasis.

14.2.15 Thymoquinone

The black cumin (*Nigella sativa*) that has a long history of medicinal use contains a phytochemical called thymoquinone (2-methyl-5-isopropyl-1,4-benzoquinone) (Padhye et al. 2008; Khan et al. 2011). In the regions of Southeastern Asia, Africa, Arab and the Mediterranean, the black cumin seeds have a notable history in traditional medicine practices. In ancient Egypt, Greece and Turkey, black cumin seeds were often used to treat many diseases and ailments (Padhye et al. 2008; Salih et al. 2009; Khan et al. 2011; Ahmad et al. 2013). The *Nigella sativa* plant seeds and oil are used for medicinal purposes, and they are known for their hepatoprotective, renal-protective, antimicrobial, antidiabetic, anticancer, antihypertensive, analgesic, anti-inflammatory, immunomodulatory, spasmolytic, gastro-protective, bronchodilative and antioxidant activities (Salih et al. 2009; Khan et al. 2011; Ahmad et al. 2013). Recently, Barkat et al. (2017) reported that administration of thymoquinone to MDA-MB-231 acts on cell cycle and inhibits progression from G1 to S phase by targeting the cell cycle-related proteins such as cyclin D1, cyclin E and p27. It also exhibits histone deacetylase (HDAC) inhibitory effects, targets p21 and Maspin and induces pro-apoptotic gene, Bax, and downregulates anti-apoptotic gene Bcl-2 (Barkat et al. 2017).

14.2.16 Vernodalin

Vernodalin is available as colourless oil, and it is classified as a sesquiterpene lactone having two active α,β-unsaturated enoate moieties. It was isolated from the plant *Centratherum anthelminticum* (L.) Kuntze, commonly known as kalajiri, somraj, black cumin or bitter cumin, is a robust leafy plant that belongs to Asteraceae family. Scientific synonyms for this plant include *Vernonia anthelmintica* and *Conyza anthelmintica*. *Centratherum anthelminticum* (L.) Kuntze is commonly found in India, Himalaya, Khasi mountains, Sri Lanka and Afghanistan and is widely used as a traditional medicine against fever, cough, diarrhoea and skin diseases in these regions (Looi et al. 2013). Vernodalin regulates MDA-MB-231 cell apoptosis through activating the expression of Forkhead box (FOX)

transcription factors and the crucial downstream targets such as Bim, p27Kip1, p21Waf1/cip1, cyclin D1 and cyclin E. In addition, we showed that FOXO3a/PI3K-Akt played a significant role in vernodalin-induced apoptosis in MDA-MB-231 breast cancer cells. Immunoprecipitation assays showed Akt kinase activity was downregulated. To further confirm the involvement of FOXO3a in reducing the cell proliferation of MDA-MB-231 cells, we silenced the FOXO3a gene protected BCs against vernodalin-induced apoptosis. *In vivo* antitumour action of vernodalin was further confirmed by examining cell proliferative markers, PCNA and Ki67, in the LA7-induced mammary gland tumour model. We also corroborated our findings *in vivo* by showing upregulation of p27Kip1 and FOXO3a and decrease in the p-FOXO3a level in vernodalin-treated breast tumour tissue. Based on the results, we suggested that PI3K-Akt/FOXOa pathway is a critical mediator of vernodalin-induced cytotoxicity, and this compound could be further developed as a potential chemopreventive or chemotherapeutic agent for BC therapy (Ananda Sadagopan et al. 2015).

14.2.17 Norstictic Acid

Norstictic acid (NA) is a depsidone-derived metabolite common in several lichen species of the genera *Usnea*, *Ramalina* and *Parmelia*. Various biological activities have been reported for NA including antioxidant, antimicrobial and cytotoxic in nature (White et al. 2014). Recently, Ebrahim et al. (2016) reported that administration of NA to MDA-MB-231 cells significantly suppressed the proliferation, migration and invasion. Also, it showed minimal toxicity to non-tumorigenic MCF-10A mammary epithelial cells. They also performed molecular modelling, Z′-LYTE biochemical kinase assay and Western blot analysis, to identify c-Met as a potential macromolecular target. Further, NA treatment significantly suppressed MDA-MB-231/GFP tumour growth of a BC xenograft model in athymic nude mice. At this juncture, they concluded that lichen-derived natural products are promising resources to discover novel c-Met inhibitors useful to control TNBC (Ebrahim et al. 2016).

14.3 Conclusions and Future Prospects

It is a well-known phenomenon that TNBC is an aggressive form of breast cancer due to the lack of receptors. The conventional chemotherapeutic agents are ineffective for the treatment of TNBC patients. Natural products are versatile molecules that were used for the treatment of various ailments. It was confirmed that bioactive natural agents can eliminate the TNBC cells *in vitro* and *in vivo* with proper mechanism of action. From the above studies, we can conclude that bioactive natural agents can be useful for the treatment of TNBC, either alone or in combination with conventional chemotherapeutic agents.

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Endophytic Microbes as a Novel Source for Producing Anticancer Compounds as Multidrug Resistance Modulators

15

Julio Alves Cardoso Filho

Abstract

Endophytes are a range of microorganisms which inhabit the plant tissues, establishing an association with their hosts during a period for nearly or all their life without causing any apparent damage. It is known that some of these microorganisms have the potential to produce a diverse range of molecules that may be a reservoir of pharmaceutical drugs with anticancer activity. Cancer is a major trouble public health in all countries of the world, and this disease has higher mortality rate. Cancer is a name given to a complex disease, which is distinguished by the development and expansion of untypical cells and can lead to death. Nowadays the multidrug resistance (MDR) in cancer chemotherapy, remains a challenge to human and animal health around the world, which in several cases makes treatment inefficient. As a result, an increased number of strategies have been encouraged in search of MDR modulators (drugs that overcome MDR capacity) from various natural resources including endophyte-derived compounds. Thus, the aim of this chapter is to present the current status of several strategies adopted to promote the identification of some of these bioactive endophyte-derived compounds such as flavonoids, coumarins, polysaccharides, and β-glucans and to demonstrate their pharmaceutical potential as MDR modulators.

Keywords

Chemotherapy · Cytotoxicity · Plant-microbe interaction · Secondary metabolites · Therapeutic drugs

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15.1 Introduction

An endophyte is defined as any organism that form latent asymptomatic infirmity restricted to healthy plants parts, for all peculiar life cycle (Rudgers et al. 2010; Perotto et al. 2013; Dutta et al. 2014; Hardoim et al. 2015; Gouda et al. 2016). Interest in endophytic organisms began in the nineteenth century with the German phytopathologist, Heinrich Anton de Bary who has proposed the term "epiphytes" to define the fungi or bacteria that inhabit on the surface of plant tissue and "endophytes" for those inhabiting inside (de Bary 1866). The term "endophyte" has been debated ever since it was proposed (Petrini 1991, Wilson 1995; Andrews and Harris 2000). Nowadays, the term endophytic has become an alternative to mutualism (Kumar et al. 2014; Hardoim et al. 2015), and rarely are phytopathogens (Mendes et al. 2013; Philippot et al. 2013; Nair and Padmavathy 2014). Endophytic microorganisms are distinctly distributed throughout the organs and tissues of plants and are aggregated with various plant structural systems (Porras-Alfaro and Bayman 2011; Specian et al. 2012; Stepniewska and Kuzniar 2013). Numerous reviews have been focused on the microorganisms that are normally linked with to the plants (Turner et al. 2013; Chaparro et al. 2014; Farrar et al. 2014; Akhtar et al. 2015; Wisniewski-Dyé and Vial 2015; Kaul et al. 2016; Sengupta et al. 2017). The most recent reviews highlight to the endophytic association, such as endophyte fungi (Vasundhara et al. 2016; Wang et al. 2016), fungal entomopathogens (Vega et al. 2009; Meyling and Hajek 2010; Qayyum et al. 2012; Quesada-Moraga et al. 2014), endophytic bacteria (Zamioudis and Pieterse 2012; Christina et al. 2013; Ahemad and Kibret 2014; Prasad et al. 2015), plant-growth-promoting bacteria, mycorrhizas (Gorzelak et al. 2015; Johnson and Gilbert 2015; Cardoso Filho et al. 2016), and rhizobia (Wang et al. 2012; Maróti and Kondorosi 2014; Gourion et al. 2015; Okazaki et al. 2016).

The endophytic communities usually have been divided into different subgroups, such as "obligate" or "facultative," which are related to known plants (Rosenblueth and Martínez-Romero 2006; Hardoim et al. 2012; Andreote et al. 2014). In the last few years, an outstanding knowledge has been increased on the endophytic biology (Firakova et al. 2007). Most endophytes are unculturable; therefore the analysis of their diversity and the molecular basis of their interactions with the plant are revealed by using molecular approaches (Arnold 2005; Sun and Guo 2012). Endophytic microbes are found in almost terrestrial biomes, from the cold regions (e.g., Arctic) to the warm tropical regions (Aly et al. 2011; Deshmukh et al. 2015). They can drive host plant demography (Finkel et al. 2011; Rudgers and Clay 2012; Yule et al. 2013), shape plant communities (Weishampel and Bedford 2006; Rudgers et al. 2010; Rillig et al. 2013), guide the community structure and the biodiversity of the aggregated organisms (Rodriguez et al. 2009), and have an impact on the phenotype and epigenome of their associated plants (Gilbert et al. 2010). Numerous types of interactions of these endophytic with their hosts can induce changes in plant metabolic pathways and provide novel nutritional or biosynthetic capacities (Yu et al. 2010; Swamy et al. 2016a). Thus, it was proposed that they help with host adaptation to environmental stress conditions (biotic and abiotic) (Yule et al. 2013; Gouda et al. 2016).

The Taxol® discovery (isolated in 1993) was idealized as a new biotechnology endophyte era, with a huge demand in the global market (Stierle et al. 1993; Kusari et al. 2014a). It is worth mentioning that the production of plant compounds (e.g., Taxol®) depends on the endophyte species related and their host plant genotypes and their interaction with another type of higher-order trophic interactions (Hartley and Gange 2009). Despite the challenges, currently an expressive range of studies have demonstrated the antimicrobial effect and antioxidant activity of endophyte-derived compounds (Strobel et al. 2004; Gupta et al. 2005; Zhang et al. 2006; Gunatilaka 2006; Staniek et al. 2008; Suryanarayanan et al. 2009; Aly et al. 2010; Yu et al. 2010; Kharwar et al. 2011; Kusari and Spiteller 2011; Pickens et al. 2011; Chandra 2012; Cragg and Newman 2013; Higginbotham et al. 2013; Kumar and Pandey 2013; Perotto et al. 2013; Suryanarayanan 2013; Bhardwaj and Agrawal 2014; Demain 2014; Kusari et al. 2014a, b; Zhang et al. 2014a; Fridlender et al. 2015; Newman and Cragg 2015; Stierle and Stierle 2015; Nicoletti and Fiorentino 2015; Chen et al. 2016a; Gouda et al. 2016). Further, endophytes offer new sources of natural compounds for exploitation with plausible applications in crop production, food, cosmetic industries, and medicine (Chandra 2012; Bishayee and Sethi 2016; Liu et al. 2017).

Cancer is the second major killer disease all over the world (Pérez-Herrero and Fernández-Medarde 2015), with a forecast of 12.7 million of new cases, and 7.6 million cancer-related deaths are related every year (Chandra 2012; Jemal et al. 2011). By the year 2025, the global is estimated to increase to 20 million new cancer cases (Gupta and Chaphalkar 2015) and 13 million of cancer deaths due to the expansion and the senescence of the population (Ferlay et al. 2015). Cancer is the name given to a collection of related diseases (Siegel et al. 2017), which is represented by the proliferation of atypical cells in one or both of the tissues/organs (Gupta and Chaphalkar 2015; Siegel et al. 2017). This abnormal growth of cells forms a mass called a tumor or a neoplasm (Gupta and Chaphalkar 2015).

In the cancer pathology, tumors (or neoplasm) are named and broadly classified into two types, the benign (or non-cancerous) and malignant (or cancerous) tumors (Wiseman et al. 1995; Scheurer et al. 2008; Hartel et al. 2011). The term neoplasm (which means new growth) is usually used in preference because it is less ambiguous (Omuro and Delattre 2007). A benign neoplasm, which projects from an epithelial surface, is called a papilloma, and often it is limited to its primary site, neither infecting neighboring typical cells nor spreading to distant body locations (Ohgaki and Kleihues 2005). On the other hand, a malignant neoplasm is capable of both infecting surrounding healthy tissue and outspread throughout the body by the circulatory or lymphatic systems (metastasis) (Ahlbom et al. 2009; Wen et al. 2010; Miyamoto et al. 2017). Only malignant neoplasms are called cancers (Blakeley 2008). Both benign and malignant neoplasms are labeled according to the category of the cell from which they occur (such as carcinoma, sarcoma, myeloma, lymphoma, and leukemia) or tissue from which they originate (e.g., lung or breast carcinomas) (Stolnicu et al. 2015). Carcinomas are malignancies of epithelial cells and are the most usually diagnosed cancers originating in the skin, lungs, breasts, pancreas, and other organs and glands (Pandya et al. 2015). Sarcomas are a type of cancers that are derived from transformed cells of the mesenchymal source (Chen

et al. 2016a). Leukemia is a condition in which the bone marrow makes too many white blood cells, and it is a complex disease driven by numerous genetic, epigenetic abnormalities (Saultz and Garzon 2016; Cagnetta et al. 2014). Lymphomas and myeloma are lymphatic system cancers (Lichtman 2010). Melanoma is a metastatic type of aggressive malignancy cancer that develops when melanocytes begin to proliferate abnormally (He et al. 2017). Melanoma has a high resistance to cytotoxic agents (Gray-Schopfer et al. 2007). Lymphomas usually are all neoplasms of lymphoid tissue, which develops when one or more errors occur on the lymphocyte level, leading to the production of abnormal cells that proliferate or live much longer than healthy lymphocytes (Doi et al. 2008; Tan and Ellis 2013).

DNA damage is considered to be the primary cause of cancer (Ceder and Elgqvist 2017). A variety of biotic and abiotic factors, such as tobacco use (esophageal squamous, lung, colorectal, stomach, liver, and pancreatic cancer), infectious organisms (liver, stomach, and cervical cancer), an unhealthy diet (overweight/obesity), physical inactivity (breast and colorectal cancer), fewer pregnancies, and genetic factors, such as inherited genetic mutations, hormone dysfunction, and immune conditions, cause DNA damage in a coordinated or sequential manner and, thus, trigger oncogenes and/or tumor inhibitor genes (Ferlay et al. 2015; Torre et al. 2015; Chen et al. 2016b; Miller et al. 2016; Ceder and Elgqvist 2017), and these factors can interact in a variety of ways to potentiate carcinogenesis (Mishra et al. 2013). Diagnosed cancers related to these factors, such as the breast cancer (1.67 million), lung cancer (1.82 million), colorectal cancer (1.36 million), esophageal cancer (482,000 thousand), gastric cancer (990,000 thousand), and pancreatic cancer (178,000 thousand), are already on the rise in economically transitioning countries (Brown and Ahnen 2015; Ferlay et al. 2015; Kushi et al. 2012).

The most usual cancer treatments include surgery (before 1955), radiotherapy (1955–1965), chemotherapy (cytotoxic drugs use), biologic therapies (immunotherapy and hormone therapy, after 1965), and targeted therapy (referred to as "personalized medicine," after 2004), which comprise the majority of therapy regimens used clinically for the treatment of cancers (Arias 2008; Joo et al. 2013; Tan and Ellis 2013; Abarrategi et al. 2016; Tan et al. 2016). The chemotherapy is more frequently the first choice for the therapy of many cancer types (Siegel et al. 2017). Nowadays, the cancer chemotherapeutic strategies usually are related to the systemic administration of a drug (Gunji et al. 2013; Al-Quteimat and Al-Badaineh 2014; Au et al. 2015; Deng et al. 2016). Nevertheless, they exhibit toxic side effects for tumor cells (cancerous cells) as well as for normal cells (Brigger et al. 2002; Altieri et al. 2008; Ullah 2008). Unfortunately, chemotherapeutic agents induce changes within normal cell types undergoing replication (Sajesh et al. 2013). Consequently, for this reason, it is essential to improve the efficacy-toxicity balance of anticancer agents, and it remains an ongoing challenge in oncology (Fanciullino et al. 2013; Deng et al. 2016). Most of the anticancer therapies usually depend on the ability of the bioactive compounds to reach their designated cellular and subcellular target sites while minimizing accumulation and side effects at non-specific sites (Raj et al. 2016). Alternatively, new technologies, strategies, and therapies are extremely necessary to better target cancer cells.

Despite advances in biotechnology research, there is still a great interest in the international research for synthetic compounds due to economic and time-consuming reasons (Pimentel et al. 2011). Recently Newman and Cragg (2016) disclosed a list of all approved drugs from 1981 to 2014, from which an expressive number of natural compounds are produced by microbes and/or endophytes, and therefore it is considered that this area of natural product research should be expanded significantly. The aim of this chapter is to provide a comprehensive information on the current status of strategies available to promote the production of bioactive compounds by endophytic microbes (e.g., fungi and bacteria) and the potential application of these endophytic compounds as anticancer agents. Moreover, an alternative approach to produce anticancer agents by some potential endophytes is discussed for the discovery of novel drugs to overcome the multidrug resistance pathogens.

15.2 Multidrug Resistance (MDR) and Cancer Chemotherapy

Cancerous cells can develop resistance to one or more drugs having the similar action of mechanisms, and this resistance type is called multidrug resistance (MDR) (Eid et al. 2015; Callaghan et al. 2014; Choi and Yu 2014). MDR is the main cancer resistance mechanism and the cause for the nonsuccess of cancer therapy (Ling 1997; Chabner and Roberts 2005; Xue and Liang 2012; Binkhathlan and Lavasanifar 2013; Long et al. 2016). MDR is a multifactorial phenomenon; consequently, multiple mechanisms could contribute to MDR (Shanmugam et al. 2016). MDR may have a multilevel structure, such as macroscopic level (resistance to host-related factors, e.g., absorption (P-gp and food), distribution, metabolism, excretion), microscopic level (resistance to tumor-related factors, e.g., evolutionary resistance, related to the alteration of drug residency in cell cancer and alteration of drug target), and microenvironmental resistance (e.g., pH, oxygen, and glucose) and mesoscopic level (resistance to tumor-host interacting factors, e.g., vascular resistance and blood viscosity) (Tiwari et al. 2011; Cherigo et al. 2015; Cui et al. 2015; Dinic et al. 2015; Long et al. 2016). For this reason, lately, an approach to search for MDR modulators (with the capacity to overlap the MDR) from natural compounds has been in place for ultimate decades (Szakács et al. 2014; Meanwell 2016; Long et al. 2016; Qiu et al. 2017).

15.2.1 Cellular Mechanisms of the MDR

The MDR is related to overexpression of three ATP-binding cassette (ABC) transporter proteins, such as P-glycoprotein (P-gp, gene symbol ABCB1), MRP2 (gene symbol ABCC2), the multidrug resistance protein 1 (MRP1, gene symbol ABCC1), and the breast cancer resistance protein (BCRP, gene symbol ABCG2) (Eid et al. 2015; He et al. 2011; Wu et al. 2011; Wong et al. 2009; Jain et al. 2009; Kuo 2009; Szakacs et al. 2006; Szakács et al. 2014). The ABC transporter proteins function as

ATP-dependent drug efflux pumps (Girardin 2006). This mechanism is known as "the neostrategy of cancer cells and tissues" (Harguindey et al. 2005).

P-glycoprotein (P-gp) is a membrane-bound efflux pump found in several tumor cells and is known as a key player in the MDR phenotype (Eid et al. 2015; El-Araby et al. 2017). P-gp is a 170-kDa phosphorylated glycoprotein located on chromosome 7 and encoded by the human MDR-1 gene (Zinzi et al. 2014). The function of P-gp is to export xenobiotics from the cells (Linardi and Natalin 2006) and deposit them into the extracellular space (e.g., in the gut and for renal and hepatic clearance) or out of the body (Aller et al. 2009). Additionally, this recognizes different chemotherapeutic agents, such as antibiotics, antimalarials, herbicides, and cancer chemotherapeutics in humans (Higgins 2007; Al-Quteimat and Al-Badaineh 2014; Abdallah et al. 2015). P-gp usually loading charged hydrophobic molecules contains multiple substrate-binding sites within the ligand-binding domain and plays an impactful function in strategy and MDR action (Aller et al. 2009; Loo et al. 2009; Solyanik 2010; Binkhathlan and Lavasanifar 2013).

The chemotherapeutic anticancer agents (usually hydrophobic molecules) are actively loaded out of the cells (that express MDR1: tumor cells or normal cells) in opposition to a concentration gradient, thus decreasing the intracellular drug aggregation and reducing the drug-mediated cell death (Eid et al. 2015; Munoz et al. 2007). Plenty of these chemotherapeutic drugs are amphipathic natural compounds, such as the taxanes (paclitaxel and docetaxel), anthracyclines (doxorubicin, daunorubicin, and epirubicin), epipodophyllotoxins (etoposide and teniposide), antimetabolites (methotrexate, fluorouracil, cytosar, 5-azacytosine, 6-mercaptopurine, and gemcitabine), topotecan, dactinomycin, and mitomycin (Piccart 2003; Thomas and Coley 2003; Kandalaft et al. 2010; Moudi et al. 2013; Edwardson et al. 2015; Deshmukh et al. 2015). Therefore, a better knowledge about the mechanisms (cellular and molecular) that induce MDR is extremely important in order to maximize the current chemotherapy systems in cancer treatment (Ullah 2008; Wu et al. 2011; Binkhathlan and Lavasanifar 2013; Meanwell 2016; Qiu et al. 2017).

15.2.2 Modulation of P-gp and Strategies to Overcome the MDR

Until now, many studies often have related to P-gp expression with MDR to cytotoxic compounds, specifically with leukemia cells. Beyond that, the existing P-gp inhibitors have demonstrated weak success in clinical cases due to the fact that chemotherapeutic drugs have restriction in potency and specificity and their relationships with other chemotherapeutic drugs (Colabufo et al. 2010; Wu et al. 2011; Zhang et al. 2013; Long et al. 2016; Meanwell 2016; Qiu et al. 2017). To make matters worse, the P-gp blockers to be used clinically show their unwanted immunosuppressive and cardiovascular effects (Nobili et al. 2012; Binkhathlan and Lavasanifar 2013; Abdallah et al. 2015).

The older generation of P-gp inhibitors (e.g., verapamil, quinidine, and cyclosporine A) failed due to their low affinity and deficiency selectivity for P-gp, resulting in the use of high doses, leading to intoxication in treated patients,

generating patterns of unexpected pharmacokinetic correlations when associated with other anticancer compounds (Bates et al. 2001; Palmeira et al. 2012; Amin 2013). The next generation of P-gp inhibitors (the second generation) was designed according to the affinity of the side activity procedure, aiming to expand the effectiveness and to reduce the toxicity (Darby et al. 2011). Unfortunately, these P-gp modulators still had limited use, because the great part of them would act as substrates of cytochrome P450 3A (Leonard et al. 2002; Modok et al. 2006). The third generation of P-gp inhibitors was idealized for overcoming MDR in cancer therapy, through QSAR (quantitative structure-activity relationships) studies and the other combinatorial chemistry approaches, with P-gp inhibitory activity (Tan et al. 2013). Meanwhile, in phase III clinical studies in 2010, they were withdrawn from circulation due to its high toxicity (Cripe et al. 2010). The P-gp efflux pump blockers may act through two different mechanisms, one of them is blocking access to the substrate site and the others are usually related to the ATPase inhibition (El-Araby et al. 2017).

Currently, a new generation of ABC inhibitors (called the fourth generation) was proposed aimed at reducing costs and time required for the discovery of a potent drug (Macalino et al. 2015; Lima et al. 2016). This generation mimics the natural compounds (Chen et al. 2010; Cherigo et al. 2015; Gomes et al. 2015; El-Araby et al. 2017), and include peptidomimetics (Hoffmann et al. 2009; Conti et al. 2011), surfactants and lipids (Ahmad et al. 2016), nanomaterial-induced autophagy (Panzarini and Dini 2014), combination therapies based on nanoparticles (Mignani et al. 2015), ligand-based drug design (LBDD) and structure-based drug design (SBDD) (Doolittle et al. 2015; Lima et al. 2016; Abdolmaleki and Ghasemi 2017; Pedrosa et al. 2017), the inhibition of MDR-associated genes by degradation of particular types of mRNA (Stege et al. 2010; Zhang et al. 2016), combinatorial chemistry (Liu et al. 2017), use of monoclonal antibodies for P-gp (Goda et al. 2006), in silico virtual screening (VS) techniques to design the new drug or the knowledge-based identification of compounds that exhibit a desired biological activity and that are not substrates of P-gp (Prado-Prado et al. 2013; Macalino et al. 2015; Zoete et al. 2016), use of multiple drug resistance blockers of ABC transporters to overcome MDR (Wu et al. 2011; Abdallah et al. 2015), utilization of nanotechnology-based formulations and nanomedicine strategies to circumvent the MDR (Patil et al. 2010; Lei et al. 2015; Herrera et al. 2016; Colby et al. 2017), inhibition of MDR using peptides (Singh et al. 2014), photodynamic therapy (Broekgaarden et al. 2015; Kralova et al. 2017), epigenetic assays for drug discovery (Gul 2017), the pharmacophore hybridization approach (Fortin and Bérubé 2013; Romagnolia et al. 2014), and protein reporter bioassay systems (Chiba et al. 2012).

15.3 Natural Products and Drug Discovery

Natural product research focused on ethnopharmacology is currently considered the most striking strategy in discovering new molecules with chemotherapy anticancer potential (Nicoletti and Fiorentino 2015; Gouda et al. 2016). The search for natural

products has historically dated back to 1550 BC, but the scientific period began in the 1950s (Chandra 2012). In a broad sense, natural product is a generic term for plants, animals (marine and terrestrial), minerals, microbes, and their secondary metabolites found in nature (Newman and Cragg 2016; Ahn 2017). In principle, the search for novel compounds is a critical step to current pharmaceutical research (Katiyar et al. 2012; Brusotti et al. 2014; Sharma and Gupta 2015; Newman and Cragg 2016; Mishra et al. 2017). To elaborate, these compounds are a complex group of products with a wide biodiversity yet specific molecular properties, when compared to the physicochemical synthetic compounds (Lachance et al. 2012). On an average, in each discovery phase, the drug cost is ~$350 million and requires the preparation and evaluation over 12 years (Tari 2012; Mayura and Amol 2015). Currently, the virtual screening (Zoete et al. 2016), combinatorial chemical techniques (Liu et al. 2017), high-throughput screening (HTS) platforms (Michael et al. 2008; Roy et al. 2010; Lynn et al. 2015), rational drug design (Sun et al. 2017), and high-throughput chemistry (Toyooka 2017) have provided powerful tools for screening large compound libraries (Balmith et al. 2017) in a cost-effective manner (Katsila et al. 2016) to discover drugs based on target-based screening (Lionta et al. 2014). Further, nanoprobes help in screening and identification of specific sites of the anticancer molecule (Li et al. 2011; Zhang et al. 2017).

15.4 Biologically Active Compounds from Endophytes and Cancer

Many natural products or bioactive compounds (e.g., alkaloids, benzopyranones, chinones, phenolic acids, flavonoids, steroids, quinones, saponins, terpenoids, tannins, xanthones, tetralones, and many others) derived from endophytic microorganisms represent over 35% of selected and pre-new drug utilization of these chemotherapeutic candidates in the period from 1981 to 2010 (Newman and Cragg 2012; Swamy et al. 2016b). Data from the 1940s until the end of 2014 indicate that 75% of these chemotherapeutic compounds are synthetic and 49% are natural products or directly derived their from (Newman and Cragg 2016; Macías-Rubalcava and Sánchez-Fernández 2017). The extracts or natural products from plants and endophyte organisms are of great value in the control of malignancies in view of their low cytotoxic activities and drug resistance. As a consequence, the natural endophyte-derived metabolites have attracted peculiar attention with the purpose of being human cancer-chemopreventive compounds and anticancer chemotherapeutic drugs (Joseph and Priya 2011; Kharwar et al. 2011; Wang et al. 2011; Chandra 2012; Kaul et al. 2012; Kusari et al. 2012; Aly et al. 2013; Bhardwaj and Agrawal 2014; Nicoletti and Fiorentino 2015; Nisa et al. 2015; Chen et al. 2016b; Jia et al. 2016; Jin et al. 2016; Imhoff 2016; Negreiros de Carvalho et al. 2016; Vasundhara et al. 2016; Zhang et al. 2016; Wu et al. 2017; Narayana et al. 2017; Sarasan et al. 2017). Theendophytic microorganisms are considered a prolific reservoir of particular anticancer compounds and a vast number of new, structurally diverse, biologically active compounds (Charlop-Powers et al. 2015) and allow a sustainable

production of these desirable compounds. From the use of the technologies described, in the last years, more and more endophyte-derived compounds have been found attractive in cancer therapeutics, due to the growth cancer mortality and the expensive cost of the cancer chemotherapy to a continued search for new better and chemotherapeutic anticancer agents (Nobili et al. 2009). Endophyte-derived metabolites commonly present multitarget activity in opposition to MDR-related metabolic enzymes, proteins, and apoptotic signaling pathways, and this may aid to revert the resistance toward chemotherapeutic compounds (Hagmann et al. 2010; Armitage and Barbas 2014; Bianchini et al. 2015; Eid et al. 2015).

15.4.1 Methods for Searching New Endophytic Compounds

Natural product drug discovery works on the basis that biological diversity is the key to chemical diversity (Singh et al. 2014). One prerequisite for the bioprospecting and discovery of new active metabolites is choosing suitable source material which significantly increases the chance of "hitting a target."

15.4.1.1 Culture-Dependent Methods

In terms of ecology, among the endophytic microorganisms, a separation can be made between biotrophic endophytes (Croes et al. 2013) and non-biotrophic endophytes (Shen and Fulthorpe 2015). Particularly, an isolation method must be adapted to the plant species and their tissues and the endophyte type studied (Chandra 2012). By the way, not all endophyte microbes are culturable by using the standard growth media (Sun and Guo 2012). Endophytic microbes (e.g., fungi and bacteria) isolated from plant tissues are affected by surface sterilization methods, and these specific growth conditions may influence on the isolate's sporulation (Venugopalan and Srivastava 2015; Vasundhara et al. 2016).

The main critical step in any cultivation standard method is the organism's isolation (Petrini 1991). Usually, an endophyte isolation procedure consists of a surface sterilization of the fragments of the plant tissues (Alain and Querellou 2009; Reinhold-Hurek and Thomas Hurek 2011), followed by crushing the sterilized fragments of plant tissues, which are later placed into nutrient selected medium (Gazis and Chaverri 2010; Qin et al. 2012; De Souza et al. 2013; Baldan et al. 2014; Shen and Fulthorpe 2015; Pei et al. 2017). Until a few years ago, limited reports were primarily due to the inability to isolate and successfully culture, e.g., marine-derived microorganisms, and have them produce secondary metabolites with relevant bioactivity, even though there have consistently been reports of bioactive extracts from extremophiles from terrestrial or marine-derived microorganisms (Al-Massarani 2014; Cherigo et al. 2015).

15.4.1.1.1 Screening Culture-Dependent Methods for Antimicrobial Activity

The crude extracts obtained from of each endophyte need firstly to be tested through *in vitro* methods for evaluating antimicrobial activity (Nasir et al. 2015), such as

agar diffusion methods (e.g., antimicrobial gradient), thin-layer chromatography (TLC) bioautography (e.g., direct bioautography and agar overlay bioassay), dilution methods (e.g., broth dilution method), time-kill curve test, adenosine triphosphate (ATP) detection methods (e.g., ATP-luminescence technology), and flow cytofluorometric protocols (e.g., antibiotic susceptibility testing by flow cytometry).

A plenty of researchers have reported that several endophytic microorganisms isolated by culture-dependent methods have shown antimicrobial activities (Gazis and Chaverri 2010; Qin et al. 2012; De Souza et al. 2013; Baldan et al. 2014; Shen and Fulthorpe 2015; Santoyo et al. 2016; Pei et al. 2017). After isolation, microbes could be manipulated by biotransformation (e.g., fermentation/co-cultivation and use of epigenetic modifiers) to improve the yields of desired compounds and to produce new analogs of active compounds by overcoming the limitations of endophytes axenic culture (Nisa et al. 2015). Nonetheless, a great number of endophytic microbes still cannot be cultured *in vitro* due to their growth limited only to specific environmental conditions from which they were isolated, leaving a gap open for drug discovery (Vasundhara et al. 2016).

15.4.1.2 Culture-Independent Methods

The literature usually reports that only 0.001–1% of the endophytic microbes that inhabit the plant tissues can be isolated and cultured by using traditional protocols (Alain and Querellou 2009; Eevers et al. 2015). The understanding of the morphology and ecophysiology of the endophytic populations in natural ecosystems is the major challenge of the twenty-first century of the biology sciences (Zhou et al. 2015). Since 1990, several molecular techniques (culture-independent methods) are used in microbial ecology (Turner et al. 2013). The utilization of molecular techniques (e.g., as DNA fingerprinting and DNA sequencing protocols) has the potential to overcome the difficulties in traditional laboratory cultivation-dependent methods (Sun and Guo 2012; Zhang et al. 2014b; Wang et al. 2016; Kaul et al. 2016). Molecular detection of endophytes into plant tissues usually involves the following steps: the extraction of total genomic DNA (including endophyte organism and the host plant) from surface-sterilized plant tissues, the amplification of DNA fragments from total DNA with endophyte-specific primers, the separation of PCR products (bands) by denaturing gradient gel electrophoresis (DGGE) and excision of nonhomologous DGGE bands representing different taxa, the cloning of PCR products, the screening of positive clones for different taxa using DNA-fingerprinting techniques, the sequencing of representative clones with different fingerprinting patterns and DGGE bands, and the theoretically identification of the sequences into several taxonomic levels based on phylogenomic techniques and sequence analysis by similarity comparison (Sun and Guo 2012; Turner et al. 2013; Hardoim et al. 2015).

15.4.1.2.1 Screening by High-Throughput DNA/RNA Technologies

The omics studies, based on genomics, transcriptomics, proteomics, metagenomics, metabolomics and metabonomics analysis methods, have developed fastly and currently play a primordial role in biological science research (Nishiumi et al. 2014;

Tsurumaru et al. 2015). The utilization of the high-throughput DNA/RNA molecular sequencing technologies (Webster and Thomas 2016) enables genome sequencing by pyrosequencing (Caporaso et al. 2012) including Illumina (e.g., HiSeq, MiSeq, Roche454 GS FLX+, SOLiD 5500 series, and Ion Torrent/Ion Proton platforms), comparative genomics (Luter et al. 2015), functional gene array (GeoChip 3.0, GeoChip 5.0, PhyloChip G3) (Tu et al. 2014; He et al. 2010; Zhang et al. 2015; Probst et al. 2015), metagenomics, metatranscriptomics, and metabolomics and metabonomics approaches for microbiome analysis (Shendure and Ji 2008; Porras-Alfaro and Bayman 2011; Loman et al. 2012; Caporaso et al. 2012; Weinstock 2012; Gowda and Raftery 2013; Aguiar-Pulido et al. 2016; Bourne et al. 2016; Jiang et al. 2016) and provides a powerful alternative to molecular studies of endophyte community in natural environments (Manter et al. 2010).

15.4.1.2.1.1 Metagenomics as a Tool for Searching New Endophytic Metabolites

Metagenomics involves analysis of total genomic sequence information from all life forms found in various ecological communities (Nováková and Farkašovský 2013; Kaul et al. 2016; Probst et al. 2015). Nowadays, the recent development of metagenomics tools and the use of high-throughput screening technologies have exponentiated the pool of useful genetic sequence information that can be screened for antimicrobial discovery (Pawar et al. 2017) and stimulated the currently impending renaissance in drug discovery and encourage the development of novel natural drug molecules (Kaul et al. 2012).

The comparative metagenomic analysis is extremely helpful for knowledge and understanding of the spectrum of the genetic and metabolic biodiversity of the endophyte microorganisms acting into the several types of symbiotic or nonsymbiotic interactions with the different taxons of plants and animals (Kaul et al. 2016). Mass spectrometry-based proteomics for community analysis (Chen et al. 2015) and the metagenomic approach are able to overcome this bottleneck by the development of culture-independent techniques (Kaul et al. 2016). Nowadays, some of these molecular methods that are being used or can be used to unravel plant-endophyte interactions (Tian et al. 2012; Cuadros-Orellana et al. 2013; Suryanarayanan 2013; Wright et al. 2013; Kaul et al. 2016; Sengupta et al. 2017) has been described recently. The bioprospecting at the public or private data bank for the sequences of interest, followed by chemical synthesis of selected genes, is a more current methodology to identify novel genes (Simon and Daniel 2017). These methods refer to total sampling the all determination of the collective set of metagenomes and metatranscriptomes of the microbial populations and must be used to the comparative ecology studies of all microorganisms that inhabit in marine biomes, terrestrial biomes, human and animal clinical samples, sludge, polluted environment, and plants (Kaul et al. 2016).

15.4.1.2.1.2 Metatranscriptomics as a Tool for Searching New Endophytic Metabolites

The metatranscriptomics involves the totality analysis of all transcriptomes associated with all groups of microbial species into the choice ecosystem and aims to understand the response of these communities of the changes (naturally induced or

not) applied to the environmental conditions studied (Sheibani-Tezerji et al. 2015; Wang et al. 2015). The metatranscriptomic analysis could provide a comparison of relative abundance of specific groups of endophytes and non-endophyte microbial communities from multiple samples (e.g., rhizosphere microbiomes and non-rhizospheric microbiomes), without polymerase chain reaction bias (Molina et al. 2012; Kaul et al. 2016). The majority of the difficulties detected in metaecological studies are due to the high complexity of the microbial community (few populations are of high frequency and many populations of low abundance), and these limitations can be prevented in metatranscriptomics studies by a selective approach on the active populations during the sample (Morales and Holben 2010).

15.4.1.2.1.3 Metabolomics as a Tool for Searching New Endophytic Metabolites

Metabolome is used to evaluate the specificities and associations of low-molecular-weight metabolites under a specific set of conditions (e.g., unknown pathological human conditions) (Hong et al. 2016). Metabolomics methods have an exceptional advanced in the last decades, and capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) has arisen as a new technology for the studies related to cellular metabolites (Yamakawa et al. 2017). Even more frustrating are the challenges associated with the characterization of metabolites from NMR (nuclear magnetic resonance) spectroscopy or MS (mass spectroscopy) spectra. The database is the Human Metabolome Database (HMDB), which contains more than 40,000 metabolite entries, thousands of metabolite concentrations, >700 metabolic and disease-associated pathways, as well as information on dozens of cancer biomarkers (Nishiumi et al. 2014; Wishart et al. 2016). Another human metabolome database is the METLIN (Bingol et al. 2015, 2016). Gowda and Raftery (2013) presented the advances in the field of metabolomics with emphasis on biomarker discovery and translational efforts, highlighting the current status, challenges, and future directions. Nowadays the metabolomics studies (for biomarker discovery) are NMR based, and have applications in the areas of toxicology, inborn errors of metabolism, cardiovascular disease, and cancer detection (Monteiro et al. 2013). The applied metabolomic to biomarker discovery has included aspects of pathobiochemistry, systems biology/medicine, and molecular diagnostics and requires bioinformatics and multivariate statistics (Griffiths et al. 2010). In metabolomics studies, model organisms can supply the samples for method development as well as the foundation of comparative phyllo-metabolomics (Reed et al. 2017).

15.4.1.2.1.4 Metabonomics as a Tool for Searching New Endophytic Metabolites

Metabonomics is related to the profiling of several metabolic compound levels, their constants, and progressive changes affected by factors such as lifestyle, diet, environment, genetic, and both beneficial and adverse pharmaceutical effects (Zennaro et al. 2017). Recently, a metabonomics methodology is based on ultra-performance liquid chromatography (UPLC) coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) to identify biomarkers to galactomannan activity (Jiang et al. 2017). As a result, this study demonstrates that a metabonomics

approach is an excellent, novel tool that can be used to evaluate the underlying therapeutic mechanisms of herb extracts. More recently, Kim et al. (2017) have used the pharmacometabolomics as an emerging "omics" field and used individual metabolic signatures to define mechanisms of action and variations in response to treatment, supporting personalized drug therapy. Navarro et al. (2016) reported the utility of pharmacometabonomics to inform IV busulfan dosing. Another study has shown that the pharmacometabonomics profiling helps in predicting toxicity in patients with inoperable colon cancer (Backshall et al. 2011). These authors suggested that metabolic profiles can describe subpopulations susceptible to adverse conditions and have a role in chemotherapy assessing in cancer patients. The pharmacometabonomics could be used to predict drug metabolism, pharmacokinetics (PK), and drug safety and efficacy, and it is complementary to pharmacogenomics (PGx) and pharmacoproteomics (Everett et al. 2013) but has the advantage of being sensitive to environmental as well as genomic factors (Everett 2016). For these reasons, the pharmacometabonomics will be considered a new tool for personalized medicine (Everett 2015). In specific cases, omics studies (e.g., genomics, metagenomics, proteomics, metatranscriptomics, metabolomics) can aid the comprehensive investigation of a compound's effect (e.g., determining the activity of bioactive metabolites) on the potentially large numbers of biological steps (Antipova et al. 2011; Gika et al. 2014; Knief 2014).

For this reason, the "omics" technologies and tools can be used to determine the role of endophytes in the plant microbiome (Lucero et al. 2011; Gao and Tao 2012). The characterization of endophytic profile in plants is essential to use these "omics" multiphasic approaches to study cultured and uncultured microbes (Pei et al. 2017). These studies based on genomics, transcriptomics, proteomics, metagenomics, metabolomics, metabonomics have been the emerging interest in microbial systematic that has built an extremely large database in NCBI (https://www.ncbi.nlm.nih.gov) and another gene bank (Chaudhary and Dahal 2017). DNA barcoding idealized by Hebert et al. (2003) represented a major step forward for the DNA-based species. The development of DNA barcode database covers the Consortium for the Barcode of Life (CBoL; http://barcoding.si.edu/) and the International Barcode of Life (Ibol; http://www.ibol.org/), Barcode of Life Data Systems database (BOLD; http://www.boldsystems.org/index.php/databases). The DNA barcoding and metabolomics could be supplemented with the use of transcriptomics and proteomics approaches (Mishra et al. 2016). Recently, Laroche et al. (2017) suggested that metabarcoding-based methodology can be used for biomonitoring both eDNA (sequencing environmental DNA) and eRNA (sequencing environmental RNA) products, as an alternative to traditional morphological-based identification to characterize biological assemblages and monitor anthropogenic impacts in marine environments. Thus, these omics methodologies would be helpful to fill these gaps and improve the knowledge and understanding about endophytism (Suryanarayanan 2013) and discourse and expose the Pandoras box to the interactions between endophytic microorganisms and host plants.

15.4.2 High-Throughput Screening (HTS) for Unknown Compound Detection

HTS technology is considered the "Rosetta stone" for unknown compound identification in currently drug discovery programs. A survey of all FDA-approved new molecular entities (NMEs) shows that natural compounds and their derivatives represent over one-third of all NMEs (Patridge et al. 2016).The strategies to bioprospection in microbial collections for orphan biosynthetic are emerging as a need within anticancer drug discovery programs, in combination with HTS and chemical dereplication of novel compounds (Genilloud 2014). A recent strategy for a quick identification of novel metabolites in complex mixtures (metabolomics with combined MS/NMR) is called SUMMIT MS/NMR, adapted for HTS procedures for use in screening of novel metabolites in biological and biomedical mixtures overcoming the need of experimental MS and NMR metabolite databases (Bingol et al. 2015). A HTS platform of microbial natural compounds for the search, screening, and selection of molecules with antibiofilm properties against *Salmonella enteritidis* was described by Paytubi et al. (2017). This HTS platform is the first validated HTS assay using microbial extract mixtures, which allowed to detect four types of activities against biofilm embedded cells. The validation of HTS assay and the primary screening protocols are indispensable to allow the selection of extracts or bioactive molecules with pharmacological action and with real possibility to be marketed (Monteiro et al. 2012).

15.4.2.1 Hyphenated Techniques in Natural Product Analysis

In this process (Ibekwe and Ameh 2015), one or more separation techniques [e.g., liquid chromatography (LC) or gas chromatography (GC)] are linked to one or more spectroscopic detection methods [e.g., ultraviolet-visible (UV-vis), infrared (IR), MS, or NMR spectroscopy]. All crude extracts or fractions (e.g., animal, plant, or microbe) often occur as a mixture of unknown compounds with several types of polarities, since their separation is a huge challenge for their screening, identification, and characterization (Elipe 2003; Antipova et al. 2011). In order to obtain the structural information of the identified compounds in a crude sample, high-performance liquid chromatography (HPLC or simply LC), GC, or capillary CE are coupled to spectroscopic detection techniques, e.g., Fourier-transform infrared (FTIR), photodiode array (PDA) UV-vis absorbance, or fluorescence emission, MS, and NMR (Patel et al. 2012). As a result, innumerable modern hyphenated techniques (e.g., CE-MS, GC-MS, LC-MS, and LC-NMR) were developed (Patel et al. 2010). However, HPLC is a very commonly used analytical technique to determine active molecules in natural crude extracts (Brusotti et al. 2014). Liquid chromatography mass spectrometry (LC-MS) is one of the most prosperous analytical method, due to its inherent selectivity and sensitivity (Qi et al. 2014). These hyphenated systems generate multidimensional data (chromatographic and spectroscopic) for online identification and dereplication purposes (Brusotti et al. 2014). In LC-MS, chemical derivatizations are frequently used to enhance the MS ionization

efficiency and selectivity, to facilitate structure elucidation, and to improve the chromatographic separation (Qi et al. 2014). The interfaces between LC systems and the MS instruments are also under continual improvement (Zhou et al. 2012). Electrospray ionization sources (ESI) are now more compatible with the higher flow rates utilized in UHPLC (ultra-high-pressure chromatography).

15.4.2.2 Dereplication of Microbial Natural Products

Typically dereplication approaches can combine the use of chromatographic and spectroscopic techniques with database bioprospecting (Pérez-Victoria et al. 2016). Dereplication is used to screen natural products from the extracts of microbial fermentation broths or plant samples (Nielsen et al. 2011; Ito and Masubuchi 2014). Chemical dereplication process is preferentially based on MS NMR spectrometry (Carrano and Marinelli 2015). Determining the basic constituents in crude mixtures using MS/MS and UV/vis spectra is getting easier with current advances in analytical equipment and versatile bioactive molecule databases (Carlson 2010; Nielsen et al. 2011). Current dereplication strategies include hyphenated techniques, such as HPLC-MS, HPLC-NMR, HPLC-NMR-MS, and HPLC-SPE-NMR (Carrano and Marinelli 2015). The point of convergence of these strategies (e.g., UV-vis profiles, chromatographic retention times, MS, NMR chemical shifts) will benefit in identifying structurally similar biomolecules.

15.4.2.3 Chemical Derivatizations of Microbial Natural Products

The bioanalysis of drugs used in the management of cancer is often complicated by the lack of selectivity and sensitivity (Sternson et al. 1988; Koehn 2008). Chemical derivatization strategies represent a more recent approach for the separation of lipids from complex mixtures, including isomeric lipids (Ryan and Reid 2016). Chemical modifications are usually applied in order to adapt their physicochemical properties, generate derivatives for structure-activity relationship (SAR) studies, and allow the synthesis of active molecular probes by conjugation of reporter tags (e.g., fluorophores, biotin) which retain bioactivity (Roblesa and Romoa 2014) and the challenges related to derivatization of each exclusive natural compound displaying broad scales of reactivity.

15.4.3 New Tools to Determining the Action of Mechanism of Bioactive Compounds

Modern drug discovery efforts have had mediocre on implementation rates with increasing developmental costs, and this factor has encouraged pharmaceutical scientists to seek innovative methodology (Isgut et al. 2018). Recent studies on the benefits of drug combinations lay the groundwork for a renewed focus on natural compounds in drug discovery (Chang and Kwon 2016; Cheung et al. 2016; Arai 2016; Fernando et al. 2016; Lindequist 2016; Nicoletti and Trincone 2016; Pereira et al. 2016; Plodek and Bracher 2016; Perkins et al. 2016; Richter et al. 2016;

Avinash et al. 2016; Fierro-Cruz et al. 2017; Zhou et al. 2017). In part, this occurred because of the current scientific mood in obtaining high-quality natural products screening modern molecule libraries (Atkins et al. 2012) or in applying modern screening assays to these libraries (Patel et al. 2014).

15.4.3.1 Target-Based Screening

Target-based screening is one of the major and more used methodologies in drug discovery (Cichonska et al. 2015; Pérez-Victoria et al. 2016; Yarla et al. 2016). Identifying the targets of bioactive molecules is the biggest obstacle in chemical biological research (Kitagawa et al. 2010). For effective bioactive small molecule bioprospecting and development into a novel therapeutic drug, a systematic screening and target protein identification are required (Cho and Kwon 2012). Currently the chemical genetics is the key point of interest, in which small molecular compounds are used as probes to differentiate the protein functions within signaling pathways (Tashiro and Imoto 2012).

The structural diversity of natural products makes them ideal screening sources for chemical inhibitors that can be used to discern the complex molecular mechanisms underlying cellular events by chemical genetic approach (Newman and Cragg 2007). There are two fundamental methods to identify chemical inhibitor targets: direct and indirect (Hart 2005). In the direct method, the target proteins bound to the inhibitor are purified and directly identified by mass spectrometry. The indirect methodology to chemical inhibitor target identification involves screening for drug candidates by profiling biological data (Kitagawa et al. 2010). Mass spectrometers are unique with potential to directly analyze any biological molecule susceptible to ionization (Crutchfield et al. 2016). The advances in the mass spectrometry-based proteomic methods have the capacity to provide the understanding of the molecular activity of natural products (Cheng et al. 2010). These may provide an extensive overview of the applications of mass spectrometry-based techniques in the identification and characterization of natural product protein interactions. Moreover, computational target fishing mines biologically annotated chemical databases as Target Hunter (http://www.cbligand.org/TargetHunter) and then maps compound structures into chemogenomic space that has been used to predict the biological targets (Wang et al. 2013).

15.4.3.2 The High-Resolution Phenotypic Profiling

The high-resolution phenotypic profiling of natural products that induced effects on the single-cell level was proposed by Kremb and Voolstra (2017). In 2015, Sandercock et al. (2015) described the identification of antitumor biologics using primary tumor models, 3-D phenotypic screening, and image-based multiparametric profiling. In this study, a practical assay for the characterization and classification of natural compounds was reported according to four major categories of biological information: (1) compound-induced perturbations of multiple cellular processes, (2) multilevel toxicity, (3) structure-activity relationships (SAR), and (4) mechanism of action (MOA) and molecular targets or off-target effects (Kremb and Voolstra 2017). This methodology has been used more than over 30,000 target

compounds and showed that small-molecule profiling can be employed to identify the compound sets with high activity and biological diversity. The creation of database based on broad biological activity screens on cellular models a "performance-diverse" compound collections (Patel et al. 2012), enlarged the identification and characterization of news MOA or biological target sites (Wawer et al. 2014). The results have suggested a basis for further analysis of chemical structure in relation to biological function, by the use of a cell-based phenotypic HTS with a specific application to antiviral drug discovery (Patel et al. 2014).

15.4.3.3 Cell-Based Phenotypic HTS

This approach cell-based phenotypic screens can result in data sets from small libraries or portions of large libraries (Patel et al. 2012) and develop accurate hit picking from these multiple data sets and propitiate one more efficient search drug discovery (Patel et al. 2014). The developed cell-based screening system by a cell line that expresses influenza viral ribonucleoprotein complex (vRNP) made it possible to identify a set of molecules that blocks the vRNA transcription/replication by using reporter protein expression from virus-like RNA as a readout and virus replication *in vitro* (Ozawa et al. 2013) and, thus, is potentially appropriate to identify vRNA transcription/replication inhibitors for many RNA viruses, especially for primary screens, by profiling their antiviral efficacy against multidrug-resistant influenza A viruses (Ma et al. 2016).

15.4.3.4 Companion Diagnostic Techniques

Companion diagnostics are *in vitro* clinical laboratory protocols idealized to predict the efficacy of a targeted cancer therapy by assessment of one or more biomarkers and to determine if a drug may or may not be effective. In this era of targeted chemotherapy, the current development of companion diagnostic techniques is fundamental for the implementation of new therapeutics (Yoo and Park 2015). The companion diagnostics began with certification by FDA (the United States Food and Drug Administration) in 1998 of an immunohistochemistry (IHC) assay (HercepTest™, Dako Denmark A/S, Glostrup, Denmark) for HER2 protein overexpression (Yoo and Park 2015). For the detection of this gene amplification, the only approved companion diagnostic devices are the NGS (next-generation sequencing) platform (MiSeqDx; Illumina, San Diego, California) by FDA (Khoury and Catenacci 2015). Gradually, the NGS and MS proteomics are confronting the traditional diagnostic assays and implementing a new sketch in companion diagnostic technology.

15.4.3.5 Biology-Oriented Synthesis

The organic synthesis of complex molecules is often laborious and time-consuming. Biological research is aided by small-molecule modulators that perturb protein activity without altering the underlying biological systems (van Hattum and Waldmann 2014). The chemical space is a big universe and cannot be exploited satisfactorily by the use of traditional synthesis methods (Wetzel et al. 2011). Navigation in chemical space is facilitated by a Scaffold Hunter (Bon and Waldmann

2010) and ChemGPS-NP (Larsson et al. 2007; Lachance et al. 2012). A Scaffold "Hunter" provides the hierarchical structural classification of small-molecule database collections in treelike arrangements, their annotation with bioactivity data, and the intuitive surfing in the webchemical space. The ChemGPS-NP (http://chemgps.bmc.uu.se/batchelor/about.php) is tuned for exploration of the regions of chemical space most likely to enclose compounds with activities and biological functions (Larsson et al. 2007). The biology-oriented drug synthesis (BIODS) was used recently to design and synthesize libraries of compounds on the skeleton of authentic drugs (marketed drugs) with more and diversified biological activities (Ullah et al. 2016). Other approaches, in biology-oriented synthesis, such as the diversity-oriented synthetic strategies (DOS) have been employed to cancer chemical biology and drug discovery (Collins and Jones 2014). The integration of in silico screening against specific targets and virtual library enumeration should be a combination to guide and maximize the contributions of DOS to the discovery of targeted anticancer therapeutics (O'Connor et al. 2012).

15.4.3.6 BioAssay Ontology (BAO)

The large amount of information generated in HTS campaigns are submitted to public repositories such as PubChem Bioassay Database (https://pubchem.ncbi.nlm.nih.gov) and ChEMBL (https://www.ebi.ac.uk/chembl) which increase at an exponential rate (Visser et al. 2011). The lack of standards to describe and annotate biological assays and screening outcomes in the domain of drug and chemical probe discovery is a critical limitation to utilize public and proprietary drug screening data (Abeyruwan et al. 2014). In 2010 (Visser et al. 2011), the BioAssay Ontology (BAO) project was created (http://bioassayontology.org). The main objectives of BAO are to enable effective integration, aggregation, retrieval, and analyses of drug screening data (Abeyruwan et al. 2014). The ontology is available online at the NCBO BioPortal http://bioportal.bioontology.org/ontologies/BAO BAO-GPCR (Vempati et al. 2012; Abeyruwan et al. 2014), and BAO leverages description logic to formalize the domain knowledge and allow the semantic integration with several other resources. These approaches allow the generation/extraction of derived ontologies (or perspectives) and analyze the activity of compounds for identification of technology artifacts (Balderud et al. 2015; Zander Balderud et al. 2015).

15.4.4 Bioactive Endophyte-Derived Compounds as MDR Modulators

The research related to anticancer drugs has focused the reversing MDR by using natural products which exhibited potential as chemosensitizers (Joseph and Priya 2011; Choi and Yu 2014; Zinzi et al. 2014; Newman and Cragg 2015; Abdallah et al. 2015; Long et al. 2016; Teng et al. 2016). Bioactive endophyte-derived substances may either be used as chemotherapeutic or chemopreventive agents with chemoprevention, as well as a reservoir of novel bioactive molecules (Chandra 2012; Kusari et al. 2012; Suryanarayanan 2013).

15.4.4.1 Flavonoids

Recent interest in these biomolecules has stimulated a large number of surveys on the health aspects of flavonoids for humans and animals (Telerman et al. 2017). Many flavonoids possess antioxidative, free radical scavenging, hepatoprotective, anti-inflammatory, and anticancer properties, while some flavonoids display antiviral properties (Kumar and Pandey 2013). Current *in vivo* studies have partially validated *in vitro* findings, suggesting that the mechanisms underlying the modulatory effects of flavonoids are complex and difficult to predict *in vivo* (Miron et al. 2017). Flavonoids have been related to inhibit ATP-binding cassette (ABC) transporters, MDR-associated proteins, and breast cancer resistance protein that contribute to the development of MDR (Miron et al. 2017). The flavonoids are appropriate candidates to improve oral drug bioavailability and chemoprevention and overcome MDR (Pick et al. 2011; Michalak and Wesołowska 2012; Li et al. 2013; Gupta et al. 2014; Xu et al. 2015; Martínez-Pérez et al. 2016).

15.4.4.2 Coumarins

Coumarins are characterized as a member of the benzopyrone family (Jain and Joshi 2012). There are four major coumarin subtypes, namely, the simple coumarins, pyranocoumarins, furanocoumarins, and the pyrone-substituted coumarins (Zhao et al. 2016). Both natural and synthetic coumarins are of great pharmacological interest and hence attracted many researchers for the discovery of novel therapeutic products (Kumar et al. 2015). Current and the major challenges about coumarins are related to the translation of traditional knowledge into new leads against cancer (Riveiro et al. 2010; Barot et al. 2015; Umashankar et al. 2015).

15.4.4.3 Polysaccharides

Friedman (2016) presented a recent review about mushroom polysaccharides. In this review, the individual and as blended mixtures against obesity, diabetes, cancer, and infectious diseases are covered, and suggestions for further research are presented. Advances in antitumor activity from *Phellinus* were reported, and mechanisms were recently reviewed in Yan et al. (2017). Studies have suggested that polysaccharides obtained from *Phellinus* sp. are more promising alternatives for cancer therapy or can act as synergies for the available anticancer drugs.

15.4.4.4 β-Glucans

The Chinese Food and Drug Administration (SFDA) approved fungal glycan-based drugs (Zhou et al. 2014). This review article provides basic clinical information of the fungal glycan-based drugs in China and also summarizes structures, functions, and animal studies of fungal glycans conducted by scientists worldwide. Chen et al. (2013) have summarized the use of β-glucans in colon cancer. The use of β-glucans was considered an innovative strategy to deliver nanoparticles containing chemotherapeutic agents and, thus, improve the therapeutic efficacy. Meng et al. (2016) reviewed the mechanisms of isolated mushroom polysaccharides, particularly (1 → 3)-β-D-glucans, and described the function in modulating the immune system and potential tumor-inhibitory effects of β-glucans. The commercial

pharmaceutical drugs from β-glucans, such as schizophyllan, lentinan, grifolan, PSP (polysaccharide-peptide complex), and PSK (polysaccharide-protein complex), have shown evident clinical results (Ren et al. 2012).

15.5 Conclusions and Future Prospects

Endophytes include a wide range of microorganisms inhabiting the plant tissues. Endophytes have been proven as a good and reliable natural source for obtaining numerous biologically active metabolites including flavonoids, coumarins, polysaccharides, and β-glucans. Several strategies are adopted to characterize these compounds with various pharmacological significance. These symbiotic microorganisms may serve as a bioreservoir for obtaining numerous pharmaceutical drugs with anticancer potential. Currently, cancer treatment is challenged by the development of multidrug resistance to cancer chemotherapy. Hence, there is a continuous search of lead molecules that can overcome multidrug resistance. In this regard, endophyte-derived compounds have been found more attractive. The scientific community has screened a great number of medicinal plants and their endophytic symbionts until now. In spite of a long list of bioactive secondary metabolites from endophytes, the production and commercialization of these compounds are still incipient. The choice of the best methodologies should include the combination of sample extraction/preparation tools and analytical techniques to isolate and characterize bioactive secondary metabolites of endophytic microbes and their host plants. Thus, the endophyte biodiversity represents an unlimited source of novel bioactive compounds with potential as drug leads.

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Traditional Medicinal Plants and Their Therapeutic Potential Against Major Cancer Types

16

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Abstract

Cancer is highly prevalent in the world and affects millions of people. Lung, prostate, colorectal, and breast cancers are the most commonly identified among cancer subjects and account for more than half of all cancer deaths. Surgery, chemotherapy, radiotherapy, and hormonal therapy are the typical treatments for cancer; however, the side effects of these treatments can be excessive and vary widely according to subject's health issues. At present, traditional herbal therapy can be used in conjunction with conventional treatments. The use of anticancer plants in medicine is becoming increasingly relevant, as they can reduce the side effects of medical treatments and improve patients' quality of life. Various traditional medical practices, including Chinese medicine, Ayurveda, Kampo, Unani, and Korean medicine, use herbs as major ingredients of their practice, and the effectiveness of these traditional medicines has been acknowledged after modern scientific testing in many cases. The records of traditional anticancer plants used

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among various tribe, races, and nationalities are abundant. For instance, Liquorice root (*Glycyrrhiza glabra*) and snake-needle grass (*Oldenlandia diffusa*) served with hot water can help patients suffering from lung cancer. Likewise, *Astragalus membranaceus*, *Podophyllum hexandrum*, *Podophyllum petaton*, and *Arctium lappa* are commonly used all around the world to treat breast cancer. Colorectal cancer is treated with ashwagandha (*Withania somnifera*), garlic (*Allium sativum*), and ginger (*Zingiber officinale*). Therefore, there is an urgent need to document these potential ethnomedicinal plants in terms of anticancer drug discovery research. The scientific justification for these plants having a recuperative function in cancer must also be established. The main objective of this chapter is to provide an overview on anticancer plants used in traditional medicine to treat lung cancer, breast cancer, colorectal cancer, and prostate cancer.

Keywords
Cancer · Drugs · Ethnomedicine · Herbal therapy · Medicinal plants

16.1 Introduction

Cancer is one of the leading causes of morbidity and mortality worldwide, responsible for 8.8 million deaths in 2015 (WHO 2017). The most common causes of cancer death are lung cancer (1.69 million deaths), colorectal cancer (774,000 deaths), and breast cancer (571,000 deaths). Globally, nearly one in six deaths is due to some form of cancer, and while there were approximately 14 million new cases in 2012, the number of new cases is expected to rise by about 70% over the next 20 years (Ferlay et al. 2014). There are many types of cancer treatments available, such as surgery, radiation therapy, chemotherapy, immunotherapy, hormone therapy, and stem cell transplants. The primary goal of treatment is to remove or reduce the cancer or to considerably prolong life. However, persistent feelings of physical, emotional, or mental tiredness or exhaustion are common side effects of cancer treatments.

Traditional medicine has been practised over generations within various societies since well before the era of modern medicine; it is based on theories, beliefs, and indigenous experience and strives to maintain health and to treat illness (Swamy et al. 2011; Kumara et al. 2012; Akhtar et al. 2014a, b; Gezahegn et al. 2015; Swamy et al. 2016). Ayurveda, Siddha medicine, Yunani medicine, ancient Iranian medicine, Islamic medicine, traditional Chinese medicine, traditional Korean medicine, African Yoruba medicine, and traditional African medicine are some of the traditional methods followed to treat cancer. The core discipline or similarity among traditional medicine practices is herbalism, the use of plants and plant extracts for medicinal purposes (Swamy et al. 2017; Mohanty et al. 2017). This type of therapy is also widely adopted alongside conventional cancer treatments (Swamy and Sinniah 2015; Aung et al. 2017).

Currently, majority of the drugs available for the treatment of cancer are mainly derived from natural resources. Many of the approved anticancer drugs by The Food

and Drug Administration (FDA), USA, are either pure phytocompounds or their derivatives (Giddings and Newman 2013). A growing interest among researchers towards decoding prospective natural compounds from plants is mainly due to their lesser side effects and more specific to target sites with numerous mechanisms of action on cancer cells (Mishra and Tiwari 2011; Gali-Muhtasib et al. 2015; Aung et al. 2017). Considering the above facts, this chapter is aimed to update the information on the use of traditional medicinal plants and their curative properties against major cancer types such as lung, prostate, colorectal, and breast cancers.

16.2 Medicinal Plants as a Source of Anticancer Compounds

Medicinal plants have attracted global attention in recent years for their hidden therapeutic potentials (Swamy et al. 2017). Many plants have been investigated, and the parts to be used, along with processing and dosage instructions, have been richly recorded in the traditional medicine handbooks of many developing countries. The *Compendium of Materia Medica* is considered as the most complete and comprehensive medical book ever written in traditional Chinese medicine. It lists all types of the plants and the other items that were believed to have medicinal properties. Ayurvedic medicine and Unani are good examples of the oldest medical systems from ancient India, and those encouraged health promotion by means of herbal compounds and special diets. The African Yoruba and Islamic medical formulary described many preparations drawn from plants and other mineral sources. However, despite the rich ethnomedicinal knowledge that lies behind the traditional uses of these herbs, current scientific evidence to validate these medicinal claims remains scant (Swamy and Sinniah 2016; Mohanty et al. 2017). Continuing the quest for plant-based natural products is critical, however, and some medicinal plants have already been studied for their anticarcinogenic and antimicrobial activities. Several medicinal values imparted by plants are correlated to the occurrence of numerous bioactive compounds such as alkaloids, polyphenols, flavones, flavonoids, terpenoids, and many more (Arumugam et al. 2016; Swamy et al. 2017). Likewise, plants exhibiting anticancer and anticarcinogenic properties are reported to contain certain phytochemicals that have an inhibitory effect on human cells, including cancer cells; the most useful are, however, not toxic to humans. Multiple researchers have identified that most plants that have demonstrated anticancer properties are herbs that contain naturally occurring antioxidants such as polyphenols and brassinosteroids (Greenwell and Rahman 2015; Swamy et al. 2016; Mohanty et al. 2017; Aung et al. 2017). In the following section, these antioxidative compounds occurring in different plant species and their therapeutical potential against major cancer types are updated.

16.2.1 Plant Polyphenols

These are important components with chemopreventive and therapeutic properties that work against cancer. Polyphenol compounds include flavonoids, tannins, curcumin, stilbene resveratrol, and gallocatechins; these are all considered to be

anticancer compounds, inducing apoptosis in various cancer cell lines (Greenwell and Rahman 2015). Some of the major polyphenols and their anticancer activity against major cancers are discussed below.

16.2.1.1 Flavonoids

Flavonoids are secondary metabolites or plant pigments used for flower coloration and can thus be found ubiquitously in plants. Various plants that have been investigated for their anticancer properties, such as the fern species and plants used in traditional Chinese medicine, including litchi leaf (*Litchi chinensis*) and garlic (*Allium chinense*), are rich in flavonoid compounds. Litchi pericarp extracts demonstrated anticancer activity on breast cancer and hepatocellular carcinomas (Greenwell and Rahman 2015), while garlic contains quercetin and isorhamnetin, types of flavonoid that can control colon cancer cells and breast cancers (Lee et al. 2012; Del Follo-Martinez et al. 2013; Hu et al. 2015; Zeng et al. 2017). Parsley (*Petroselinum crispum, P. sativum*) seed extract kills breast cancer cells (Farshori et al. 2013), while black tea (*Camellia sinensis)* has been reported to contain theaflavin, thearubigins, and catechins that inhibit free radical generation (Balentine et al. 1997) and thus help to prevent chronic diseases such as cancer and cardiovascular problem (Łuczaj and Skrzydlewska 2005). Flavonoids are known to be physiologically active agents in plants and are thus becoming of high interest scientifically for their health benefits. Flavonoids manifest cytotoxicity on cancer cells and offer high free radical scavenging activity through intrinsic and extrinsic signalling pathways. They also inhibit and alter regulation of proteins and other agents that may contribute to the survival of cancer cells (Valko et al. 2006).

16.2.1.2 Tannins

Tannins, an astringent organic compound, are toxic to many microorganisms due to inhibition of oxidative phosphorylation (Scalbert 1991). It is responsible for the puckering feeling in the human mouth following the consumption of unripe fruits or tea (Vidal et al. 2004). Pomegranates (*Punica granatum*) have been widely studied for their potent antioxidants, which include ellegitannin, elegiac acid, and punicalagin, which are commonly known for their anti-proliferative and apoptotic effects on colon cancer cell lines (Seeram et al. 2005). Herbs such as cloves (*Syzygium aromaticum*) can also play important role in preventing and treating some cancers. Cervical, breast, prostate, and oesophageal cancer cells were killed within 24 h after the application of clove oil (Dwivedi et al. 2011). It was also found effective in treating lung cancer by means of in situ cell proliferation (Banerjee et al. 2006). Tarragon (*Artemisia dracunculus*) extract is indicated as cytotoxic to breast cancer though regulation of certain proteins that induce apoptosis (Obolskiy et al. 2011). Tarragon also contains potent compounds such as sakuranetin and 6-methoxycapilliarisin that appear to have anticancer effects on oesophageal cancer by inducing DNA damage in the cancerous cells (Hong and Ying 2015). Cumin (*Cuminum cyminum)* has been used since ancient times, and some studies have shown it to have a cytotoxic effect to colorectal cancer cell lines (Prakash and Gupta 2014; Al-Snafi 2016). Thyme (*Thymus vulgaris)* essential oil has been claimed to demonstrate cytotoxic activity

on head and neck squamous cell carcinomas by interrupting reproduction at the transcriptional level of cancer cells, with effects such as interfering in N-glycan biosynthesis and extracellular signal-regulated kinase 5 signalling (Sertel et al. 2011). Cinnamon (*Cinnamomum* sp.) is most commonly known as culinary spice, yet it is also used for its medicinal properties in treating gastrointestinal sickness. Cinnamon bark extract was evaluated for anticancer ability, and it was found to induce apoptosis in HepG2 cells (liver cancer cells) after 24 h at certain dosages (Varalakshmi et al. 2014). An extract of cinnamon, 2′-hydroxycinnamaldehyde, shows several antitumour effects on oral cancer cell lines; this is demonstrated by anti-proliferative activity of apoptotic cells and inhibition of tumour mass growth (Kim et al. 2010).

16.2.1.3 Curcumin

This compound is a yellow pigment produced by plants, mostly by those in the ginger family (Zingiberaceae). Curcumin has enormous potential in terms of cancer prevention and treatment, and numerous studies and reviews described it as a potent antioxidant and anti-inflammatory agent (Aggarwal et al. 2003; Agrawal and Mishra 2010). It inhibits biochemical activity, restraining overexpression of some signalling pathways and regulating the expression of tumour suppression genes (Creţu et al. 2012). Temu kunci, or galangal (*Boesenbergia pandurata*), is a rhizome generally used in cooking that can also be prepared to treat diarrhoea and mouth ulcers. It has been proven non-toxic to human skin fibroblast cells and offers protective effects against colon cancer (Kirana et al. 2007). Turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*) are two plants that contain an abundance of curcumin and which have been investigated for their therapeutic properties. One piece of research, for example, showed that ethanolic extract of turmeric showed anti-melanoma activity against malignant melanomas (Danciu et al. 2015).

16.2.1.4 Stilbene Resveratrol

It is present in several dietary materials such as grapes (*Vitis vinifera*) (Burns et al. 2002). Stilbene compounds such as trans-astringin and trans-piceatannol are also found in wine, and these show potential in terms of anticancer chemopreventive activity (Waffo-Téguo et al. 2001). Ban-Zhi-Lian (*Bauhinia racemosa*) was also examined and found to have significant anticancer activity on lung cancer, ovarian cancer, prostate cancer, and breast cancer cell lines, as well as aiding the treatment of leukaemia. Extracts of this plant contain anthraquinone, saponins, terpenoids, and alkaloids that show 90–99% cell growth inhibitory activity on various human cancer cell lines (Mishra et al. 2013). Pirandai (*Cissus quadrangularis*) has been used to treat skin diseases; herbal extracts of pirandai showed significant anticancer activity on skin cancer cell lines, thanks to the release of cytochrome c, which induces apoptosis (Bhujade et al. 2013). Ethanolic extracts of pirandai, which contain various secondary metabolites such as phenols and tannins, demonstrated anticancer activity on breast cancer (Ruskin et al. 2014). Mugga (*Eucalyptus* sp.) has been traditionally used for a variety of medical purposes, including cancer treatment. One piece of research showed that this plant presented weak to moderate cytotoxicity to non-lymphoma tumour cell lines and potent anticancer activity to lymphoma tumour cell lines (Bardaweel et al. 2014).

16.2.1.5 Gallocatechins

Gallocatechins are found in green tea, redcurrants, gooseberries, and marrowfat peas. The consumption of these suggested reducing the risks of various cancers such as those of the bladder, prostate, and oesophagus (Greenwell and Rahman 2015). Tea is one of the most popular beverages in the world, and most of the cultures whose diets are rich in green tea have proportionately fewer cases of certain cancers (Sivakumar et al. 2010). Green tea (*Camellia sinensis* var. *assamica*) polyphenols, particularly the gallocatechins, are important antioxidants that offer chemopreventive effects against cancer (Takeo 2015). Another piece of research showed that consumption of green tea was closely related to a reduction in the risk of deaths due to cancer (Jankun et al. 1997). Redcurrant (*Ribes rubrum*) in freeze-dried form shows preventive functions in terms of many cancer cell lines; this is expressed through cell cycle arrest and the interruption of gene expressions of cell division enzymes (Lim 2012). The potent inhibition of cancer cell lines such as stomach, prostate, intestine, and breast cancer cell lines offers promising anti-proliferative protection against cancer cell lines (Boivin et al. 2007).

16.2.2 Brassinosteroids

These compounds are potent hormones that arise from naturally occurring compounds found in plants; these play a role in promoting high-growth activity and enhance resistance and tolerance to disease and stress. They are found at all young vegetative tissues throughout the plant kingdom (Bajguz and Tretyn 2003). Recently, these compounds were proven to inhibit cell growths in breast cancer cells without any side effects to non-tumour cell growth. This was achieved through interactions with the cell cycle machinery (Steigerová et al. 2010). Another study on the anti-angiogenic activity of brassinosteroids showed that they can react with human steroid receptors and produce novel combinations of activators and inhibitors to inhibit anti-genesis by reducing the mobility of tumours to prevent metastasis (Rárová et al. 2012). A key specification in anticancer treatments is for the agent not to be cytotoxic to normal cells and be cell specific to cancer cells. Another piece of research also found that brassinosteroids inhibited both breast and prostate cancer cell lines by arresting cancer cells in the G1 phase cell cycle and inducing apoptosis without affecting normal cell growth (Malíková et al. 2008).

16.3 Natural Anticancer Compounds and Pharmaceutical Industry: Current Status

Natural products have traditionally been important sources of pharmaceuticals. New technologies to produce synthetic chemistry products by conventional pharmaceutical industrial means have met with some drawbacks. The disadvantages of conventional medicine are that sickness often can't be completely treated, only temporary control can be offered as a result, and most importantly, patients with

cancer can't be cured by taking medication. To treat cancer, surgical treatments, radiotherapy, and chemotherapy are used. The benefit of these treatments is the removal of cancer cells; these are systemic treatments and are widely adopted. However, these treatments often cause traumatic effects, as well as being expensive, and they may shorten or risk the survival of the cancer patient in some cases. To date, there is no medical approach to completely cure cancer, and therefore the pharmaceutical industry has begun to pay attention to natural products to find new bioactive substances. In terms of cancer treatment, there are many benefits to traditional medicine. Many people believe that herb consumption is less toxic, with minimal side effects, although, of course, this depends on the herb being consumed. Traditional medicine treatments aim to enhance the patient's immune system and to inhibit the recurrence or metastasis of cancer cells. However, there is a large degree of uncertainty around herb-based medication; many new compounds isolated from natural products have only been tested in regional or local healing practices, and many are adopted without thorough testing or clinical evidence, with minimal or no industrial processing; few of these compounds or functional herbs are ever been registered and marketed (Fabricant and Farnsworth 2001).

Several notable examples of herbs that have been proven to control certain cancers effectively have been thoroughly studied, clinically attested, and are now processed in the pharmaceutical industry. These are kanglaite, Pacific yew, happy tree, and triphala. Kanglaite (*Coix lacryma-jobi*) is commonly known as Job's tear. Those have received significant attention in the global health market as the first drug derived from a traditional Chinese herbal remedy that has been shown to successfully control cancer cells and notably improving patient immune function. This herb was registered as KLT in China, the USA, and Russia. It is prepared in a fat emulsion formulation, and it is administered via intravenous injection and intraarterial perfusion. The injection of this herbal extract has been undertaken on more than 200,000 patients to treat cancer in China. Clinical trials on breast carcinomas, lung cancer, liver cancer, oesophageal cancer, pancreatic cancer, kidney cancer, gastric cancer, colorectal cancer, ovary cancer, and prostate cancer show improved therapeutic effects and evidence of prolongation of life (Normile 2003; Lu et al. 2008; Li et al. 2009; Zhu et al. 2009; Shan et al. 2012; Zhan et al. 2012).

A striking example among anticancer herbs is the Pacific yew (*Taxus brevifolia*). This plant contains the antitumour agent taxol, which was first isolated in 1971 (Wani and Taylor 1971). The plant is a potentially limited source that was already becoming scarce when its chemotherapeutic potential was realised. It was approved for medical use in 1993 (Fischer and Ganellin 2010). Large-scale production and commercialization have promoted plant extract production using a semi-synthetic pathway or attempt to use plant extracts from alternative species of *Taxus*, which can then be converted into taxol (Witherup et al. 1989). This compound was commercially developed by Bristol-Myer Squibb; taxol is the generic name, and the brand name for the commercial version is Paclitaxel. The chemotherapy drug paclitaxel (taxol) is used in breast, ovarian, and lung cancer treatments (McGuire et al. 1996; Sandler et al. 2006; Miller et al. 2007).

Happy tree (*Camptotheca acuminata*) is a tree native to China that is used as a cancer treatment in traditional Chinese medicine. It was discovered in 1966 as part of a systematic screening of natural products for anticancer drugs (Wall et al. 1966; Carte et al. 1990). Camptothecin was isolated from bark and stem of this tree, which showed remarkable anticancer activity in preliminary clinical trials (Potmesil and Pinedo 1994). Further clinical research found it to affect human malignancies such as colorectal and ovarian cancer (Shimada et al. 1993; Slichenmyer et al. 1993; Takimoto et al. 1998). Camptothecin is now manufactured by many pharmaceutical companies of India such as Brios Pharma, Altavista Phytochemicals Private Limited, and Green India Herbs.

Triphala, otherwise known as tree fruits, is an Ayurvedic herbal formula consisting of three plants: Amalaki (*Emblica officinalis*), Bibhitaki (*Terminalia bellirica*), and Haritaki (*Terminalia chebula*). The active compounds in triphala are gallic acid, chebulagic acid, and chebulinic acid (Naik et al. 2006; Gupta 2012). It is taken to promote internal cleansing in all stagnating conditions and to improve digestion; it is generally consumed as a food supplement rather than taken as a medication to treat cancer. Triphala is a product of the Himalaya Drug Company, India, and is sold solely for digestive care. In terms of clinical use, triphala aqueous extract has been tested for cytotoxic effects on many cancer cell lines, such as breast cancer (Sandhya et al. 2006); the anticancer activity of triphala appears to be part of a synergistic effect of the combination of plant extracts; each extract was also tested to see whether it induced apoptosis, had antimutagenic effects, or activated cell death programme on cancerous cells (Wongnoppavich et al. 2009). There is a great deal of research evidence to show that triphala can treat cancer *in vitro*; however, more clinical research is required before triphala can be considered as a treatment for cancers.

16.4 Traditional Medicinal Plants in the Treatment of Major Cancer Types

16.4.1 Lung Cancer

16.4.1.1 Astragalus (*Astragalus membranaceus*)

This is one of the most widely used herbs in traditional Chinese medicine; it is known in Mandarin as *Huangqi*. It is a general immune booster and thus helps to reduce the immune suppressing effects of chemotherapy, as well as enhance the effects of cisplatin and carboplatin during chemotherapy (Guo et al. 2012). Research has found astragalus to increase effectiveness of platinum-based chemotherapy on cancer patients with compromised immune systems as a result of said chemotherapy (McCulloch et al. 2006). The Chinese Materia Medica recommendations for astragalus doses are 9 to 15 g/day (used in combination with other herbs), and the prescription may vary according to the severity of the illness. Patients taking astragalus supplements experienced enhanced recovery times and improved survival rates due to the reduction in chemotherapy toxicity; this is therefore a promising

adjunctive treatment for cancer. This herb is revered for its ability to boost the immune system by promoting white blood cell count and by encouraging proliferation of antibodies to strengthen antiviral immunity (London 2010).

16.4.1.2 Adenophora (*Radix adenophorae*)

This is a Chinese herb "nan sha shen", also known as American silver to proot, which is used to nourish the yin. It is commonly used for the lungs, to clear up lung heat, dissolve phlegm, and relieve coughs (You Li 2010). According to traditional Chinese medicine, Adenophora is sweet, slightly bitter, and cold, and its main functions are to treat coughs, bronchitis, and pulmonary infections (Sahashi 2005). It is often used in herbal cough remedies for children (Liu 2009). Adenophora is considered safe and can be found in almost all herbal shops. The American herbal products association has given it a class 1 rating, means it can safely be consumed when used appropriately (Gardner and McGuffin 2013).

16.4.1.3 Liquorice Root (*Radix glycyrrhizae*)

It is known in Mandarin as "gancao". In Chinese medicine, it is commonly combined with other herbs in a single prescription to tone the lungs and spleen and to relieve coughs and shortness of breath (Wang et al. 2013). It can mitigate the effects of various foods, herbs, drugs, and chemical poisonings (Fiore et al. 2005). In decoction for lung relief, it is paired with Folium Mori "Sang ye" and other herbs, to moisturise the lungs (McNamara and Song 1995). It is clinically proven to accelerate mucus secretion and sooth lung inflammation (Aly et al. 2005). From a research perspective, a substance extracted from liquorice root, licochalcone A, has been shown to have antitumor activity in lung cancer (Shibata et al. 1991; Asl and Hosseinzadeh 2008).

16.4.1.4 Poria (*Wolfiporia extensa*)

This is known in Mandarin as "fu ling". It is actually a fungus whose filaments are stored and used as medicine. It has been used medicinally in China for long-term health care by consuming it on daily basis (McNamara and Song 1995). Traditional Chinese medicine believes this herb is sweet and tasteless in flavour and neutral in terms of properties. One of its main uses is to relieve choking coughs caused by phlegm (Wu and Fischer 1997). It has also been used in various herbal combinations to treat diarrhoea, kidney inflammation, and gastrointestinal tract bleeding (Huang 1998). Poria extract contains triterpene and polysaccharide fractions that have demonstrated anticancer action through the mechanism of down regulation of nuclear factor-kappa B activity and its signalling pathway, as well as having anti-angiogenesis properties and inducing apoptosis (Poucheret et al. 2006; De Silva et al. 2012). The cytotoxicity of poria in formulations against lung cancers has been proven efficient (Leem 2015).

16.4.1.5 Snake-Needle Grass (*Oldenlandia diffusa*)

This is known as "bai hua she she cao". This herb has demonstrated as anticancer and chemopreventative effects in both laboratory and animal studies (Wong et al.

1996; Song et al. 2004). The herb reduces lung metastasis when orally administered (Gupta et al. 2004). Studies reported anti-inflammatory effects through the reduced production of tumour necrosis factor alpha, interleukin-6, and prostaglandin-2, all of which are commonly over expressed in mesothelioma cancers (Yoshida et al. 1997; Gupta et al. 2004). Although several lab and animal studies have shown this herb to have anticancer effects, clinical investigation in humans has failed to produce similar evidence.

16.4.1.6 Asparagus Root (*Asparagus officinalis*)

Wild asparagus root is known as "tian men dong" in traditional Chinese medicine. It is used to relieve asthma, suppress coughing, and promote expectoration (Huang 1998). It is held to be sweet and bitter in flavour and cold in nature, nourishing the lungs and moistening dryness (McNamara and Song 1995). Though studies conducted on asparagus root to examine its biological effects have only been conducted on animals, the evidence so far shows anticancer activity against leukaemia and lung cancer by means of the inhibition of tumour necrosis factor alpha (Huang et al. 2008).

16.4.1.7 Jin Fu Kang

It is a blend of 12 herbal extracts formulated against lung cancer by researchers at the Shanghai University of Traditional Chinese Medicine, China. It has been shown that lung cancer patients who receive a combination of jin fu kang and chemotherapy survive at higher rates. It is also commonly used to improve blood circulation and alleviate pain (Liu et al. 2000). It contains Pueraria root, notopterygium, frankincense, myrrh, earthworm, salvia root, ligusticum root, white peony root, and wingless cockroach. The administration method involves 5-gram sachets, dissolved in lukewarm boiled water, and taken orally after a meal, one to two sachets each time, twice a day (McNamara and Song 1995). However, this medication is not suitable for pregnant women or patients with gastric ulcers or hypertension (Yang et al. 2009). It was developed at the Shanghai University of Traditional Chinese Medicine specifically for the treatment of lung cancer. The formula has been clinically tested for decades and was approved by the Chinese Drug Administration in 1999. Lung cancer patients undergoing chemotherapy who also take jin fu kang show increased survival rates when compared to those undertaking chemotherapy treatment alone (Liu and Sun 2007).

16.4.1.8 Yang Zheng Xiao Ji

It is a formulation of 14 herbs used to treat cancer in the traditional Chinese medicine. It contains extracts of *Radix astragali*, *Fructus ligus, Fructus ligustrilucidi*, *Radix ginseng*, *Ganoderma lucidum*, *Rhizome curcuma*, *Rhizoma atractylodes*, and *Hedyotis diffusa*. It is also used to improve the appetite and immune system. It has been found to restrain tumours from spreading through inhibiting cell adhesion and migration, and it has a marked effect on angiogenesis (Jiang et al. 2012). In combination with chemotherapy, it has been shown to increase survival rates and reduce side effects (Jiang et al. 2015).

16.4.2 Breast Cancer

16.4.2.1 Garlic (*Allium sativum*)

For many centuries, garlic has been used to treat a range of illnesses (Gupta et al. 2004). Garlic oil contains a sulphur-holding substance known as ajoene oil or allicin. This oil has been found to control cancer cell production. The anticancer activity of garlic is thus due to its high levels of organic sulphides and polysulphides. The mechanism behind the antitumour activity triggered by stimulating the lymphocytes and macrophages is that they kill cancerous cells and interfere with tumour cells' metabolism. The administration method involves taking 5–6 cloves of crushed garlic, around 5 g/day (Kapoor 2000). The crushed cloves should sit for at least 15 min to release the allinase enzyme before consumption (Shareef et al. 2016).

16.4.2.2 Purple Coneflower (*Echinacea* sp.)

This herb was used by native Americana to cure wounds and infections. It is processed into either comminute or powdered substances, to serve as a tea for oral use or packaged into solid dosage form. Then dry extract is extracted using ethanol and encapsulated into 170–470 mg capsules. In powder form, it is packed 250 mg/capsule (Gupta et al. 2004). This herb is also sometimes served as a liquid tincture (Barnes et al. 2005).

16.4.2.3 Burdock (*Arctium lappa*)

Burdock is a plant that is sometimes used as a food. The root, leaf, and seeds are also used to make medicine. It has been used to treat variety of ailments, including diabetes and hair loss, and it is said to kill germs and reduce fever. It is also used for high blood pressure and sometimes to increase the sex drive. In traditional European medicine, this herbal preparation can be comminute, powdered, made into a tincture, and used as a soft extract. A European powdered capsule contains 350 mg/capsule, and the dose for liquid extracts is 25–50 drops, 1–3 times/day. For tinctures, 50 drops, 1–3 times/day, are recommended. The dry extract dose is 1 g/day (Gupta et al. 2004). As an Ayurvedic medicine, 40 mg/tablet of powered burdock is served. Its roots are also used in therapeutic remedies (Poucheret et al. 2006).

16.4.2.4 Green Tea (*Camellia sinensis*)

The unfermented cut young dried leaves of *Camellia sinensis* are used to produce various types of teas. They contain less than 2% by weight of caffeine and are generally taken by mouth to improve mental alertness and thinking (Zaveri 2006). Herbal preparations can be either comminuted or powdered. Green tea is rich in flavonoid compounds and amino acids (Wang et al. 2000), and green tea is well documented in European herbal medicine as a remedy for sickness. In capsule form, each contains 250–465 mg, and 2 tablets/day are recommended (Gupta et al. 2004). However, population research suggests that drinking green tea is not linked to a reduced risk of breast cancer in Asian people, though green tea may have different protective effects in people depending on their genotypes. However, drinking green tea does seem to be linked with a reduced risk of breast cancer recurring (Wu et al. 2003; Zhang et al. 2007).

16.4.2.5 Ginseng (*Panax ginseng*)

Ginseng can refer to either white or red ginseng, and herbal preparations vary according to the type. In European herbal medicine, ginseng is powdered into 300 mg/capsule, with 2–3 capsule/day dosage (Lust 2014). For ginseng in dry extract, 15 ml of oral liquid is given once daily. Each 15 ml of oral liquid contains 140 mg of dry extract (Kapoor 2000). American ginseng is also listed as an ingredient in some soft drinks, and its oil and extracts are used in soap and cosmetics (Gupta et al. 2004). There is a little evidence in terms of clinical research to support ginseng treating breast cancer (Shin et al. 2000). However, research conducted in China suggests that patients treated with any form of ginseng maintained better psychological condition. Thus, it might be more appropriate to say it has benefits in terms of supporting and minimising damage from the cancer drug tamoxifen in treatment (Cui et al. 2006).

16.4.2.6 Ashwagandha (*Withania somnifera*)

This is a common herb in Ayurvedic medicine, and it is known as Indian ginseng. Traditionally, the berries and leaves are applied externally to tumours or tubercular swellings. The roots are powdered and mixed in milk and honey to treat burns and wounds. Extracts of Ashwagandha are taken as a supplement in 300–500 mg/tablets, 3 times/day (Kapoor 2000). Ashwagandha can also be taken as powder served with milk (Mishra et al. 2000).

16.4.2.7 Mahogany (*Dysoxylum binectariferum*)

This is used in Ayurvedic medicine for its anti-inflammatory and immune regulatory properties (Kapoor 2000). Several pieces of research have shown evidence of its potential in terms of anticancer activity (Cragg and Newman 2005; Jain et al. 2014). This plant contains the compounds required to act as a substitute for the synthetic flavopiridol medication used to control breast cancer (Kumara et al. 2012). The juice of mahogany is thus served as traditional medication to cure cancer (Kapoor 2000).

16.4.2.8 Bearberry (*Vaccinium macrocarpon*)

The fruits are edible and juiced for medicinal purpose by herbal practitioners. Bearberry tea is a traditional herbal treatment in northern Europe and Eurasia. It is served in traditional medicine to cure free radicle damage in the body and to prevent breast cancer. The most prominent function of bearberry juice is in treating urinary tract infections and kidney stones (Lust 2014).

16.4.3 Colorectal Cancer

16.4.3.1 *Aloe vera* (Synonym: *Aloe barbadensis*)

A. vera is an herbal remedy promoted as treating a variety of illness (Lust 2014). These two herbal remedies act together as a means to cleanse the colon and eliminate toxins which could accumulate in the digestive tract causing disease; they also function as an anti-inflammatory agent (Kapoor 2000).

16.4.3.2 Celandine (*Chelidonium majus*)

It is a member of poppy plant family. The parts that grow above the ground are used to make medicine(Lust 2014) and can be comminuted into a tea infusion or taken as around 1.2–3.6 g in a tincture with 45% ethanol or 2–4 ml of a 1:10 preparation three times/day (Kapoor 2000). Celandine has been used to treat scurvy and promote diuretic activity (Gilca et al. 2010; Dumbravă et al. 2008). This herb is also used to treat asthma (Vavrečková et al. 1996). Celandine extract can be used externally as eye drops in suitable organic solvents. It is also applied to the skin to treat bleeding wounds, swollen joints, and warts (Kapoor 2000).

16.4.3.3 Ginger (*Zingiber officinale*)

It is an Ayurvedic herb and used in many Indian dishes (Kapoor 2000). Inflammation markers that have been proved in clinical research to act as precursors to colon cancer can be reduced significantly by the consumption of ginger powder or ginger roots (Abdullah et al. 2010). A powerful anti-inflammatory, ginger soothes and heals the digestive tract (Stoilova et al. 2007). Based on research findings, ginger can decrease the level of inflammatory markers in the gut tissue; increased inflammation and chronic inflammation in the gut are highly associated with developing precancerous lesions or cancerous polyps (Abdullah et al. 2010), and thus ginger has been suggested as one of the best home remedies for the prevention of colon cancer.

16.4.3.4 Turmeric (*Curcuma longa*)

In Ayurvedic practices, this has been used as attempted treatment for a variety of internal disorders such as indigestion, throat infection, and the common cold (Kapoor 2000). Curcumin is the active ingredient in the turmeric, and research has demonstrated that when colon cancer cells are pre-exposed to curcumin and then treated with silymarin, the cells undergo a higher rate of cell death (Akram et al. 2010). Some herbalists use turmeric to prevent and treat colon cancer. There is no absolute administration of turmeric dosage to treat colorectal cancer, but the low cancer rate in India may be related to turmeric consumption in most people's diets (Potter et al. 1993; Mohandas and Desai 1998).

16.4.4 Prostate Cancer

16.4.4.1 Cannabis Oil (*Cannabis sativa*)

Cannabis is a plant that has been used for medicinal purposes for a many years. It is commonly known as marijuana, hemp, or cannabis (Lust 2014). Cannabis has many bioactive compounds; the cannabinoids have been identified as potential powerful natural cures for prostate cancer (Sarfaraz et al. 2005). Most prostate cancer patients have an inhibitor of DNA binding 1 (ID-1) gene that is the causal factor for the aggressive and uncontrolled multiplication of cancer cells (Ouyang et al. 2002). Scientifically, cannabinoids were found to inhibit the ID-1 gene and reduce the number of cancer cells. Cannabis is also helpful in reducing cancer-related side effects (Hermanson and Marnett 2011). However, some crude cannabis oils may be

hallucinogenic; therefore, the stalks and sterilised seeds of this plant should be used to produce hempseed oil, which is less hallucinogenic and without cannabinoids. This hemp oil is used to control inflammation and reduces neurological problems. The traditional medicine suggests consumption of 60 g or 60 ml/day for up to 90 days (Lust 2014). However, consumption of cannabis or hemp oil can lower the blood pressure, and it is not advisable to ingest cannabis directly. The oil can also have a bad taste that lingers in the mouth (Ruixing et al. 2008).

16.4.4.2 Saw Palmetto (*Serenoa repens*)

This plant's ripe fruit is used to make medicine. This plant is rich in fatty acids and phytosterols (Lust 2014). It has been used in traditional medicine to treat benign prostatic hyperplasia and to decrease the symptoms of an enlarged prostate (Tacklind et al. 2012). This herb has repeatedly been shown, in multiple studies, to reduce prostate cancer inflammation and enlargement. It appears to be completely safe to use, with no side effects. It also works for over 90% patients after 4–6 weeks (Champault et al. 1984). Traditional medicine suggests a dose of 320 mg of saw palmetto, daily for 2 months before prostate surgery to minimise the risk of surgery and blood loss (Lust 2014).

16.4.4.3 Cayenne Pepper (*Capsicum annuum*)

It is also known as the cow-horn pepper, guinea spice, red hot chili pepper. Cayenne is used in cooking, as a powder or in its whole form. Cayenne consumption dilates blood vessels and speeds the metabolism due to high levels of capsaicin (Lust 2014). Capsaicin has a profound anti-proliferative effect on human prostate cancer, as well as inhibiting tumour necrosis factor and inducing apoptosis through regulation of many gene expressions (Mori et al. 2006). Capsaicin was found to increase the levels of certain proteins involved in apoptosis and also reduced the number of prostate-specific antigens (Scher et al. 1999). Other benefits include helping to prevent ulcers, opening and draining congested nasal passages, and reducing cell damage that can lead to diabetic complications (Sánchez et al. 2007; Ramos-Torres et al. 2015). Herbalists suggest 400 mg/day, three times/week of capsaicin to treat prostate cancer (Kapoor 2000).

16.4.4.4 Stinging Nettle (*Urtica dioica*)

This is a plant grows in North America, Europe, and Africa that has been used as an herbal remedy for thousands of years. The name comes from the stinging sensation that arises when parts of the body brush against the plant's hairy stems and leaves (Lust 2014). Its leaves have been used successfully to reduce symptoms associated with prostatitis in Europe for over a decade. Prostatitis is a noncancerous condition that causes the prostate gland to enlarge, making urination difficult (Shoskes 2002). Nettles can be eaten on their own or as an ingredient in foods; the leaves must be cooked or steamed to destroy the hairs, which contain a number of irritating chemicals (Lust 2014).

16.4.4.5 Black Seed (*Nigella sativa*)

This plant's seeds have often been used to make medicines to treat headache and toothache (Lust 2014). It also manifests some potent antitumour and anticancer properties (Khan et al. 2011; Randhawa and Alghamdi 2011). This plant has been studied extensively in terms of treating cancer, as many herbalists adopt this natural treatment for patients with prostate problems (Gilani et al. 2004).

16.4.4.6 Lycopene

This is a naturally occurring chemical that manifests as a red pigment contained in common foods such as tomatoes, pink grapefruits, guava, and watermelon (Giovannucci 1999). This is a very strong antioxidant that has been found to prevent and even reverse the progression of prostate cancer, as well as treating benign prostatic hyperplasia. In a recent study, 30 mg a day of lycopene showed curative results in prostate cancer. For best results, supplements are recommended alongside eating and drinking plenty of lycopene-containing food and juices (Jatoi et al. 2007). Earlier research showed that taking a specific combination of lycopene, selenium, and saw palmetto by mouth for 8 weeks reduced pain in men with prostate swelling and pelvic pain more significantly than saw palmetto alone (Feifer et al. 2002).

16.5 Medicinal Plants: Safety, Toxicity, and Adulteration Issues

Herbal products have been used as medicines for centuries, and their use has continued into the current age. In recent years, herbal medicine has once more become popular, and it is widely adopted in Western culture; this has drawn the attention of the research community to the potential of the herbs used in Asian and African traditional medicines also. Using herbal medicines as a primary treatment or as an adjunct treatment to pharmaceutical remedies is a feasible approach, but the latter combination may be the preferred choice to benefit from the synergistic effects of both treatments, which may yield an improved curative effect and minimise the side effects of conventional treatment (Girard and Vohra 2011).

Although many herb users reported the benevolence of traditional treatments in terms of preserving good health, treating sickness, and mitigating the side effects of conventional medication, most herbal remedies have not been extensively investigated in clinical settings (Bateman et al. 1998). Those, caution should be taken when using herbs for fear of aggravating health conditions; the safety of patients must be the first priority. Clinical effort should be expended to consider all the advantages and hazards to a patient in order to achieve best recuperative results. Herbal medicines may pose harm to patients for several reasons, including allergic reactions, drug-herb interactions, adulteration of products, and contamination of substances (Girard and Vohra 2011).

16.5.1 Allergic Reactions

Some patients may suffer an adverse effect to any medication. This is more commonly found in in asthma sufferers, pregnant woman, and children. Allergic reactions to herbal remedies may be due to allergens present in some pollen or other plant parts. Plants belonging to the genus, *Echinacea*, can cause stinging of the tongue or transient burning sensations if eaten uncooked or semi-cooked, and some patients suffer from pollen-induced asthma on use. Although the benefits of *Echinacea* species are well documented in traditional practice, the most common side effect is an upset stomach (Damato et al. 2007). Other people may be truly allergic to this herb, developing symptoms such as rashes, worsening of asthma symptoms, and even anaphylaxis (trouble breathing). Patients are at higher risk of having a reaction to *Echinacea* if they are allergic to Asteraceae family plants such as ragweed, marigolds, and chrysanthemums (Bauer 1998). *Aloe vera* was reported to cause abdominal pains and diarrhoea and to be potentially carcinogenic when taken with other medication such as cardiac glycosides and antiarrhythmic agents (Blumenthal 2000). Liquorice root (*Glycyrrhiza glabra*) may cause hypokalaemia, hypertension and arrhythmias, and oedema in some patients (Mumoli and Cei 2008). Saw palmetto can cause gastrointestinal upsets, diarrhoea, gynecomastia, and paroxysmal atrial fibrillation (Ernst 2002). Burdock may cause allergic reactions in people sensitive to certain flowers and herbs, again including chrysanthemums, marigolds, and daisies, causing rashes on the skin. It can slow down blood clotting and may increase the risk of bleeding in people with bleeding disorders, as well as increasing the risk of bleeding during and after surgery. Taking burdock might lower sugar levels, which is potentially dangerous in diabetes patients who are already under medication to lower blood sugar levels (Chan et al. 2011).

Large doses of ashwagandha can upset the stomach, causing diarrhoea and vomiting. It can also cause more serious side effects in some people, such as abnormal heart rhythms, breathing problems, and sedation. Unprocessed celandine can cause mucous membrane and skin irritation. Direct consumption may cause severe irritation of the stomach and intestines, and liver damage and acute hepatitis have been reported (Benninger et al. 1999; Gilca et al. 2010). Side effects of taking cannabis oil include memory loss and random thoughts. It causes an increased heart rate and imbalances in the body (Hall and Degenhardt 2009; Volkow et al. 2014). Stinging nettle (*Urtica dioica*) leaf may cause burning and itching or even a rash on sensitive skin. It may also cause low blood pressure, fluctuation of blood sugar levels, and digestive discomfort (Setty and Sigal 2005). Some patients reported that it also encourages bleeding and can cause uterine contractions. It is thus risky to take stinging nettle if pregnant or breast feeding (Westfall 2001). It may also interact with some other medications and is not recommended for patients taking blood thinning diuretics, blood pressure control medication, and anti-inflammatory drugs. It also interacts with alpha-blockers and finasteride (Calahan et al. 2016).

16.5.2 Drug-Herb Interaction

From the perspective of drug-herb interactions, herbal products can frequently have drug interactions with prescription medications. Some herbal medication is privately processed and not verified or licenced by authorities; such products are not labelled with safety warnings. Herbs with potent chemicals may breakdown drugs' components in a patient's body, causing side effects from prescription medication, and blocking the intended therapeutic effects of drugs. Echinacea is commonly known for its interactions with drugs, slowing down the breakdown of caffeine and causing side effects including nervousness, headaches, and insomnia (Bauer 1998). Saw palmetto, which is popular in treating prostate cancer, is not complimentary with finasteride. Saw palmetto can also interact with warfarin to cause bleeding and slow blood clotting. If saw palmetto is taken with oestrogen or oral contraceptives, the effectiveness of these hormones could be reduced (Rowland and Tai 2003). Ginseng is used for improving vitality but was reported to have many drug interactions; it should not be used with anticoagulants, insulin, or oral hypoglycaemics (Luo and Luo 2009). Garlic is a culinary flavouring agent, but it has an additive effect with warfarin that affects blood sugar level and blood clotting in patients; garlic may also react with clopidogrel (Vaes and Chyka 2000). Green tea, a popular drink in China and Japan to prevent stomach disorders, interferes with the activity of some blood thinners such as warfarin. Ginger is also another example of culinary herb that prolongs bleeding and may have interactions with warfarin and aspirin that cause iris bleeding (Vaes and Chyka 2000).

16.5.3 Adulterants in Herbs

Adulteration usually refers to non-compliance with health and safety standards, making the herbs directly harmful or reducing their potency so that they are harmless but ineffective. The most commonly used form of adulteration by manufacturers is the addition of undeclared materials that are cheaper than the declared substances (Ko 1998; Swamy and Sinniah 2016; Mohanty et al. 2017). The chemical and biomedical analysis methods used by authorities to detect chemical adulterants in herbal medication are liquid chromatography, gas chromatography, flow injection, and capillary electrophoresis. Several review papers are discussed on herbal screening to identify such additives (But 1994). Ginseng is now banned by the Food and Drug Administration, USA, as it frequently contains dexamethasone (a corticosteroid) and chlorpheniramine. Dexamethasone can cause immunity impairment, increase blood sugar levels, and cause psychiatric complications (Calahan et al. 2016). Corticosteroids are the most common adulterant reported in many Chinese herbal medicines (Ernst 2002). A report from Taiwan showed that 1/5 of samples investigated were contaminated with at least one conventional pharmacological adulterant. This reveals that adulterants are potentially a large problem for traditional medicine (Ernst 2002).

16.5.4 Contaminated Herbal Medication

There has been an increasing concern over food safety and traceability, and in terms of herbal medication, while herbs are used for the prevention of ailments and treating sickness, the lack of safety and potential toxicity of several herbs available on the market have shaken some people's confidence in the field. Many research projects screening for heavy metals found lead and arsenic to be major problems in herbal preparations (Mitchell-Heggs et al. 1990; Swamy and Sinniah 2016). Heavy metals such as zinc, copper, iron, and manganese are essential nutrient in micro quantities; however, above certain permissible limits, the presence of these heavy metals in herbal medication can become toxic and potentially lethal to sensitive patients (Byard 2010; Dghaim et al. 2015). Some Chinese herbal medicine is reported to cause heavy metal poisoning, where metals exceed acceptable standard consumption for daily intake (Ernst 2002). In Ayurvedic medicinal herbs, lead, mercury, and arsenic contamination have been reported (Saper et al. 2004). Herbal remedies are rapidly gaining popularity throughout the world, but there is dissatisfaction with the production standards currently used. Most people have the conceptual idea that herbal preparations are natural and therefore intrinsically harmless; in fact, improper herbal prescriptions, unintended drug interactions, adulterants, and unregulated heavy metal content in herbs can be very powerful and potentially lethal issues that make them less safe than conventional medicines (Ahmad et al. 2006).

16.6 Herbal Medicine: A Brief Insight into Economic Value

The prevalence of ethnomedicine in developing countries is greater than in developed countries, thanks to local belief and a general acceptance of the ideology that herbs are natural medicines; however, ethnomedicine is often perceived as unreliable, and less clinical evidence is available for it in developed countries. The explanation of why ethnomedicine is still widely practised in many places is most people cannot afford modern medicine and have no access to pharmaceutical health care (Yager et al. 2008). Over 80% of the African population rely on traditional African medicine even though this is not developed to a comparable standard to modern pharmaceutical products, and many Africans suffer from shortage of critical drugs and die from curable diseases (Elujoba et al. 2005; WHO 2014). For developing or ethnomedicine rich countries, development of native medicinal plants could be an important step towards improving accessibility of effective pharmaceuticals, as well as boosting the economic status of those countries (Ahmad et al. 2006).

Based on the findings of Global Industry Analysis on the global herbal product industry, Singapore's herbal products sales reached SGD 299 million and grew by 3% in 2016. This could be due to the good reputation and quality of the products available there, which are less processed and have fewer side effects. An example of a successful traditional medicine entrepreneur is Cerebos Pacific, Singapore, the leading player, whose signature product is Brand's Essence of Chicken. The World Health Organization (WHO) notes that herbal medicine can be turned into wealth.

In Saudi Arabia, herbal products are experiencing stronger demand, and this industry has an estimated annual growth of 9%. Other examples of successful traditional medicine companies are Mondelez Eastern Europe and Middle East & Africa FZE. In the United Arab Emirates (UAE), they are keen on herbal alternatives for daily household and herbal remedies, boosting sales of natural health products. The leading player in UAE is Ricola. For Japan, herbal products experienced 1% growth in 2016, with a sales value of JPY382 billion. Miki Corp is the Japanese leading player in terms of herbal products, and it has forecast sales in 2021 of JPY392 billion. For New Zealand, natural health and supplementary products based on herbs are currently being debated in parliament. This is likely to invite more herbal manufacturers to invest there in the future to produce licenced products. In Sweden, herbal products experienced 3% growth in 2016, Cloetta Sverige remains the sales leader in this herbal industry, but it is potentially prone to slightly weaker growth due to consumer scepticism surrounding the efficacy of herbal products. In many Asian countries, such as Thailand, Vietnam, Hong Kong, and the Philippines, there is a general strong acceptance of herbal products. Most of the herbal products in these countries are expected to increase sales over time due to stable demand and product acceptance.

In general, the herbal medicine market is estimated to reach USD107 billion in 2017 in the global market (Montes and Zapata Jr. 2012). China and India monopolise the herbal product industry, with their shares in the global market reaching a value of USD 60 billion. Chinese medicines are standardised and have authenticity in the global market. China has also created a database of its ancient knowledge of herbs, adding a modern flavour. Its budget recently included USD 3.6 million for a project to screen both conventional chemical compounds and medicinal herbs to boost drug invention. Hong Kong invested USD 64 million to construct Institute of Chinese Medicine, and Taiwan proposes USD 1.5 million to develop the Taiwan Chinese Medicinal Herb Industry (Mukherjee 2015).

16.7 Conclusions and Future Prospects

Although substantial progress has been made in the fields of cancer research towards fast track diagnosis and chemotherapy, it still remains as one of the menace causing the highest death across the globe. Hence, there is an urgent need to take measures to curb and prevent cancer. Treatments using chemically derived drugs have limitations as they cause toxicity effects on normal tissues and extend many health problems. In this regard, alternative therapies using natural anticancer agents obtained from plants are more attractive and desired. Plant-based compounds are effective against different cancer types with lesser side effects and induce multiple mechanisms of action against the target cells. Plant metabolites such as phenolic compounds, flavonoids, alkaloids, and brassinosteroids are reported to possess superior anticancer properties. These compounds are known to destroy tumour cells by various ways, for example, inducing antioxidant activities, inhibiting cancer cell growth, and inducing apoptosis process. Various plant-derived compounds such as

paclitaxel, podophyllotoxin, camptothecin, etc. are already in the market for the treatment of various cancer types, and few more novel drug molecules are in the discovery phases. A recent progress in the fields of nanobiotechnology has shown some hope to cure cancer effectively by conjugating various nanoparticles with plant anticancer compounds. Nanoparticles are very useful in controlling the sustained release of anticancer drugs and thus help to develop novel drugs targeting specific tissues with negligible side effects of treatments.

In the face of increasing use and a fast-growing market for herbal medicine and other herbal health-care products, some core issues or weaknesses of herbal medicine are still to be faced; these are generally related to the safety, efficacy, and quality of herbal products. To be more specific, the correct identification of herbal materials and pharmacologically active constituents, along with standardization in production, is the key to gaining trust from the modern medical community, which is required to improve the prevalence of herbal medicine aside from public demand for herbs as supplements. In the present situation, for herbal medicine to be legalised and synchronised with modern medicine, policymakers and health professionals must be presented with additional scientific evidence on the quality, efficacy, and safety of such remedies by researchers. Taking this into consideration, it may be concluded that herbal medicine offers good future prospects and may emerge as good option for the treatment of cancers, subject to further research. However, utilisation of plant-derived anticancer agents needs better management to meet the global demands.

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Platelet-Derived Growth Factor Receptor (PDGF-R) as the Target for Herbal-Based Anticancer Agents

17

Manjula Devi Ramamoorthy and Kannan Narayanan Dhiraviam

Abstract

In the preceding few years, there has been an exponential development in the field of herbal medicine against cancers due to their natural origin and relatively less adverse reactions than chemotherapy. For quite some time, plant-based molecules have been a source of therapeutic agents, and they keep on playing a crucial part as new investigational drugs against different illnesses including malignancy. The herbal products interact with a wide assortment of proteins, for example, catalysts, transcription factors, inflammatory cytokines, and gene products, correlated with cancer cell survival, invasion, proliferation, migration, and angiogenesis. Most fatal illnesses are caused by alterations of nearly 500 unique genes. The vast group of protein tyrosine kinase functions as a component of signaling pathways and tends to be fundamental for the transforming activity of normal cells into cancerous cells. In which, platelet-derived growth factor receptor (PDGF-R) has a prominent part in the initiation of cell signaling pathways. Platelet-derived growth factor (PDGF) isoforms and their receptors play a vital role in the direction of development and survival of cancer cells. In diseased condition, upgraded motioning of this receptor is the trademark. In particular cases, the persistence of PDGF signaling is fundamental to the survival of malignant cells. Thus, hindrance of PDGF receptor signaling has turned out to be valuable for curing patients with certain rare tumors. The advancement of receptor tyrosine kinase inhibitors that can obstruct diseases caused by abnormalities in this signaling pathway is considered as a promising methodology for the drug development. These drug revelation endeavors have created inhibitors and low molecular weight therapeutics coordinated against the ATP-restricting locales of

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protein kinases. The aim of this chapter is to discuss the production of plant secondary metabolites that might block tumor metastasis by targeting PDGF-R phosphorylation.

Keywords
Metastasis · Angiogenesis · Plant secondary metabolites · Platelet-derived growth factor · Receptor · Tyrosine kinases

17.1 Introduction

A disease which undergoes uncontrolled cell growth and proliferation that can affect any part of the body is known as cancer (Sakarkar and Deshmukh 2011). Despite all diseases, it remains a primary cause of death around the world (Siegel et al. 2016). The development and evolution of a tumor are vigorous and triggered by long-term alterations in the fundamental sequences and acquiring of persistent characteristics that authorize the progression of malignancy (Hanahan and Weinberg 2000). In past decades, cancer research has enormously expanded because of the increased number of cancer-related deaths around the world. As indicated by the IARC (International Agency for Research on Cancer), cancer affected about 14.1 billion individuals and caused 8.2 million deaths around the globe in 2008. In the future, 16,85,210 new disease cases and 595,690 tumor deaths are predicted to occur (Torre et al. 2015). General cancer frequency patterns are steady in women; however, they declined by 3.1% every year in men, a lot of which is a direct result of decreased number of prostate cancer cases due to available early diagnosis. Till now, more than 100 types of cancers have been illustrated. Breast and lung cancers are the most often analyzed cancers and are the leading cause of mortality for men and women, respectively (Martel et al. 2012). Prostate malignancy is the most frequently diagnosed disease among men, and lung tumor is one of the main sources of deaths among women in both developed and underdeveloped nations. Other recurrently analyzed cancer types include liver, stomach, and colorectal cancers among men. In women, stomach, cervix uteri, and colorectal cancers are very frequent (Center et al. 2012). Cancer cells differ from normal cells in view of their characteristics as irregular cells that develop beyond their standard limits and can hit adjacent parts of the body and spread to different organs to generate auxiliary tumor (Fidler 2002). This unique feature of cancer cells is defined as metastasis. Metastasis is the principal cause of misery and mortality in most cancers encouraged with the help of neo-angiogenesis. A tumor cell can propagate up to the size of 1–2 mm without the aid of vascularization. Enlargement further than this point necessitates an expansion in the quantity of new functional vessels to give satisfactory supplies of supplements (Weiss 2000). Hence, in this manner, the progression of angiogenesis is a basis for the essential tumor advancement and in addition metastasis. This relationship between tumor development and angiogenic activity is highlighted by several findings that the number of growth factors has transforming ability. However,

further than the regulation of primary tumor size, angiogenesis has a significant effect on the capacity of neoplasm to metastasis (Risau 1997). On the off chance that a tumor does not have adequate vascularization, hypoxia occurs, and it encourages the expression of several pro-angiogenic factors. Balance among pro- and anti-angiogenic factors manages tumor vascularization. There are several angiogenic factors that play a major role in this process, namely, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), endostatin, angiostatin, and thrombospondin (Papetti and Herman 2002). Among these, VEGF and PDGF assume a vital part and influence angiogenesis. Tumor cells secrete these angiogenic factors and prompt a concentrated gradient which drives angiogenesis. In this manner, aggressive angiogenic treatment could end up being a successful method for managing scattered illness. Surgery, chemotherapy, and radiotherapy treatment are considered as the most widely recognized strategies for disease treatment. Despite the fact that every one of these strategies is extremely feasible for malignancy treatment they cause severe reactions when used (Yang et al. 2012). The primary issue related to all of these treatment strategies is progressive resistance of tumor cells against treatment. Imperviousness to the majority of accessible anticancer agents, for example, anthracyclines and taxanes, and its expanding frequency are the first hindrance in the present treatment (Wang et al. 2012). Characteristic phytochemicals are considered as a primary resource for potential chemopreventive and chemotherapeutic agents (Torre et al. 2015). These therapeutic agents have assumed a critical part in health care in both ancient and present-day times. In recent times, remarkable changes have been paid to the profoundly compelling phytochemical cancer prevention agents from natural sources (Liu and Butow 2006). Studies have revealed that among 65 drugs enlisted against cancers during 1981–2002, 48 drugs such as vinblastine, vincristine, vinorelbine, vindesine, paclitaxel, podophyllotoxin, docetaxel, topotecan, irinotecan, doxorubicin, epirubicin, daunorubicin, and idarubicin were obtained from natural sources (Mukherjee et al. (2001). The aim of this chapter is to discuss about the plant secondary metabolites that might block tumor metastasis by targeting PDGF-R phosphorylation.

17.2 Importance of Angiogenesis in Metastatic Events

Metastasis arises when hereditarily unsteady cancer cells adjust to a tissue microenvironment that is far off from the primary tumor. Several steps are involved in the biological cascade of metastasis including cellular detachment from membrane, increased motility and invasiveness, passage and continued existence in circulation, exit into new tissue, and eventual colonization of a distant site (Fig. 17.1). All of these create a physiological barrier to the spread of cancer cells, and the cancer cells must cross these obstacles in order to succeed in metastasis process. The growth of tumor and its metastasis depend on the process of angiogenesis (Folkman 1990). Angiogenesis is a multistep process including the separation of endothelial cells from the basement membrane and pericytes, invasion and migration across the

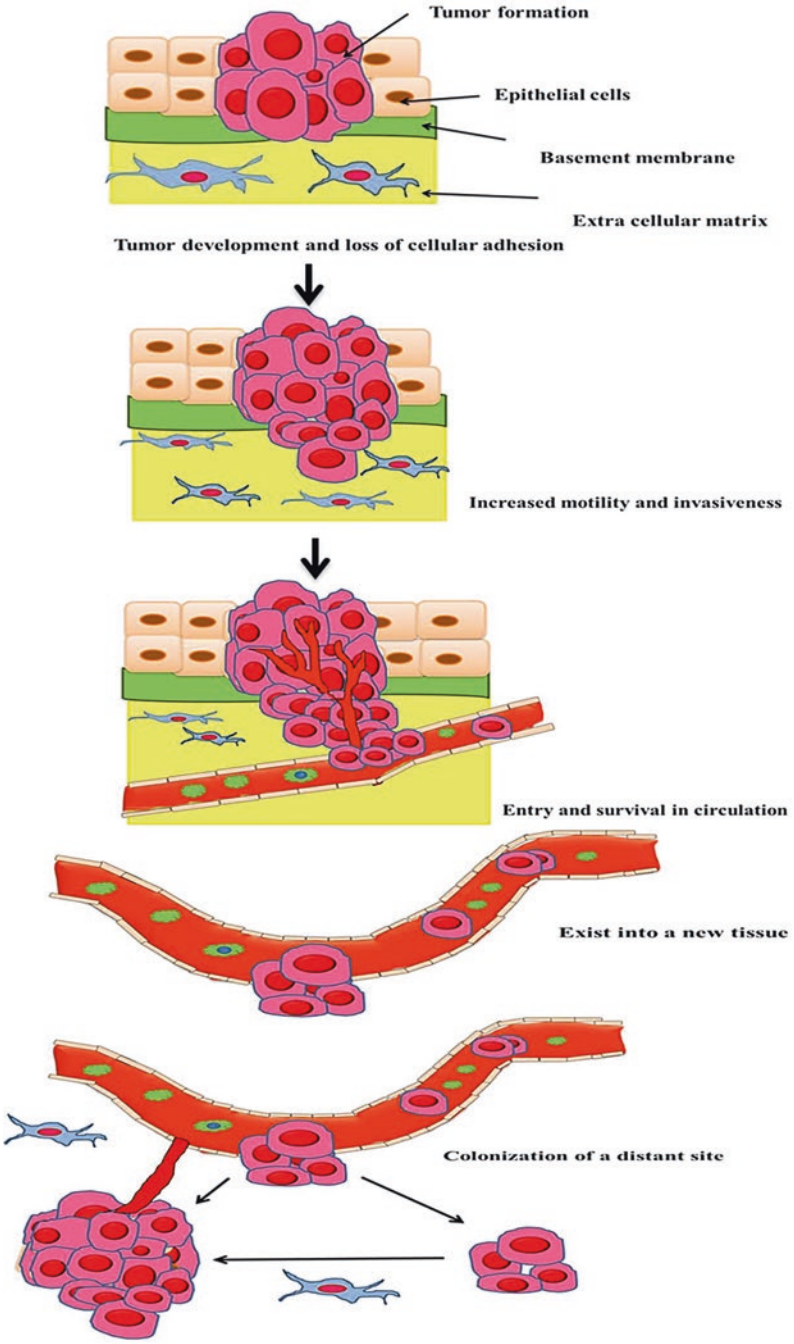

Fig. 17.1 Progression of metastasis in tumor cells

basement membrane, and eventually expansion to form the tumorous body. Endothelial cell activation decides the fate of cells whether the cells are in normal or pathological condition (Ribatti and Djonov 2012). This continuous process relays on the harmony among several molecules including angiogenic and inhibitory molecules discharged by both host normal and cancer cells. There are quite a number of angiogenic molecules that have been recognized, namely, the transforming growth factors (TGF), angiopoietins, tumor necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF), interleukins, members of the vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) families. These factors were considered as a positive regulation of angiogenesis. Apart from this, endogenous inhibitors of angiogenesis including endostatin, angiostatin, and thrombospondin were also regarded as regulatory molecules in angiogenesis (Bergers and Hanahan 2008). These signals prompt endothelial cells to differentiate into tip cells, which later start to move and endure at the developing vessel's central front (Cao et al. 2011). This endothelial cell differentiation was considered as a critical process in tumor condition since the quantity of tip cells is restricted resulting in a well-structured vasculature in normal condition. In tumor angiogenesis, this system is disordered by generating abundant pro-angiogenic signals or due to the absence of angiogenic inhibitors which promote the unnecessary tip-cell arrangement and relocation of endothelial cells which is not the expected orderly physiology related to a healthy vasculature (Cao et al. 2004; Tobler et al. 2008; Helfrich et al. 2009). From this, anti-angiogenic therapeutics can prompt standardization of the tumor vasculature possibly expanding the adequacy of cytotoxic agents, on account of a superior perfusion of the tumor. In accordance with this theory, aggressive angiogenesis has critical impact in malignancy treatment. Among these several angiogenic factors, PDGF assumes an imperative part in both typical and neurotic conditions. In typical state, PDGF demonstrates tremendous impact in embryonic advancement and controls tissue homeostasis in the adult. Overactivity of PDGF signaling is related to the improvement of a few dangerous malignant diseases described by unreasonable cell expansion.

17.3 Platelet-Derived Growth Factor (PDGF) and Its Isoforms

PDGF is a foremost mitogen for fibroblasts, smooth muscle cells, and other cells, and the PDGF family is constituted by four homodimers (AA, BB, CC, DD) and one heterodimer (AB) that are substantially secreted from platelet α-granules (Chou et al. 2007; Perros et al. 2008; Khoshkam et al. 2015). It's a group of four cysteine-knot category growth factors (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) which control the development of connective tissue cells, for example, fibroblast and smooth muscle cells. Eight cysteine moieties were ideally conserved between two subunits in PDGF dimer. Additionally, six more molecules engage with disulfide bonds (Chen et al. 2013). By following up on mesenchymal cells, PDGFs mainly direct embryonic improvement, particularly the arrangement of blood vessels and organs (Andrae et al. 2008). All PDGFs have a regular structure with the distinctive

growth factor domain required for the binding of receptors, dimerization of subunits, and activation. Besides, PDGF holds N- and C-terminal polypeptide arrangements linked in regulating the biological activities of the factors and their aptitude to associate with extracellular matrix (Fredriksson et al. 2004a). PDGF-A is synthesized as polypeptides of 196 and 211 amino acid buildups because of alternative splicing, and PDGF-B consists of 241 amino acids, while PDGF-C and PDGF-D chains have 345 and 370 amino acid residues, respectively (Su et al. 2008). Both PDGF-A and PDGF-B possess short N-terminal augmentations that experience intracellular proteolytic process for initiation, while both PDGF-C and PDGF-D chains show a unique protein domain, the alleged CUB space, as a feature of their N-terminal expansions. In the C-termini, both PDGF-C and PDGF-D basically are short of amino acid sequence augmentation, while both former PDGFs show for the most part fundamental successions required in association with the extracellular matrix (Chiara et al. 2004; Wardega et al. 2010).

17.3.1 PDGF Genes and Receptors

The genes relevant for the A and B chain are situated on chromosome 7 and 22, respectively. PDGF genes are arranged in a comparative way with seven exons (Bonthron et al. 1988). Exons with their respective roles are represented in tabular form (Table 17.1). PDGF isoforms apply their impacts on target cells by triggering structurally related protein tyrosine kinase receptors. There are two kinds of receptors for PDGFs, PDGF-Rα and PDGFR-β, with a molecular scope of 170 and 180 KDa, respectively (Andrae et al. 2008; Bonner 2004). These receptors are part of the class III receptor tyrosine kinases (RTKs), a family of five individuals including PDGF-Rα and PDGF-Rβ; KIT, FMS, and FLT3 have diverse articulation model and physiological function (Lemmon and Schlessinger 2010). Like all RTKs, the PDGF-R group of receptors has a particular structural design that uses the extracellular area to perceive ligands, a solitary transmembrane helix to pass supplementary contribution from external to the cell, and an effector tyrosine kinase domain that reacts to the extracellular stimuli and experiences phosphorylation to initiate downstream signaling. PDGF-Rα signal controls gastrulation and the progression of a number of organs like the lung, digestive tract, skin, testis, kidney, bones, and neuroprotective tissues. PDGFR-β signaling is better perceived as a fundamental

Table 17.1 List of platelet-derived growth factor (PDGF) exons with their respective role

Exon number	Significant role
Exon 1	Signal sequence
Exons 2 and 3	Encode precursor sequence that is removed during processing
Exons 4 and 5	Encode most of the mature protein
Exon 6	Encode COOH-terminal sequence that may be removed during the maturation of B chains
Exon 7	Noncoding

controller of early hematopoiesis and blood vessel configuration (Andrae et al. 2008; Ostman 2004). While PDGF–PDGF-R signaling appears essential throughout the developmental stages, the expression of both PDGFs and PDGFRs is firmly controlled in adulthood. Each receptor constitutes intra- and extracellular domain. The extracellular element comprises five Ig-like domains among which the ligand binds to three outermost Ig-like domains. Intracellular segment of the receptor encloses tyrosine kinase domain with distinctive inserted sequences without homology to kinases (Yang et al. 2008a). The PDGF receptor structures were more comparable with those of the colony-stimulating factor (CSF-1) receptor and stem cell factor (SCF) receptor. The gene responsible for α-receptor is confined on chromosome 4q12: This is near the genes for SCF receptor and VEGF receptor 2. β-receptor gene is present on chromosome 5 close to CSF-1 receptor gene. Activation of PDGF-R can succeed via an aberrant way through various G protein-coupled receptors. For example, the mitogens like angiotensin II and dopamine activate tyrosine phosphorylation through the transactivation of PDGFR-β (Heeneman et al. 2000; Gill et al. 2010). Factor VII associated with tissue factor additionally stimulates an unremarkable PDGFR-β phosphorylation and firmly potentiates chemotaxis actuated by a small measured level of PDGF-B. The transactivation system of PDGF-R does not engage with the release of PDGF ligands by difference to the transactivation of other RTKs like EGF receptor (Gao et al. 2006). The PDGF receptors can also be activated through other growth factors such as VEGF-A. This mitogen can act on both PDGFR-α and PDGFR-β and hence regulate the mesenchymal cell migration through PDGF-R signaling (Ball et al. 2007). This interaction between VEGF and PDGF-R was examined by Pennock and kazlauskas (2012), and they confirmed that VEGF is a weak agonist only at supraphysiological concentrations; otherwise, it acts as a stronger PDGF antagonist.

17.3.2 Dimerization: A Key Event in Receptor Activation

Signaling of PDGF via PDGF-Rs uses the regular procedure for RTKs, which includes ligand-prompted receptor dimerization and the resulting receptor structural modification that is linked to the activation of the intracellular tyrosine kinase domain (Lemmon and Schlessinger 2010). Tyrosine kinase receptor commencement is a ligand-instigated receptor dimerization, which juxtapose the intracellular component of the receptor and permits trans-autophosphorylation of tyrosine residues in between the dimer and receptors. The dimeric molecule PDGF can combine with the two receptors at the same time and frame an extension between the receptors. Despite the spanning impact of PDGF, the dimeric receptor complex is additionally balanced out by direct receptor–receptor interaction interceded by Ig domain 4 (Omura et al. 1997). PDGF isoforms have their cell impacts by instigating homo- or heterodimeric buildings of α- and β-tyrosine kinase receptors, bringing about cell development, chemotaxis, rearrangement, and avoidance of apoptosis. Both α- and β-receptors are expressed in overlapping domains; however, with distinct cell types, the level receptors at the cell surface can be changed by external stimuli. Due to their dimeric

nature, the PDGF isoforms possess two receptor-binding epitopes (Fredriksson et al. 2004b). In this way, the PDGF molecules bind two receptor molecules simultaneously. α-Receptor binds both A and B chains, while β-receptor binds just the B chain with high affinity; hence, the distinctive isoforms of PDGF can incite a diverse combination of receptor dimers. PDGF-AA stimulates αα-receptor dimers, and PDGF-AB induces αα- or α, β-dimers, whereas PDGF-BB is responsible for all three conceivable mix of dimers (Andrae et al. 2008).

17.3.3 Mechanism of PDGF-R Autophosphorylation

The autophosphorylation of PDGF receptors gives out two imperative functions: (1) The conformation of intracellular part of the receptor changes in order to activate the kinase; (2) autophosphorylation permits the docking sites for SH2 domain-containing signaling molecules. The kinase receptor is left in dormant form by three mechanisms: (1) The activation loop present in the kinase domain is probably to be folded over the catalytic cleft; (2) the juxtamembrane part of the receptor is probably going to be collapsed in a loop which limits the access to the active site; (3) the C-terminal tail of the receptor is most likely curled over the kinase domain (Baxter et al. 1998). As a result of autophosphorylation, the conformation of kinase domain changes and got activated. Furthermore, the autophosphorylated residues in PDGF receptors can act as a binding site for SH2 domain-containing molecules. These include the signaling molecules with intrinsic enzymatic activities such as src family, the GTPase-activating protein (GAP) for Ras, the SHp-2 tyrosine phosphatase, and phospholipase C-γ (PLC-γ) (Heldin et al. 1998). Moreover, the receptor binds and activates signal transducers and activators of transcription (STATs) and can act as a transcription factor in the nucleus after the translocation followed by activation. Finally, the receptors bind the adapter molecule which lacks intrinsic enzymatic activities but can form complexes with other signaling molecules. In addition, the PDGF receptors bind other adapters like Shc, Nck, Ck, and GAB which mediate interaction with a plethora of different downstream signaling molecules (Heldin 2013). The activation of these signaling pathways leads to cell proliferation and survival as well as reorganizes actin and cell migration. The extensive cross talk between the different signaling pathways makes it difficult to assign individual pathways to specific responses; in a cell-type- and context-dependent manner, several signaling pathways contribute to the cellular responses.

17.3.4 Significance of PDGF Signaling in Cancer

The PDGF signaling is highly organized and modulated. There are certain mechanisms used to assume the PDGF signaling in normal as well as in malignant cells. In PDGF-stimulated cells, reactive oxygen species were produced in PI3-kinase-dependent pathway which further inhibits tyrosine phosphatase by reacting with a cysteine residue in the active site (Bae et al. 2000). Another mechanism includes the

PDGF signaling that will dephosphorylate and inactivate Erk MAP-kinase, and removal of this phosphatase enhances Erk MAP-kinase activation (Jurek et al. 2009). There are more than a few perceptions supporting the thought that upregulated PDGF signaling can constrain tumorigenesis (Pietras et al. 2003). In specific tumors, PDGF or PDGF receptor genes are transformed; on the other hand, their expressions are increased. The PDGF receptor genes have also been found to be mutated in certain malignancies. These mutations cause the replacement of amino acids in the receptor region to activate kinases. PDGF receptor genes have been found in gene rearrangement in certain leukemias. Enhancement of PDGFR-α has additionally been seen in oligodendrogliomas, esophageal squamous cell carcinoma, and course intimal sarcomas. It makes the cells susceptible to stimulation by lowered amount of PDGF, or if the number of receptors becomes high enough, signaling may occur in PDGF-independent manner. An activating deletion mutation in the PDGFR-α gene has also been detected in a human glioblastoma. During tumorigenesis, epithelial tumors may undergo epithelial-mesenchymal transition (EMT), which is related to expanded invasiveness and metastasis. During EMT, PDGF receptor expression by the tumor cells increases so that epithelial tumors that initially did not respond to PDGF may become responsive to PDGF stimulation. The expression of PDGF isoforms is also part of the EMT program, which may enhance PDGF receptor signaling by autocrine stimulation (Jechlinger et al. 2006).

PDGF created by tumor cells or non-tumorigenic cells, like endothelial cells and macrophages, can act on non-tumor cells in solid tumors. Thus, pericytes surrounded by blood vessels and fibroblasts and myofibroblasts in the stroma convey PDGF receptors and react to PDGF. Pericytes rely on PDGF created by endothelial cells and have an important role during angiogenesis. PDGF stimulation of fibroblast and myofibroblast in the stroma adds to the expanded interstitial fluid pressure (IFP) in tumors. The increased IFP is an obstacle in the chemotherapeutic treatment of tumors since it decreases transcapillary flow and drug uptake (Heldin et al. 2004). The actuality is that PDGF-R signaling is mostly overactive in tumors, hence prompting treatment of these various malignancies with PDGF/PDGF receptor antagonist. During tumor progression, tumor cells acquire some of the mutations, some of which drive tumorigenesis. It has been observed that tumor cells often become addicted to the signaling pathways that are activated by mutational events and inhibition of such pathways induces tumor cell death in apoptosis (Weinstein and Joe 2008). On the other hand, regrowth of tumor often occurs after some time due to the appearance of various types of resistance mechanism.

17.3.5 PDGF-R Inhibitors

The contribution of PDGF signaling in malignant diseases as well as certain nonmalignant diseases has prompted the improvement of various sorts of antagonists of PDGF signaling that now are under preclinical and clinical evaluation (Wang et al. 2014). The developed inhibitors include antibodies, DNA aptamers, or soluble extracellular parts of the receptor that bind PDGF isoforms and thus prevent their

binding to signaling receptors (Jayson et al. 2005). On the other hand, antibodies or other binders can focus on the receptors and keep their enactment or advance their degradation. These types of antagonists have the advantages of being reasonably specific; however, they are expensive and cumbersome to administer (Shen et al. 2007). Another kind of opponent is low molecular weight inhibitors of the receptor kinases. Several potent inhibitors of PDGF receptor kinases including sunitinib, imatinib, sorafenib, pazopanib, and nilotinib have been developed in recent times. None of these inhibitors are accurate; they all have their characteristic profiles of inhibition of different other kinases. Thus, imatinib inhibits the stem cell receptor (Kit) and Ab1 kinases in addition to the inhibition of PDGF receptor kinases. Sunitinib inhibits vascular endothelial growth factor (VEGF) receptors and F1 t3, while sorafenib has an inhibitory profile similar to sunitinib but also inhibits the serine/threonine kinase Raf (Socinski 2011).

17.4 Anti-angiogenic Therapy

The multifarious progression involved in the angiogenic scheme affords many targets for therapeutic applications. However, the idleness in this mechanism causes the likelihood resistance to the healing agents (Jensen et al. 2009). The agents that target angiogenesis may act in two different ways: The agent can work on the development of angiogenic molecule synthesis including the inhibition of mTOR, heat shock protein 90 (HSP90), or cyclooxygenase (COX). These factors can inhibit the cell progression via cell growth or metastasis (Zhong et al. 2000; Mabjeesh et al. 2002). Apart from these angiogenic factors, angiogenic receptors predominantly tyrosine kinase inhibitors are the main target in anti-angiogenic therapy. The therapeutic agents including sorafenib, pazopanib, sunitinib, regorafenib, and axitinib are the most popular tyrosine kinase inhibitors. These compounds are as of now being utilized as a part of the treatment of many harmful illnesses extending from lung, breast, gastric, hepatocellular, colorectal, and neuroendocrine tumors and glioblastoma (Gotink and Verheul 2010; Mihaly et al. 2012; Grothey et al. 2013). Among these therapeutic agents, sorafenib potentially inhibits the PDGF-mediated autophosphorylation of PDGFR-β and the VEGF-mediated autophosphorylation of VEGFR-2 and VEGFR-3 in HAoSMCs (Wilhelm et al. 2004). Even if all of these mentioned inhibitors have been commercialized, the lack of specificity corresponding to the side effects has been seen as a disadvantage when seeking the most promising anti-angiogenic agents. This contemplation makes the researchers focus on the plant-based metabolites against malignant diseases.

17.5 Plant-Derived Bioactive Compounds as PDGF-R Inhibitors

The complexities are linked with angiogenesis including the deregulation of multiple cell signaling pathways and their specific downstream targets. So far the anti-angiogenic molecules used failed in the clinical trials as they target a single

component of cell signaling. These molecules were designed to focus on the growth factor or receptor molecules which play a significant role in the normal physiological process; however, consequently, they cause some side effects (Lu et al. 2016). In this phenomenon, the beneficial effect of plant-derived molecules to arrest tumor angiogenesis is high efficacy with little or no toxicity at the prescribed therapeutic doses. Additionally, the finding from various studies has shown that plant-based drugs are more potent anti-angiogenic compounds than synthetic molecules with respect to their specificity (Yehya et al. 2017). The mechanism involved in the anti-angiogenic activity of plant-derived compounds from different sources will be discussed here.

17.5.1 Evodiamine

The fruit of *Evodia rutaecarpa* Benth. is a standout among the most prominent and multipurpose herbs utilized in China for pharmacological application (Fei et al. 2003). Phytochemical analysis demonstrated that evodiamine, an indole alkaloid, exists in elevated amounts in Chinese herb evodia. This has wide-ranging biological activities with vasodilatory, anti-obesity, anticancer, and anti-inflammatory effects (Jiang and Hu 2009). Evodiamine represses PDGF-BB-induced vascular smooth muscle cell proliferation in a dose-dependent manner. It inhibits cell cycle progression by suppressing the activation of p38 and Erk/MAPK pathway and ameliorates ROS generation (Yang et al. 2008b; Heo et al. 2009; Yang et al. 2013). Restraint of mitogenesis and p38/p Erk action may fill in as the molecular basis for the capacity of evodiamine. Evodiamine causes continued initiation of Erk/MAPK signaling pathway in 3T3-L1 and essential preadipocytes prompting a potent inhibitory impact for adipogenesis (Wang et al. 2008). The consequence of this investigation showed that evodiamine hindered vascular smooth muscle cell (VSMC) multiplication by stifling cell cycle movement, p38 MAPK and Erk 1/2 enactment, and generation of ROS. Moreover, evodiamine offers the potential of treating cardiovascular infections connected with the strange expansion of vascular smooth muscle cells.

17.5.2 Epigallocatechin Gallate (EGCG)

Green tea constitutes the rich amount of EGCG which aids in cancer chemoprevention (Fujiki et al. 1998). EGCG improved the impacts of ginseng compound in the restraint of colon tumor cell development, showing that green tea could be a successful synergist with an anticancer agent for malignancy chemoprevention. It obstructs the PDGF-initiated proliferation and migration of rodent pancreatic stellate cells (Masamune et al. 2005). The soluble and plasma membrane-integrated EGCG straightforwardly communicates with PDGF-BB and in this way puts off precise receptor binding promoting the inhibitory impacts of EGCG on platelet-derived growth factor-incited cell signaling and mitogens (Weber et al. 2004).

17.5.3 Curcumin

Curcumin is a dynamic element of turmeric, an outstanding Indian zest that is obtained from the plant *Curcuma longa* dried roots. Curcumin hindered PDGFR-incited proliferation of human hepatic myofibroblasts (Zheng and Chen 2006). The activated mechanism by curcumin in PDGF signaling is as follows: Curcumin decreases the level of tyrosine phosphorylation of PDGFR-β and EGF-R; represses the action of ERK, JNK, and PI3/AKT; reduces cell growth; and induces apoptosis dose-dependently (Kunnumakkara et al. 2008). Moreover, curcumin interferes with PDGF signaling via relieving its inhibitory effect on PPARγ gene expression to reduce the cell growth; it also promotes the expression of PPARγ genes (Zhou et al. 2007).

17.5.4 Resveratrol

Resveratrol, a noteworthy polyphenol occurring in different plants such as grapes and peanuts, has appeared to be required in cell reinforcement, anti-proliferative, anti-inflammatory, and chemopreventive activities. Various potential medical advantages, including decreased danger of malignancy and coronary illness, are believed to be related to the utilization of resveratrol. It can successfully and proficiently repress endothelial cell multiplication and migration, with little cell toxicity in the HUVEC and ARPE19 lines (Cao et al. 2010). Also, there have been perceptions of inhibitory consequences for smooth muscle cell migration (Venkatesan et al. 2009) and tumor necrosis factor-alpha-incited monocyte adhesion and migration (Kim et al. 2007). As of late, it was recognized that the restraint of PDGF-BB-actuated cell migration by resveratrol and the particular inhibitors PDGF-R, PI3K, MEK, or p38 in wound healing test agreed with diminished enactment of PDGF-BB-incited PDGFR-β, PI3K/Akt, ERK, and p38 phosphory-lation in Western blot investigation, recommending that resveratrol hinders cell migration through the inhibition of PI3K/Akt, PDGFR-β, and MAPK cascade (Chan et al. 2013).

17.5.5 Ellagic Acid (EA)

Ellagic acid is a naturally occurring phenolic constituent present in natural products and nuts, most elevated amounts of which are found in raspberries (Daniel et al. 1990). EA is considered as a potent anticarcinogenic and antimutagenic compound. EA shows anti-angiogenic property by repressing PDGF-R movement and phosphorylation of its substrate. It can intrude with endothelial cell-associated VEGR-2 phosphorylation bringing about the restraint of the downstream signaling activated by this receptor and in the hindrance of two key events fundamental in angiogenesis, i.e., EC movement and morphogenic separation into capillary-like structure. In parallel, EA indicated robust inhibitory activity against perivascular cells through its restraint of PDGF-R action and signaling prompting hindrance of VSMC relocation (Labrecque et al. 2005).

17.6 Conclusions and Future Prospects

Plant-derived compounds gain much importance toward cancer therapy, and till now several signaling pathways were elucidated in the regulation of cancer metastasis. Among this, RTKs play a significant role in metastasis-associated signaling pathways. RTKs such as PDGF-R have a dual mechanism along with VEGF which mediates cancer cell regulation. Hence, the molecules which act against PDGF-R have the ability to inhibit the major growth factor VEGF also. The discovery of new plant-derived bioactive compounds as an inhibitor of angiogenic growth factors or their receptor has become radically important for the development of anti-angiogenic moieties in cancer therapeutics. Nevertheless, perceptiveness of the specific mechanism induced by phytochemicals is still completely unaddressed. The examination of these pathways under which the angiogenic inhibitors act is crucial in drug design. Thus, researchers focus on the further understanding of the molecular level of bioactive compounds with respect to their particular signaling pathway.

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Plant Cell and Organ Culture as an Alternative for the Production of Anticancer Compounds

18

Hosakatte Niranjana Murthy, Vijayalaxmi S. Dandin, Kadanthottu Sebastian Joseph, So-Young Park, and Kee-Yoeup Paek

Abstract

Plants are the reservoirs of various valuable secondary metabolites like camptothecin, podophyllotoxin, and ginseng saponins which are used as anticancer agents. Plant cell and organ cultures are competent to synthesize and accumulate many of these compounds and can be used as a source for extraction of such compounds. Different strategies have been applied for overaccumulation of secondary metabolites in plant cell cultures which include screening and selection of high yielding cell lines, optimization of nutrient media, and elicitation. In the present chapter, some of the anticancer compounds derived from plant cell and organ cultures are described, and various strategies for improving the accumulation of useful secondary metabolites in cell and organ cultures and methods for the large-scale production using bioreactors are highlighted.

Keywords

Adventitious root culture · Bioreactor cultures · Cell culture · Hairy root culture · Secondary metabolites

18.1 Introduction

Cancer is a disease which arises when a cell starts disobeying the regulatory systems of cell cycle, which control the cell proliferation and circumvention of the controls which regulate the events of cell cycle that will result in division of cells in

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an uncontrolled manner. These cells are not self-limited in growth due to genome instability and invade the adjacent tissues leading to malignancy and spread to other tissues. Cancer may arise due to the activation of a proto-oncogene to become oncogenic or the failure in functioning of tumor suppressor gene (Bertram 2001). The International Agency for Research on Cancer (IARC, an agency of World Health Organization) estimates that there is an occurrence of 28 different types of cancers in 184 countries and 14.1 million people are affected by various cancers such as lung, breast, colorectal, prostrate, cervical, and uterine cancers (Ferlay et al. 2015). Cancer cure and prevention therefore remains as a high priority for cancer research across the world as it is one of the leading causes of human mortality. Tumor growth and progression depend on angiogenesis, a process of new blood vessel formation from pre-existing vascular endothelium. The newly formed blood vessels facilitate the tumor growth. Therefore, inhibiting angiogenesis has been a potential cancer therapeutic option (Wanga et al. 2015). The current clinical treatment includes surgery, radiation, and chemotherapy. However, chemotherapeutic agents induce several side effects (Nussbaumer et al. 2011). To overcome the side effects of chemotherapy, it is essential to design nontoxic natural cancer therapeutic drugs. It has been recognized that the plant products serve as the reservoir of diverse chemical compounds for treating human ailments. Among them, several of the plant-derived bioactive compounds have been found to be clinically useful anticancer agents, and 60% of currently used anticancer agents come from plants and microbes (Cragg and Newman 2005). Presently, the plant-derived anticancer agents in clinical use are paclitaxel (commercial name Taxol) obtained from various species of *Taxus* genus (originally paclitaxel was obtained from the Pacific yew, *Taxus brevifolia* Nutt., family Taxaceae), camptothecin obtained from *Camptotheca acuminata* Decne (family Nyssaceae), podophyllotoxin obtained from many species of *Podophyllum* genus (*Podophyllum peltatum* L. commonly known as the American mandrake and Mayapple; *Podophyllum emodii* Wallich from Indian subcontinent) (Cragg and Newman 2005), and ginsenosides or ginseng saponins obtained from *Panax* species (originally ginsenosides were obtained from the ginseng, *Panax ginseng* C.A. Meyer, family Araliaceae) (Nag et al. 2012). The plant cell and tissue culture technology offers an imperative method for producing such high-value compounds. This chapter lays emphasis on the chemical nature, source, and uses of plant-derived anticancer compounds, and it also underscores the recent steps forward for enhancing the production of camptothecin, paclitaxel, podophyllotoxin, and ginsenosides by the optimization of important parameters for bringing in an efficient bioprocess in cell and tissue culture.

18.1.1 Paclitaxel

Paclitaxel (commercial name, Taxol) a complex diterpene alkaloid (Fig. 18.1) is naturally obtained from *Taxus* species (family Taxaceae). Paclitaxel has been proved as highly effective in the treatment of various types of cancers, since it acts as a microtubule-stabilizing agent to protect against disassembly. Paclitaxel was

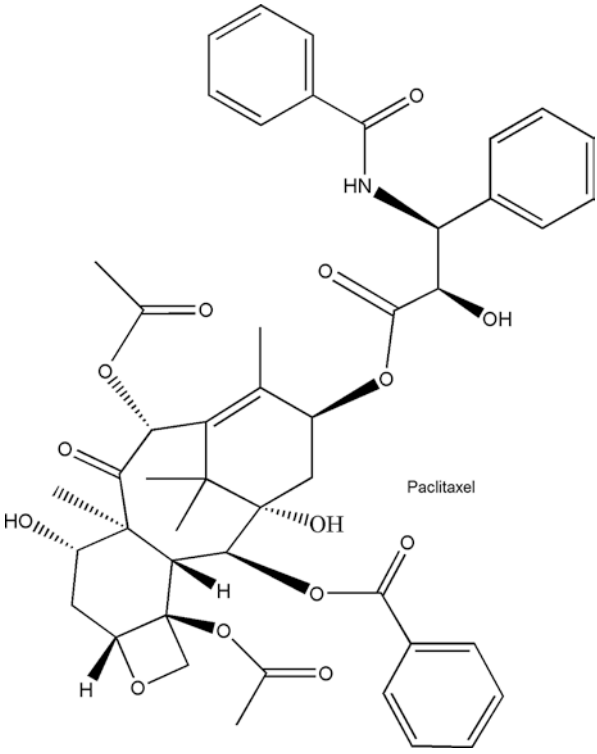


Fig. 18.1 Structures of paclitaxel

developed by the National Cancer Institute, USA, as a drug for cancer therapy and used for the treatment of refractory ovarian cancer, metastatic breast and lung cancer, and Kaposi's sarcoma (Srivastava et al. 2005). The natural source of paclitaxel is the bark of several *Taxus* species; however, the cost of extraction is very high since the concentration of paclitaxel accumulation is very low (0.02% of dry weight) and also entails the destruction of natural resources (Cusido et al. 2014). Even though, paclitaxel can be chemically synthesized, but this process is not commercially viable. Plant cell cultures have been developed for the production of paclitaxel by Phyton Biotech in 1995, and in 2004 the FDA has approved the use of plant culture supply of paclitaxel/Taxol (Leone and Roberts 2013).

18.1.2 Camptothecin

Camptothecin (CPT) is a monoterpene indole alkaloid (Fig. 18.2) which is isolated from the Chinese plant, *Camptotheca acuminata* (Nyssaceae) (Wall et al. 1966). CPT is used in cancer treatment since it is a potent inhibitor of DNA topoisomerase I, which leads to DNA damage and the apoptosis in cancer cells. Studies have shown that CPT itself is not suitable for clinical application since it has low water solubility and certain side effects; therefore, water-soluble CPT derivatives such as topotecan

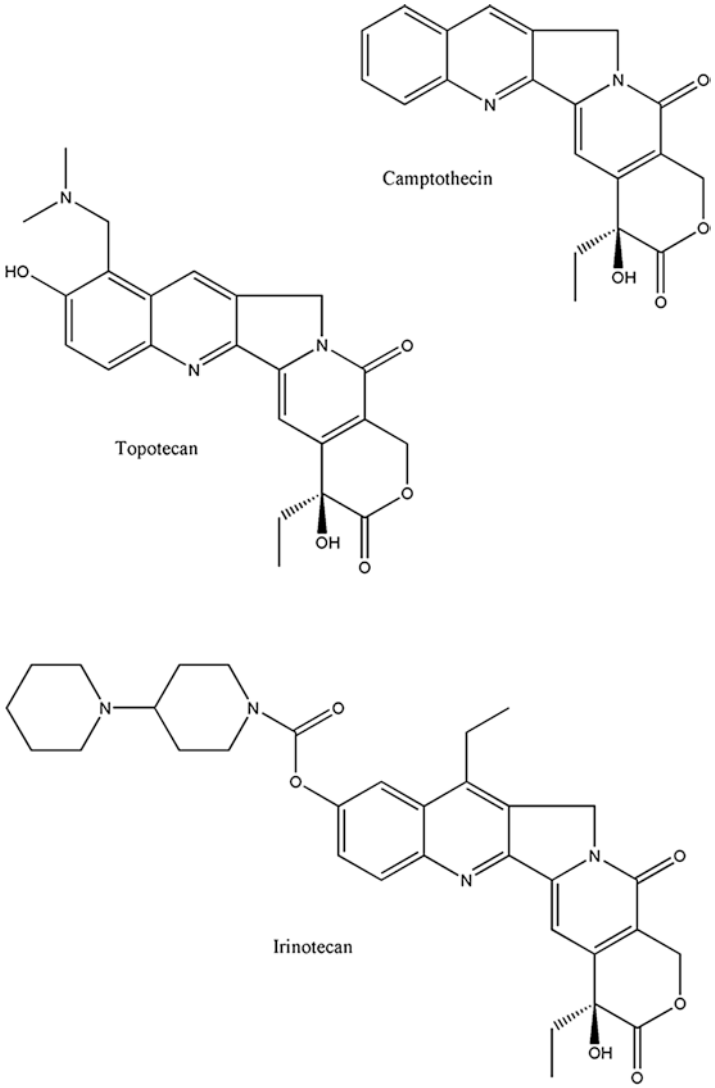


Fig. 18.2 Structure of monoterpene indole alkaloids

and irinotecan (Fig. 18.2) were synthesized and have been successfully used for the treatment of ovarian, lung, and colorectal cancers, and CPT has been approved by the Food and Drug Administration (FDA) of the USA. Currently, topotecan and irinotecan are all synthesized from natural camptothecin which is mainly extracted from *Camptotheca acuminata* (Beegum et al. 2007). Subsequently, CPT was also recognized and extracted from other plant species such as *Ervatamia heyneana* (Gunasekera et al. 1979), *Melliodendron megacarpum* (Arisawa et al. 1981), *Nothapodytes foetida* (Govindachari and Viswanathan 1972), and *Ophiorrhiza* species (Beegum et al.

2007). However, the extraction of CPT from plants is limited because of low yields (about 1 mg/g dry weight) and scanty natural resources (Lopez-Meyer et al. 1994), and scientists have used biotechnological ways especially cell culture methods for the production of CPT and its derivatives (Kai et al. 2015).

18.1.3 Podophyllotoxin

Podophyllotoxin (PTOX) is an aryl-tetralin lignan (Fig. 18.3) and has been originally isolated from *Podophyllum peltatum* L. (American podophyllum or Mayapple; family Podophyllaceae). Later, it is also isolated from several species like *P.*

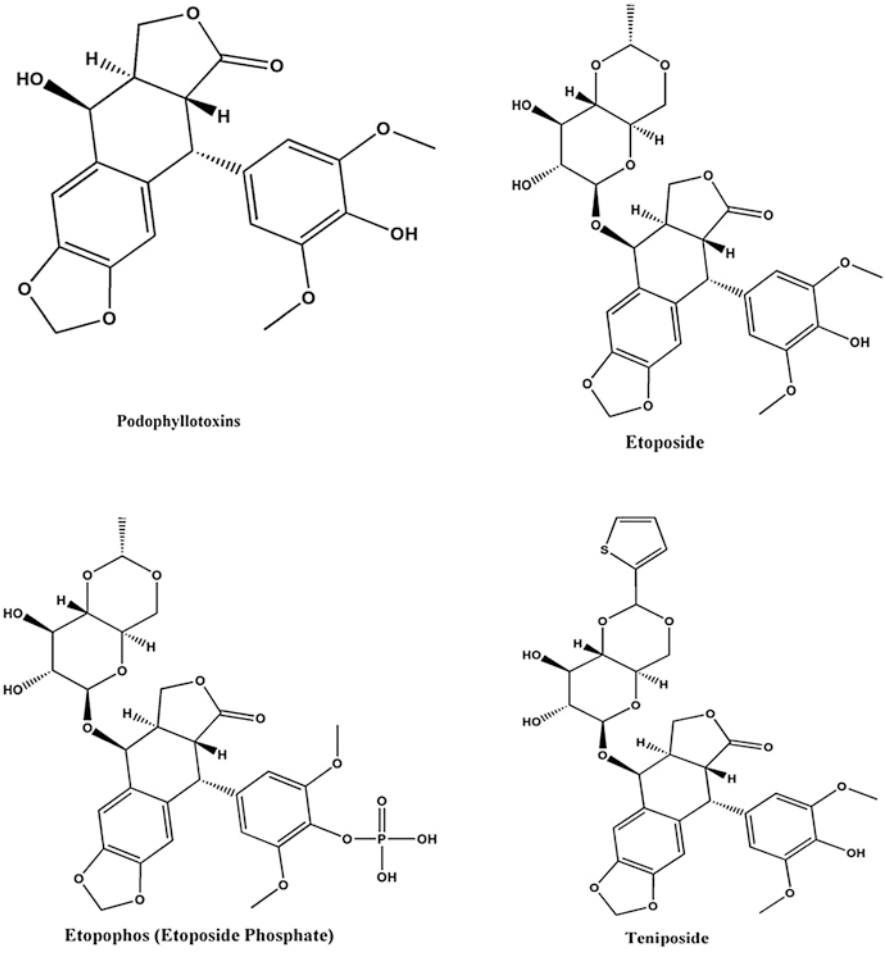
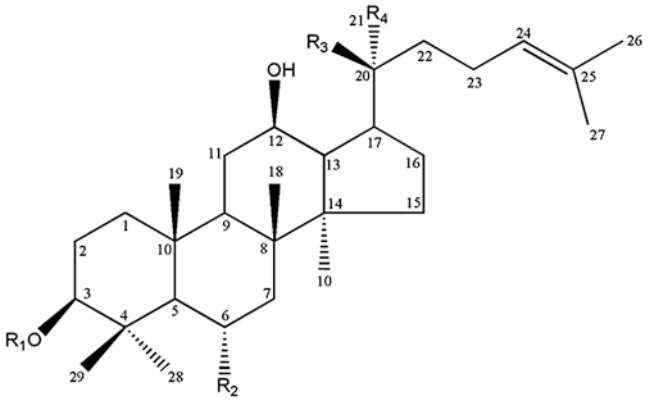


Fig. 18.3 Structure of podophyllotoxins and semisynthetic antineoplastic drugs (etoposide, Etopophos, and teniposide)

hexandrum Royle (Indian podophyllum) and *P. pleianthum* (Taiwanese podophyllum). PTOX has also been reported in other plants such as *Linum* spp., *Callitris* spp., *Juniperus* spp., *Thuja* spp., *Hyptis* spp., *Thymus* spp., *Teucrium* spp., *Nepeta* spp., *Dysosma* spp., *Diphylleia* spp., and *Jeffersoniana* spp. (Ionkova 2007; Yousefzadi et al. 2010). PTOX shows strong cytotoxic activity against various cancer cell lines. However, PTOX is too toxic for the treatment of neoplastic diseases in humans; it is used as a precursor for chemical synthesis of semisynthetic antineoplastic drugs, etoposide, Etopophos, and teniposide (Fig. 18.3), which are successfully used as antitumor agents (Holthuis 1988; Cragg and Newman 2005). Podophyllotoxin derivatives are used in the treatment of lymphomas, acute leukemia, and testicular, lung, ovarian, bladder, and brain cancer (Srivastava et al. 2005). *Podophyllum* spp. are the major source of PTOX, and their availability is limited in nature, and some species are categorized as endangered. Moreover, the chemical synthesis of podophyllotoxin is an expensive process; therefore, biotechnological production of podophyllotoxin using plant cell and tissue cultures has been preferred by various research groups (Farkya et al. 2004).

18.1.4 Ginsenosides

Ginseng is a common name for a group of plants belonging to genus *Panax* (family Araliaceae) which includes *P. ginseng* (Asian ginseng), *P. notoginseng* (notoginseng), and *P. quinquefolius* (American ginseng). The bioactive compounds of ginseng are triterpenoid glycosides or dammarane saponins which are commonly referred to as ginsenosides or ginseng saponins (Murthy et al. 2014a; Kim et al. 2015). Ginsenosides are classified into three groups, namely, protopanaxadiol group (PPD group), protopanaxatriol group (PPT group), and ginsenoside Ro, based on the aglycone skeleton present in them, which is derived from oleanolic acid group (Fig. 18.4). Ginsenosides Rb1, Rb2, Rc, and Rd belong to PPD group, while ginsenosides Re, Rf, Rg1, Rh1, and notoginsenoside R1 belong to the PPT group. The major saponins of Asian ginseng are ginsenoside Rb1, Rb2, and Rg1; those in notoginseng are ginsenosides Rb1, Rd, Rg1, and notoginsenoside R1; and American ginseng possesses ginsenosides Rb1, Rd, and Re (Wang et al. 2015). After oral consumption of natural ginseng, saponins are biotransformed to their metabolites by enteric microbiome before absorbed by the intestine. Usually Rb1, Re, and Rd ginsenosides are converted into Rg3 and compound K, which exhibit potent anticancer activity (Fig. 18.5) (Tawab et al. 2003; Nag et al. 2012; Li et al. 2014; Wang et al. 2015). Field cultivation of ginseng generally involves 4–6 years, and accumulation of ginsenosides in cultivated plants (roots) is affected by environmental factors such as soil, climate, and biological factors. To overcome these problems, cell and organ cultures methods have been opted for rapid and large-scale production of ginseng biomass and ginsenosides (Wu and Zhong 1999; Paek et al. 2009; Murthy et al. 2014a; Thanh et al. 2014a, b; Kim et al. 2015).



Group	Saponin	R_1	R_2	R_3	R_4
PPT	Rg_1	H	*O*-glc	*O*-glc	CH_3
	Re	H	*O*-glc^2→^{1}rha	*O*-glc	CH_3
	Rh_1	H	*O*-glc	OH	CH_3
	20*S*-Rg_2	H	*O*-glc^2→^{1}rha	OH	CH_3
	20*R*-Rg_2	H	*O*-glc^2→^{1}rha	CH_3	OH
PPD	Rb_1	glc^2→^{1}glc	H	*O*-glc^6→^{1}glc	CH_3
	Rc	glc^2→^{1}glc	H	*O*-glc^6→^{1}araf	CH_3
	Rb_2	glc^2→^{1}glc	H	*O*-glc^6→^{1}arap	CH_3
	Rb_3	glc^2→^{1}glc	H	*O*-glc^6→^{1}xyl	CH_3
	Rd	glc^2→^{1}glc	H	*O*-glc	CH_3
	Rg_3	glc^2→^{1}glc	H	OH	CH_3
	F_2	glc	H	*O*-glc	CH_3
	CK	H	H	*O*-glc	CH_3

Fig. 18.4 Structure of ginsenosides

18.2 Plant Cell and Organ Cultures as a Source of Secondary Metabolites

Plant cell and organ culture strategies offer ideal conditions for continuous production of high-value secondary metabolites that can be extended to commercial production of important pharmaceutical compounds including anticancer compounds. They are advantageous compared to natural resources or chemical synthesis as they provide (1) a steady production system, independent of environmental/geographical variation constraints; (2) efficient production of targeted compounds; (3) production of high-quality target compounds free of undesirable chemical contaminants like

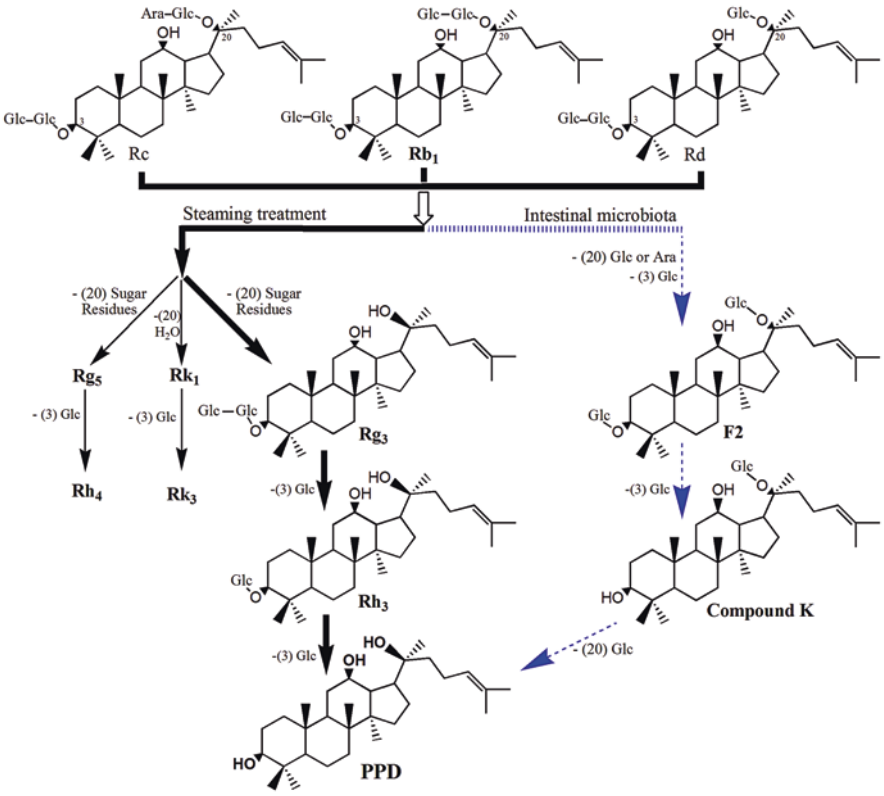


Fig. 18.5 Conversion of Rb1, Re, and Rd ginsenosides into Rg3 and compound K by microbes

agricultural chemicals; (4) easy scale-up process after establishment of chemical, physical, and other controlling parameters; (5) uncomplicated extraction of targeted compounds, devoid of aggressive solvents and multistep process (Wilson and Roberts 2012; Davies and Deroles 2014; Murthy et al. 2014b). Various culture techniques employed successfully for the biomass as well as metabolite production are (1) cell suspension cultures, (2) hairy root cultures, (3) adventitious root cultures, and (4) shoot cultures. However, the fundamental method followed for the *in vitro* production of secondary metabolites has been a cell suspension culture (Deroles 2009; Nosov 2012; Thanh et al. 2014a, b), and the success rate is based on the plant species used for the culture. Nevertheless, this approach is not suitable for various systems in which secondary metabolite accumulation needs cell differentiation (Deroles 2009). Only the differentiated cells or organs such as hairy roots (Georgiev et al. 2012), adventitious roots (Murthy et al. 2008, 2016), embryos and plantlets (Jeong et al. 2005; Park et al. 2005; Shohael et al. 2014), and shoots (Praveen et al. 2009; Dandin and Murthy 2012) could involve in secondary metabolism which leads to accumulation of secondary products. Recent development in cell and organ cultures reveals that production of secondary metabolites is a two-step process.

Control over biomass accumulation is achieved in first step, and synthesis of secondary metabolites is achieved in the second step (Murthy et al. 2014b). Parameters controlling the biomass accumulation such as selection of high productive cell lines and media optimization involving alteration of factors such as macro and minor salts, nitrogen ratio, type and amount of carbohydrates, and phytohormone composition can be improved during the initial step of culture establishment. Physical conditions, such as aeration, temperature, light, pH, and agitation rate, also influence biomass accumulation, and they should be set during the first stage of biosynthetic process (Fig. 18.6) (Nosov 2012; Davies and Deroles 2014; Murthy et al. 2014b). Parameters affecting synthesis and accumulation of secondary metabolites such as elicitation, nutrient feeding, precursor feeding, permeabilization, and immobilization could be adopted in the second stage of culture process (Fig. 18.6). New approaches for enhancement of plant cell culture productivity is based on overexpression or suppression of certain genes involved in secondary metabolism which is popularly referred to as metabolic engineering (Davies and Deroles 2014; Ludwig-Muller 2014). Commercial production of plant secondary metabolites is adoption of

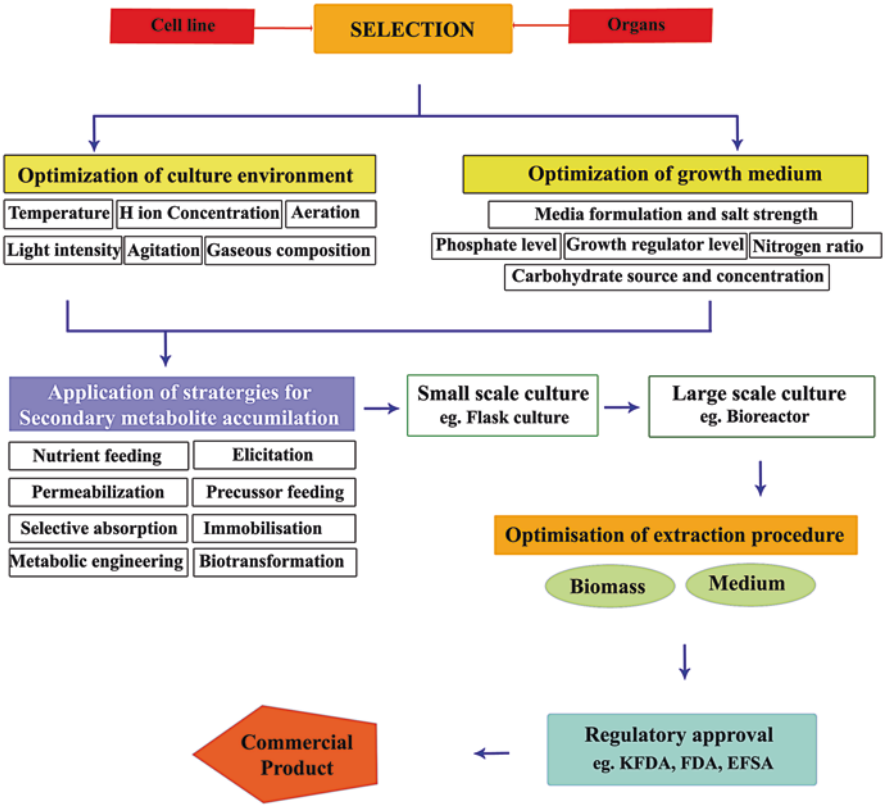


Fig. 18.6 Schematic representation for the production of anticancer compounds from plant cell and organ cultures

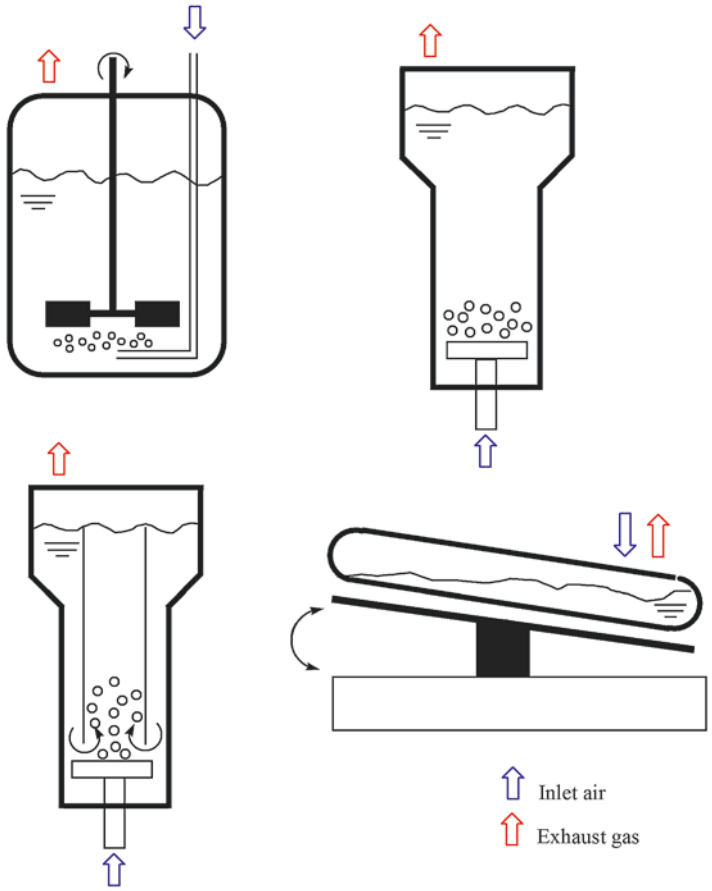


Fig. 18.7 Bioreactors used for plant cell cultures

scale process which involves selection of bioreactor type and design. Various bioreactors have been designed and developed for cultivation of plant cells and organs including mechanical stirred bioreactors, airlift/bubble-column or balloon-type bioreactors, fixed-bed bioreactors, wave-mixed bioreactors, and disposable bioreactors (Fig. 18.7) (Weathers et al. 2010; Steingroewer et al. 2013; Georgiev 2014; Lehmann et al. 2014). The choice of bioreactors depends on the type of culture and production systems. For example, for the cell suspension cultures, stirred tank bioreactors, airlift bioreactors, and bubble bioreactors are suitable, whereas, for hairy root, adventitious, and embryo suspension cultures, airlift, bubble, or nutrient mist bioreactors are found to be appropriate (Fig. 18.8). For shoot cultures, temporary immersion bioreactors or Ebb and flood bioreactors are desirable. Mode of operation of bioreactors has a vital role as the accumulation or production of secondary metabolites varies in different cultures. Some plant secondary metabolites are accumulated in

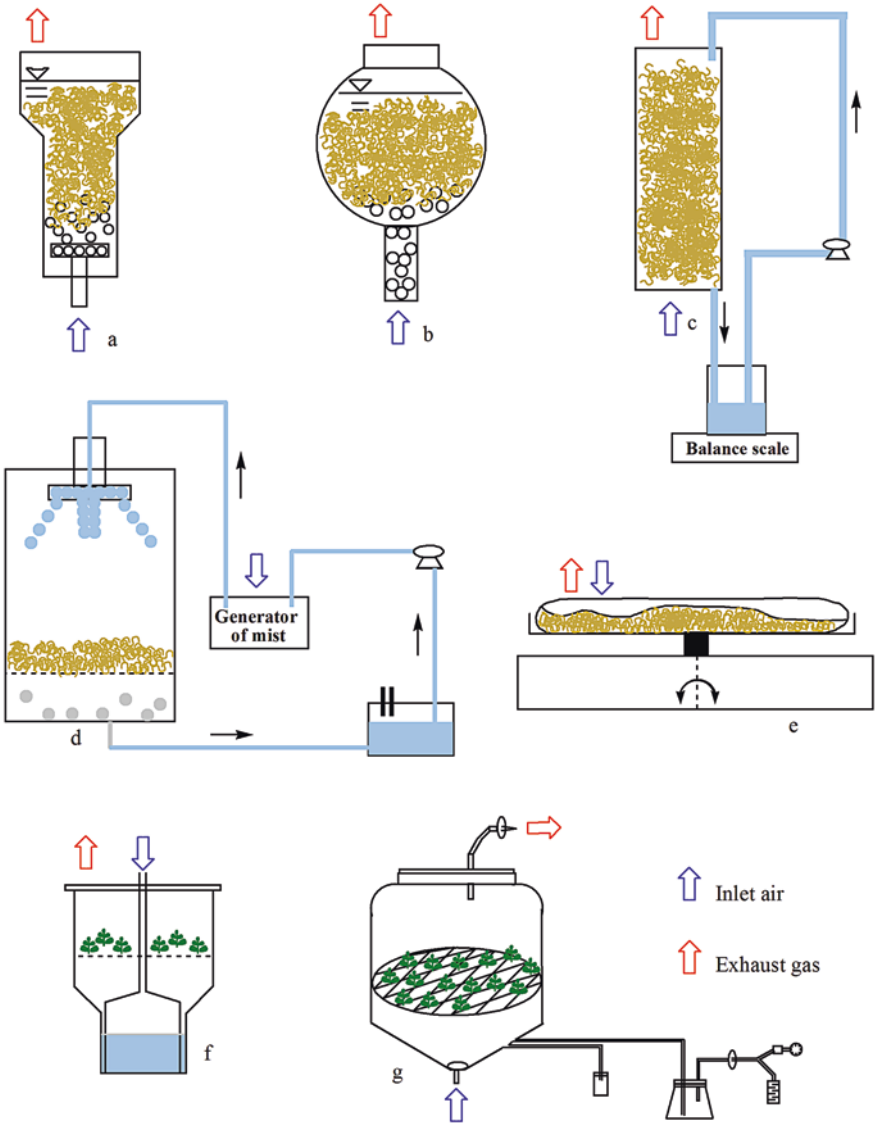


Fig. 18.8 Airlift, bubble, or nutrient mist bioreactors used for hairy root, adventitious, and embryo suspension cultures

the vacuole of cultured cells and organs which necessitates the maintenance of batch cultures and harvesting of the biomass for secondary metabolite extraction. For certain cultures, where cells and organs release the secondary products into the medium, a continuous culture system is adopted. Permeabilization and two-phase system are employed, and later, the medium is extracted to harvest the product.

18.2.1 Production of Paclitaxel from Cell Culture

Paclitaxel was originally extracted and identified by Wani et al. (1971) from the inner bark of *Taxus brevifolia* (the Pacific yew tree); later it was reported from several *Taxus* species. Paclitaxel accumulation in natural plants is very low (about 0.02% of dry weight), and its extraction cost is extremely high. Furthermore, the use of inner bark for paclitaxel production involves heavy destruction of trees; a single tree yields paclitaxel enough for about only 1 dose of drug. Although paclitaxel can be prepared by chemical synthesis (total synthesis and semisynthesis using more abundant taxoids), the process is not commercially viable. The most promising approach for sustainable production of paclitaxel and related taxoids is provided by plant cell cultures, and extensive research work has been carried out in cell cultures of *Taxus* species (Zhong 2002; Malik et al. 2011; Cusido et al. 2014). Among the *Taxus* species, effective paclitaxel yielding cell cultures can be established with respect to the species *Taxus baccata* (Malik et al. 2011), *Taxus media* (hybrid of *Taxus baccata* and *Taxus cuspidata*; Baebler et al. 2002; Bonfil et al. 2003), *T. cuspidata* (Fett-Neto et al. 1995; Li and Tao 2009), *T. brevifolia* (Collins-Pavao et al. 1996), *T. chinensis* (Luo and He 2004) and *T. globosa* (Tapia et al. 2013). Induction of calli on semisolid medium and establishment of cell suspension is the first step for the production of paclitaxel in different species of *Taxus*. Different explants, viz., cotyledons, hypocotyls, root or young seedlings, stems, and needles, have been used for callus induction. Various basal media such as Murashige and Skoog (1962), Gamborg (Gamborg et al. 1968), Shenk and Hildebrandt (1972), and Woody Plant Medium (McCown and Lloyd 1981) have been used for the initiation and maintenance of callus cultures (Malik et al. 2011). The medium has been supplemented with various phytohormones as well as organic supplements and additives, including casein hydrolysate, mannitol, polyvinylpyrrolidone, ascorbic acid, and amino acids to stimulate the growth and the proliferation of callus. Among all the phytohormones, 2,4-dichlrophenoxyacetic acid (2,4-D) in combination with kinetin (Kn) is the best combination for callus induction, growth, and maintenance.

Cell suspensions are initiated by inoculating friable calli into liquid medium, and various researchers showed that cell suspensions of *Taxus* species could able to produce paclitaxel and related compounds under optimized *in vitro* conditions (Zhong 2002; Tabata 2004; Franse 2007; Exposito et al. 2009). The optimization of plant secondary metabolite accumulation in cell suspension is a tedious process, since in most cases the best conditions for growth are different from those for the production of bioactive compounds. For example, working with media for *Taxus* cell suspension cultures for paclitaxel production, Cusido et al. (2002) tested the effects of two types of basal media (Gamborg and Woody Plant Medium) and sources of sugar (sucrose and sucrose + fructose), auxin [2,4-D and 1-napthaleneacetic acid (NAA) and picloram], and cytokinin [Kn, zeatin, 6-benzylaminopurine (BAP), and meta-Topolin] and developed as many as 48 possible culture media combinations. Improvement of productivity of paclitaxel in cell cultures was achieved by adopting various approaches/strategies including screening and selection of high yielding cell lines, optimization of culture media for growth of cells and production

of metabolites, optimization of culture conditions, elicitation, precursor feeding, immobilization, and two-phase culture. The preliminary approach in establishing a long-term *Taxus* cell cultures is thus the selection of a fast-growing cell lines capable of producing paclitaxel. Brunakova et al. (2004) reported the great variability among the callus cultures of *T. baccata* which were originating from the same mother plant. They selected nine well-growing callus lines, of which only one showed highest accumulation of paclitaxel (23.2 μg/g DW). Dark incubation of cultures is suitable for the growth and accumulation of biomass and secondary metabolites compared to 16 h light/8 h dark photoperiod (Brunakova et al. 2004; Wickremesinhe and Arteca 1993). Experiments of Cusido et al. (2002) showed that paclitaxel production is dissociated from cell growth and that it is recommendable to work with a two-stage system in which plant cells are first cultured in a medium optimized for growth and then transferred to the production medium (Cusido et al. 2002). Cells were cultured in Gamborg medium supplemented 1% sucrose, 0.5% fructose, 2.0 mg/l NAA, and 0.1 mg/l BAP for growth and then transferred to a medium with 3% sucrose, 2 mg/l picloram, and 0.1 mg/l Kn for accumulation of paclitaxel (Cusido et al. 1999; Palazon et al. 2003a). The growth and production of secondary metabolites from cultured cells depend greatly on the source of carbon employed, its concentration, and on the biosynthetic pathway or process involved, as well as requirements by different species. It was demonstrated that addition of fructose to moderately productive *T. baccata* cell cultures at day 10 significantly improved the accumulation of biomass in the later part of culture and even accumulation of paclitaxel (Hirasuna et al. 1996).

Elicitation has been applied as an important strategy for enhancing the paclitaxel in cell cultures of *Taxus* species (Wu et al. 2001; Yukimune et al. 1996). Jasmonic acid and its methyl esters have been used to increase the production of paclitaxel in cell cultures of *T. baccata* (Yukimune et al. 1996; Moon et al. 1998; Bonfill et al. 2006), whereas, methyl jasmonate was useful for paclitaxel production in the cell cultures *T. canadensis* (Semger et al. 2006) and *T. cuspidata* (Mijalili and Linden 1996). Other elicitors such as vanadyl sulfate, silver nitrate, cobalt chloride, arachidonic acid, ammonium citrate, and salicylic acid and polysaccharides of bacterial and fungal origin have also been used to improve paclitaxel production in *Taxus* cell cultures individually or in combination, and they have a pronounced effect on yield when applied synergistically. For instance, methyl jasmonate (10 mg/l) and salicylic acid (100 mg/l) along with *Rhizopus stolonifer* fungus (25 mg/l) was shown to improve paclitaxel production 16-fold when added at day 25–30 of culture to a culture medium (Khosroushahi et al. 2007). Feeding of cultures with precursors or sugars was another approach tested by some researchers. Fett-Neto et al. (1994) improved the paclitaxel production of *T. cuspidata* following feeding of phenylalanine, benzoic acid, and N-benzoylglycine, which are paclitaxel side-chain precursors. Choi et al. (2000) achieved a high level of paclitaxel production in *T. chinensis* through intermittent feeding of 3%, 1%, and 2% sucrose on days 0, 7, and 21, respectively.

Immobilization is one of the useful approaches for increasing the production of secondary metabolites. Immobilized cells have several advantages over freely

suspended cells including better cell to cell contact, high cell concentration per unit volume, and protection from shear stress (Brodelius and Mosbach 1982). Calcium alginate was used for immobilization of *T. baccata* cells (Bentebibel et al. 2005) and found threefold increments in production of paclitaxel. Mirjalili and Linden (1995) and Linden et al. (2001) studied the effects of gaseous environment of culture vessels especially on different concentrations and combination of oxygen, carbon dioxide, and ethylene on cell growth and paclitaxel production in suspension cultures of *T. cuspidata*. By means of several factorial design experiments in shake flasks, they suggested that 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide, and 5 ppm ethylene was ideal gas mixture composition for the production of paclitaxel. Effect of osmotic stress on paclitaxel production was investigated in cell suspension cultures of *T. chinensis* (Kim et al. 2001). The optimal paclitaxel production was achieved at an initial concentration of 60 g/l sucrose. Even osmotic pressure generated by mannitol or sorbitol, polyethylene glycol enhanced the paclitaxel production.

Application of two-phase culture system was another method for hyperaccumulation of paclitaxel in cell suspension cultures of *Taxus* species (Collins-Pavao et al. 1996). Secondary metabolites produced by cells are either accumulated in vacuoles of the cells or they are released into culture medium. Accumulation of secondary products inside the cell and/or release of such compounds into the cultures may inhibit the metabolic pathways. Therefore, it is essential to introduce another in situ extracting phase into cultures which can be useful for improving the production of metabolites as well as the process that facilitates the product purification. For in situ product removal, nonionic sorbents such as Amberlite XAD (a solid sorbent) were used in cell cultures of *T. cuspidata* on day 16, which enhanced the accumulation of paclitaxel by 40–70% (Kwon et al. 1998). Wang et al. (2001) used 10% (v/v) dibutyl phthalate during the late-log phase was useful for enhanced production of paclitaxel with minimal growth inhibition in *T. chinensis* cell suspension.

The scale-up cultures have been established by various groups of scientists, and several designs have been suggested for *Taxus* cell cultures (Zhong 2002; Franse 2007; Malik et al. 2011). Pneumatically mixed and stirred tank (Srinivasan et al. 1995); Wilson-type (Pestchanker et al. 1996); balloon-type bubble, bubble column, with split-plate internal loop, with concentric draught-tube internal loop, with fluidized bed and stirred tank (Son et al. 2000), and airlift (Navia-Osario et al. 2002a, b); turbine stirred tank (Cusido et al. 2002); and turbine stirred tank, tower airlift, and wave (Bentebibel et al. 2005) bioreactors have been successfully used for cultivation of *Taxus* cells for the production paclitaxel (Table 18.1). Bentebibel et al. (2005) used stirred, airlift, and wave bioreactors for the production of paclitaxel from freely suspended as well as immobilized cells of *T. baccata* and obtained optimal paclitaxel production in immobilized cells (43.43 mg/l at day 16, fivefold higher than free cells) in the stirred tank bioreactors, followed by wave and airlift bioreactors. Son et al. (2000) cultivated the *T. cuspidata* cells in balloon-type bubble bioreactor (BTBB), a bubble-column bioreactor (BCB), a BCB with a split-plate internal loop, a BCB with concentric draught-tube internal loop, a BCB with a fluidized bed reactor, and two different models of stirred tank reactors. Among the bioreactors used,

Table 18.1 Paclitaxel production from cell suspension cultures using bioreactor for *Taxus* species

Species	Type of bioreactors	Culture strategy	References
T. baccata	5 l stirred tank bioreactor	Use of free and immobilized cells	Bentebibel et al. (2005)
	4 l airlift bioreactor		
	2 l wave reactor		
	20 l airlift bioreactors	–	Navia-Osorio et al. (2002a, b)
	1 l pneumatically mixed and stirred tank bioreactors	Kinetic study	Srinivasan et al. (1995)
T. chinensis	1 l bubble-column reactor	Ethylene addition	Pan et al. (2000)
	1 l airlift bioreactor	Methyl jasmonate elicitation + sucrose feeding + ethylene addition	Dong and Zhong (2002)
	10 l stirred bioreactor	Methyl jasmonate elicitation	Wang and Zhong (2002b)
	1 l airlift bioreactor	Repeated methyl jasmonate elicitation	Wang and Zhong (2002b)
T. chinensis	2.5 l airlift bioreactor	Precursor feeding + sucrose addition	Yuan et al. (2001)
	20 l stirred tank reactor with two chambers of 10 l each with membrane in the center	Co-cultivation strategy – *Fusarium mairei* was cultured in one tank and *Taxus* cell in another tank and both were separated by membrane	Li et al. (2009)
T. cuspidata	600 ml Wilson-type bioreactor	Kinetic study	Pestchanker et al. (1996)
	20–500 l balloon-type bubble bioreactors	Medium replacement or feeding	Son et al. (2000)
T. media	5 l stirred bioreactor	Two-stage culture + methyl jasmonate elicitation + precursors	Cusido et al. (2002)
T. media	7 l mixed type of airlift bioreactor	–	Syklowska-Barank and Furmanowa (2005)
T. wallichiana	20 l airlift bioreactors	–	Navia-Osorio et al. (2002a, b)
T. yunnanensis	16 l stirred tank bioreactor	Ag^{+}, chitosan and methyl jasmonate elicitation	Zhang et al. (2002a)

BTBB was found suitable for *T. cuspidata* cell cultures, and cell doubling time was 12 days with 30% inoculum density. They adopted medium feeding strategy after determining the levels of sugars and medium conductivity of spent medium. With such study models, they adopted large-scale cultures of 500 l (BTBB) capacity and obtained approximately 3 mg/l paclitaxel and 74 mg/l total taxanes after 27 days of

culture. A highly efficient bioprocess technique for the production of paclitaxel with *T. media* was developed by adopting two stage cultures, viz., elicitor treatment and precursor feeding. Cell suspensions were maintained until cells entered stationary growth phase (12 days) and the second stage being the production medium supplemented with methyl jasmonate (220 μg g^{-1} fresh weight) along with mevalonate (0.38 mM) and N-benzoylglycine (0.2 mM). Under these conditions, 21.12 mg/l of paclitaxel were obtained after 8 days of culture in the production medium. Currently, bioreactor technology has been applied at commercial level (up to 75,000 l) for the production of paclitaxel and related taxanes by ESCA genetic (CA, USA), Phyton (NY, USA), Samyang Genex (Taejon, Republic of Korea), and Phyton Biotech (Germany) (Zhong 2002; Wink et al. 2005; Franse 2007).

Metabolic engineering is one of the strategies for making cell cultures as commercially viable method. The genes 10-deacetylbaccatin III-10-*0*-acetyltransferase (*dbat*) and 3′-*N*-debenzoyl-2′-deoxytaxol-*N*-benzoyltransferase (*dbtnbt*), which are involved in paclitaxel biosynthesis and intracellular taxane accumulation, were cloned into *T. baccata*, and their expression was studied in callus and cell cultures (Katkovcinova et al. 2008; Brunakova and Kosuth 2009). The *dbat* and *dbtnbt* genes that were expressed at higher levels were shown with callus growth and paclitaxel accumulation. Research work on construction of vectors and cloning of other useful genes involved in paclitaxel biosynthetic pathway is in progress.

18.2.2 Production of Camptothecin from Cell and Organ Culture

Biosynthesis and accumulation of camptothecin (CPT) was initially reported in *Camptotheca acuminata* and later in other plants species including *Camptotheca lowreyana* and *C. yunnanensis* (Li et al. 2002), *Ervatamia heyneana* (Gunasekera et al. 1979), *Merrilliodendron megacarpum* (Arisawa et al. 1981), *Mostuea brunonis* (Dai et al. 1999), *Nothapodytes foetida* (Govindachari and Viswanathan 1972), *Ophiorrhiza* species (Beegum et al. 2007), and *Pyracantha klaineana* (Zhou et al. 2000). Organ-, species-, and genotype-specific accumulation of CPT were reported in natural plants of these species (Table 18.2). Highest amount camptothecin accumulation was reported in leaves of *Camptotheca acuminata* (0.4–0.5% dry weight; Lopez-Meyer et al. 1994) compared to other organs. Since relatively low amounts of CPT are isolated from natural plants of *C. acuminata*, plant cell culture has been extensively explored for the production of CPT. Callus and cell suspension cultures were established in *Camptotheca acuminata* showed accumulation of lower quantity of CPT in (0.04–0.41%) (van Hengel et al. 1992; Zhang et al. 2002b; Ma 2007). Wiedenfeld et al. (1997) raised callus and organ cultures of *C. acuminata*, and they cultured callus on composition of Murashige and Skoog (1962), Delfel-Smith (Delfel and Smith 1980), Nitsch and Nitsch (1969), and Gamborg (Gamborg et al. 1968) media with various growth regulator supplements and observed accumulation of CPT ranging from 0.2 to 0.23%, 0.10 to 0.12%, and 0.11 to 0.12%, respectively, in callus, plantlets, and shoots. Media optimization studies revealed that a nitrogen concentration of 70 nM in the Murashige and Skoog medium gave highest biomass

Table 18.2 Camptothecin contents in different plant species and cell/organ cultures

Plant species	Plant parts/cell and organ culture	Amount of CPT % dry weight	References
Camptotheca acuminata	Leaves	0.40–0.50	Lopez-Meyer et al. (1994)
	Seeds	0.30	
	Bark	0.18–0.20	
	Yong leaves	0.24–0.30	Li et al. (2002)
	Hairy roots	0.10	Lorence and Nessler (2004)
	Callus	0.20–0.23	Wiedenfeld et al. (1997)
	Plantlets	0.10–0.12	
	Shoots	0.11–0.12	
C. lowreyana	Young leaves	0.39–0.55	Li et al. (2002)
	Old leaves	0.9–0.11	
C. yunnanensis	Young leaves	0.25–0.44	
	Old leaves	0.059	
Ervatamia heyneana	Wood and stem bark	0.13	Gunasekera et al. (1979)
Merrilliodendron megacarpum	Leaves and stem	0.053	Arisawa et al. (1981)
Mostuea brunonis	Whole plant	0.01	Dai et al. (1999)
Nothapodytes foetida	Stem wood	0.14–0.24	Aiyama et al. (1988)
		0.14	Padmanabha et al. (2006)
	Stem bark	0.30	Govindachari and Viswanathan (1972)
	Plant	0.05	Yamazaki et al. (2003)
	Stem bark	0.23	Padmanabha et al. (2006)
	Leaves	0.08	
	Root bark	0.33–0.77	
	Root wood	0.18	
	Seeds	0.50	Roja and Heble (1994)
	In vitro regenerated shoots	0.075	
	Cell suspension cultures	0.11%	Fulzele et al. (2001)
	Leaves of *in vitro* regenerated plants	0.028–0.082	Dandin and Murthy (2012)
	Stems of *in vitro* regenerated plants	0.021–0.037	
	Callus	1.30	Thengane et al. (2003)
	Callus	0.2–0.3	Ciddi and Shuler (2000) and Sundravelan et al. (2003)
Ophiorrhiza alata	Hairy roots	7.85	Yu-ut et al. (2011)
O. kuroiwai	Hairy roots	0.022	Asano et al. (2004)
O. liukiuensis	Hairy roots	0.083	
O. mungos	Whole plant	0.001	Tafur et al. (1976)
O. pumila	Young roots	0.10	Saito et al. (2001)
	Leaves	0.03	
	Hairy roots	0.10	

(continued)

Table 18.2 (continued)

Plant species	Plant parts/cell and organ culture	Amount of CPT % dry weight	References
O. rugosa	Albino plants	0.10	Vineesh et al. (2007)
	Normal plants	0.03	
Pyrenacantha klaineana	Stems	0.005	Zhou et al. (2000)

accumulation of *C. acuminata* cell suspension cultures, while a NH_4^+/NO_3^- molar ratio of 5:1 (total of 40 mM nitrogen) gave optimum CPT yield (Pan et al. 2004). Similarly supplementation of altered NH_4^+/NO_3^- was suitable for the production of withanolides in cell suspension cultures of *Withania somnifera* (Murthy and Praveen 2012; Praveen and Murthy 2011, 2013). Hairy roots were induced in *C. acuminata* using *Agrobacterium rhizogenes* strains ATCC 15834 and R-1000, and hairy roots were cultured in Gamborg's medium containing 3% sucrose and were able to synthesize 0.10% of CPT. Even though callus, cell, and organ cultures of *C. acuminata* have accumulated comparatively lesser amounts of CPT, there is a scope for medium optimization and to enhance accumulation of CPT. *Nothapodytes nimmoniana* and *Ophiorrhiza pumila* were another two plant species which were accumulating CPT in higher quantity, and in these two species, various workers have established callus and cell culture system. Mature trees of *Nothapodytes nimmoniana* accumulated CPT at a concentration of 0.075% and 0.5% in the shoots and seeds, respectively (Roja and Heble 1994). The cell and organ cultures of *N. nimmoniana* produced much lower CPT than natural plants (Roja and Heble 1994; Fulzele et al. 2001; Dandin and Murthy 2012). However, more recently established callus cultures of *N. nimmoniana* have been found to produce higher levels of CPT even higher than the natural stand (Ciddi and Shuler 2000; Sundravelan et al. 2003; Thengane et al. 2003). These reports suggest the selection of superior genotype/s and cell or calli clones is essential for the *in vitro* production of CPT from cell and organ of *N. nimmoniana.* In cell cultures of *Ophiorrhiza pumila*, however, cell biomass was not able to synthesize the CPT (Kitajima et al. 1998). Hairy roots were induced in *O. pumila* (Saito et al. 2001), and hairy root cultures could be able to accumulate higher biomass and CPT. The amount of CPT accumulated by hairy root biomass was comparable to natural roots of *O. pumila* (0.10% dry weight). Hairy roots were also induced in *O. alata*, *O. kuroiwai*, and *O. liukiuensis*; higher accumulation of CPT was reported in hairy root cultures (Asano et al. 2004; Saito et al. 2001; Sudo et al. 2002; Yu-ut et al. 2011) (Table 18.2).

Elicitation is one of the important strategies for hyper-accumulation of secondary metabolites in cell and organ cultures (Murthy et al. 2014a, b). Various kinds of abiotic elicitors such as metal ions, ultraviolet (UV) radiation, and biotic elicitors like pectin or cellulose, chitin, chitosan, glucans, and phytohormones, namely, jasmonic acid and salicylic acid, have been used successfully in cell and organ cultures (Namdeo 2007; Murthy et al. 2014a, b). CPT production was increased in cell cultures of *Camptotheca acuminata* with the addition of elicitors such as methyl jasmonate, jasmonic acid, and yeast extract. A similar enhanced effect was seen after

the addition of methyl jasmonate to *Panax ginseng* cell cultures and *Eleutherococcus sessiliflorus* embryogenic suspension cultures (Thanh et al. 2005; Shohael et al. 2008). Thirty-fold increment of CPT was reported (1.17 mg/g fresh weight) with the addition of Cu^{++} ions ($CuCl_2$ was used as source) in the medium (Gu et al. 2006). Similarly positive effect of salicylic acid and UV-B on accumulation of CPT was on records (Pi et al. 2010). *Ophiorrhiza pumila* hairy root culture medium was found to contain enhanced amounts of CPT, which could be increased with the addition of a polystyrene resin Diaion HP-20 to the medium (Saito et al. 2001). The addition of Diaion HP-20 into the medium increased the secretion of CPT in the medium which is useful for isolation and purification of CPT from the medium itself. Bioreactor production of CPT by hairy root cultures (3 L capacity) of *O. pumila* was reported by Sudo et al. (2002), and CPT accumulation was comparable to cell cultures in shake flasks. Despite these advances with cell and organ cultures of *Camptotheca*, *Nothapodytes*, and *Ophiorrhiza*, still the commercial production of CPT is not feasible. Systematic approaches such as strain improvement and medium and culture environment optimization should be worked out in these systems. The identification of enzymes involved in biosynthesis of CPT and adoption of metabolic engineering strategies will undoubtedly impact the production of CPT by cell and organ cultures.

18.2.3 Production of Podophyllotoxin from Cell and Organ Culture

Cell, callus, and tissue cultures were established in different plant species including *Callitris drummondii*, *Juniperus chinensis*, *Linum album*, *Linum strictum* ssp. *strictum*, *Podophyllum hexandrum*, and *P. peltatum* in which PTOX was reported by several investigators and all of them were accumulating PTOX in different levels (Table 18.3). Callus induction and presence of PTOX was first demonstrated in *P. peltatum* by Kadkade (1981, 1982). Subsequently, callus was induced in *P. hexandrum* using seedlings grown *in vitro* (van Uden et al. 1989). Callus was also initiated from leaves of *Callitris drummondii* and from stem and leaves of young seedlings of *L. album* on different media (van Uden et al. 1990b; Smollny et al. 1992; Muranaka et al. 1998).

Cell suspension cultures of *Linum album* were established in Murashige and Skoog medium supplemented with NAA, which accumulated 0.5% lignans containing podophyllotoxin and other products (Smollny et al. 1998). *Podophyllum hexandrum* cell suspension was also initiated by van Uden et al. (1989), Heyenga et al. (1990), and Chattopadhyay et al. (2001) in Gamborg as well as in Murashige and Skoog medium. The cultures which were raised by Chattopadhyay et al. (2001) in Murashige and Skoog medium with IAA required 30 days for biomass accumulation, and these cultures could produce 4.9 mg/l PTOX. Several researchers have attempted *in vitro* propagation and raising the plantlets of *Podophyllum* which could be used as a source of PTOX. A reliable protocol for propagation and cultivation of *P. hexandrum* was developed by Armugam and Bhojwani (1990) and Nadeem et al.

Table 18.3 Podophyllotoxin production from cell and organ cultures of *Podophyllum* and other species

Plant species	Cell or organ culture	Amount of PTOX % dry weight	References
Callitris drummondii	Callus, cell suspension	0.02	van Uden et al. (1990b)
Juniperus chinensis	Callus culture	0.01	Muranaka et al. (1998)
	Callus	0.09	Premjet et al. (2002)
Linum album	Callus	0.30	Smollny et al. (1992)
	Cell suspension	0.50	Smollny et al. (1998)
	Cell suspension	0.11	Empt et al. (2000)
	Cell suspension	0.77	van Furden et al. (2005)
	Cell suspension	0.86	Federolf et al. (2007)
	Transformed callus	1.25	Farkya and Bisaria (2008)
	Hairy roots	0.62	Farkya and Bisaria (2008)
	Hairy roots	0.51	Baldi et al. (2008)
	Cell suspension	0.06	Baldi et al. (2008)
Linum strictum	Hairy roots	0.06	Vasilev and Ionkova (2004)
Podophyllum hexandrum	Cell suspension	0.09	Heyenga et al. (1990)
	Callus	0.07	Chattopadhyay et al. (2003a)
	Cell suspension	0.86	Chattopadhyay et al. (2003b)
Podophyllum peltatum	Callus	0.38	Kadkade (1981, 1982)
	Embryos	0.01	Anabazhagan et al. (2008)
	Adventitious roots	0.03	Anabazhagan et al. (2008)

(2000). The plants regenerated using rhizome tip explants of *P. peltatum* on Murashige and Skoog medium supplemented with benzyladenine and activated charcoal. The PTOX content in regenerated plantlets was similar that found in the wild (Moraes-Cerdeira et al. 1998). Genetically transformed *P. hexandrum* were obtained by embryo transformation, using different strains of *Agrobacterium rhizogenes*, stains A4, 15,834, and K599. The transformed calli cultures were reported to accumulate three times higher PTOX compared to control (Giri et al. 2001). Hairy root cultures of *Linum flavum* have been established and reported to accumulate 1% (dry weight) 5-methoxypodophyllotoxin and its glycoside derivatives (van Uden et al. 1992). Hairy root cultures were also developed by Oostdam et al. (1993) in *Linum flavum*, and such hairy root clones could able to accumulate about 1.5–3.5% (dry weight) of 5-methoxypodophyllotoxin.

Various methods for improvement of PTOX yield in cell cultures of *P. hexandrum* have been attempted by many researchers. The effect of nitrogen and phosphate levels on suspension cultures have been investigated by Chattopadhyay et al.

(2003b); Murashige and Skoog medium supplemented with 60 mM nitrogen having 1:2 ammonium to nitrate ratio, 60 g/l glucose, and 1.25 mM phosphate was found to be congenial for the accumulation of PTOX. The Plackett-Burman design is one of the statistical methods to identify relatively significant media components from the different selected variables (Kalil et al. 2000). Such method was applied to optimize the media and culture conditions in *Podophyllum hexandrum* cell cultures for growth of biomass and accumulation of PTOX. The optimized conditions were found to be medium pH 6.0, 1.25 mg/l of IAA, 72 g/l glucose, and 8 g/l inoculum size. With such optimized conditions, a maximum of 20.3 g/l of dry biomass and 0.071 mg/l PTOX was obtained (Chattopadhyay et al. 2003b).

Precursor feeding was one the procedure followed for increased accumulation of PTOX and related compounds in cell and organ suspension cultures. Application of the amino acid L-phenylalanine to cell cultures of *Linum flavum* resulted in a three- to fivefold increase in the levels of 6-methoxypodophyllotoxin (van Uden et al. 1990c). Similarly, the addition of precursor, coniferyl alcohol, and β-cyclodextrin to *Podophyllum hexandrum* cell suspension cultures increased the yield of PTOX four-fold. Hairy roots of *Linum flavum* and *Podophyllum hexandrum* were cocultivated in dual flasks and bioreactors. PTOX concentrations in co-cultures were increased by 240 and 72% in dual shake flasks and bioreactors, respectively, when compared to single cultures. It was shown that coniferin provided to *Linum flavum* hairy roots acts as a precursor for accumulation of PTOX by *Podophyllum hexandrum* cells (Lin et al. 2003). Enhancement of cell permeabilization facilitates the release of secondary metabolites into the culture medium and also improves further synthesis and accumulation in the cultured cells. Permeabilization with 0.5% isopropanol led to a partial release of PTOX into the culture medium (van Uden et al. 1990a). Attempts have been made to increase the accumulation of PTOX by elicitation treatments in *Linum* cell cultures. However, elicitors such as methyl jasmonate, hydrogen peroxide, and salicylic acid or *Phytophthora megasperma* treatments increased the PTOX accumulation in *Linum* sp. (Arroo et al. 2002).

Optimization of culture environment was used as a useful strategy for biomass and metabolite accumulation, and the effect of temperature, light/dark treatment, medium pH, and agitation of cultures have been worked out in cell cultures of *Linum* sp. and *Podophyllum* sp. *P. hexandrum* cell growth was better at 20 °C, while *Callitris drummondii* exhibited a temperature optimum of 26 °C for the accumulation of PTOX (van Uden et al. 1990c; Chattopadhyay et al. 2002). Dark suspension cultures of *P. hexandrum* accumulated 0.1% (day weight basis) PTOX during the stationary phase of growth cycle that was three to four times more than light grown cultures (van Uden 1993). In suspension cultures of *L. album* grown in light and dark, the maximum concentration of lignans achieved was up to 0.2% and 0.5% (dry weight basis), respectively (Smollny et al. 1998). A medium pH of 6.0 was beneficial for high biomass and PTOX accumulation in *Podophyllum hexandrum* cell cultures (Chattopadhyay et al. 2002). *P. hexandrum* cells growing in shake flasks were found to be sensitive to hydrodynamic stress generated by changing rotational speeds (Chattopadhyay et al. 2002). A rotational speed of more than 150 rpm was reported to be deleterious and led to reduction of cell viability, and

agitation speed of 100 rpm was ideal for biomass accumulation and PTOX production in cell suspension of *P. hexandrum* (Chattopadhyay et al. 2002).

Chattopadhyay et al. (2003a) cultured *Podophyllum hexandrum* cells in 3 l stirred tank bioreactors, optimized various process parameters, and studied the effect of batch, fed batch, and continuous cultures. They have reported the accumulation of 53 g/l of biomass and 48.8 mg/l of PTOX with continuous mode of operation. Likewise, *L. album* cell suspensions were cultured successfully in a 20 l bioreactor, which resulted in an overall optimal product yield of 0.2% PTOX (Arroo et al. 2002).

18.2.4 Production of Ginsenosides from Cell and Organ Cultures

Cell and organ cultures have been explored extensively for rapid and large-scale production of ginsenosides since field cultivation of ginseng needs 4–6 years and quality control is a difficult process (Wu and Zhong 1999; Paek et al. 2009; Murthy et al. 2014a, b; Kim et al. 2015) (Table 18.4). Extensive work has been carried on cell cultures of *Panax notoginseng* and adventitious root cultures of *P. ginseng*, and cell or root lines which are good for biomass and ginsenoside accumulation were selected. Various medium and physical parameters influencing the ginsenoside yield such as type of nutrient medium, salt strength, type and levels of growth regulators, types and levels of carbohydrate/s, inoculum density, levels of major elements such as nitrogen and phosphate, hydrogen ion concentration, agitation, aeration, light, and temperature have been worked out systematically (Murthy et al. 2014a, b; Kim et al. 2015).

Medium components such as sugar levels have dramatic effects on ginsenoside yield with cell suspension cultures of ginseng, for example, the feeding of sucrose from 20 to 40 g/l in *Panax notoginseng* suspension culture promoted 2.3-fold production of total ginsenosides (Zhang et al. 1996a), whereas, in the case of *P. ginseng*, sucrose feeding of 60 g/l enhanced 3.5-fold accumulations of ginsenosides compared to control containing 30 g/l sucrose in the medium (Akalezi et al. 1999; Wu and Zhong 1999). Even feeding of cultures with sorbitol along with casein hydrolysate also increased 3.5-fold of total ginsenosides. The increased phosphate concentration in medium from 0 to 1.25 mM was reported to enhance the sevenfold productivity of ginsenosides in suspension cultures of *P. notoginseng* and *P. quinquefolius* (Zhong and Zhu 1996; Liu and Zhong 1998). *P. ginseng* cell cultures also accumulated enhanced amount of ginsenosides (fourfold increment compared to control) with the initial addition of 6.0 μM copper to the medium. Furthermore, a higher rate of nitrate to ammonium can also improve the yield of ginsenosides in cell cultures of *P. ginseng*, P. *notoginseng*, and *P. quinquefolius* (Zhang et al. 1996b, Paek et al. 2009).

Elicitation with methyl jasmonate (200 μM) could be able to enhance the accumulation of ginsenosides particularly PPD type of ginsenosides in cell cultures of *P. ginseng* and *P. notoginseng* (Thanh et al. 2005; Wang et al. 2005; Hu and Zhong 2007). Similarly, the addition of 10 μM N,N′-dicyclohexylcarbodiimide to *P.*

Table 18.4 Production of ginsenosides from cell and organ cultures of *Panax* species

Plants	Type of cultures	Strategy adopted	Amount of ginsenoside produced	References
Panax ginseng	Cell suspension in flasks	Addition of 0.42 mM phosphate	643 mg/l	Liu and Zhong (1998)
	Cell suspension in flasks	Addition of 6% sucrose	275 mg/l	Akalezi et al. (1999)
	Cell suspension in flasks	0.2 M sorbitol and 0.5 g/l casein hydrolysate	1130 mg/l	Wu et al. (2005)
	Cell suspension	Elicitation by addition of 100 μM vanadate	5.5 mg/g DW, 55 mg/l	Hunag and Zhong (2013)
	Cell suspension in flasks	Elicitation by addition of 10 μM N, N′-dicyclohexylcarbodiimide	33 mg/g DW, 36 mg/l	Huang et al. (2013)
	Cell suspension in 5 l bioreactor	Elicitation by addition of 200 μM methyl jasmonate for last 10 days of culture	3.33 mg/g DW, 100 mg/l	Thanh et al. (2005)
	Adventitious root culture in flasks	Elicitation by addition of 100 μM methyl jasmonate	48 mg/g DW, 364 mg/l	Kim et al. (2007)
	Adventitious root culture in flasks	25 μM IBA and elicitation by addition of 100 μM methyl jasmonate	50 mg/g DW, 500 mg/l	Kim et al. (2007)
	Adventitious root culture in flasks	Elicitation by addition of 2 mg/l jasmonic acid	25 mg/g DW, 255 mg/l	Yu et al. (2002)
	Adventitious root culture in 3.5 l airlift bioreactor	Addition of polyunsaturated fatty acid	7.9 mg/g DW, 87 mg/l	Dewir et al. (2010) and Wu et al. (2009)
	Adventitious root culture in 5 l airlift bioreactor	Addition of ethephon and 100 μM methyl jasmonate	30 mg/g DW, 270 mg/l	Bae et al. (2006)
	Adventitious root culture in 5 l airlift bioreactor	Addition of IBA	8.09 mg/g DW	Paek et al. (2009)
	Adventitious root culture in 5 l airlift bioreactor	Addition of 5% sucrose	10.02 mg/g DW, 83.6 mg/l	Paek et al. (2009)
	Adventitious root culture in 5 l airlift bioreactor	Addition of 10.25 mM NO_3^-/NH_4^+ (18:5.0)	9.89 mg/g DW, 83.6 mg/l	Paek et al. (2009)
	Adventitious root culture in 5 l airlift bioreactor	Addition of 100 μM methyl jasmonate	40–50 mg/l; 160–480 mg/l	Kim et al. (2004)

(continued)

Table 18.3 (continued)

Plants	Type of cultures	Strategy adopted	Amount of ginsenoside produced	References
P. ginseng	Adventitious root culture in 5 l airlift bioreactor	Addition of 10–25 µM Cu	3–4 mg/g DW, 26 mg/l	Paek et al. (2009)
	Adventitious root culture in 1000 l airlift bioreactor	Addition of 100 µM methyl jasmonate	40 mg/g DW, 378 mg/l	Paek et al. (2009) and (Murthy 2014a, b)
	Hairy root culture in flasks	–	2.5–5.4 mg/g DW	Mallol et al. (2001)
	Hairy root culture in flasks	Elicitation by addition of 1.2% (w/v) Tween 80	–	Liang et al. (2015)
	Hairy root culture in flasks	Elicitation by addition of 200 µM methyl jasmonate	27 mg/g DW	Kim et al. (2009)
	Hairy root culture in wave bioreactor	Medium exchange every 14 days	145.6 mg/l	Palazon et al. (2003b)
P. notoginseng	Cell suspension in flasks	Addition of 1.25 mM phosphate	30 mg/g DW, 980 mg/l	Zhong and Zhu (1996)
	Cell suspension in flasks	Addition of 4% sucrose	177 mg/l	Zhang et al. (1996a)
	Cell suspension in flasks	Addition of 60 mM NO_3^-/NH_4^+ (60:0)	859 mg/l	Zhang et al. (1996b)
	Cell suspension in flasks	Elicitation by addition of 6.0 µM Cu	43 mg/g DW, 119 mg/l	Zhong and Wang (1996)
	Cell suspension in flasks	Elicitation by addition 200 µM methyl jasmonate or 2-hydroxyethyl jasmonate	20–28.9 mg/g DW, 167–241 mg/l	Hu and Zhong (2007) and Wang and Zhong (2002a)
	Cell suspension in 30 l centrifugal impeller bioreactor	High density cell culture	30 mg/g DW, 2.1 g/l	Zhang and Zhong (2004)
	Cell suspension in 1 l bioreactor	Elicitation by addition of 200,200 µM methyl jasmonate	23 mg/g DW, 530 mg/l	Wang et al. (2005)
	Adventitious root culture in flasks	–	45 mg/g DW, 120 mg/l	Gao et al. (2005)

(continued)

Table 18.3 (continued)

Plants	Type of cultures	Strategy adopted	Amount of ginsenoside produced	References
P. quinquefolius	Cell suspension in flasks	Addition of 1.25 mM phosphate	960 mg/l	Liu and Zhong (1998)
	Cell suspension in flasks	–	29 mg/g DW, 197 mg/l	Kochan and Chmiel (2011)
	Hairy root culture in flasks	–	3–10 mg/g DW	Mathur et al. (2010) and Kochan et al. (2012)
	Hairy root culture in 10 l bioreactor	–	6 mg/g DW	Kochan et al. (2012)
P. japonicus	Cell suspension in flasks in 500 l bioreactor	–	5–49 mg/g DW	Kochkin et al. (2013)
	Hairy root culture in flasks	–	60 mg/g DW	Zhang et al. (2010)

ginseng cell cultures was also beneficial for higher accumulation of ginsenosides (Huang et al. 2013). Cell suspension cultures of *P. notoginseng* were also established using stirred tank bioreactors (Wu and Zhong 1999). However, yield and productivity of ginsenosides were comparatively low compared to flask scale cultures. Large-scale cell suspension culture of *P. ginseng* is feasible in 500 l capacity drum and balloon-type airlift bioreactors, and by culturing 60 g/l inoculum in Murashige and Skoog medium supplemented with 7 mg/l IBA and 0.5 mg/l kinetin, 30 g/l sucrose for 30 days, 187 kg and 400 kg fresh biomass, and 6.2 kg and 13.3 kg dry biomass, 7.86 mg/g DW and 7.75 mg/g DW total ginsenosides could be achieved (Thanh et al. 2014a, b).

Adventitious roots were induced in *Panax ginseng* cultured in small-scale to large-scale cultures after establishing the culture parameters and bioprocess strategies (Murthy et al. 2014a, b, 2016). Adventitious root cultures were regarded as advantageous over cell cultures for higher biomass and ginsenoside production because of their stability in physical and chemical conditions during culture process. Adventitious roots were induced from the root explants of *P. ginseng* on Murashige and Skoog medium supplemented with growth regulators (Son et al. 1999; Yu et al. 2000). Adventitious root suspension cultures were established in Murashige and Skoog medium supplemented with 19.68 μM IBA and 3% sucrose in flask and 3 l capacity airlift bioreactors. The suitability of the medium, salt strength, growth regulator type, combination and concentration, type and concentration of carbohydrate, nitrogen source and its concentration, physical conditions such as light and temperature, agitation, and aeration have been investigated methodically by different researchers (Table 18.4). Influence of gaseous composition of oxygen, carbon dioxide, and ethylene on adventitious root cultures of *P.*

ginseng was well documented by Jeong et al. (2006). Air supplied to the bioreactors was enriched with 30 and 40% oxygen, 2.5 and 5% carbon dioxide, or 10 and 20 ppm of ethylene, and only enrichment with 40% oxygen enhanced biomass and ginsenoside accumulation compared to conventional sterilized air. *Panax ginseng* adventitious root cultures were fed precursor squalene which was beneficial on biomass and metabolite productivity (Sivakumar et al. 2006), whereas medium replenishment method was employed by Jeong et al. (2008) which resulted in a 26% increase in dry biomass (28.7 g/l) and an 8.3% increment in ginsenoside content (4.92 mg/g DW). These results demonstrate that precursor feeding and medium replenishment are useful bioprocess strategies for improving secondary metabolite production. Adoption of such culture strategies helped in establishment of large-scale adventitious root cultures of *P. ginseng* in 10,000 l airlift bioreactors and 850 kg of fresh biomass and 85.4 kg of dry biomass, and 40 mg/g DW ginsenoside productivity could be achieved with the operation batch culture of 45 days duration (Murthy et al. 2014a, b). Hairy root cultures were also established by infecting ginseng explants with *Agrobacterium rhizogenes*, because of their stability, quick growth and biomass accumulation, and flexibility for scale-up production using bioreactors (Mallol et al. 2001; Palazon et al. 2013b; Woo et al. 2004; Yu et al. 2005). Overproduction of ginsenosides by metabolic engineering was an alternative approach followed by many researchers. For example, *PgHMGRI*, *PgFCAS*, *PgDDS*, *PgSS1*, and many other genes involved in triterpenoid biosynthetic pathway have been cloned to ginseng which has improved the accumulation of ginsenosides in transgenic hairy root lines and plants (Kim et al. 2015). These results suggest that identification of additional functional enzymes and their genes and cloning of such genes involved in biosynthesis of ginsenosides will certainly help in large-scale production of useful saponins.

18.3 Conclusions and Future Prospects

Despite the effective methods developed for the production of anticancer compounds such as paclitaxel, camptothecin, podophyllotoxin, and ginsenosides through cell and organ culture for large-scale production, the commercial exploitation of anticancer compounds produced *in vitro* has certain setbacks related to the factors affecting the biomass and metabolite accumulation especially chemical, physical, and bioprocess parameters which are needed to be overcome. Metabolic engineering may help to overcome such obstacles and may enhance and modify the production of such pharmaceutically valuable secondary compounds. This approach is imperial in reducing the catabolism of desired compounds and increased expression of the rate limiting enzymes and effective expression of all the genes involved in the pathway, or it may be a useful tool in identification of key enzymes and the genes responsible for key enzymes, cloning of such genes, and their overexpression in the selected clones in other plant species which possess potent anticancer compounds.

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Plant-Derived Extracts and Compounds: An Alternative Therapy Against Breast Cancer

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Abstract

Breast cancer is among the second overall cause for death in women. In 2012, 1.7 million new cases of breast cancer have been reported worldwide. Emerging modern cancer therapy advocates the usage of alternative natural sources for cure and prevention of breast cancer. Plant sources are most widely used for naturally derived anticancer agents. Plant crude extracts, plant-derived compounds, and secondary metabolites have shown anticancer healing or protective property. Thus, the aim of this chapter is to summarize the various plant sources having the potential to be used as an anti-breast cancer agent.

Keywords

Alkaloids · Anticancer agents · Breast cancer · Plant-derived compounds/extracts · Secondary metabolites

19.1 Introduction

Breast cancer is second overall cause for women mortality worldwide. According to National Cancer Institute Surveillance Epidemiology and End Results (SEER) database, it was estimated in 2016 that 2,46,660 women in the United States will be diagnosed with invasive breast cancer, and 61,000 women will be diagnosed with in

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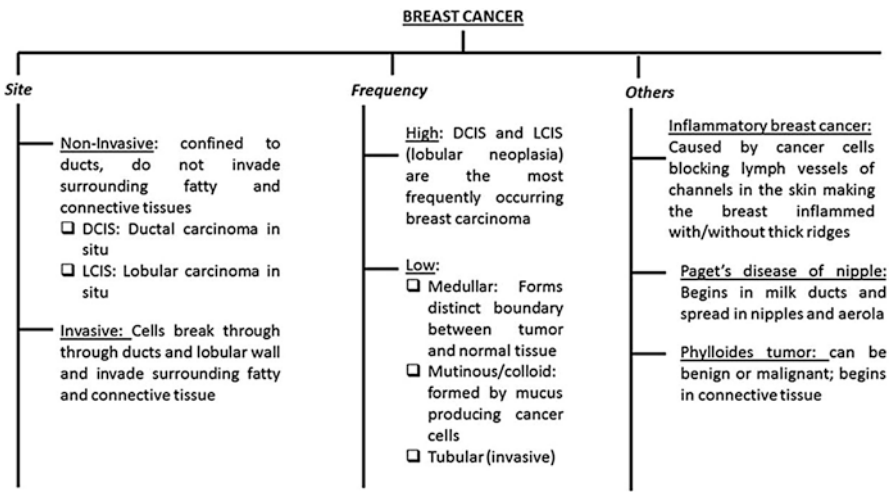

Fig. 19.1 Classification of breast cancer

situ breast cancer, and estimated 2600 men in the United States will be diagnosed with breast cancer. Occurrence of tumor in breast is tissue-specific; some occur in ducts known as ductal cancers (ACS 2016); some occurs in cells that line the lobules known as lobular cancers, while some begin in other tissues. Breast cancer can be classified in diverse ways; Fig. 19.1 gives an overview of the same adapted from Sharma et al. (2010). Numerous factors may lead to development of breast cancer like genetic causes, hormonal causes, and/or lifestyle causes. In context of hormone receptors, breast cancer can be endocrine-receptor positive, triple positive, or triple negative. ER-positive breast cancer responds toward the estrogen or progesterone receptors. Triple-positive carcinomas are those which respond to estrogen and progesterone as well as overproduce HER2, while triple-negative carcinomas are those which don't have hormone receptors neither overproduce HER2.

Conventional treatment of breast carcinomas includes surgery like lumpectomy, mastectomy, or breast-conserving surgery (Sharma et al. 2010). Radiation therapy and chemotherapy treatments are given to the patients (Grover et al. 2017; Diaby et al. 2015). Recent advances in treatment of breast carcinoma include usage of nanotechnology, gene therapy, oncogene inactivation, augmentation of tumor suppressor genes, cell-targeted suicide, chemoprotection approach, virus-mediated oncolysis, and immunomodulation (Tanaka et al. 2009; Osborne et al. 2004; Wang and Liu 2003; Disis and Park 2009). Although most advanced conventional therapies are used for treatment of breast carcinoma, they have serious side effects, or their role limits to only extending the life span for few years. Thus, there is a need to carry out alternative therapies, concepts, or approaches for prevention of breast cancer. Plant compounds and extracts are most promising candidates as they have been reported with medicinal traits with varied applications (Oskoueian et al. 2011;

Uche and Aprioku 2008; Sharma et al. 2012; Prasad et al. 2012). The aim of this chapter is to summarize the various plant sources having the potential to be used as an anti-breast cancer agent.

19.2 Plant Compounds and Extracts: Inhibitors of Breast Cancer

Plants belonging to *Fabaceae* or *Papilionaceae* family were used to catch and kill fish in the nineteenth century; this ingredient later was identified as isoflavonoid. Commercial use of plant for cancer treatment dates back in the1960s. According to National Cancer Institute, US Department of Agriculture (USDA) collected samples of Pacific yew's bark (*Taxol brevifolia*) which had the potential to fight carcinomas of breast, lung, and ovary (Weaver 2014). After years of research and clinical trials, FDA approved Taxol (http://www.biotopics.co.uk/ jsmol/taxol.html) as anti-breast cancer compound in 1994 (Ferlini et al. 2003; Wani et al. 1971; Fuchs and Johnson 1978). From then till now, hundreds of drugs are discovered including vinblastine (Fig. 19.2), vincristine (Fig. 19.3), and alkaloids. Quest of new anticancer drugs originated from plant source is a very active research field due to single- and multidrug resistance of tumor cells. Thus, such drugs can be used in increasing efficacy of chemotherapy considerably (El-Sayed and Cordell 1981; El-Sayed et al. 1983; Wall and Wani 1995; Leveque and Jehl 1996). Compounds/extracts of plants are those compounds which take part in plant defense mechanisms. Moreover, certain plant mutualistic fungi like arbuscular mycorrhizae have been reported to enhance plant defense via various mechanisms (Bora and Lokhandwala 2016). It

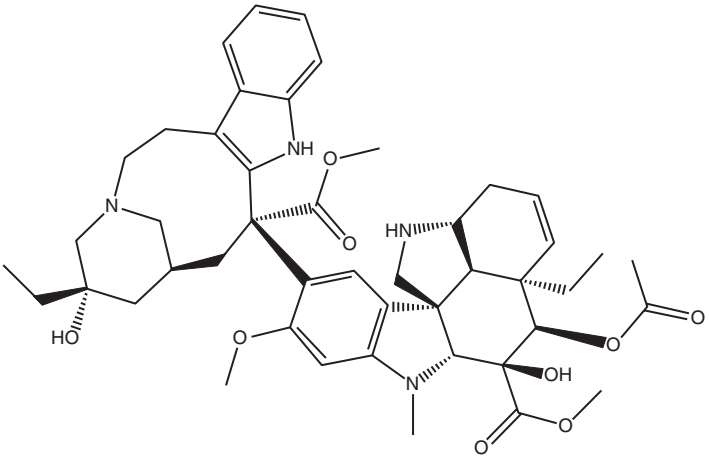

Fig. 19.2 Structure of vinblastine

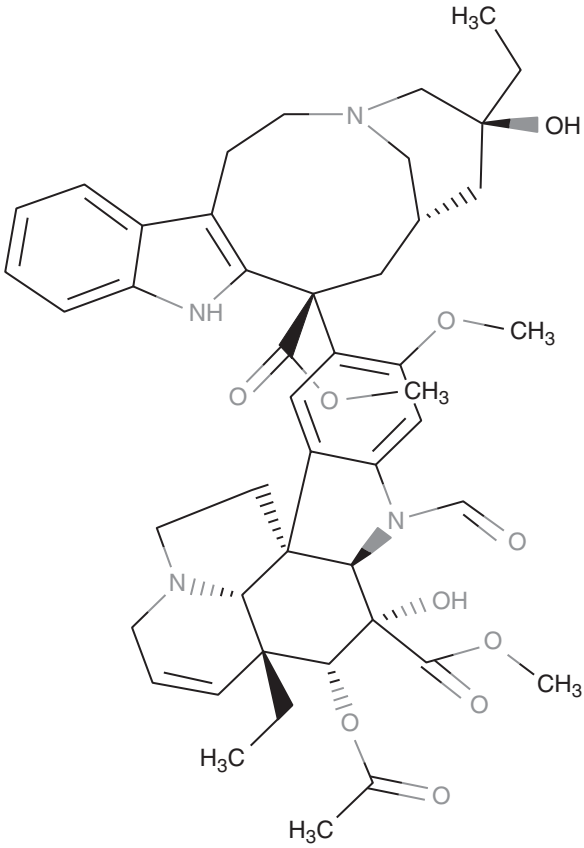

Fig. 19.3 Structure of vincristine

has been reported that colonization of this fungus increases the production of phenols, flavonoids, and alkaloids (Soares et al. 2005; Guo et al. 2010; Lingua et al. 2013) apparently, the compounds used in breast cancer prevention. However, experimental evidences are required to support the speculation.

19.2.1 Plant Extracts

19.2.1.1 Apple Extracts

Apple (*Malus domestica*) are rich in phytochemicals and major contributor of dietary phenols and dietary flavonoids. Apples are widely consumed, and their consumption has been linked to prevent numerous chronic diseases including coronary heart disease, asthma, type II diabetes, and cancer (He and Liu 2008; Yang and Liu 2009). Recent study in Italy, on apple intake and the risk of different cancers, suggests an inverse relationship between apple consumption and risk of various cancers including breast cancer (Gallus et al. 2005). Another study suggested that extracts of whole apple prevent mammary cancer in a dose-dependent manner in rats (Liu

et al. 2005). Further, the antiproliferative activity of triterpenoids, organic acids, and flavonoids isolated from apple peel was investigated (He and Liu 2008). This study showed that flavonoids, namely, quercetin and quercetin-3-O-β-D-glucopyranoside, isolated from apple peel have potent antiproliferative activity against MCF-7 cells. The antiproliferative activity of whole apple extract in combination with quercetin 3-β-D-glucoside against MCF-7 human breast cancer cell was tested. The results showed that the apple extracts in combination with quercetin 3-β-D-glucoside demonstrated a synergistic effect in MCF-7 cell proliferation (Yang and Liu 2009). Further, *in vivo* bioactivity of lead anticancer compounds and extracts isolated from apple and their effects in combination need to be tested.

19.2.1.2 *Morinda citrifolia* Extracts

Morinda citrifolia L. (Indian mulberry) is a medicinal plant in Southeast Asia and has antibacterial, analgesic, and hypotensive properties. All parts of the plants, including the roots, bark, leaves, and fruits, have been demonstrated to possess active compounds that have great medicinal values. The methanol extract of fruits of *Morinda citrifolia* was tested for cytotoxicity activity against breast cancer cell line. A concentration of 0.1 mg/mL of crude extract showed cytotoxic activity against breast cancer (MCF-7) cell lines at 29%. (Arpornsuwan and Punjanon 2006). Moreover, the fruit juice or puree is also reported to inhibit or prevent metastasis and destroy metastasized mammary or breast cancer cells (Wang et al. 2005).

19.2.1.3 Cruciferous Plant Extract

Vegetables belonging to Brassicaceae family including broccoli, brussels sprouts, cabbage, and cauliflower are good source of phytochemicals (Ju et al. 2000). Consumption of vegetables of Brassicaceae family has inverse relationship with cancer incidences in numerous sites, including breast cancer (Chang et al. 2006). Indole-3-carbinol (I3C) and 3,3′-diindolylmethane (DIM) are indole and indole-derivative compound, respectively. I3C is an active compound of cruciferous plants, and DIM is acid condensation product of I3C, and the latter has antiangiogenic activity (Chang et al. 2006). Both of these compounds arrest breast cancer cells in G1 phase and cause apoptosis of these cells (Hong et al. 2002a, b; Cover et al. 1998, 1999). In one study the antiproliferative activity of crude *Brassica oleracea* juice was investigated on MCF-7 (ER+) and MDA-MB-231 (ER−) breast cancer cell lines (Brandi et al. 2005). The data showed that active compounds present in the edible part of *B. oleracea* can inhibit ER+ and ER-human breast cancer cells growth considerably by activating apoptosis and necrotic pathway (Brandi et al. 2005). In another study the estrogenic and antiestrogenic activity of ethyl acetate extracts of freeze-dried cabbage (FDC), freeze-dried fermented cabbage (FDS), and acidified brussels sprouts (ABS) were investigated. The results of this study showed that the extract acts bifunctionally; at lower concentration they function as an antiestrogen and at higher concentration they work as an estrogen agonist (Ju et al. 2000). Thus, these studies suggest that cruciferous vegetables have potential chemopreventive agents and can moderate cancerous effects.

19.2.1.4 Citrus Plant Extracts

Citrus fruits are grown worldwide and have been used for various medicinal purposes and health supplements (Lv et al. 2015). In current decade, numbers of studies have been done on citrus secondary metabolites and its bioactivities and to develop new chemotherapeutic and complementary medicine. Citrus-derived secondary metabolites include alkaloids, carotenoids, coumarins, flavonoids, limonoids, phenolic acids, and essential oils and have anticancer, anti-inflammatory, antioxidant, cardioprotective, and neuroprotective properties (Lv et al. 2015). The anti-mammary tumor effect of grapefruit juice and orange juice was investigated in rats (So et al. 1996). The study showed that orange juice delayed the onset of mammary tumor and reduced tumor burden, whereas grapefruit juice was not able to inhibit tumor growth in female rats (So et al. 1996). Similar study was done to investigate the anti-mammary tumor effect of double strength orange and grapefruit juice in female rats (Guthrie and Carroll 1998). The data showed that double strength orange juice was more effective in inhibiting mammary tumorigenesis as compared to double strength grapefruit juice (Guthrie and Carroll 1998).

19.2.1.5 Grape Extracts (Red Wine)

Grapes are reliable source of various phytochemicals, including anthocyanins and resveratrol. Previous studies have suggested cardiovascular benefits and cancer chemopreventive activities of grapes (Pezzuto 2008; Levitsky and Dembitsky 2014). It is a major source of polyphenols, and earlier studies showed the antiproliferative effect of red wine polyphenols on human breast cancer cells (Nakagawa et al. 2001; Nifli et al. 2005). The antiproliferative effect of flavonoid fractions of red wine had been investigated on breast cancer cells lines and normal human mammary epithelial cells (Hakimuddin et al. 2004) They concluded that the flavonoid fractions of red wine were selectively less cytotoxic to normal human mammary epithelial cells (HMEC) and non-tumorigenic MCF-10A cell lines and showed maximum growth inhibition toward MCF-7 breast cancer cells. Further, the mechanism of antiproliferative action of red wine polyphenol fractions was examined. The data suggested that polyphenol disrupts calcium homeostasis which may disturb mitochondria functions and further cause membrane damage, and these subsequent events result in selective cytotoxicity toward MCF-7 breast cancer cells (Hakimuddin et al. 2006).

19.2.1.6 Coffee Extracts

Coffee is one of the most widely consumed beverage in the world. Many epidemiologic studies have been done to understand the relationship between coffee intake and breast cancer risk and have produced conflicting results (Larsson et al. 2009; Li et al. 2011, 2013; Fagherazzi et al. 2011; Oh et al. 2015). Some studies suggest that there is no association between coffee intake and development of breast cancer (Larsson et al. 2009; Fagherazzi et al. 2011; Li et al. 2013), while some studies suggest that the association is affected by type of hormone receptor status of the breast cancer (Li et al. 2011; Oh et al. 2015). One study showed that there is significant decrease in breast cancer risk with higher coffee intake in estrogen receptor (ER)

negative (Li et al. 2011). Another study indicates that coffee intake significantly decreases the risk of overall and estrogen receptor-positive (ER+)/progesterone receptor-negative (PR-) breast cancer (Oh et al. 2015). Thus, if coffee consumption is linked with breast carcinoma, development or treatment is still a matter of research.

19.2.1.7 Tea Extracts

It is the most commonly consumed beverage in the world. Black tea consumption is prevalent in Northern Europe, North America, and Western Asia, while green tea consumption is more common in Eastern Asia (Oh et al. 2015). Tea contains polyphenols and other components which have been described as antiestrogenic, antiangiogenic, antiapoptotic, antioxidant, prooxidant, and tumor inhibitory, and other potentially chemopreventive properties (Shrubsole et al. 2009). Many studies examined the association of tea intake and risk of breast cancer; however the results are contradictory (Ganmaa et al. 2008; Larsson et al. 2009; Boggs et al. 2010; Fagherazzi et al. 2011; Oh et al. 2015). In meta-analysis of 13 papers which investigated populations in 8 countries showed that the association between black tea intake and risk of breast cancer is inconsistent; a negative association was found between black tea consumption and breast cancer risk in 8 case-control studies; however in 5 cohort studies there was a moderate significant increase in breast cancer risk with black tea consumption (Sun et al. 2006). In the Swedish Mammography cohort study, the association of black tea consumption with the incidence of overall breast cancer, and study in context of estrogen receptor (ER) and progesterone receptor (PR) status of the tumor, has been investigated. The findings of this study suggest that black tea consumption may increase the risk of ER+/PR+ tumors (Larsson et al. 2009). The Swedish Women's Lifestyle and Health study showed that tea consumption is positively associated with the risk of overall and ER+/PR+ breast cancer (Oh et al. 2015). No correlation between tea drinking and overall breast cancer risk was observed in the Nurses' Health Study (Ganmaa et al. 2008), in the Black Women's Health Study (Boggs et al. 2010), and in French cohort study (Fagherazzi et al. 2011) .

Anticancer effect of crude green tea extract was studied on 472 patients with I–III stages of breast cancer for 7 years. The results showed that increased consumption of green tea reduced the number of axillary lymph node metastases in premenopausal patients with stage I and II. Further, increased green tea intake was linked with reduced relapse of stage I and II breast cancer (Nakachi et al. 1998). Similarly, in another meta-analysis investigation, it was shown that there is an inverse relationship between intake of green tea and breast cancer risk (Sun et al. 2006). The anti-breast cancer effect of green tea extract with tamoxifen was investigated *in vitro* and *in vivo*. The study showed that green tea extract increased tamoxifen-induced antiproliferation in ER+ MCF-7, T47D, and ZR75 human breast cancer cells *in vitro*. Further, the mice treated with the combination of green tea extract + tamoxifen had decreased tumor size and more apoptosis in tumor tissue as compared to the mice treated with either agent alone (Sartippour et al. 2006). Another large population-based case-control research performed in Shanghai

showed that green tea intake may be weakly correlated with a reduced breast cancer risk. However in one study, the relationship between green tea intake and incidence of breast cancer remained unclear (Ogunleye et al. 2010).

19.2.1.8 South African and Palestinian Plants Extract

A collaborative research program between the Council for Scientific and Industrial Research (CSIR) in South Africa and the National Cancer Institute (NCI) in the United States was started in 1999 to evaluate anticancer activity of South African plants. In this program, a total of 7500 plant extracts were screened for *in vitro* anticancer activity against 3 human cancer cell lines including MCF-7 (Fouche et al. 2006). Based on biological activity (total growth inhibition, TGI), the extracts were divided into four categories: inactive (TGI >50 μg/ml), weak (15–50 μg/ml), moderate (6.25–15 μg/ml), and potent (TGI < 6.25 μg/ml) (Fouche et al. 2006; Fouche et al. 2008). Extracts which showed moderate and potent activity were further tested against a panel of 60 human cancer cell lines including breast cancer lines at the NCI. Out of 7500 plant extracts, 50 of them were active extracts (TGI < 15 μg/ml; moderate and potent). However, none of them have potent activity against MCF-7 cell line, and only 20 extracts showed moderate activity (Fouche et al. 2008). Organic extracts of 24 Palestinian traditional medical plants were tested for their anticancer activity against 3 cancer cell lines including MCF-7 and MDA-MB23 (Kaileh et al. 2007). However, none of them showed potent and moderate activity against MCF-7 and MB23 human breast cancer cell lines.

19.2.2 Plant-Derived Compounds

19.2.2.1 Flavonoids

Flavonoids are produced from phenylalanine and tyrosine together with acetate units (Heller and Forkman 1995), and they participate in light-dependent phase of photosynthesis in plants during which they catalyze electron transport (Das 1994). There are numerous reports that consumption of dietary flavonoids is effective against treatment of several types of cancer like lung, pancreas, and ovary. Genistein, principal isoflavonoid of soja, and quercetin, a flavonoid present in skin of apples and red onions, have been shown to inhibit tumor cell growth (Kuzumaki et al. 1998; Gibellini et al. 2011; Russo et al. 2016; Srivastava et al. 2016). Mode of actions of specific flavonoids has not yet been revealed, but there is a generalized idea that radical scavenging activity of electron donor flavonoids can aid in tumor-preventing effects (Orhan et al. 1999; Miliauskas et al. 2004; Kumar and Pandey 2013; Treml and Šmejkal 2016). Blocking angiogenesis, modulating MDR1 activity, and inhibiting cyclin-dependent kinases (CDKs) are some of the mechanisms by which flavonoids aid in tumor prevention.

Among the most extensively studied flavonoids, flavopiridol, catechins, genistein, and quercetin are known to possess anticancer activities and hence prevent cancer. Flavopiridiol (Fig. 19.4) causes cell cycle arrest, induces apoptosis, inhibits angiogenesis, and potentiates chemotherapy (Kaur et al. 1992; Carlson et al. 1996;

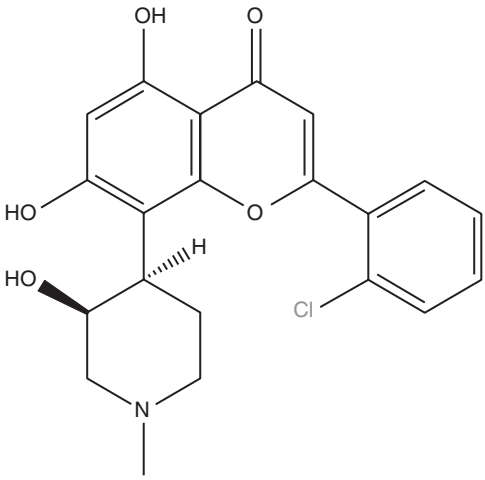

Fig. 19.4 Structure of flavopiridol

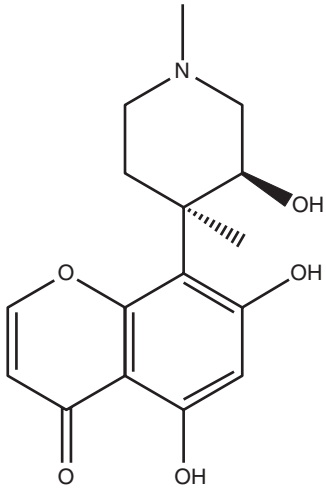

Fig. 19.5 Structure of rohitukine

Brusselbach et al. 1998; Parker et al. 1998; Schrump et al. 1998). Flavopiridol was the first CDK inhibitor to be tested in clinical trials (Tan and Swain 2002). Flavopiridol is a semisynthetic flavone derivative of natural anti-inflammatory and immunomodulatory alkaloid rohitukine. It also known as alvocidib, HMR 1275, and/or L86-8275 (Cragg and Newman 2005). Rohitukine (C16H19NO5; 5,7-dihydroxy-8-(3-hydroxy-1-methyl-4-piperidinyl)-2-methyl-4H-chromen-4-one) (Fig. 19.5), isolated from *Amoora rohituka*, *Dysoxylum ectariferum*, and *Schumanniophyton problematicum* (Safia et al. 2015). Rohitukine showed moderate cytotoxicity against human HL-60 promyelocyctic leukemia and HCT-116 colon

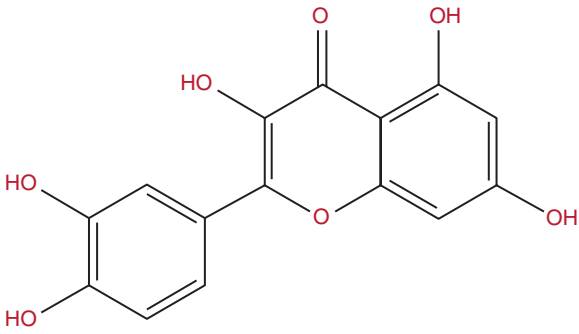

Fig. 19.6 Structure of quercetin

cancer cells (Ismail et al. 2009). Rohikutine is structurally identical to it's semisynthetic derivative flavopiridol, except there is a replacement of a methyl group by the chlorophenyl moiety at position 2 (Takada and Aggarwal 2004). Rohitukine also helped in inducing apoptosis and modulation of immune response. Flavopiridol showed arrest of G1 phase of cell cycle independent of functional p53 in breast carcinoma MCF-7 cell line (Carlson et al. 1996).

Quercetin (Fig. 19.6) is a flavonoid found in considerable amounts in some fruits, vegetables, and grains. It exerts both cell-protecting and cancer cell-killing effects due to its antioxidant and metal-chelating properties and prooxidant property as it generates ROS inducing DNA damage. It is reported that quercetin induced p21, a CDK inhibitor, and arrested cell cycle in G1 or G2/M phase in MCF-7 breast cancer cell lines (Choi et al. 2001). Jeong et al. (2009) reported that quercetin may activate caspase proteins, modify microtubules, and increase stress proteins expression ultimately leading to apoptosis. As quercetin is a dietary flavonoid, evaluation of its potential anticancer activities should be done comparing plasma levels of ingested natural compounds with those used in cancer studies. In most of the *in vitro* cancer studies, quercetin was tested at high concentrations (10–200 μM), although its effects were very mild. For instance; 94% of SK-Br3 cells were still active even after 4-day incubation at 10 μM quercetin concentration. Studies have been done to analyze the metabolism of quercetin in blood plasma in apples and onions. Hollmann et al. reported that after 24 hrs of applesauce and apple peel (68 ± 13 mg quercetin equivalents) and onion (325 ± 7 μmol of quercetin) consumption, only 0.6 μM quercetin from apple and 0.74 μM from onion rested in blood plasma. So, it is evident that major fraction of flavonoid is either metabolized or absorbed by tissues, though no tissue-specific studies substantiate that. Major flavonoid found in apple peel was not quercetin but quercetin-3-O-β-D-glucopyranoside and quercetin-3-O-β-D-galactopyranoside. Quercetin-3-O-β-D-galactopyranoside and quercetin inhibited proliferation of HepG2 cell lines with EC_{50}values of 41 and 49 μM, while for MCF-7 with EC_{50} values of 137 and 24 μM, respectively (Hollman et al. 1997).

Numerous studies on other hand also lead to double-face nature of antioxidants. Although free radicals may scavenge oxygen radical, they may also improve cell survival aiding in restoring ATP synthesis and prevention of non-apoptotic cell death (Levitsky and Dembitsky 2014). These evidences put health benefits of these scavengers in commotion. Another example in this regard to whole class of

flavonoids per se is associated with structural resemblance to cell estrogen. Medicinal plants flavonoids are weak phytoestrogens mimicking the effects of estrogens. It can be postulated on basis of epidemiological data and *in vitro* studies done on ER-positive and ER-negative breast cell lines that effect of phytoestrogens is dependent on their blood concentrations (Rice and Whitehead 2006).

19.2.2.2 Plant Sterols and Alkaloids

Alkaloids vincristine and vinblastine (also known as "tubulin-interactive agents") were found efficient in breast cancer treatment. They are isolated from Madagascar periwinkle *Catharanthus roseus* or *Vinca rosea*. Cytotoxicity by vinca alkaloids is due to their disruptions of microtubules consisting mitotic spindle apparatus causing metaphase arrest (Himes 1991). However, these agents have effect on both malignant and nonmalignant cells as microtubules are involved in many non-mitotic functions. Per mole of tubulin binds to two binding sites of vinca alkaloid (Correia and Lobert 2001). There are 16–17 reversible high affinity binding sites located at the end of microtubule; binding to vinca alkaloids interrupts microtubule congregation. Improper assembly of microtubules and decreased growth rate produce "kinetic cap" and suppress microtubule function (Jordan et al. 1992). Vacca et al. (1999) reported that 0.1 to 1.0 pmol/L of vinca alkaloids blocked essential steps in angiogenesis like endothelial proliferation, chemotaxis, and spreading on fibronectin; but normal fibroblasts and lymphoid tumors remained unaffected.

Plant sterols (PS)/carbon steroid alcohols (Otaegui-Arrazola et al. 2010) are vital components of plant cell membranes and are key components of plant plasma membrane microdomains. Campesterol, stigmasterol, and β-sitosterol are most abundant PS in human diet (Roche et al. 2008). Biochemical and molecular effects of PS make them strong candidates for breast cancer therapy; there are compelling evidences for the same (Grattan Jr 2013). Estrogen is a well-recognized mediator of breast cells growth, and it also plays key role in etiology of breast cancer (Begg et al. 1987; Jordan 2000; Finlay-Schultz and Sartorius 2015). Increased amounts of estrogen are a potential risk factor for breast cancer (Gutendorf and Westendorf 2001; Rossouw et al. 2002; Hankinson et al. 2004; Touillaud et al. 2005). Touillaud et al. (2005) also stated that intake of β-sitosterol relates to greater occurrence of estrogen receptor-positive (ER+) than estrogen negative (ER-) breast cancer (OR 0.49; 95% CI 0.18–0.98). β-Sitosterol binds competitively to both α and β forms of ER. While, plant stanols and stanol esters failed to stimulate estrogen-responsive growth in MCF-7 lines (Baker et al. 1999). However, an evidence from reporter gene array studies in human breast cancer lines conforms PS as a weak selective estrogen receptor modulators (Gutendorf and Westendorf 2001).

19.3 Conclusions and Future Prospects

Breast cancer prevention and treatment can be made more effective by co-applying both traditional and modern approaches. Those plant extracts which are considered safe would help cells to survive under wrecked intracellular machinery; contrary to those efficient conventional methods which kills both healthy and cancerous cells.

So in this case, combination of both methods would be fruitless. Ultimately, the synergistic or individual effect of plant extracts or compounds cannot be reasonably explained until researchers know the exact composition and dosage value of apparently active ingredients in plants via clinical studies and phase trials.

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20 Micropropagation and Conservation of Selected Endangered Anticancer Medicinal Plants from the Western Ghats of India

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Abstract

Globally, cancer is a constant battle which severely affects the human population. The major limitations of the anticancer drugs are the deleterious side effects on the quality of life. Plants play a vital role in curing many diseases with minimal or no side effects. Phytocompounds derived from various medicinal plants serve as the best source of drugs to treat cancer. The global demand for phytomedicines is mostly reached by the medicinal herbs from the tropical nations of the world even though many plant species are threatened with extinction. India is one of the mega diverse countries of the world due to its ecological habitats, latitudinal variation, and diverse climatic range. Western Ghats of India is one of the most important depositories of endemic herbs. It is found along the stretch of south western part of India and constitutes rain forest with more than 4000 diverse medicinal plant species. In recent times, many of these therapeutically valued herbs have become endangered and are being included under the red-listed plant category in this region. Due to a sharp rise in the demand for plant-based

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products, this rich collection is diminishing at an alarming rate that eventually triggered dangerous to biodiversity. Thus, conservation of the endangered medicinal plants has become a matter of importance. The conservation by using only in situ approaches may not be sufficient enough to safeguard such a huge bioresource of endangered medicinal plants. Hence, the use of biotechnological methods would be vital to complement the ex vitro protection programs and help to reestablish endangered plant species. In this backdrop, the key tools of biotechnology that could assist plant conservation were developed in terms of *in vitro* regeneration, seed banking, DNA storage, pollen storage, germplasm storage, gene bank (field gene banking), tissue bank, and cryopreservation. In this chapter, an attempt has been made to critically review major endangered medicinal plants that possess anticancer compounds and their conservation aspects by integrating various biotechnological tools.

Keywords
Anticancer · Conservation · Endangered · Micropropagation · Seed bank

20.1 Introduction

Herbal medicines have been served as therapeutic agents for thousands of years and still continue to be an important lifesaving drugs for mankind. Plants have been indispensable in treating diverse form of diseases including cancer. The increasing consciousness on the side effects and toxicity linked to synthetic drugs has encouraged the popularity and the demand for herbal drugs (Swamy et al. 2017). As reported by the World Health Organization (WHO), nearly 80% of the global people depend on herb-based medicines to cure health problems (Kumara et al. 2012; Swamy and Sinniah 2015). About 85% of the folk medical practices employ plant raw materials, crude extracts, or pure phytocompounds (Swamy et al. 2016; Arumugam et al. 2016; Mohanty et al. 2017). The increased global demand for the flora is leading to their overexploitation, habitat loss, and non-judicious use from the wild and adulteration of supplies (Swamy and Sinniah 2016). In addition to this, medicinal plants are highly affected by climatic change and other natural calamities; many species of the medicinal plants have become rare, threatened, or endangered. India is a major center harboring a diverse range of medicinal plants. It constitutes about 8% of world's biodiversity area, and it is one among 12 mammoth biodiversity hot spot nations of the world (Sharma and Thokchom 2014). In India, about 34 major hot spot regions have been recognized. Among them, the Western Ghats and the Eastern Himalaya regions are the top most biodiversity areas (Krishnan et al. 2011; Sharma and Thokchom 2014). India has a very unique biogeographic locations including the coldest place (Nubra valley), dry cold desert (Ladakh), alpine, temperate and subtropical areas (Himalayas), wet humid tropic regions (Western Ghats), rain forests (Meghalaya), dry desert (Rajasthan), and mangrove forests (Sundarbans) (Sharma and Thokchom 2014).

The Western Ghats (between 8°20′-40′ N and 73,077′ E) of India is found in the states of Goa, Maharashtra, Karnataka, Kerala, and Tamil Nadu (Krishnan et al. 2011; Aadhan and Anand 2017). It has spread over a bio reserves of more than 1600 km. The Western Ghats is one of the richest sources of endemic flora comprising nearly 7000 plant species of peninsular India. The altitudinal range of this region is up to 2800 m and possesses a highly varying climatic condition and several edaphic factors (Krishnan et al. 2011). A diverse range of medicinal plant species found in the Western Ghats includes diversity of ferns, lichens, algae, shrubs, climbers, herbs, and trees including both annual and perennial types. The Western Ghats area is a source of approximately 500 herbs that are primarily used in various folk medicines. Among them, nearly 50 plant species are highly valued for their bioactive constituents used in the treatment of several human ailments including cancer (Sarvalingam and Rajendran 2016). Medicinal herbs of the Western Ghats region are the source of phytocompounds having huge demand in the global pharmaceutical market. For example, the compounds such as camptothecin, berberine, L-Dopa, reserpine, and forskolin are isolated from *Nothapodytes foetida*, *Coscinium fenestratum*, *Mucuna pruriens*, *Rauvolfia serpentina*, and *Coleus forskohlii*, respectively. However, only few such plants have been explored for their medicinal values, and still there is a large scope for the scientific validation of the other available indigenous medicinal plants in this region.

Cancer has been a constant battle and global burden throughout the world. Cancer is a multi-disease wherein abnormal malignant growth of body tissue or cell occurs. Cancer cells have the ability to invade the adjacent normal cells and transform them into cancerous growth called as malignant tumors. While, non-cancerous growth is called as benign tumor. In recent years, cancer has become a menace to the world population causing maximum deaths and is increasing continuously over the last few years. A recent report by WHO Cancer Factsheet (Siegel et al. 2016), about 8.8 million deaths due to cancer related diseases have been reported globally is in the year 2015. Most frequently diagnosed cancer types include breast, liver, lung, stomach, and colorectal cancer (Torre et al. 2015; Ferlay et al. 2015). The cause of this multi-disease is mainly due to environment, obesity, poor diet, absence of physical activities, microbial infections, and the consumption of tobacco and alcohol (Plummer et al. 2016). Though, cancer can be treated using surgery, radiation therapy, and/or systemic therapies such as immune, chemical, and hormonal therapies, they are ineffective in curing the disease completely. In many cases, the treated patients feel uncomfortable due to several side effects (Yue et al. 2017). Therefore, current investigations have promoted the use of safer anticancer agents derived from natural sources (Yin et al. 2013). The history of using plant-based anticancer drugs starts way back from the 1950s with the isolation of cytotoxic compounds such as vinblastine, vincristine, and podophyllotoxin. Vinca alkaloids (vinblastine and vincristine) are very effective against the leukemia and the Hodgkin's disease. Many of the traditionally used herbs are known to possess anticancer properties, and, hence, exploration of such plants has yielded several effective anticancer compounds available today (Swamy et al. 2015; Greenwell and Rahman 2015). Plant-based drugs are the best suited as they cause lesser side effects

in the treated cancer patients. About 60% of the currently available commercial anticancer compounds are mainly derived from plants (Yue et al. 2017). The occurrences of several herbal species in the Western Ghats have been known for various therapeutical applications and are well documented (Aadhan and Anand 2017). However, their utilization as anticancer agents is yet to be explored to the maximum extent. While, a large number of flora with anticancer potential from this region have been enrooted from their natural habitats to fulfill the ever raising demand for their herb materials for both domestic consumption and for export. This has severely affected the natural sources leading to species depletion (Corlett and Primack 2008; Sarvalingam and Rajendran 2016). In specific, medicinal plants contribute to health, income, agroforestry system, and culture identity and livelihood security, thus forcing for their conservation. As estimated by the global body, IUCN (International Union for Conservation of Nature and Natural resources), a total of 3,40,000 plant species covering about 12.5% of the world's flora are under severe threat. In this chapter, major anticancer compounds containing endangered plant species of the Western Ghats of India are reviewed. In addition, biotechnological approaches such as micropropagation and cryopreservation to conserve these endangered species are also discussed.

20.2 Endangered Anticancer Plants of the Western Ghats and Their Properties

The Western Ghats of India is comprised of subtropical and tropical rain forests and harbors several highly valued medicinal plants. However, numerous species have disappeared and became extinct in this region due to various factors including deforestation, habitat destruction, unscientific mining, and environmental changes (Corlett and Primack 2008). A thorough understanding of these economically and ecologically important endangered medicinal plants is necessary to promote and conserve biodiversity in the region. The following sections provide basic information on the botany, chemistry, biological activities of selected endangered anticancer plants.

20.2.1 *Acorus calamus* Linn.

A. calcamus is one of the important herbs known for its immense medicinal potential. It is an aromatic herb with the creeping rhizome locally known by various names like Bachh and Sweet Flag, belongs to taxonomic family Acoraceae (Meena et al. 2010). It has a diverse range of pharmacological properties including the treatment of diseases like cancer, ulcer, hepatitis, spasm, schizophrenia, gout, arthritis, and anorexia (Singh et al. 2011; Ranjan et al. 2016). It is the source of innumerable phytoconstituents such as glycosides, tannins, alkaloids, saponins, flavonoids, phenols, and lectins. The essential oils obtained from this herb constitutes several volatile compounds such as calamene, calameone, calamenenol, α-pinene, β-pinene,

p-cymene, camphene, eugenol, methyl eugenol, methyl isoeugenol, eugenyl acetate, isoeugenol, azulene, eugenol methyl ether, calamol, asaronaldehyde, dipentene, terpinolene, camphor, 1,8-cineole, caryophyllene, α-asarone, and β-asarone (Palani et al. 2009; Haghighi et al. 2017). Due to these biologically active phytocompounds, *A. calamus* is having a high demand, and thus overexploited. The seed set in this plant is seen rarely and inadequate; therefore, vegetative propagation is the chief method of propagation. The plant extracts have been documented to possess anticancer activity against various human carcinoma cells (Chaturvedi and Chaturvedi 2016; Haghighi et al. 2017).

20.2.2 *Aristolochia indica* Linn.

A. indica, an endangered plant species with immense medicinal importance, belongs to Aristolochiaceae family. It is locally called by names, Garudakkodi, Eswaramooli, Iswaberusa, and Ishrmul. It is a shrub with a long twining stem and is also known as "worm killer" because of its antihelminthic properties. This plant species is listed in a red data list of South Indian medicinal herbs. It is aromatic in nature and used to treat fever, cholera, ulcer, skin diseases, leprosy, snakebite, and cancers (Dey and De 2011; Anilkumar et al. 2014). Some of the major phytoconstituents of *A. indica* include ishwarane, aristolochen, ishwarone, aristololactam N-β- D-glucoside, 6β-hydroxy-stigmast-4-en-3-one, 3β-hydroxy-stigmast-5-en-7-one, aristolochine alkaline, isoaristolochic acid, allantoin, pinocarvone, and α-pinene (Dey and De 2011; Kuo et al. 2012). Various solvent extracts of this plant possess antiproliferative activity against MCF-7, the human breast cancer cell line (Anilkumar et al. 2014; Subramaniyan et al. 2015). Likewise, aristolochic acid isolated from the ethonolic whole plant extract has shown to prevent oral cancer in Albino rats induced by 4-nitroquinoline 1-oxide (Mariappan 2012).

20.2.3 *Clerodendrum serratum* (Linn.) Moon.

C. serratum (family, Verbenaceae) is commonly known as bharangi (Poornima et al. 2015). It is widely spread across tropics, subtropics, and deciduous forests of the Western Ghats of India. As per the traditional claims, the roots and leaves of bharangi have prodigious therapeutic value. The plant is useful both internally and externally for various medicinal trails (Nagdeva and Singh 2012). Ethnomedicinal importance of the plant has been reported in various folk medical practices including Ayurveda, Unani, and Siddha for treating many serious health issues such as typhoid, syphilis, jaundice, hypertension, and cancer (Poornima et al. 2015). Some of the chief constituents found in the plant are D-mannitol, hispidulin, cleroflavone, apigenin, scutellarein, serratagenic acid, acteoside, verbascoside, oleanolic acid, clerodermic acid, γ-sitosterol, β-sitosterol, cholestanol, clerosterol, campesterol, and 24-ethylcholesterol (Poornima et al. 2015). Traditionally, it has been also used as antirheumatic, antiasthmatic, febrifuge, encephalagia, and ophthalmia. The roots

of *C. serratum* are also used as antioxidant, antibacterial, and antifungal. Besides these the antimicrobial value of this herbal plant has also been reported in its stems and leaves. These reports are very encouraging and indicate that herb should be studied more expensively for its therapeutic benefits. The plant root extract is shown to prevent Dalton's Ascetic Lymphoma in mice (Zalke et al. 2010; Nagdeva and Singh 2012).

20.2.4 *Coscinium fenestratum* (Goetgh.) Colebr.

This plant being endemic to the Western Ghats of India is critically endangered and listed in the red list of threatened plants of India (Ramasubbu 2010). It is a dioecious woody climber belonging to Menispermaceae family. It is one of the commercially traded medicinal herbs used in over 60 different Ayurveda medicine preparations. It is used for treating a variety of health problems such as skin diseases, ulcers, inflammation, eye disorders, hypertension, diabetes, jaundice, and snakebites (Madhavan et al. 2014). *C. fenestratum* stem extract is known to possess an insulin-stimulating compound, berberine, and, thus, responsible for hypoglycemic activities. Also, the compound ecdysterone found in the leaf extract of this plant is known to have several pharmacological activities such as anabolic, adaptogenic, antidiabetic, hepatoprotective, antitumor, and immunoprotective activities (Madhavan et al. 2014). Studies have shown that berberine isolated from *C. fenestratum* possess an antiproliferative effect on lung (NCI-H838), colorectal (HCT-116), and acute myeloid leukemia (HL-60) cell lines (Rojsanga et al. 2010; Tungpradit et al. 2010, 2011). *C. fenestratum* crude water extract showed the cytotoxic effect on human metastatic squamous cell (HN31) carcinoma of the pharynx (Potikanond et al. 2015). They concluded that the cell toxicity is linked to the modulation of signal molecules (p38, MAPK, p53, pAkt), resulting in the increased cell inhibition and apoptosis.

20.2.5 *Curculigo orchioides* Gaertn.

The plant belonging to the family Hypoxidaceae is a small endangered plant species found in the Western Ghats. This plant is used as one of the major ingredient in the folk medicine (Ayurveda) preparations such as Kali or Shyah-Musali used for rejuvenation (Singh et al. 2006). It is a perennial shrub and has elongated and short fleshy roots. Its rhizome is 1 ft in length and pulpy. The plant is rich in flavone glycosides. Its tubers contain cycloartane-type triterpene glycosides called curculigo saponins and curculigosides. They also contain phenyl glycosides, orcinol glycoside, corchioside A, hentriacontanol, and an alkaloid, lycorine (Singh et al. 2006). The shrub is highly beneficial for a number of ailments, and it is an important ingredient of many Ayurvedic preparations. It is reported to have immunostimulatory, aphrodisiac, hepatoprotective, antioxidant, antidiabetic, and anticancer activities (Venukumar and Latha 2002; Singh et al. 2006; Chauhan et al. 2010). An investigation was carried out to evaluate the tumor decreasing capacity of cyclophosphamide, a chemical

antineoplastic drug when combined with the methanolic extract of *C. orchioides* in Dalton's lymphoma ascites-induced tumor model (Murali and Kuttan 2015). The results revealed the synergistic effects of the plant extract and cyclophosphamide which enhance the anticancer activities and reduced the toxic side effects of the synthetic drug, cyclophosphamide. Likewise, in another recent study, its antiproliferative activity against the human cervical cancer (HeLa) cells was evidenced by Xia et al. (2016). According to them, antitumor effect was mainly due to the induction of apoptosis and enhancement of immune functions.

20.2.6 *Decalepis hamiltonii* Wight & Arn.

D. hamiltonii is one of the most important endangered woody medicinal climbing members of Asclepiadaceae family (Sharma and Shahzad (2014)). It is distributed in the Eastern and Western Ghats of peninsular India. It has been extensively used in the folk medicines for a wide range of ailments like stomach disorders and gastric ulcers and to stimulate appetite (Sharma and Shahzad 2014). Its tuber is used in a food and health drinks (Vedavathy 2004) and reported with antimicrobial, antipyretic, antiulcer, antidiabetic, antioxidant, anti-inflammatory, chemoprotective, cytoprotective, insecticidal, neuroprotective, and hepatoprotective activities (Reddy and Murthy 2013). *D. hamiltonii* root extract has been shown to have antitumor effect (Srivastava et al. 2007). In another study, treatment of *D. hamiltonii* was shown to inhibit the progression of metastasis lung cancer (Shathish and Guruvayoorappan 2014). The administration of the plant extract altered pro-inflammatory cytokine secretion and repressed the nuclear translocation of p50 and p65 subunits of NF (nuclear factor) κB in cancer cells (B16F-10).

20.2.7 *Gloriosa superba* L.

G. superba, a perennial semi-woody herbaceous climber of the Liliaceae family, is used as an Ayurvedic medicinal herb to treat various diseases (Ashokkumar 2015). The medicinal properties of the plant are due to presence of alkaloids, mainly colchicine and gloriosine. The rhizomes and seeds contain colchicine, isoperlolyrine, and related tropolane alkaloids, β-sitosterol and its glucoside, 2-hydroxy 6-methoxy benzoic acid. It is used for curing gout, arthritis, inflammation, rheumatism, ulcers, skin diseases, bleeding piles, leprosy, snakebites, and impotency (Jana and Shekhawat 2011). The hydroalcoholic extract of *G. superba* evaluated against the lung cancer cell lines showed anticancer activity (Santny 2016).

20.2.8 *Hemidesmus indicus*

H. indicus, also popularly known as "Anantmul," is a semierect shrub belonging to the family Asclepiadaceae. It is widely distributed throughout India and known as

"God of Medicine" and is used in a popular drug formulations of the Ayurveda system of medicine to treat dysentery, diarrhea, skin diseases, syphilis, dyspepsia, leukoderma, diuresis, burning of body, chronic fever, and asthma and is a blood purifier (Austin and Herbals 2008). Pharmacological studies carried out with its extract and purified compounds indicated that this plant possesses antioxidant, hepatoprotective, antiulcer, antimicrobial, hypoglycemic, antihyperlipidemic, otoprotective, analgesic, anti-inflammatory, and immunomodulatory activities (Kainthla et al. 2006). Methanolic extracts of *H. indicus* roots showed a remarkable anticancer potential against the breast cancer (MCF 7) cell lines (Papiya et al. 2010; Cherku et al. 2016).

20.2.9 *Leptadenia reticulata* (Retz.) Wight & Arn.

L. reticulata, frequently known as Jivanti, belongs to Apocynaceae family. It is spread across the tropics and subtropics of India, Burma, Nepal, Sri Lanka, Africa, the Philippines, Cambodia, and Mauritius. However, due to overexploitation, its natural habitat has been under threat and, hence, considered as endangered species. More recently, due to higher demand for its raw material from the pharmaceutical and nutraceutical industries, the plant has been encouraged to cultivate commercially in some parts of India. The plant is traditionally used to cure human problems such as hematopoiesis, tuberculosis, cough, emaciation, dyspnea, burning sensation, fever, dysentery, and cancer (Mohanty et al. 2017). It is used as a revitalizing and rejuvenating agent in Ayurveda medical practice. The plant contains several biologically active compounds, namely, α-amyrin, ferulic acid, diosmetin, β-sitosterol, luteolin, hentricontanol, stigmasterol, simiarenol, reticulin, and leptaculatin. The plant extracts are used in the several commercial brands of herbal preparations (Speman, Calshakti, Envirocare, and Chyawanprash). *L. reticulata* extracts have shown an effective activity against Dalton's ascites lymphoma in mice and inhibited different cancer cell lines *in vitro* (Sathiyanarayanan et al. 2007; Mohanty et al. 2014, 2017).

20.2.10 *Nothapodytes nimmoniana* (Graham) Mabb.

N. nimmoniana (synonyms: *Mappia foetida* and *Nothapodytes foetida*) belongs to the family Icacinaceae. It is an endemic medicinal plant of the Western Ghats and distributed fragmentally (Ankad et al. 2015; Prakash et al. 2016). It is having a high value due to the occurrence of an anticancer compound, camptothecin and 9-methoxycamptothecin (Pai et al. 2010; Ankad et al. 2015; Prakash et al. 2016). Because of this reason, *N. nimmoniana* plant is been traded indiscriminately from this region and, thus, included in vulnerable category by the International Union for Conservation of Nature (IUCN) (Aravind et al. 2005; Ankad et al. 2015). Studies have shown that the phytocompounds of *N. nimmoniana* exhibit various pharmacological activities including antihuman immunodeficiency virus, antimalarial, and antineoplastic activities (Pai et al. 2010, 2013). Moreover, the plant possessing

camptothecin is known to inhibit adenoviruses, herpes viruses, papovaviruses, and parvoviruses (Pantaziz et al. 1999; Yamazaki et al. 2003; Prakash et al. 2016). The samples of *N. nimmoniana* collected from the Western Ghats have yielded the highest camptothecin content (1.337 g/100 g dry bark powder) (Pai et al. 2013). Camptothecin, an alkaloid compound, is formerly obtained from *Camptotheca acuminate,* a Chinese tree belonging to Nyssaceae family. In recent years due to high value for this drug worldwide has made to explore in other plants such as *N. nimmoniana* and *Ophiorrhiza* species (*O. pumila*, *O. mungos*, *O. alata*, *O. liukiuensis*, *O. rugosa*, *O kuroiwai*, and *O. prostrata*). However, the major plant source for camptothecin is *C. acuminata* and *N. foetida* even today. Camptothecin exhibits superior anticancer activities by inducing various inhibitory pathways such as inducing rapid fragmentation of nucleic acids, inhibiting topoisomerase enzymes, etc. in different types of cancer cells (Park and Cheng 2005; Das et al. 2016; Raveendran 2015; Prakash et al. 2016).

20.2.11 *Ophiorrhiza mungos* L.

It is an endangered medicinal plant of the Western Ghats and is commonly known as Mongoose plant that belongs to the family Rubiaceae. Its distribution is very limited to some regions of the Eastern and Western Ghats. Traditionally, the roots are used against cancer and snakebite; the root bark is having sedative and laxative properties. The roots are used for the treatment of. It is half woody, erect, smooth, and up to 30 cm in height. The roots contain cytotoxic quinoline alkaloid, camptothecin (CPT), a highly valued anticancer compound (Baskar 2010). Though, *Camptotheca acuminata* and *Nothapodytes nimmoniana* plants contain higher content of CPT, *Ophiorrhiza* species are appreciated due to the fact that it is herbaceous plant and can be cultivated on a large scale (Kaushik et al. 2015). The plant possessing CPT and Luteolin-7-O-Glucoside is reported to have potential anticancer activity (Baskar 2010; Krishnakumar et al. 2012; Krishnan et al. 2014).

20.2.12 *Rauvolfia serpentina* L. Benth. ex Kurz.

R. serpentina, commonly known as "sarpagandha," belongs to the family Apocynaceae and its medicinal usage dates back to several thousand years. The dried roots of *R. serpentine* are composed of several biologically active phytocompounds such as reserpine, deserpidine, rescinnamine, ajamalacine, neoajmalin, ajmaline, serpentine, and α-yohimbine (Verma and Verma 2010; Kumari et al. 2013). As a sedative, the roots are used in controlling anxiety, high blood pressure, epilepsy, and insomnia disorders of central nervous system and schizophrenia (Panda et al. 2012; Lobay 2015). It is used in snakebite, insect stings, mental disorders, and cancer treatment (Kumari et al. 2013). The indole alkaloid compound, reserpine, obtained from *R. serpentina* is reported to have an effective antiproliferative activity against various cancer cell lines and recommended for its further use in the cancer chemotherapy (Abdelfatah and Efferth 2015).

20.3 Micropropagation and Conservation of Anticancer Plants of the Western Ghats

Plant species which are in danger of extinction or endangered in this region are discussed in this section with a special reference to their bioactive compounds. Plant tissue culture is very useful in conserving the biodiversity of rare and endangered medicinal plant species that produce recalcitrant seeds and play a huge role mass multiplication and germplasm conservation. Also, this biotechnological approach facilitates the production of various pharmacologically valued plant products (Cruz-Cruz et al. 2013). This section is mainly focused on the micropropagation and conservation of the above mentioned highly threatened anticancer plants from the Western Ghats region of India.

20.3.1 *Acorus calamus* Linn.

A simple and high frequency *in vitro* regeneration protocol was established for *A. calamus* using rhizomes as explants (Rani et al. 2000). Murashige and Skoog (1962) (MS) media fortified with 4 mg/l BAP (6-benzyl amino purine) and 0.5 mg/l IAA (indole-3-acetic acid) resulted maximum multiple shoots induction frequency. The regenerated shoots readily rooted when transferred directly to soil within 14 days. All plantlets were effectively established in the field. Later, Verma and Singh (2012) reported a rapid *in vitro* regeneration procedure for *A. calamus* using naturally grown rhizome explants. The highest propagation of shoots (4.4) was recorded on MS media contained with 0.5 mg/l NAA and 2 mg/l BAP where about 90% of rhizome explants exhibited proliferation rate. *In vitro* rooting was better observed in ½ strength MS medium fortified with 1.0 mg/l IBA (indole-3-butyric acid) with a maximum number of roots (5.0) within 15 days. The plantlets with roots were effectively acclimatized in pots added with sterilized sand and soil mixture (1:3) and after transfer to field conditions recorded 75% survival rate. Sandhyarani et al. (2011) used dual-phase culture system to clonally propagate triploid *A. calamus* plant. In their study, MS medium overlaid by liquid fraction of the same medium was shown to favor highest shoot proliferation rate with supplementation of NAA (0.5 mg/l) and BAP (2.0 mg/l). *In vitro* microshoots rooted well on MS media supplemented with 2.0 mg/l IBA. In another study, Lee and Han (2011) observed that the seeds of *A. calamus* showed 100% germination rate on the basal MS medium. The seedlings showed varied morphogenetic response when cultured on MS media contained with various types and concentrations of cytokinins and auxins. Nearly 100% shooting rate was obtained on the MS medium supplemented with BAP or TDZ (N-phenyl-N′-[(1,2,3-thidiazol-5-yl) urea]). MS media with 4 mg/l BAP showed 5.4 shoots, while 1 mg/l TDZ-supplemented MS media resulted with 11 shoots, and the results were significantly higher compared to control treatments with no added plant growth regulators. The highest rooting was evidenced on MS media consisting of 1 mg/l NAA (naphthalene acetic acid). The transfer of regenerants to the greenhouse showed 95% survival rate after acclimatization. More

recently, Quraishi et al. (2017) developed a simple and medium term (up to 1 year) *in vitro* conservation protocol for *A. calamus* by encapsulating its microrhizomes via synthetic seeds production. *In vitro* regenerated microtubers were used to produce synthetic seeds by encapsulating in calcium alginate beads. The stored synthetic seeds at 10 °C under dark condition recorded 100% survival rate when re-cultured after 1, 3, or 6 months of the storage, while about 80% synthetic seeds survived after 12 months of storage on 33.3 μM BAP-supplemented MS media. The use of 4.9 μM IBA in ½ MS media recorded the highest microrhizomes formation. *In vitro* regenerants were successfully acclimatized and transferred to field with a good survival rate.

20.3.2 *Aristolochia indica* Linn.

Siddique et al. (2006) reported indirect organogenesis of *A. indica* using axillary shoots. MS media fortified with 2.0 mg/l KN (kinetin) and 1.0 mg/l BAP recorded the highest callus induction rate (95%). When calli were subcultured on MS media added with KN (1.5 mg/l) and BAP (2.5 mg/l) exhibited better shoot regeneration rate (95%) and rooted well in 1 mg/l KN contained MS media. Likewise, Pattar and Jayaraj (2012) developed a reproducible procedure for *A. indica* by using leaf and nodal explants. Both explants induced callus on MS media supplemented with 0.8 mg/l BAP and upon subculturing calli on MS media added with 0.8 mg/l BAP and 0.5 mg/l NAA induced shoots. *In vitro* rooting was found better on 0.8 mg/l NAA-supplemented MS media after for 4 weeks. The nodal explants showed better morphogenetic response with 95% regeneration frequency compared to leaf explants (85%). All micropropagated plants exhibited superior growth properties in the field. Micropropagation protocol was established for *A. indica* by using nodal explants (Shah et al. 2013). Schenk and Hildebrandt medium added with 10 μM adenine sulfate followed by BAP and TDZ induced better *in vitro* shoot multiplication rate. While, SH media (Schenk and Hilderbrandt 1972) added with NAA (10.0 μM) recorded the highest *in vitro* rooting response. About 70–80% survival was observed when micropropagated plants of *A. indica* were transferred to field.

20.3.3 *Clerodendrum serratum* (Linn.) Moon.

Sharma et al. (2009) used nodal stems to rapidly propagate *C. serratum in vitro*. They employed 0.25 mg/l each BAP and IAA in addition with 15 mg/l adenine sulfate to regenerate multiple shoots. Further, addition of 2-chloroethyltrimethyl ammonium chloride (0.5 mg/l) with adenine sulfate (30 mg/l) enhanced the shoot proliferation rate with an average of 4.98 shoots within 28 days of culture. Microshoots showed *in vitro* rooting in ½ MS media added with 1 mg/l IPA (indole-3-propionic acid). The transferred regenerants in the field grew normally and flowered within 10 months. Micropropagation of *C. serratum* protocol was reported by Vidya et al. (2012) using nodal and stem explants on Lloyd and McCown (1980)

medium added with different plant growth regulators. LM media supplemented with 1.5 mg/l BAP and 0.3 mg/l NAA induced the highest number of *in vitro* shoots from stem derived calli. On the other hand, nodal explants recorded direct organogenesis on 0.5 mg/l BAP-supplemented LM media. Microshoots rooted well 100% rooting frequency in ½ strength LM medium containing 0.5 mg/l NAA. The regenerated plantlets were successfully hardened and transferred to field with a survival rate of 88%.

20.3.4 *Coscinium fenestratum* (Goetgh.) Colebr.

Micropropagation protocol for *C. fenestratum* was reported by Senarath (2010). In the study, multiple shoots formation was better achieved by culturing epicotyl explants on MS medium fortified with KN (1.0 μM) and 2,4-D (0.25 μM). Repeated subculturing on the same media promoted the growth and increased the number of shoots and length. Microshoots showed 100% *in vitro* rooting frequency in ½ MS media containing 2.5 μM IBA. Ex vitro acclimatization resulted with 66.7% of the established plantlets which showed 100% survival when transferred to the field. In another study, Khan et al. (2008) reported the induction of callus by petiole and leaf explants of *C. fenestratum* on 2 mg/l 2,4-D- and 2 mg/l BAP-supplemented media. Further, callus was analyzed to contain about 18-fold higher berberine content (1.78% dry weight) in comparison to the field-grown *C. fenestratum* plants. Likewise, Jayakumaran Nair et al. (1992) also extracted the compound berberine from callus and suspension cultures of *Coscinium fenestratum* established using leaf petiole explants. The use of 2,4-D (1 μM)- and NAA (10 μM)-supplemented MS media yielded higher cell biomass (212.0 ± 0.18 mg dry weight) containing about 4.05% of berberine content (dry cell biomass). At Tropical Botanical Garden and Research Institute, India has preserved embryos of *C. fenestratum* using the cryobank (TBGRI 2009–2010).

20.3.5 *Curculigo orchioides* Gaertn.

Francis et al. (2007) established *in vitro* regeneration protocol for *C. orchioides* using apical meristems. MS media contained with 1.5 mg/l BAP, 100 mg/l adenine sulfate, and 3% sucrose recorded the highest shoot regeneration frequency. Further, addition of IBA or IAA improved multiple shoot induction rate. However, Nagesh (2008) reported the synergistic influence of BAP (1 mg/l) and KN (1 mg/l) on multiple shoots formation from *C. orchioides* shoot tips. While on the same media, rhizome discs showed high shoot formation frequency compared to shoot tips. *In vitro* rooting was better evidenced in ½ MS media with 1 mg/l IBA (Nagesh et al. 2008). In another study, rooting medium supplemented with 0.5 mg/l BAP or KN in combination with 2,4-dichlorophenoxyacetic acid (2, 4-D) (0.5–3 mg/l) induced embryogenic callus from rhizome discs (Nagesh et al. 2010). Direct organogenesis was observed by Suri et al. (2009) by culturing leaf and underground stem explants. Leaf explants induced 4 shoots while stem explants induced 10 shoots on B5

medium fortified with BAP (4.4 μM). Microshoots transferred to ½ MS media supplemented with varied concentrations of auxins were efficient in inducing *in vitro* roots. Sharma et al. (2009) advised the use of arbuscular mycorrhizal fungi (*Glomus geosporum* and *G. microcarpum*) for better acclimatization of the micropropagated plantlets. Micropropagated plants have shown about 90% of survivability in most of the mentioned reports. In a study, Nagesh et al. (2009) successfully encapsulated *in vitro* shoot buds of *C. orchioides* in a sodium alginate beads for short-term conservation. The encapsulated buds showed survivability of 68% on MS media contained with 10% (v/v) coconut water up to 50 days stored at 4 °C.

20.3.6 *Decalepis hamiltonii* Wight & Arn.

A rapid *in vitro* propagation method was developed *D. hamiltonii* through shoot multiplication using shoot tip explants (Giridhar et al. 2005). They obtained the highest multiple shoots (6.5 ± 0.4) on MS medium contained with 4.9 μM isopentenyladenine (2iP). However, both KN (4.7 μM) and zeatin (9.1 μM) combined with IAA (0.6 μM) are also effective in inducing 5.1 ± 0.4 and 5.0 ± 0.4 multiple shoots, respectively. The addition of 0.3 μM gibberellic acid (GA_3) to the media further elongated the shoots, and the shoots were rooted on MS medium containing 9.8 μM IBA. The regenerants' successful field transfer was achieved in rooted plantlets. Recently, Samydurai et al. (2016) reported the micropropagation of *D. hamiltonii* through indirect organogenesis using cotyledonary explants. MS media containing 0.5 mg/l BAP and 0.5 mg/l KN recorded the highest callus formation percentage (82.0%). About 4.6 shoots were induced from the callus with a mean shoot length of 6.9 cm on 1.0 mg/l BAP and 0.1 mg/l GA_3, while only 3.8 shoots with 5.8 cm of shoot length were induced from the callus cultured on 1.0 mg/l each IAA and GA_3. The use of ½ MS medium supplemented with 0.4 mg/l IBA recorded the highest number of *in vitro* roots (38.2) per shoot with a length of 11.8 cm. The well-rooted plantlets showed about 97.5% of survivability after the hardening stage. *In vitro* microrhizomes were obtained from the callus derived from *D. hamiltonii* leaf discs (Thangavel et al. 2014). MS medium containing 2 mM BAP and 6 mM NAA was found more suited to induce calli from leaf discs. Later, callus was further differentiated to form microrhizomes on MS media fortified with 4 mM IBA and 8 mM NAA. A cluster of about 20 microrhizomes was formed within 90 days of culture. The use of 0.05% yeast extract and 0.05% polyvinylpyrrolidone in the above media was effective in enhancing the formation of microrhizomes.

20.3.7 *Gloriosa superba* L.

Sayeed Hassan and Roy (2005) micropropagated *G. superba* using axillary and apical buds of naturally grown plants. About 92% of the explants showed regeneration capacity with the formation of 4 shoots on MS media fortified with BA (1.5 mg/l) and NAA (0.5 mg/l). Adding 15% (v/v) coconut water and 2 g/l activated charcoal further enhanced multiple shoots formation up to 15 per culture. IBA (1 mg/l)- and

0.5 mg/l IAA-supplemented MS media were effective in inducing *in vitro* roots. The hardened plantlets showed a survival rate of 85–90%. Later, a rapid, efficient, and improved *in vitro* clonal propagation protocol was established. Shoot tips cultured on MS media containing 2.0 mg/l BAP and 0.5 mg/l NAA recorded maximum regeneration rate (76.6%) and multiple shoots (1.2). The use of MS media added with 1.0 mg/l NAA, 0.5 mg/l IBA, and 3% (w/v) sucrose formed the highest rooting frequency (66.6%) within 20 days. Acclimatization of plantlets in soil/sand/vermicompost (2:1:1) showed the highest survival rate of 93.3% in the field condition (Yadav et al. 2015). *In vitro* tuberization of *G. superba* was reported by Selvarasu and Kandhasamy (2012) in which the use of MS media fortified with BAP (4.0 mg/l) and NAA (1.0 mg/l) induced 100% primary and secondary tubers.

20.3.8 *Hemidesmus indicus*

Indirect organogenesis of *H. indicus* was reported by Sreekumar et al. (2000). They observed a better caulogenic response and regeneration of shoots (9.37) when nodal segments were cultured on ½ MS medium fortified with BAP (2.22 μM) and NAA (1.07 μM). *In vitro* rooting was evidenced on 9.2 μM IBA, and the well-rooted plantlets were hardened in pots with a survival rate of 96%. Ramulu et al. (2003) established a simple protocol for *in vitro* regeneration of *H. indicus.* They observed that root segments of aseptic seedlings cultured on MS medium containing 3 mg/l BAP and 0.5 mg/l NAA produced the highest multiple shoots (5.02 ± 1.01). For *in vitro* rooting, shoot buds were cultured on ½ MS medium which was sufficient enough to form healthy roots. The well-rooted plantlets were moved to pots contained with sand and vermiculite (1:3) under a mist chamber until 3 weeks for acclimatization which showed about 85% survival rate. A large scale multiplication and conservation protocol for *H. indicus* was developed by Shekhawat and Manokari (2016) by using nodal segments. MS media added with BAP (2.0 mg/l) responded 100% regeneration frequency and produced 9.0 ± 0.53 multiple shoots with 5.2 ± 0.25 cm length. The repeated subcultures further enhanced the number of shoots on MS media containing BAP at 1.0 mg/l, KN at 0.5 mg/l, and IAA at 0.1 mg/l. *In vitro* rooting response was better evidenced on ¼ strength MS media fortified with IBA at 3.0 mg/l. Microshoots were found to induce the formation of 62.0 ± 0.54 roots per shoot. While, *ex vitro* rooting by dipping the cut ends of shoots in IBA at 400 mg/l for 5 min showed about 96% rooting frequency with 45.0 ± 0.48 roots per shoot. Interestingly, 98% of ex vitro rooted clonal propagates survived in comparison to the *in vitro* rooted regenerants which showed only 91% survival rate upon transfer to the field.

20.3.9 *Leptadenia reticulata* (Retz.) Wight & Arn.

In vitro culturing of nodal segments of *L. reticulata* on MS medium supplemented with BAP (9 μM) and IAA (0.6 μM) induced 3–4 shoots (Arya et al. 2003). Further,

subculturing of microshoots on the fresh medium having BA (2.2 μM) and IAA (0.6 μM) enhanced the multiple shoots. Shoots were pretreated with 123 μM each IBA and NAA to induce ex vitro rooting. The established plants showed good morphological properties in the field. While, Shekhawat et al. (2006) used MS medium supplemented with BAP (5.0 mg/l) to induce multiple shoots from nodal explants. *In vitro* shoots readily roots in MS media contained with IBA at 200 mg/l. In a study Hariharan et al. (2002) established somatic embryogenesis and *in vitro* regeneration of *L. reticulata* plants using leaf explants. MS medium containing BAP (2.0 mg/l) and NAA (0.5 mg/l) induced embryogenic callus which later resulted in embryoids upon subculturing on MS medium devoid of plant growth regulators. The embryoids showed germination on MS medium containing 1.0 mg/l KN. Likewise, Martin (2004) used BAP-supplemented MS media to induce embryogenic callus from the callus derived from shoot tip and nodal segments of *L. reticulata* on MS media fortified with BAP (8.87 μM) and IBA (2.46 μM). The growth of embryos was evidenced when embryogenic calli were suspended in the liquid media. Half strength MS media with GA_3 (1.44 μM) and BAP (0.44 μM) was effective in converting embryos to plantlets. Well-rooted plantlets were shifter to poly cups, and their successive transfer to the field condition showed about 80% survival rate. However, Parabia et al. (2007) have recommended the use of IBA (1 mg/l) and KN (10 mg/l) in MS media to induce callus from nodal segments. Later, culturing calli on NAA- (1.5 mg/l) and KN (10 mg/l)-supplemented media differentiated into shoots. Likewise, Sathyanarayana et al. (2008) have stated the use of MS media with NAA (2.68 μM) and BAP (2 μM) to induce embryogenic calli. Calli when added into the basal MS liquid medium differentiated into shoots which rooted readily in ½ MS media having 4.90 μM of IBA. A simple and cost-effective direct organogenesis protocol for *L. reticulata* was established by culturing nodal segments on MS media contained with 0.25 mg/l each BAP and KN (Sudipta et al. 2011, 2014). Further, the use of 2% table sugar was found to be effective enough to respond *in vitro* morphogenesis in *L. reticulata* explants similar to that observed when treated with 2% sucrose (Sudipta et al. 2013).

20.3.10 *Nothapodytes nimmoniana* (Graham) Mabb.

Usually, *N. nimmoniana* seeds fail to germinate easily; they are pretreated to enhance the germination rate under *in vitro* condition. Most studies have advised to presoak seeds in the sterilized distilled water overnight and, later, excise the zygotic embryos aseptically and inoculate on media added with GA_3 which facilitates better seedling growth (Tejavathi et al. 2011; Anuradha et al. 2011). The combined use of 2,4-D and BAP favors callus induction and higher yield of camptothecin content (Fulzele et al. 2001; Sundravelan et al. 2004). Clonal propagation and conservation protocol for *N. nimmoniana* was proposed by Rajasekharan et al. (2010). According to them, seed embryos induced calli on MS media contained with 0.91 μM TDZ (thidiazuron) and 3% sucrose. On the same media, regenerating calli induced somatic embryos and multiple shoots after 3 weeks of culture. An average of 70

somatic embryos were developed per tube and measured 5–15 mm long. However, somatic embryos were inseparable and relatively small in size and elongated further with shoots of 2–19 mm in length when cultured on the basal MS medium after 30 days. *In vitro* rooting initiated better with the use of 1 mg/l IBA. Khadke and Kuvalekar (2013) regenerated plantlets through the direct somatic embryogenesis by using leaf and stem explants cultured on MS media supplemented with 0.5 mg/l TDZ and 10% coconut water. Leaf explants produced about 23.3 ± 1.52 somatic embryos, while stem explants resulted with 11.5 ± 0.83 somatic embryos. In another study, an efficient direct regeneration protocol for *N. nimmoniana* was established from leaf and nodal explants (Amilineni et al. (2016). They reported the use of MS media with 8.87 μM BAP to induce multiple shoots. Shoots that elongated and proliferated further when cultured on MS media was added with 4.44 μM BAP and 0.87 μM GA_3. Rooting was achieved by using ½ MS media containing IAA (4.9 μM). Micropropagated plants were morphologically similar and showed 90% survival rate in the field and produced camptothecin content in the range of 0.08–0.2%. Likewise, a clonal propagation method was established by using embryos of *N. nimmoniana* (Prakash et al. 2016). The use of BAP (0.2 mg/l) and IBA (0.1 mg/l) synergistically influenced on the differentiation of multiple shoots. The shoot regeneration frequency was about 90.6% with a mean shoot number of 10.24 produced within 8 weeks. About 92% of the microshoots showed rooting in ½ MS medium containing activated charcoal (0.05%) and fortified with 0.1 mg/l each NAA and IBA. All the regenerated plantlets were found uniform both genetically and biochemically.

Radha et al. (2012) used simple desiccation method to cryopreserve the zygotic embryos of *N. nimmoniana*. The embryos dissected out from seeds were subjected to dehydration under laminar airflow with an interval of 30 min up to 210 min. The desiccated embryos were later packed in cryovials (2 ml) and moved to liquid nitrogen (at −196 °C). After storing, the embryos were retrieved from the vial and cultured on the germination medium for the recovery. They observed that the embryonic axes exhibited 86.67% germination when not desiccated which possessed 55.7% moisture content. However, 66.67% germination was evidenced when dehydrated for 120 min which reduced the moisture content to 19.6%. After 1 week, about 60% of the desiccated (120 min) and liquid nitrogen-treated embryonic axes induced shoot and root which later developed into seedlings within 20 days of culture on growth regulator free media.

20.3.11 *Ophiorrhiza mungos* L.

In vitro propagation method was established for *O. mungos* through seedling (shoot) cultures (Jose and Sateeshkumar 2004). MS media contained with 2.22 μM BAP initiated maximum shoots (10.4 ± 1.72) after 3 weeks of culture. Further, subculturing favored shoot proliferation rate, and the shoot elongated (1.27 ± 0.12 cm) within 2 weeks with the addition of 1.44 μM GA_3. IBA (12.3 μM)- and NAA (1.07 μM)-supplemented MS media induced the highest root formation frequency. All

regenerated plants survived in the pots containing 1:1 ration of top soil and sand mixture. In an attempt to *in vitro* regenerate *O. mungos*, surface sterilized fruits were grown on MS media supplemented with varied levels of phytohormones to initiate callus and multiple shoots (Namdeo et al. 2012). MS media containing BAP (2 ppm), IAA (2 ppm), and GA (1 ppm) recorded higher callus biomass, while MS medium fortified with picloram/TDZ/GA_3 (1:2:1) induced maximum number of shoots (25) and length (6.5 cm) after 30 days of culture. Interestingly, whole *in vitro* plants contained the maximum camptothecin content (0.0768% w/w), compared to the naturally grown *O. mungos* plants (0.0030% w/w). The minimum camptothecin was recorded in adventitious buds with only 0.0026% (w/w). Likewise, an efficient direct organogenesis protocol was established to rapidly multiply *O. mungos* using terminal and axillary buds derived from *in vitro*-raised seedlings (Kaushik et al. 2015). Explants inoculated on MS medium containing 0.25 mg/l each BAP and KN was reported to induce 63.1 ± 1.35 shoots with a length of 2.8 ± 1.15 cm within 28 days. The use of GA_3 (1.0 mg/l) further enhanced the multiplication rate and increased the shoot length by 2.33-fold within 3 weeks. *In vitro* rooting frequency (92.13%) was achieved better in ½ strength MS medium supplemented with activated charcoal (100 mg/l). All rooted plantlets showed a higher survival rate of 95% after acclimatization. Further, molecular markers confirmed the genetic uniformity among the *in vitro*-raised plants with the mother plant, which suggest the true to type nature. In addition, high performance liquid chromatography analysis confirmed the production of camptothecin from *in vitro* raised plants which was comparable to that detected in the mother plants. This suggested the uniformity in the chemical profile.

20.3.12 *Rauvolfia serpentina* L. Benth. ex Kurz.

Goel et al. (2009) observed a high rate of *in vitro* shoots proliferation on MS media containing 1.0 mg/l BAP and 0.1 mg/l NAA. For rooting, MS media containing 1.0 mg/l NAA were found more effective. *In vitro* regenerants exhibited high survival rate of 90–95% under glass house condition. Micropropagated plants were genetically identical to that of the mother plants and contained optimum levels of phytoconstituents such as reserpine, ajmalicine, and ajmaline. While, Harisaranraj et al. (2010) report the use of 2.0 mg/l BA and 0.25 mg/l NAA for better *in vitro* shoot multiplication. According to Susila et al. (2013) MS medium supplemented with 0.1 mg/l NAA and 2.5 mg/l BAP induces higher shoot proliferation rate (92%), and the use of ½ MS media added with 0.4 mg/l NAA and 0.1 mg/l IBA is good for achieving maximum *in vitro* rooting. Senapati et al. (2014) have reported about 96% of shoot bud proliferation with an average 13.3 shoots measuring 8.2 cm in length on MS media containing 2 mg/l BAP, 0.25 mg/l NAA, and 3% sucrose. About 73.3% of rooting frequency was observed in ½ MS media supplemented with 0.5 mg/l IBA. The well-rooted plantlets recorded with 90% survival rate, and they were genetically identical with their donor plants as revealed by DNA markers.

For the first time, Ray and Bhattacharya (2008) have reported the cryopreservation protocol for *R. serpentina* using PVS2 (plant vitrification solution) vitrification of *in vitro*-derived nodal segments measuring 0.31–0.39 cm in length. Their results showed that explants precultured in 0.5 M sucrose for 7 days under diffuse light at 4 °C, preloaded in PVS2 for 30 min and 45 min, produced the highest viable (66%) cultures after cryopreservation. Thus, this method can be effectively used for conserving *R. serpentine* germplasms for a long period.

20.4 Conclusions and Future Prospects

Medicinal plants are crucial for the discovery of novel drugs for treating diverse forms of diseases including cancer. As synthetic drugs used to cure cancer disease possess toxicity and side effects, plant-based drugs are much appreciated. The paramount resource to treat cancer is by using anticancer compounds derived from the medicinal plants. Worldwide, one third of the plants are diminishing at an alarming rate by improper and destructive collection from wild sources. There is an immense need to conserve these medicinal plants for the future generations by adapting the most appropriate methods or through biotechnological strategies. In this regard, the micropropagation technique is very useful to mass propagate and conserve several endangered plant species that are difficult to cultivate or propagate by conventional approaches. A collective approach of suing in situ and ex situ conservation methods supported by advanced technologies will be very effective in saving these highly valued medicinal plants. In addition, modern techniques of molecular biology and genetics can support to develop a simple and more efficient regeneration systems and to conserve plant materials through pollen banking, seed banking, or storing in liquid nitrogen. As predicted, there will be a sharp increase in the global demand for the herbal products to fulfill the primary healthcare needs. Hence, it is utmost important to conserve the medicinal plants to ensure their availability to use and develop new drugs. Studies related to conservation of these endangered anticancer plants of the Western Ghats of India are very meager, and hence, future research should focus toward the establishment of more reliable and a simple *in vitro* propagation and cryoconservation protocols to restore these endangered species for future generations. All organizations including government and nongovernmental institutes, pharmaceutical industries, and other research institutes should work together to conserve and sustainably utilize these medicinal plants.

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Biotechnological Approaches for the Propagation of Anticancer Plants and the Production of Vital Compounds

21

Sandra Gonçalves and Anabela Romano

Abstract

Plants are a tremendous source of bioactive compounds with significance in many fields from medical to agrochemical. Among plant bioactive compounds, there are some important anticancer compounds clinically used in cancer therapy or used as model compounds for chemical synthesis of potent analogs. Although these compounds have great pharmacological value, their exploitation is limited by the slow-growing nature of the plant species, the low production yields, and unpredictable variability. Moreover, due to the over collection, some anticancer species are in risk of extinction. Plant cell and tissue culture techniques have emerged as sustainable options for the propagation and conservation of medicinal plants also providing an environmentally friendly alternative method for the production of metabolites, when natural supply is limited. The aim of this chapter is to summarize the most relevant work focusing on the use of biotechnological approaches to the propagation of plants with anticancer properties and the production of valuable compounds. Our literature survey demonstrated that efforts have been mainly concentrated in species producing the compounds camptothecin, podophyllotoxin, taxol, vinblastine, and vincristine. An intensive research has been conducted on the optimization of growth conditions and application of several strategies to improve the production of anticancer compounds (particularly by elicitation) and on the elucidation of biosynthetic pathways and their regulation. In spite of all these investigations and the advantages of the production of plant compounds *in vitro*, there are only few examples of the production of anticancer compounds on an industrial level, and further in-depth studies are still required.

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Camptothecin · Cell suspensions · Hairy roots · Metabolic engineering · Micropropagation

21.1 Introduction

Plants have been used since ancient times having an important role in traditional medicine. They are a remarkable source of biologically active compounds useful as therapeutics (Kolewe et al. 2008; Swamy et al. 2016; Mohanty et al. 2017). The development of identification and isolation techniques induced a new trend in the area of natural compounds allowing the discovery of numerous plant-derived compounds in the last 200 years. Nowadays, about 70–95% of the population in most of the emerging nations still uses folk medicines (Robinson and Zhang 2011; Swamy and Sinniah 2015; Arumugam et al. 2016), and around 25–28% of modern medications are obtained from medicinal plants (Samuelsson 2004). Interestingly, over 60% of anticancer drug molecules are mainly derived from medicinal plants (Cragg and Newman 2005; Gordaliza 2007). The exploration for anticancer compounds from plants started in the 1950s when the vinblastine and vincristine (alkaloids) from *Catharanthus roseus* (L.) G. Don and podophyllotoxin from *Podophyllum* spp. were discovered. In 1960, the United States National Cancer Institute initiated a program that steered to the discovery of several new chemotypes with cytotoxic properties (Cassady and Douros 1980) such as camptothecins and taxanes (Cragg and Newman 2005).

Some clinically important plant compounds like camptothecin, colchicine, demecolcine, docetaxel, podophyllotoxin, taxol, vinblastine, or vincristine are used as chemotherapeutic agents to stop cell division or acting as cytotoxic agents (Khani et al. 2012; Fridlender et al. 2015). Table 21.1 summarizes the main plant-derived compounds with anticancer properties produced by using biotechnological approaches, and Fig. 21.1 shows their chemical structure. Some of these compounds have a very high market price, for instance, 1 kg of vincristine costs about 20,000 USD and 1 kg of taxol costs ~5 million USD (Wink et al. 2005).

Chemical synthesis using natural products as templates is economically viable for compounds with simple chemical structures. For metabolites with complex structure, as some important anticancer compounds (Fig. 21.1), with multiple rings and chiral centers, the synthesis is prohibitively expensive (Kolewe et al. 2008). In these cases, alternative methods of supply are required to meet the world demand for natural anticancer compounds. Plant cell and tissue culture systems appear to be a viable option to provide a biologically feasible alternative methods for producing various bioactive secondary metabolites when natural supply is limited (Kolewe et al. 2008; Khani et al. 2012).

The use of plant cell and tissue culture techniques to produce useful bioactive metabolites has several advantages over field cultivation, among them: the production is consistent, predictable, and independent of climate or ecological factors,

Table 21.1 Plant-derived compounds with anticancer properties produced by using biotechnological approaches

Compound(s)	Plant species	Group	Mechanism of actions	Observations
Camptothecin	*Camptotheca acuminata*, *Nothapodytes foetida*, *Nothapodytes nimmoniana*, and *Ophiorrhiza pumila*	Monoterpene indole alkaloid	Inhibits the action of DNA topoisomerase I	It has low water solubility and side effects. Thus, used for clinical purposes. The chemical modification of its derivatives (e.g., topotecan and irinotecan) is currently used in chemotherapy
Podophyllotoxin	*Podophyllum* spp. and *Linum* spp.	Aryltetralin lignan	Binding the microtubules and causing mitotic arrest in metaphase	Podophyllotoxin is toxic for human cells and is a precursor of semisynthetic antineoplastic drugs (e.g., etoposide, etopophos, and teniposide)
Taxanes (taxol)	*Taxus* spp. and *Corylus avellana*	Diterpene alkaloids	Stabilize the microtube lattice by inducing the internal rearrangements of the tubulin dimmers	Taxol is used for the treatment of refractory metastatic ovarian cancer and also used as a supplement for plant cell cultures for its supply
Vinblastine and vincristine	*Catharanthus roseus*	Terpene indole alkaloids	Inhibit the formation of microtubes by the binding with tulin, leading to cell cycle arrest and apoptosis	Vincristine is a very expensive compound (1 kg cost is about USD 20,000), and the annual world market is about USD 5 million

generated continuously throughout the year and without seasonal constraints, cultures are free of microbes and insects avoiding the use of herbicides and pesticides, cultures can be established in any part of the world independently of the plant growth requisites, and isolation is usually more rapid and efficient (Murthy et al. 2014; Ochoa-Villarreal et al. 2016). Moreover, biotechnology opens the opportunity to apply traditional or metabolic engineering strategies to promote the accumulation of desired compounds by *in vitro* cultures. *In vitro* culture products can be used as models of intact plants, and cell cultures can be radiolabeled to trace metabolically secondary products (Khani et al. 2012). *In vitro* propagation techniques also facilitate the rapid multiplication of true-to-type plants and could be a great contribute for

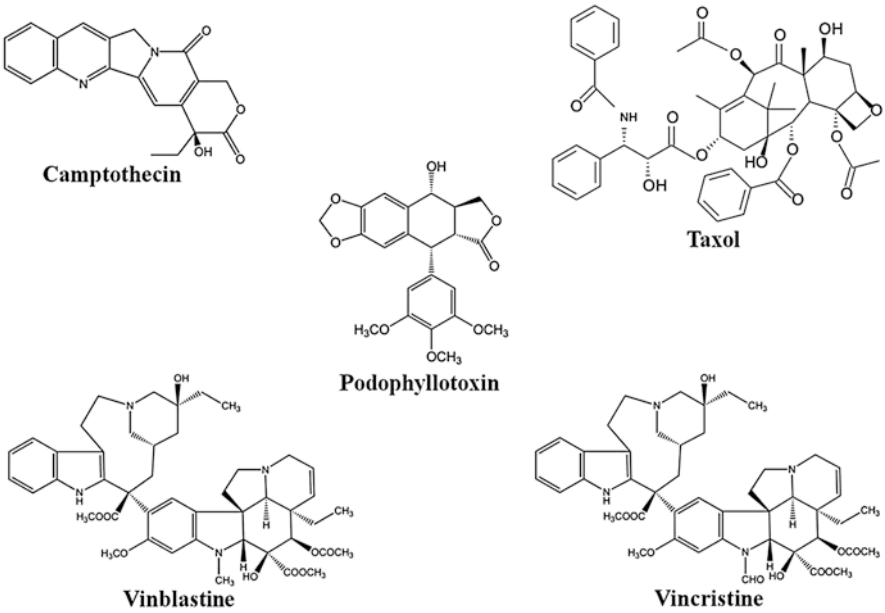

Fig. 21.1 Chemical structures of some anticancer compounds produced by biotechnological approaches

species conservation. This is of the utmost importance since an indiscriminate collection of wild plants has led to the extinction of several therapeutically valued species. Overall, the use of plant tissue culture techniques allows the mass propagation of medicinal plants and the sustainable production of anticancer compounds. The aim of this chapter is to summarize and discuss the most relevant work focusing on (1) the use of *in vitro* propagation methods to the large-scale cultivation of plant species with anticancer properties, (2) the use of plant cells/tissues to produce valued anticancer compounds *in vitro*, and (3) the different biotechnological approaches adopted to improve their production.

21.2 *In Vitro* Propagation of Anticancer Plant Species

The massive and indiscriminate collection of plant material as a source of anticancer compounds has threatened the survival of some medicinal species. Recent years have witnessed the use of tissue culture techniques for propagation and conservation of medicinal plants, and numerous publications reporting *in vitro* propagation protocols for anticancer plants have been proposed. *In vitro* propagation allows the rapid mass multiplication and the recovery of true-to-type plants within a small period of time (Kumara Swamy et al. 2010; Kaushik et al. 2015; Swamy and Sinniah 2016). This technique is particularly useful for plants difficult to propagate by conventional techniques or with slow propagation rates. Thus, *in vitro* propagation is particularly important in the case of endangered species allowing the rapid

Table 21.2 Summary of plants having anticancer properties propagated *in vitro*

Plant species	Explant(s) type (method)	Compound(s) produced	References
Camptotheca acuminata	Axillary buds and shoot tips	Camptothecin	Liu and Li (2001)
Heliotropium indicum	Hypocotyl segments of aseptically germinated seedlings and nodal sections of plants grown in a glasshouse (organogenesis)	Indicine N-oxide	Datta et al. (2003)
Podophyllum peltatum	Cotyledon explants of zygotic embryos (somatic embryogenesis)	Podophyllotoxin	Kim et al. (2007)
Ophiorrhiza mungos	Axillary and terminal buds of *in vitro*-raised seedlings (organogenesis)	Camptothecin	Kaushik et al. (2015)
Ophiorrhiza prostrata	Internode and leaf explants (organogenesis)	Camptothecin	Martin et al. (2008)
Ophiorrhiza rugosa	Axillary meristems	Camptothecin	Roja (2008)
Magnolia dealbata	Leaf explants (organogenesis)	Honokiol and magnolol	Domínguez et al. (2010)
Ophiorrhiza alata	Leaf and node explants from germinated seeds (organogenesis)	Camptothecin	Ya-ut et al. (2011)
Nothapodytes nimmoniana	Nodal explants from germinated seeds (organogenesis)	Camptothecin	Dandin and Murthy (2012) and Prakash et al. (2016)
Rhinacanthus nasutus	Leaf explants (organogenesis)	Naphthoquinones and rhinacanthins	Cheruvathur et al. (2012)
Podophyllum hexandrum	Zygotic embryos (somatic embryogenesis)	Podophyllotoxin	Rajesh et al. (2014)
Salacia chinensis	Nodal explants	Mangiferin	Chavan et al. (2015)
Jeffersonia dubia	Sucker explants	Berberine	Jeong and Sivanesan (2016)

multiplication without affecting the natural resources (Verpoorte et al. 2002; Swamy et al. 2009; Prakash et al. 2016; Mohanty et al. 2017).

Some selected examples including plants producing the important anticancer compounds camptothecin (Liu and Li 2001; Martin et al. 2008; Roja 2008; Ya-ut et al. 2011; Dandin and Murthy 2012) and podophyllotoxin (Kim et al. 2007; Rajesh et al. 2014) are summarized in tabular form (Table 21.2). A number of studies were reported for the species *Nothapodytes nimmoniana* (J. Graham) Mabb. [syn. *N. foetida* (Wight) Sleumer], a source of camptothecin (Prakash et al. 2016). The populations of this species are under heavy exploitation pressure due to the indiscriminate collection for camptothecin extraction. Plant tissue culture techniques provide alternatives for plant clonal propagation and camptothecin production. Plant propagation

has been mainly performed through direct regeneration and several explant types (e.g., shoot tips, nodal segments, leaves, hypocotyls, cotyledons, immature embryos); medium formulations and culture conditions have been tested, and it was observed that the production of camptothecin was affected by culture conditions (Isah and Mujib 2015; Kaushik et al. 2015; Prakash et al. 2016). *In vitro* propagation protocols have been also developed for plant species producing less investigated anticancer compounds. Datta et al. (2003) described the first *in vitro* propagation protocol for *Heliotropium indicum* L., a species that produces the anticancer compound indicine N-oxide. More recently Cheruvathur et al. (2012) described the first detailed study on *in vitro* propagation for the anticancer plant *Rhinacanthus nasutus* (L.) Kurz, a source of naphthoquinones and rhinacanthins.

In vitro propagation of plants can be attained by three different methods: meristem culture, organogenesis, and somatic embryogenesis (George and Debergh 2008). In meristem culture, nodal segments or apical buds are used to produce shoots without the involvement of a callus phase (Pati et al. 2006). This method is typically divided in the following stages (George and Debergh 2008): *in vitro* culture establishment, multiplication of shoots, rooting, and acclimatization. Somatic embryogenesis and organogenesis involve the formation of embryos or adventitious shoots, respectively. This can occur indirectly when shoots or embryos are regenerated from callus or a cell culture (indirect organogenesis or indirect embryogenesis) or directly without the callus intervention (direct organogenesis or direct embryogenesis). Organogenesis is the most used method to propagate anticancer plant species [e.g., *Heliotropium indicum* L., *Ophiorrhiza prostrata* D. Don., *Magnolia dealbata* Zucc., *Ophiorrhiza alata* Craib, *Ophiorrhiza mungos* Linn., *N. nimmoniana*, or *Rhinacanthus nasutus* (L.) Kurz.], although some studies also report plant propagation through meristem culture using axillary or apical buds as explants (*Camptotheca acuminata* Decaisne, *Ophiorrhiza rugosa*, and *Salacia chinensis* L.) or somatic embryogenesis (e.g., *Podophyllum peltatum* L., *Podophyllum hexandrum* Royle Mansf.) (Table 21.2).

One of the main advantages of micropropagation is the production of true-to-type plants (Kumaraswamy and Anuradha 2010; Sudipta et al. 2014). Some authors analyzed micropropagated plants obtained with molecular markers and confirmed that they maintain the genetic integrity even after prolonged periods under *in vitro* conditions (Cheruvathur et al. 2012; Chavan et al. 2015; Isah and Mujib 2015; Kaushik et al. 2015; Prakash et al. 2016). Overall, our literature survey indicates that plant regeneration through *in vitro* methods is an efficient mean for the large-scale propagation of many anticancer plants allowing high propagation rates of clonal plants that can support production of biocompounds and conservation strategies.

21.3 Production of Anticancer Compounds in Organ Cultures

As addressed in a next section of this chapter, in many cases plant cell cultures are the preferable biotechnological way to produce valuable secondary metabolites. Nevertheless, secondary metabolites are a product of differentiation, and, therefore,

the use of organized tissues like roots, shoots, or embryos instead of undifferentiated cell cultures is required in some cases (Verpoorte et al. 2002). Intact plants of *C. acuminata* contain around 0.2–5 mg/g dry weight (DW) of camptothecin, while calli and suspension cultures produced only 0.002–0.004 mg/g DW or lesser (Lopez-Meyer et al. 1994). Also the hairy roots of *Ophiorrhiza pumila* showed high capacity to produce camptothecin (0.1% DW) although the callus culture failed to produce this compound (Saito et al. 2001).

Several reports describe the production of important anticancer compounds in organ cultures, namely, shoots, roots, and somatic embryos. El-Sayed and Verpoorte (2007) recorded the production of indole alkaloids in several tissues of *C. roseus* depends on cell differentiation and organogenesis. Aslam et al. (2010) quantified the content of the indole alkaloid vinblastine in various *in vitro*-produced tissues of the same species, namely, embryogenic and nonembryogenic calli, different stages of embryogenesis, and somatic embryos derived from plantlets and field-grown plantlets, and concluded that the production is age and tissue dependent. Rajesh et al. (2014) observed high content of podophyllotoxin in germinated somatic embryos as compared to germinated zygotic embryos of *Podophyllum hexandrum* Royle. However, *Podophyllum* spp. are difficult to establish *in vitro*, and the cultures' capacity to produce podophyllotoxin is frequently low (Yousefzadi et al. 2010a), making the economic exploitation unviable (Bhattacharyya and Chattopadhyay 2015). These difficulties led to the study of another group of plants, *Linum* spp., that proved good sources of podophyllotoxin, and several approaches have been successfully used for increasing the production capacity (Malik et al. 2014).

Roja (2008) analyzed the camptothecin content in different parts of *O. rugosa* plants produced *in vitro*. There was a substantial rise in the content of this compound in the leaves (450-fold) compared to *in vivo* plant tissues. The tissue culture-raised plantlets of *O. mungos* contained 0.0438 ± 0.18% of camptothecin which was comparable to that of mother plant (0.043 ± 0.16%) (Kaushik et al. 2015). *In vitro* shoots of *N. nimmoniana* produced a content of camptothecin equivalent or significantly greater than the *in vivo* plants (Dandin and Murthy 2012). The content of camptothecin in stems and leaves of clonally propagated plants of *N. nimmoniana* was found to be 0.026% w/w and 0.0013% w/w, respectively (Prakash et al. 2016). These are very promising observations and constitute a renewable source for the production of this important anticancer compound without destroying the plant. More recently, studies from Gopalakrishnana and Shankar (2014) suggest that the shoot cultures of *O. rugosa* var. *decumbens* are also a good alternative for the production of camptothecin.

There are also studies reporting other less investigated anticancer compounds in *in vitro* produced tissues. Jeong and Sivanesan (2016) observed berberine in *Jeffersonia dubia* shoots and callus cultures. Honokiol and magnolol, two antitumor agents, were produced in aerial and underground parts of micropropagated *M. dealbata* plants in higher contents than in wild plants (Domínguez et al. 2010). *In vitro* regenerated plants of *S. chinensis* showed higher mangiferin content (429.10 ppm) than field-grown plants (353.30 ppm), although in this case the greater content was observed in undifferentiated callus cultures (593.87 ppm) (Chavan et al. 2015).

Hairy root cultures are well-recognized as a good option for *in vitro* production of secondary metabolites due to their high level of cellular differentiation, and, therefore, the production of anticancer compounds in hairy roots is addressed separately in the next section of this chapter.

21.4 Production of Anticancer Compounds in Hairy Root Cultures

Hairy root cultures offer new prospects for producing valuable plant compounds under *in vitro* conditions (Chandra and Chandra 2011). Hairy roots are induced by infecting plants with a gram-negative soil bacterium, *Agrobacterium rhizogenes*. During this infection, a small fragment of DNA (T-DNA) from the *Ri* (root-inducing) plasmid of the bacterium is transferred to the host plant where it integrates with the plant genome. Hairy roots have the ability to synthesize a range of compounds in addition to several other advantages such as increased levels of cell differentiation, rapid growth, genetic and biochemical stability, and maintenance facility (Chandra and Chandra 2011). Another important advantage is to accumulate metabolites that are usually amassed in other parts of the plant (aerial parts). Secondary metabolite production in hairy root cultures is affected by different factors like light, temperature, and CO_2 and O_2 concentrations, and scaling up in bioreactors is a strong option for the production of anticancer compounds (Chandra and Chandra 2011; Khani et al. 2012). When selecting a bioreactor, various factors need to be taken into consideration such as nutrient accessibility and uptake, oxygen and hydrogen consumption, mixing, and shear sensitivity. From the various bioreactors used, gas-phase, liquid-phase, or hybrid reactors are the most used for culturing hairy roots (Srivastava and Srivastava 2007).

There are several works on the production of anticancer compounds in hairy roots, particularly aryltetralin lignins (Baldi et al. 2008; Farkya and Bisaria 2008; Kumar et al. 2012; Bahabadi et al. 2014; Cong et al. 2015), indole alkaloids (Li et al. 2011; Rizvi et al. 2015, 2016; Hanafy et al. 2016), and camptothecin (Saito et al. 2001; Yamazaki et al. 2003; Lorence et al. 2004; Ya-ut et al. 2011), among others (Table 21.3). The optimization of the several steps of the *Agrobacterium*-mediated transformation process (Rizvi et al. 2015), and the study of the effect of different parameters, namely, culture medium composition (Farkya and Bisaria 2008; Hanafy et al. 2016), aeration (Baldi et al. 2008), and other physical factors (Jose et al. 2016) on the production of anticancer compounds, has been the subject of many studies. Also, the use of elicitors such as signaling molecules (Li et al. 2011), fungal elicitors (Kumar et al. 2012; Bahabadi et al. 2014), or UV-B light (Binder et al. 2009) showed to be a good approach to enhance the production of compounds in *C. roseus* and *Linum* spp. hairy roots (Table 21.4). Hairy root cultures are important not only for the production of anticancer compounds but also to investigate secondary metabolism. Cong et al. (2015) used hairy root cultures to study the kinetics of aryltetralin lignin accumulation in *L. album* and *L. flavum*, and Rizvi et al. (2016) produced transgenic hairy root cultures of *C. roseus* to understand the regulation of indole alkaloids.

Table 21.3 Some common anticancer compounds produced in hairy root cultures

Plant species	Compound(s)	References
Ophiorrhiza pumila	Camptothecin	Saito et al. (2001) and Yamazaki et al. (2003)
Camptotheca acuminata	Camptothecin	Lorence et al. (2004)
Catharanthus roseus	Vincristine and vinblastine	Li et al. (2011), Rizvi et al. (2015, 2016) and Hanafy et al. (2016)
Ophiorrhiza alata	Camptothecin	Ya-ut et al. (2011)
Angelica gigas	Pyranocoumarins (decursin and decursinol)	Park et al. (2012)
Linum spp.	Aryltetralin lignans (podophyllotoxin and 6-methoxy podophyllotoxin)	Baldi et al. (2008), Farkya and Bisaria (2008), Kumar et al. (2012), Bahabadi et al. (2014), and Cong et al. (2015)
Plumbago rosea	Plumbagin	Jose et al. (2016)

Table 21.4 Examples of biotechnological strategies used to improve the production of compounds with anticancer properties

Plant species	Compound(s)	Strategy	Culture type	References
Taxus baccata	Taxol	Immobilization	Cell suspension cultures	Bentebibel et al. (2005)
Taxus cuspidata	Taxol	Elicitation (methyl jasmonate)	Cell suspension cultures	Kim et al. (2005)
Taxus baccata	Taxol	Elicitation (methyl jasmonate, salicylic acid, and fungal elicitor)	Cell suspension cultures	Khosroushahi et al. (2006)
Taxus yunnanensis (syn. *Taxus wallichiana*)	Taxol	Elicitation (heat shock)	Cell suspension cultures	Zhang and Fevereiro (2007)
Ophiorrhiza prostrata	Camptothecin	Elicitation (methyl jasmonate and acetyl salicylic acid)	Adventitious root cultures	Martin et al. (2008)
Catharanthus roseus	Terpenoid indole alkaloids	Elicitation (UV-B stress)	Hairy root cultures	Binder et al. (2009)
Nerium oleander	Oleandrin	Optimization of culture medium and precursor feeding	Cell suspension cultures	Ibrahim et al. (2009)

(continued)

Table 21.4 (continued)

Plant species	Compound(s)	Strategy	Culture type	References
Camptotheca acuminata	Camptothecin and 10-hydroxycamptotencin	Elicitation (several elicitors)	Cell suspension cultures	Pi et al. (2010)
Linum album	Podophyllotoxin	Elicitation (salicylic acid)	Cell suspension cultures	Yousefzadi et al. (2010b)
Catharanthus roseus	Terpenoid indole alkaloid	Elicitation (sodium nitroprusside)	Hairy root cultures	Li et al. (2011)
Linum album	Podophyllotoxin and 6-methoxypodophyllotoxin	Elicitation (fungal elicitor)	Hairy root cultures	Kumar et al. (2012)
Taxus baccata	Taxanes	Optimization of basal medium	Cell suspension cultures	Kajani et al. (2012)
Nothapodytes nimmoniana	Camptothecin	Optimization of culture medium	Cell suspension cultures	Karwasara and Dixit (2013)
Taxus globosa	Taxanes	Optimization of culture medium and elicitation (methyl jasmonate)	Cell suspension cultures	Tapia et al. (2013)
Linum album	Podophyllotoxin and 6-methoxypodophyllotoxin	Elicitation (fungal elicitors)	Hairy root cultures	Bahabadi et al. (2014)
Plumbago rosea	Plumbagin	Elicitation (jasmonic acid, yeast extract, and auxins)	Cell suspension cultures	Silja et al. (2014)
Taxus cuspidata	Taxol	Elicitation (jasmonic acid)	Cell suspension cultures	Patil et al. (2014)
Nothapodytes foetida	Camptothecin	Gamma irradiation	Callus cultures	Fulzele et al. (2015)
Taxus globosa	Taxanes	Elicitation (methyl jasmonate) and immobilization	Cell suspension cultures	Osuna et al. (2015)
Ophiorrhiza mungos	Camptothecin	Elicitation (yeast extract and silver nitrate)	Cell suspension cultures	Deepthi and Satheeshkumar (2016)
Taraxacum officinale	Taraxasterol and taraxerol	Elicitation (methyl jasmonate and β-cyclodextrin)	Root callus suspension cultures	Sharma and Zafar (2016)
Corylus avellana	Taxanes	Elicitation (silver nanoparticles)	Cell suspension cultures	Jamshidi and Ghanati (2017)

21.5 Production of Anticancer Compounds in Cell Suspension Cultures

Undifferentiated cell suspension culture systems also suit good to produce valuable secondary metabolites on a larger scale, because it is easier to scale-up (Kolewe et al. 2008; Ochoa-Villarreal et al. 2016). In comparison with whole-plant extraction or other chemically synthetic approaches, plant cell culture technology is a competitive method for metabolite production for pharmaceutical applications (Dicosmo and Misawa 1995). To obtain suspension cultures, firstly explants excised from the plant material are cultured in suitable conditions to produce a callus culture (a mass of undifferentiated cells). Then, the calli are transferred to liquid media, and the cultures are incubated under controlled conditions and agitation. Under appropriate environments, these undifferentiated cells can be stimulated to regenerate plants. The selection of the plant species, the tissue for callus initiation, and the process to be used are important factors when initiating cell suspension cultures (Murthy et al. 2014). Moreover, it is important to select a highly productive cell line.

The use of bioreactors for large-scale cultivation of bioactive compounds is an adequate technology due to its time and cost reduction and the consistent production between batches without significant variations and geographical constraints. The production of taxol is a successful example; it has been scaled up in bioreactors to supply part of the demands of Bristol-Myers Squibb Company (Wink et al. 2005). To obtain high production yields, it is important to use an appropriate bioreactor, and several factors must be taken into consideration in that choice, namely, flow, mixing efficiency, shear pattern, and oxygen transfer (Eibl and Eibl 2008). Some examples of bioreactors utilized to produce secondary metabolites are the stirred tank, the pneumatic, and the disposable. One of the most important bioreactor types used for commercial production of anticancer compounds is the stirred-tank bioreactor. In addition to the bioreactor selection, other operational variables need to be optimized during plant cell suspension cultures, namely, cell growth and compound productivity and quality. The main factors that influence cell growth that need to be adjusted are nutrient uptake, production kinetics, transfer of heat and oxygen, and fluid hydrodynamics (Huang and McDonald 2009).

Many investigations have been conducted on the use of plant cell suspension cultures for the production of secondary metabolites including important anticancer compounds, like taxanes from *Taxus* spp. (Cusido et al. 2014), terpenoid indole alkaloids from *C. roseus* (Almagro et al. 2015), camptothecin from *C. acuminata* among other species (Karwasara and Dixit 2013), podophyllotoxin from *Podophyllum* and *Linum* spp. (Malik et al. 2014), berberine from *Coptis japonica*, and shikonin from *Lithospermum erythrorhizon* (Khani et al. 2012). In addition, recent studies also demonstrated the potential of hazel (*Corylus avellana* L.) to produce taxol biotechnologically as an alternative to Taxus spp. (Gallego et al. 2017).

21.6 Strategies to Improve the Production of Compounds in Cell Suspension Cultures

For commercial exploitation it is imperative to obtain high yields and consistent secretion of bioactive metabolites in cell suspension cultures. Even selecting a highly productive cell line, the production yields are not always the required. Furthermore, cell cultures frequently lose their production efficiency after long periods of cultivation. Thus, many researchers have been focusing on the utilization of various strategies to stimulate the production of secondary metabolites and obtain efficient yields including traditional and metabolic engineering strategies (Khani et al. 2012).

21.6.1 Traditional Strategies

Manipulation and optimization of numerous chemical and physical factors, like culture medium composition, elicitation, nutrient and precursor feeding, inoculum density, environment factors, agitation, immobilization, and biotransformation, are some of the traditional strategies used to increase biomass accumulation and plant metabolite production (Murthy et al. 2014; Ochoa-Villarreal et al. 2016). Growth conditions *in vitro* vary according to the species and type of explants used, and, therefore, the optimization of growth conditions is sometimes a laborious process. In addition, biomass accumulation and secondary metabolite synthesis are usually a two-step process in which both steps need to be optimized independently (Murthy et al. 2014). Several studies have been conducted with the aim of optimizing growth conditions, especially culture medium composition, for the production of anticancer compounds, and most of them are related to the nitrogen source, phosphate, growth regulators, and inoculum density (Ibrahim et al. 2009; Kajani et al. 2012; Karwasara and Dixit 2013; Tapia et al. 2013).

Elicitation is probably the most remarkable technique to improve secondary metabolite production in plant cell cultures. This strategy involves the use of biotic or abiotic elicitors to enhance biosynthesis paths and consequently metabolite production. Elicitors are compounds that stimulate upregulation genes, some of them targeting secondary metabolite genes associated with defense response to environmental stimuli (Kolewe et al. 2008). Examples of biotic elicitors are the polysaccharides from plant cell walls, plant growth regulators, microorganisms based chitin, chitosan, or glucans, glycoproteins, etc. Jasmonic acid, a stress-signaling molecule which is a natural occurring hormone that induces the biosynthesis of defensive or protective metabolites (e.g., proteins and secondary metabolites). Several authors studied the effect of jasmonic acid and methyl jasmonate in the production of anticancer compounds (Table 21.4), namely, taxol and other taxanes (Kim et al. 2005; Khosroushahi et al. 2006; Tapia et al. 2013; Patil et al. 2014; Osuna et al. 2015; Sharma and Zafar 2016), camptothecin (Martin et al. 2008), and plumbagin (Silja et al. 2014). Other elicitors like salicylic acid have also been used to increase the production of taxol (Khosroushahi et al. 2006), camptothecin (Martin

et al. 2008; Pi et al. 2010), and podophyllotoxin (Yousefzadi et al. 2010a, b). Treatment of *L. album* cells with salicylic acid (10 μM) increased podophyllotoxin production over three times than the control (Yousefzadi et al. 2010b). Recently, Jamshidi and Ghanati (2017) observed that elicitation of *C. avellana* cells with silver nanoparticles augmented the yields of taxol and baccatin III (taxanes), and their contents reached 378% and 163% of the control, after 24 h of treatment. Indeed, the use of nanoparticles as a new approach of elicitation has gained impetus in the last years. The treatment with fungal elicitors is also a common approach used to enhance the production of anticancer compounds (Khosroushahi et al. 2006; Kumar et al. 2012; Bahabadi et al. 2014; Deepthi and Satheeshkumar 2016). There are also several works reporting the use of abiotic elicitors to improve the production of anticancer compounds. Abiotic elicitors including UV (ultraviolet) irradiation, temperature, heavy metals, and chemical additives disturb the integrity of cell membranes (Zhao et al. 2010). UV treatment increased camptothecin (up to 11-fold) and 10-hydroxycampthotencin (25-fold) production in plant cell cultures of *C. acuminata* (Pi et al. 2010). The production of this compound in callus cultures of *N. foetida* also enhanced with the application of gamma irradiation (Fulzele et al. 2015). Zhang and Fevereiro (2007) observed that heat shock treatment significantly improved the production of taxol by *T. yunnanensis* cell suspension cultures. Overall, the production increases with elicitation, depending on its nature and elicitor's concentration, as well as on several other factors such as the plant species, cell line, and development stage (Cusido et al. 2014).

The immobilization of plant cells in gel matrices (e.g., alginate) is another strategy used to increase metabolite production that is achieved by using the surface immobilization or the gel entrapment method (Murthy et al. 2014). This strategy is mainly used to avoid problems of cell aggregation and low shear resistance (Dörnenburg and Knorr 1995). Some of the benefits of this technique are extended cell viability in the stationary stage, simplified downstream processing, a higher cell density in small-scale bioreactors with low cost and contamination risks, reduced shear stress, improved product/metabolite amassing, enables the use of flow-through reactors with greater flow rates and reduction of fluid viscosity (Dicosmo and Misawa 1995). There are few studies applying these strategies for producing anticancer compounds. Bentebibel et al. (2005) obtained one of the highest yields of taxol using the immobilization of *Taxus baccata* L. cells. More recently, Osuna et al. (2015) studied the influence of methyl jasmonate in both free and immobilized cell cultures of *T. globosa* Schltdl. in the production of several taxanes (taxol, 10-deacetyltaxol, 10-deacetylbaccatin III, baccatin III, and cephalomannine) but observed that alginate immobilization did not generally enhance the production of these compounds.

Another strategy to increase the production of plant metabolites is based on the capacity of cultivated cells for biotransformation of exogenously supplied compounds through different reactions, namely, hydroxylation and glycosyl conjugation, among others. The supplied compounds may be of synthetic origin and not necessarily natural intermediaries of plant metabolism (Murthy et al. 2014). This process is probably one of the most commercially realistic strategies although the

high cost of precursors could make the process sometimes unviable (Dicosmo and Misawa 1995). Biotransformation of taxadienes by *Ginkgo biloba* cells yields a series of structurally different polyoxygenated taxadienes with significant activity against human lung adenocarcinoma cells (A549) and its taxol-resistant subclone, A549/taxol cells (Zou et al. 2008).

21.6.2 Metabolic Engineering

Manipulation of biosynthetic pathways is an attractive strategy for further improvement of high yields in plant cell cultures and involves the identification and manipulation of the genes encoding the critical and rate-limiting enzymes in the pathway. The biosynthetic processes are only known for few molecules, and thus, the elucidation of many secondary pathways is an exciting area of research. This approach involves the introduction of specific genes into the plant genome to enhance a metabolic biosynthetic step or to close unwanted pathways (Cusido et al. 2014). The major limitation to metabolic engineering is the availability of information of the biosynthetic pathways.

There have been important advances on the elucidation of the biosynthetic pathways of anticancer compounds essentially indole alkaloids from *C. roseus* and taxol. Among them taxol is probably the most investigated compound, and studies from Onrubia et al. (2014) demonstrated a perfect link between the expression of several genes and the production of taxanes. As reviewed by Cusido et al. (2014), there are several attempts of applying metabolic engineering approach for producing taxanes by *Taxus* species. Several studies demonstrated that elicited *Taxus* cell cultures are an excellent platform to investigate taxane biosynthesis (Cusido et al. 2014). Usually, an increased levels of secondary metabolites are produced by plant cells as a defense mechanism to external factors. This is a consequence of the increased levels of gene expressions that control the biosynthetic pathways. Lenka et al. (2015) demonstrated the elicitation of the taxol production by methyl jasmonate which is regulated at the level of transcription. Comparing the transcriptome of elicited with not elicited cells is an effective strategy to discover genes involving in plant metabolism (Cusido et al. 2014). Using jasmonate-elicited *T. baccata* cell cultures and complementary DNA-amplified fragment length polymorphism, Onrubia et al. (2014) identified taximin, a novel regulator of taxol biosynthesis (Onrubia et al. 2014). More recently, using the same approach, Ramírez-Estrada et al. (2016) identified several genes as putative candidates for gene coding for enzymes that involve in unidentified steps of biosynthesizing taxane. Authors confirmed that one of these genes encodes the β-phenylalanine-CoA ligase. Although the expression of known genes or gene-encoding transcription factors in cell cultures of *Taxus* species have been extensively studied, the biosynthetic pathway of taxol is not fully elucidated so far (Cusido et al. 2014). The metabolic engineering efforts conducted to improve taxane production in *Taxus* cells demonstrated that this is not an easy process due to the difficulty of genetically transforming these gymnosperm plants in addition to their slow growth capacity (Cusido et al. 2014). Different

approaches have been equated to overcome these difficulties, namely, the use of transformed ginseng roots or transgenic bacteria (Cusido et al. 2014).

Intensive research has been also conducted on the biosynthesis of indole alkaloids from *C. roseus*, and the regulation of their pathways was recently reviewed by Almagro et al. (2015). Several authors studied how elicitors (e.g., methyl jasmonate, fungal filtrate, yeast extract) affect indole alkaloid biosynthesis. Guo et al. (2013) investigated the regulatory effects on tabersonine, vindoline, and vinblastine biosynthesis by the addition of various elicitors. Results showed that acetyl-CoA and tryptophan were the most significant factors in the biosynthesis of vinblastine. Few studies report the genetic transformation of this species. Two major strategies are used to increase the production of indole alkaloids: the overexpression of transcription factors and genes of the biosynthetic pathways and the overexpression of genes in other organisms (Almagro et al. 2015). Overall, an obvious association is noticed between the expression profile of the genes and the production of these compounds (Almagro et al. 2015).

There are few studies related with camptothecin and podophyllotoxin production. Recently Cui et al. (2015) increased more than 50% the production of camptothecin in transgenic hairy root cultures of *O. pumila*. Yousefzadi et al. (2010b) showed that salicylic acid increases podophyllotoxin production in *L. album* cultures probably by modifying the expression of key genes of the phenylpropanoid pathway. Authors observed that salicylic acid regulated genes involved in the first steps of the biosynthesis of this compound without affecting genes involved in later steps of the process. This selective action in these genes can be further used to control the production of podophyllotoxin. Despite the high added value of plant anticancer compounds and the numerous investigations conducted, their biosynthesis is not fully understood, and the use of metabolic engineering strategies to enhance their production is still little applied (Lalaleo et al. 2016).

21.7 Conclusions and Future Prospects

Plants are a rich source of secondary metabolites with a wide spectrum of biological activities that can be used for industrial applications, namely, agrochemicals and pharmaceuticals. There are several plant species containing compounds with cytotoxic properties, but the clinical anticancer activity was proven only for few of these compounds that are used in therapy or used as intermediates for chemical synthesis of potent analogs (e.g., podophyllotoxin and camptothecin derivatives, indole monoterpene alkaloids, and taxanes). These compounds have several common characteristics: they are produced in small amounts, they have complex chemical structures, and their biosynthetic pathways are complex and not completely elucidated. Thus, the production of these compounds at industrial scale has become an active field of research. In this sense, the production of these compounds in plant cell and organ cultures is an attractive alternative of supply. The major advantage of this alternative is the production of anticancer compounds in controlled conditions and the possibility of using biotechnological strategies and genetic tools to increase

their production. Moreover, the application of plant cell and tissue culture techniques allows the rapid mass multiplication and the recovery of true-to-type plants within a short span of time which is particularly important in the case of endangered species as is the case of several medicinal plants with anticancer activity. In many cases cell suspension cultures are the preferable biotechnological way of producing valuable secondary metabolites. However, secondary metabolite production is under strict metabolic regulation and tissue-specific localization, and, therefore, the use of differentiated tissues like roots, shoots, or embryos instead of cell cultures is required in some cases. Hairy root cultures have a high level of cellular differentiation and great genetic or biochemical stability and, thus, have proven to be a good option for *in vitro* production of secondary metabolites, including some anticancer compounds (e.g., indole alkaloids).

Several research groups around the world have focused on studies aiming on the optimization of biomass growth conditions and on the application of biotechnological strategies to increase production yields. Studies are mainly focused on the production of taxanes from *Taxus* sp., terpenoid indole alkaloids from *C. roseus*, camptothecin from *C. acuminata* among other species, and podophyllotoxin from *Podophyllum* and *Linum* species. It has been possible to enhance the production yields through the manipulation of empirical factors involved in plant cell and organ culture. Several factors including nutrients, carbon source, plant growth regulator levels, or culture environmental conditions can be optimized. Moreover, production yields can be considerably improved with the application of several biotic and abiotic elicitors. Also, several studies have been focused on the elucidation and regulation of biosynthetic pathways. The use of elicitors to activate genes involved in metabolic pathways is one of the most used strategies to increase the biotechnological production of anticancer compounds. It is now an accepted fact that the plant cell and organ cultures provide a biosustainable alternative platform for the production of valuable anticancer compounds. In spite of all the advantages of this technique and the significant advancements in the last years, there are only few examples of the production of plant anticancer compounds on an industrial level. More studies on the elucidation and regulation of biosynthetic pathways are required allowing the further generation of transgenic cell lines for production of vital compounds. Also synthetic biology opens new perspectives of biotechnological production of vital compounds based on the transfer of biosynthetic pathways to microorganisms.

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An Alternative Approach for Anticancer Compounds Production Through Plant Tissue Culture Techniques

22

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Abstract

Higher plants produce various anticancer secondary metabolites (colchicine, camptothecin, combretastatin, paclitaxel, plumbagin, podophyllotoxin, psoralen, vincristine, vinblastine, etc.). The indiscriminate harvesting of these plants from the wild for the metabolites and inadequate efforts for cultivation led to a decrease in natural populations. However, these metabolites/compounds exist in low quantities, and it is economically not feasible to obtain them in large scale. Moreover, the accumulation of these metabolites varies from its geographical and environmental conditions. Alternatively, economically feasible production strategies should be investigated in order to overcome these problems and to overproduce the metabolites of therapeutic importance. To this perspective, advances in plant cell and tissue cultures, mainly culturing of cells/tissues, suspension cultures, precursor feeding, hairy root cultures, and bioreactors using cell suspensions/hairy root cultures are evaluated as the feasible and cost-effective alternative means for the production of economically important compounds. The present chapter summarizes the latest techniques/strategies for the overproduction of anticancer metabolites using plant cell and tissue culture approaches.

Keywords

Anticancer compounds · Bioreactors · Cambial meristematic cells · Hairy root cultures · Plant tissue cultures

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22.1 Introduction

Cancer is one of the leading causes of death in the world. It has been reported by the World Health Organization (WHO) that about 8.8 million people died worldwide in 2015 due to cancer, nearly 1 in 6 of all the deaths globally. In 2016, unfortunately 595,690 people died due to cancer out of 1,685,210 new cases of cancer diagnosed. Currently, there are many types of treatments available for cancer. However, a patient receives the treatment based on the cancer type and how advanced it is. The available treatments include chemotherapy, radiation therapy, immunotherapy, surgery, etc. However, these treatments are not free from side effects. For example, radiation therapy affects healthy cells also along with cancer cells, while plant-based drugs are successfully employed for the treatment of cancer with lesser side effects (Somasundaram et al. 2010). In 1960s vinblastine and vincristine are the two natural anticancer compounds introduced into the market to treat Hodgkin's lymphoma and acute childhood leukemia, respectively. Likewise another plant-based anticancer compound paclitaxel is used to treat breast cancer and ovarian cancer, while camptothecin is used to treat gastric, rectal, colon, and bladder cancers. Another important natural compound, podophyllotoxin confers a great efficacy against lymphosarcoma and Hodgkin's disease (Nissen et al. 1972). Discovery and introduction of these compounds to the market supported further drug discovery programs utilizing natural resources or products. However, the cost of these compounds in the market appears to be very high. For example, vincristine 1 kg costs around 20,000 US$, and annual world market cost for this compound is about 5 million US$. Similarly, the demand and cost of camptothecin and podophyllotoxin (etoposide, a semisynthetic derivative of podophyllotoxin) seem to be in the range of vincristine (Khani et al. 2012; Atanas et al. 2015; Shiv et al. 2016).

From the past decades, mankind depends on plants as a source of pharmaceuticals to treat various cancers. Plant-based drugs (natural products) play a significant role in the treatment of cancer. However, these compounds are produced in plants at very minute quantities, and hence, there is no adequate supply of these compounds owing to increased number of cancer incidences in recent years. Thus, there is a high demand for these plant-based anticancer compounds globally. Biotechnological approaches using plant cell cultures are the attractive and alternative means for the product enhancement of plant-derived anticancer compounds (Ramachandra Rao and Ravishankar 2002). These approaches offer a defined production system, which ensures the production of novel compounds and stable supply of products without any alteration in the quality and yield. To overcome this supply crisis, many scientists have adopted various plant biotechnology protocols/techniques for the enhanced production of anticancer compounds (Table 22.1). In fact, there have been many plant biotechnology techniques used for mass production of anticancer compounds (Fig. 22.1). Presently, the demand for plant-derived anticancer compounds is steadily increasing, but their production in parent plants in the wild is at below the required level (Fig. 22.2). Therefore, plant cell and tissue cultures will be a

Table 22.1 Summary of various techniques used to enhance the production of anticancer compounds

Plant name	Anticancer compound	Culture type	Yield	References
Taxus cuspidata	Taxol	Cell suspension cultures	12.2 mg/l	Mirjalili and Linden (1995)
T. baccata	Paclitaxel	Bioreactor cultures	1.5 mg/l	Srinivasan et al. (1995)
T. media	Paclitaxel; baccatin III	5 l Stirred reactor	21.1 mg/l	Cusido et al. (2002)
			56.0 mg/l	
Camptotheca acuminata	Camptothecin	Hairy root culture	1 mg/g DW	Lorence et al. (2004)
Taxus media	Paclitaxel	Cell suspension cultures	1.25 mg/g DW	Baebler et al. (2005)
T. baccata	Paclitaxel	Airlift bioreactor (4 l);	12.03–20.79 mg/l;	Bentebibel et al. (2005)
	Baccatin III	Wave bioreactor (2 l);		
		Stirred bioreactor (5 l)	7.78 mg/l;	
			5.06 mg/l	
T. baccata	Taxol	Precursor feeding	13.75 mg/l	Khosroushahi et al. (2006)
P. peltatum	Podophyllotoxin	Adventitious roots	0.588 mg/g DW	Anbazhagan et al. (2008)
O. prostrata	Camptothecin	Adventitious root cultures	0.19%	Martin et al. (2008)
Liunm mucronatum	Podophyllotoxin	Hairy roots	5.78 mg/g DW	Samadi et al. (2014)
O. mungos	Camptothecin	*In vitro* regeneration	0.0768%	Namdeo et al. (2012)
Psoralea corylifolia	Psoralen	Precursor feeding	2.5 mg/g FW	Mohammad Parast et al. (2014)
Plumbago rosea	Plumbagin	Embryogenic cell suspension cultures	1 mg/g DW	Silja et al. (2014)
Plumbago rosea	Plumbagin	Adventitious root cultures	1.23%	Silja and Satheeshkumar (2015)
Catharanthus roseus	Vinblastine and vincristine	Callus	0.5623 and 0.1651 μg/g DW	Iskandar and Iriawati (2016)
Ophiorrhiza mungos	Camptothecin	Cell suspension cultures	0.8 mg/g DW	Deepthi and Satheeshkumar (2016)

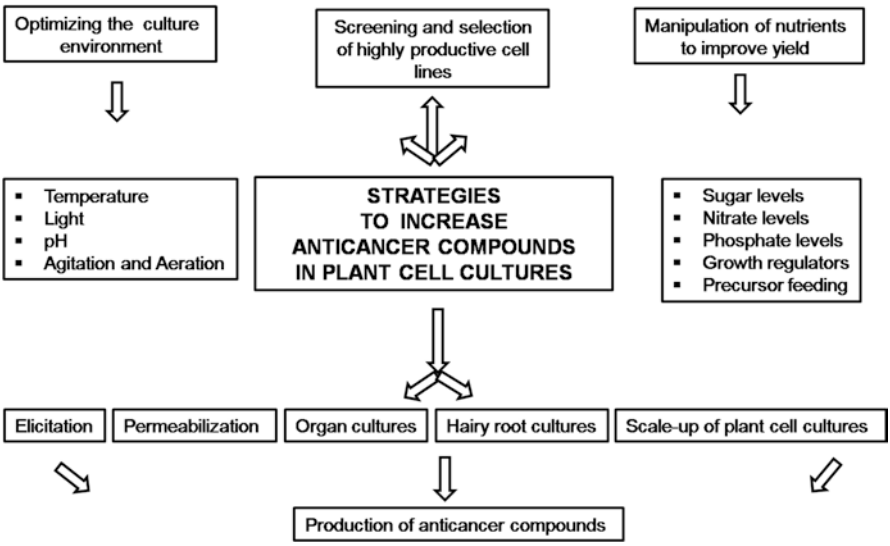

Fig. 22.1 Strategies to increase anticancer compounds through plant cell cultures

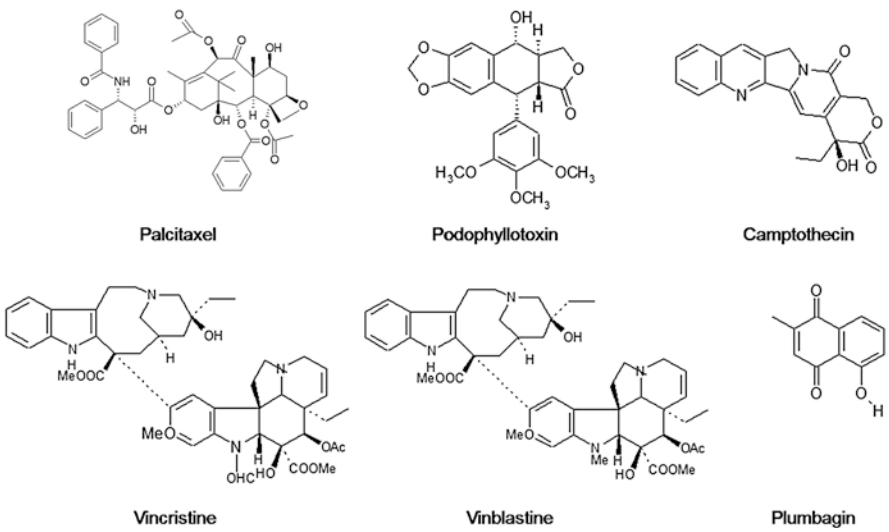

Fig. 22.2 Some important plant-derived anticancer compound structures

promising, cost-effective, and viable alternative for the increased production of anticancer compounds in a commercial level. The present chapter summarizes the latest techniques/strategies for the overproduction of anticancer metabolites using plant cell and tissue culture approaches.

22.2 Approaches to Overproduce Anticancer Compounds Production Using Plant Cell Culture

22.2.1 Highly Productive Cell Lines Selection and Screening

Plant cells *in vitro* culture systems exhibit genetic variation and heterogeneity in which the physiological and expression profiles of the respective natural products can be different. Hence, highly productive cell line selection for the establishment of stable and prolific plant metabolites can be imperative. Detection of highly productive cell lines is generally done through visual selection, mutant selection, and clonal selection (Remotti et al. 1997; Shiba and Mii 2005; Liang et al. 2006). These are the techniques for the detection of highly productive cell lines. The accumulation patterns of natural products in the similar plant can be genotype specific, and hence, the selection of a high-yielding clone appears to be critical. Berlin and Sasse (1985) had surveyed various techniques utilized for describing the variants recovered from cell and organ cultures. There would be a possibility of decreased or complete loss of the secondary metabolite-producing ability during repeated subcultures. This might appear because of the genetic instability associated with the somaclonal variation. Deepthi and Satheeshkumar (2016) had isolated high-yielding cells through an aggregate cloning method in cell cultures of *Ophiorrhiza mungos* L. for the enhancement of camptothecin. Formation of a new cell wall further substantiates the consideration of the produced clones as true colonies (Dougall 1987).

22.2.2 Nutrient Manipulation to Increase Yield of Anticancer Compounds

For the production enhament of secondary compounds in cell and tissue cultures, many approaches are in use, including manipulating the composition of media (Karwasara and Dixit 2011), augmentation of plant growth regulators (Karwasara et al. 2011), and slight changes in the concentrations of carbon source, nitrogen source, nitrate-to-ammonia ratio, phosphate, and micronutrients. These manipulations bring out tremendous results with respect to cell growth and accumulation of desired phytochemical content. Further, developing favorable culture conditions is of prime importance for the growth and biosynthetic efficiency of the *in vitro* cultured cells. Basic knowledge about a particular secondary metabolite biosynthesis and regulation that influence the productivity of cultured cells is required to standardize culture conditions for optimizing the production. This involves standardization of plant growth regulator(s) concentration and composition and also composition of culture medium.

22.2.2.1 Carbon Source

Sugars such as glucose, fructose, and maltose are tried in plant tissue culture media to induce cell growth as well as secondary metabolite production. Among these, sucrose is considered as the efficient source of carbon for inducing cell

proliferation, as the disaccharide sucrose is a balanced carbon source. Sucrose supplemented in the medium not only supports for cell proliferation and growth but also influences the biosynthesis and accumulation of plant metabolites in culture (Wang and Weathers 2007). For the production of camptothecin in suspension culture of *Nothapodytes nimmoniana*, 5% (w/v) sucrose was found to be optimal at the tested concentration range of 2–7% (w/v) (Karwasara and Dixit 2013). Similarly, Rajesh et al. (2014) evaluated the effect of different carbon sources on podophyllotoxin production in adventitious root culture of *Podophyllum hexandrum.* Among the carbon sources tested, 6% sucrose resulted in a maximum accumulation of podophyllotoxin (4.8 mg/g DW). Supplementation of sucrose 20 g/l to cultures at a later stage improves the taxane production along with cell growth and concluded that an addition of 30 g/l sucrose at the initial stage of culture improved the cell growth and even the cell growth reached to stationary phase and significantly increased the taxane production (Wang et al. 1999). Addition of 30 g/l sucrose at day 20, 36 mg/l taxol is produced in a two-stage culture of *T. chinensis* (Wang et al. 2001).

22.2.2.2 Nitrogen Source and Nitrate-to-Ammonia Ratio

Nitrogen has a greater influence on the metabolism of the plant cell and greatly contributes for the growth and development of a plant (Chen et al. 2003). It is useful in the biosynthesis of nitrogen-containing substances such as amino acids, proteins, nucleic acids, and other nonprotein nitrogen metabolites. There are different types of media that contain nitrogen source in the form of nitrate and ammonium. The ratio of nitrate to ammonia has a significant influence on the cell proliferation, growth and development, biosynthesis, and product accumulation of metabolites. High nitrate (NO_3) and low ammonium (NH_4) supplemented in the medium at a ratio of 10:20 resulted in maximum podophyllotoxin (5.8 mg/g DW) accumulation in adventitious root culture of *P. hexandrum* and induced maximum adventitious roots (24.3/explant) and biomass accumulation (5.1 g FW) after 2 months of culture (Rajesh et al. 2014). Whereas, in cell suspension cultures of *N. nimmoniana*, increasing the concentration of nitrate and ammonium (NO_3^-/$NH4^+$) at a ratio of 1:5 (with 60 mM total nitrogen) promoted camptothecin production (48.7 μg/g DW), while increased nitrate favored cell growth only and increased ammonium favored camptothecin biosynthesis (Karwasara and Dixit 2013). In the root cultures of *O. mungos* Linn., it was reported that low NO_3^-/$NH4^+$ ratio (10:50 mM) is required for maximum camptothecin production, while for biomass production high ratio of $NO3^-$/$NH4^+$(40:20 mM) is required (Deepthi and Satheeshkumar 2017). This shows that nitrate-to-ammonia ratio in the medium plays an important role in metabolite accumulation and cell growth. So in order to attain product enhancement of anticancer compounds in plant cell and tissue cultures, standardization of nitrate-to-ammonia ratio is one of the prerequisites.

22.2.2.3 Phosphate

Phosphate is one of essential inorganic nutrients in plant cell culture medium for the growth and production of metabolites. For example, lower phosphate concentration

in the medium not only decreased the cell growth but also camptothecin accumulation (Deepthi and Satheeshkumar 2017). A similar effect of low phosphate in the medium for adventitious root culture of *Podophyllum hexandrum* was also observed for the production of podophyllotoxin (Rajesh et al. 2014). Phosphate (KH_2PO_4) supplemented at 1.25 mM concentration induced 27.1 adventitious roots per explant with biomass 5.7 g FW and 1.42 g DW. Whereas, increased phosphate concentration (0.5 mM) impeded the biomass accumulation and camptothecin production in *N. nimmoniana* cell cultures (Karwasara and Dixit 2013).

22.2.2.4 Plant Growth Regulators

Providing stress to the culture media in the form of plant growth regulator concentration and combination shows positive influence on the accumulation of plant metabolites. Generally plant growth regulators influence the callus formation, modulation of callus initiation, dedifferentiation of cells, cell division, and secondary metabolite production. It has been reported that one of the plant growth regulators 2,4-D supplemented in the medium reduces the secondary metabolite content; hence it is supplemented at very low concentrations for secondary metabolite production. However replacing 2,4-D with NAA showed better results on secondary metabolite accumulation. For example, cultures of *Nothapodytes foetida* in presence of 2,4-D resulted in low levels of camptothecin accumulation, while in the presence of NAA increased the amounts of camptothecin and its accumulation (Fulzele et al. 2001). NAA at higher concentrations in *O. rugosa* var. decumbens cultures increased the camptothecin production, while the combination of NAA with 6-BAP induced more camptothecin production rather than when they have supplemented alone (Vineesh et al. 2007). Increasing the BAP concentration to NAA inhibited the camptothecin production in *N. nimmoniana* callus cultures. However other auxin IBA had shown the positive influence on camptothecin accumulation at higher concentrations (Isah and Mujib 2015). The superiority of NAA over 2,4-D on plumbagin production was also reported in *Plumbago indica* hairy roots (Gangopadhyay et al. 2011). In contrast to this, plumbagin production was affected in the presence of NAA in *Drosophyllum lusitanicum* (Nahalka et al. 1996). The negative influence of NAA on camptothecin production in *Catharanthus roseus* cell cultures was also reported by Whitmer et al. (1998). High cytokinin (BAP) presence in the medium decreased the plumbagin content, while at lower concentrations the same cytokinin (BAP) showed better results for the plumbagin production. Abscisic acid supplemented in the medium induced about five times more paclitaxel production in cell suspension cultures of *T. chinensis* compared to control cultures with no abscisic acid (Luo et al. 2001). Plant growth regulators show significant effect on plant metabolite production in a concentration-dependent manner. Sometimes higher concentrations of NAA gives better results for secondary metabolite formation in comparison to 2,4-D. In conclusion, for the production/enhancement of plant metabolites (anticancer compounds), plant growth regulator(s) concentration and combination is one of the critical factors that plays a major role in cell cultures. So, the ideal concentration and combination of plant growth

regulators has to be standardized in order to increase the production of anticancer compounds in plant cell and tissue cultures.

22.2.2.5 Precursor Feeding

Another important approach for the product enhancement of anticancer metabolites is the addition of intermediate metabolites (precursors) of the respective biosynthetic pathway to the cell cultures. A precursor is generally an intermediate that is enzymatically converted into another compound or desired metabolite. Therefore the addition of these intermediates (precursors) to the cell cultures might result in increasing of the desired metabolite. Exogenous supplementation of precursors to the medium induces a subset of genes associated with secondary metabolite biosynthesis, modulates their expression, and promotes the accumulation of desired metabolites. Addition of phenylalanine to the medium resulted in a 5.6-fold increase in taxol (13.75 mg/l) production compared to the respective control cultures (Khosroushahi et al. 2006). Similarly, in *Taxus baccata* cell cultures, taxol content was increased from 5.2 to 13.1 μg/g (DW) with the addition of 1 mM phenylalanine (Cusido et al. 1999). Similarly addition of phenylalanine in cell cultures of *Linum flavum* resulted in fivefold increase in 6-methoxypodophyllotoxin (Uden van et al. 1990). Coniferyl alcohol supplemented in combination with cyclodextrin resulted in fourfold increase in podophyllotoxin in *P. hexandrum* cell cultures (Woerdenbag et al. 1990). With the addition of 2.5 mg/l cinnamic acid, psoralen content was increased from 1.93 to 2.50 mg/g FW in callus cultures of *Psoralea corylifolia* (Mohammad Parast et al. 2014).

22.2.3 Optimizing the Culture Environment

It is known that basal medium composition, pH, temperature, and light have a major influence on cell growth and secondary metabolite accumulation in plant cell tissue cultures. Therefore, optimizing the culture environment will certainly enhance the biomass and metabolite accumulation in plant cell cultures.

22.2.3.1 pH

One of the crucial factors for anticancer compounds production is pH of the culture medium. Changes/manipulation of pH determines the release of intracellular alkaloids into the culture medium (Asada and Shuler 1989). Alkaloids are stored in the vacuole when synthesized in cell suspension cultures. It has been showed that culture medium pH and intravacuolar pH determine the storage capacity of alkaloids (Neumann et al. 1983). In general cell culture medium pH is adjusted in between 5 and 6 units; however, during sterilization by autoclaving medium, pH drops by 0.6–1.3 units. For the production of podophyllotoxin and cell growth in *P. hexandrum* cell culture, the optimal pH is 6.0 (Chattopadhyay et al. 2002b). Similarly, Rajesh et al. (2014) also reported that pH in the range of 5–6 favored the production of podophyllotoxin and biomass accumulation in adventitious root culture of *P. hexandrum*.

22.2.3.2 Temperature

Temperature is another important factor that influences the production of plant metabolites. Generally plant cell cultures are maintained at a temperature of 25 ± 2 °C. However, increasing the temperature to 29 °C favored the paclitaxel production (Choi et al. 2000). Interestingly temperature maintained at 24 °C favored the biomass accumulation in suspension culture of *Tsuga chinensis* but at 29 °C inhibited cell growth. Therefore, to reduce the negative influence of temperatures on cell growth initially, the temperature was maintained at 24 °C, and later cultures were shifted to 29 °C. Maintaining these temperatures, paclitaxel production was optimized for a maximum yield of 137.5 mg/l and an average production of 3.27 mg/l over a period of 42 days.

22.2.3.3 Light

Light intensity and regime is another important factor that influences the production of biomass and metabolites in culture. In *T. cuspidate* cell cultures, white light strongly inhibited the production of taxol and baccatin III. While in dark-grown callus and suspension cultures, three times higher yield of these metabolites was observed compared to cultures maintained in the presence of light (Fett-Neto et al. 1995). Similarly, podophyllotoxin accumulation favored dark conditions in cultures of *P. hexandrum* and *P. peltatum* (Kadkade 1982; Chattopadhyay et al. 2002a; Anbazhagan et al. 2008). Likewise, camptothecin concentrations in *Camptotheca acuminata* leaves significantly increased under blue light and 50% shading treatments (Liu et al. 2015; Hu et al. 2016).

22.2.3.4 Aeration and Agitation

Aeration and agitation of cultures appear to play a crucial role in the production of anticancer compounds. Increasing either aeration rate or negligible gas transfer has a negative influence on growth and anticancer compounds production. So, it is essential to evaluate the adequate amount of oxygen requirement of the cell cultures (Zhong et al. 1992). Gases such as oxygen (O_2), carbon dioxide (CO_2), and ethylene are also important in plant cell cultures (Lee et al. 2010). Respiration and metabolism are carried out by O_2; the main metabolic component in cell growth is CO_2, while ethylene is believed to be a gas component produced by the plant in response to environmental stress (Khani et al. 2012). The importance of nutrients, inoculum density, and oxygen supplementation for the production of paclitaxel in *Linum album* cell cultures has been suggested by Garden (2004). *Linum album* cell cultures maintained in a bioreactor (stirred tank with a capacity of 5 l) by optimizing aeration conditions, 183.6 mg/l podophyllotoxin yield was achieved (Baldi et al. 2008). Likewise, Luo et al. (2001) reported that 40–60% dissolved oxygen allowed a 7.2 mg/l paclitaxel production in a bioreactor with a six-flat-bladed turbine in cell suspension cultures of *T. chinensis*. Increased CO_2 (10%) levels inhibited the production of paclitaxel while at low headspace oxygen concentration promoted the paclitaxel in *T. cuspidate* cell cultures (Mirjalili and Linden 1995). An optimized combination of these three gases for the production of paclitaxel is 5 ppm ethylene, 10% (v/v) O_2, and 0.5% (v/v) CO_2 as evaluated

and suggested by Mirjalili and Linden (1995). Camptothecin production was achieved in cultures of *O. mungos* Linn. maintained at 90 rpm continuous agitation (Deepthi and Satheeshkumar 2016).

22.2.4 Elicitation

In nature, plants create a vast defense system by producing secondary metabolites against various environmental stresses (pathogen attack). Similarly, in *in vitro* conditions, plant cells and tissues respond to trigger the synthesis of secondary metabolites when challenged with similar elicitors from pathogens or of synthetic origin (Giri and Zaheer 2016). Elicitors are the signaling molecules; depending on their molecular origin, they may be either biotic or abiotic and physical or chemical factors (Angelova et al. 2006). At low concentrations, elicitors evoke the production of enhanced levels of commercially important metabolites. Elicitation of important natural products through cell cultures leads to not only marking down the process time but also enhance the desired product yield. From past decades, many authors have reported the effect of various elicitors such as oligosaccharides, biotic or abiotic, glycoproteins, yeast, and plant signaling molecules on plant metabolite production. For example, 13.3-fold increase in camptothecin was observed in *O. mungos* Linn. cell cultures elicited with yeast when compared to the control cultures (Deepthi and Satheeshkumar 2016). Likewise the treatment of *C. acuminata* cell suspension cultures with sorbitol resulted in increased production of camptothecin (5 mg/l) (Yang et al. 2017). About 1.130 mg/g DW plumbagin was produced in *Plumbago rosea* L. cultures after challenging with NAA (Silja et al. 2014) and in adventitious root cultures established from leaf explants of *P. rosea* (Silja and Satheeshkumar 2015). Taxol is a plant diterpenoid, a well-known anticancer compound as it is a unique poison of microtubular cell system (Woo et al. 1994). To avoid its supply crisis, many people have focused toward elicitation strategies for the synthesis of taxol and taxol-like derivatives. Methyl jasmonate, a plant stress signaling molecule synthesized through octadecanoid pathway, tangled in the production of anticancer plant metabolites through plant cell and tissue cultures. Suspension cultures of *T. chinensis* var. mairei elicited with 100 μM methyl jasmonate accumulated more amounts of taxol (Wang et al. 2004). Similarly, paclitaxel was overproduced from 0.4 to 48 mg/l with 100 μM methyl jasmonate (Yukimune et al. 2000). Taxol production through methyl jasmonate is well known, but the mechanism of how methyl jasmonate is involved in the synthesis of taxol is not clearly understood. Even though different elicitors induce different types of anticancer compounds with different amounts, one has to consider the concentration of a suitable elicitor, time of addition and interaction of the elicitor in the cultures, and age of the cultures (Namdeo 2007). These parameters play a significant role in the production of anticancer compounds in plant cell cultures using elicitors.

22.2.5 Permeabilization

The metabolites produced by the cell culture systems are stored in the vacuoles of the cells. Therefore for the ease of recovery and purification, it is essential to release the produced metabolite into the culture medium (Cai et al. 2012). This product removal further facilitates the continuous production of the metabolite by relieving the feedback inhibition, and thus improvement in the consistent production can be expected. Till date there have been numerous attempts for the permeabilization of plant cells in cultures for the release of metabolites (Trejo-Tapia et al. 2007; Siu and Wu 2014). To achieve the desired results there are different strategies followed, like change of culture medium pH, transferring culture to medium that contains no phosphate, electroporation and using the permeabilizing agents like chitosan and dimethyl sulfoxide that affect cell membrane permeability. Using these strategies the metabolites produced by the cells in cultures are made to get released into the culture medium. Moreover, definite concentration and time period of addition of permeabilizing agents to the media are the few factors to be considered so as to avoid the inhibition of cell growth. Utilization of 10% dibutyl phthalate as a permeabilizing agent was found superior for the release of taxol into the medium compared to hexadecane and decanol. While combining the selective permeabilizing agent with sucrose, feeding selectively increased the taxol production to sixfold higher (Wang et al. 2001).

22.2.6 In Situ Adsorption and Two-Phase Culture System

In general, a decrease in product formation is observed in the intact cell cultures. Further, storage of the produced metabolite at internal sites such as vacuoles also makes the situation very critical for the extraction and becomes economically not feasible. In addition, the following factors such as higher volatile nature of the metabolites produced, feedback inhibition, and product degradation because of lower stability also limit the maximum accumulation of plant metabolites in cell cultures (Berger 1995). A concomitant strategy is adopted for the product removal continuously either through solvent extraction or using an adsorbent makes it feasible for harvesting or isolating the desired metabolites from the culture containers. This strategy not only decreases the intrusion between growth of the cell and product accumulation but also reduces the product degradation from the producing cell lines due to the release of enzymes, and environmental conditions, and finally minimizes the downstream processing events (Freeman et al. 1993; Malik et al. 2013). Harmonious effect of in situ product removal with other general methods of enhanced metabolite production such as immobilization, elicitation, precursor feeding, and permeabilization can further potentiate the product enhancement of desired metabolites through cell and tissue cultures of plants.

Through in situ adsorption using styrene-divinylbenzene resin (Diaion® HP-20), plumbagin production has been enhanced by precursor feeding in root cultures and obtained 1.4- and 1.6-fold, respectively, higher than L-alanine alone-fed cultures or

untreated control root cultures (Jaisi and Panichayupakaranant 2017). Taxol production in *T. cuspidata* can be enhanced from 40 to 70% by utilizing a nonionic adsorbent, Amberlite® XAD-4 (Kwon et al. 1998). Likewise, Pavao et al. (1996) had established two-phase culture systems in *T. brevifolia* for partitioning taxol released in the second phase. Tricaprylin, an eight-carbon triglyceride, partitioned the secreted taxol into the culture medium.

22.3 Organ Cultures

Product enhancement through differentiated cultures has an advantage over undifferentiated cells. It has been said that during plant development presence of some of the desired metabolites is confined to a particular tissue or organ. Production of anticancer compounds through *in vitro* regenerated plants has tremendous importance because of high production in differentiated tissues and more stability in organ cultures (Roja 1994). A study by Sankar-Thomas and Lieberei (2011) suggested the low profile of camptothecin levels in undifferentiated calli compared to differentiated tissues of *C. acuminata* organ cultures. Thus biosynthesis of camptothecin requires tissues or organs of differentiated stages. The study supported the use and advantages of organ cultures over undifferentiated cultures. Similarly, higher levels of camptothecin (66–111%) accumulated in the regenerated *O. pumila* plants as compared with that in the wild plant. Likewise, 15 μg/g tissue dry weight vinblastine was produced in multiple shoots of *C. roseus* (Miura et al. 1988). Two-month-old plantlets produced 0.11%–0.36% taxol in *T. canadensis* and 0.01%–0.1% in *T. baccata* (Zhiri et al. 1994), while *T. baccata* shoot tip cultures produced taxol from 0.03 to 0.46 mg/kg (Jaziri et al. 1990). In addition to shoot cultures, root cultures are also promising to produce plant metabolites. The highest amount of camptothecin was obtained from the roots of early-stage seedlings of *C. acuminata* Decaisne (Nyssaceae) and also reported the decrease in camptothecin content in aged seedlings (Valletta et al. 2007). Similarly, Kaushik et al. (2015) evaluated the camptothecin yield in micropropagated plants of *O. mungos* and found that tissue culture-raised clones had 0.0438 ± 0.18% of camptothecin content, while the mother plant contained 0.043 ± 0.16%. This showed a similar chemical profile between wild-grown and micropropagated plants. More recently, maximum camptothecin content (0.12% w/w) was found to occur in the root samples of *in vitro* regenerated *N. nimmoniana*. Further, it has been reported that camptothecin content varied in plant parts (leaves, stems, and roots). The amount of camptothecin in leaves and stem was reported to be 0.0013% w/w and 0.026% w/w, respectively (Prakash et al. 2016). Moreover, the compounds produced through root cultures are very difficult to harvest and show slower growth. Therefore, an alternative method is required to produce root-derived compounds, and as of now, hairy root cultures are the best system to produce root-derived compounds which are described in the following sections.

22.4 Adventitious Root Cultures

Adventitious root system is an alternative to the conventional tissue culture methods for studying the biochemical events occurring during the secondary metabolite production and also to understand the effect of various factors on the normal metabolic pathways of the culture system. They can be produced under natural or various stress conditions and also induced by mechanical damage or tissue regeneration. Hence, it can be considered as more natural for not containing any chimeric DNA. Adventitious roots can be produced using any plant part (node, stem, leaf, non-pericycle tissues, or any other organ) as an explant. Adventitious root cultures have more genetic stability than any other culture systems (Casson and Lindsey 2003). They can also be used as a source of food storage organs and site of synthesis and accumulation of produced natural products. Adventitious roots exhibit higher multiplication and biomass accumulation and show an elevated secondary metabolite biosynthesis (Murthy et al. 2014). Product enhancement of desired metabolites depends on changes in the culture conditions by using different elicitors and growth promoters, precursors. etc. The metabolites thus produced by adventitious cultures can be cost-effective and used as antioxidants, food ingredients, pharmaceuticals, and therapeutic agents. An efficient culture system for the product enhancement in *Andrographis paniculata* adventitious roots has been evaluated for the andrographolide production (Praveen et al. 2009). Compared to callus suspension cultures, adventitious root cultures showed an increase in andrographolide amount. A cytotoxic quinoline alkaloid, camptothecin, and its derivatives were used for the treatment of cancer. The anticancer property of camptothecin is exhibited by its reversible binding to the topoisomerase-1 enzyme and inhibits the DNA replication by stabilizing the enzyme/DNA complex (Deepthi and Satheeshkumar 2016). Martin et al. (2008) successfully established the adventitious root culture system which has been evaluated as a promising approach for the production of camptothecin in *O. prostrata*. In root cultures of *O. mungos*, 3.4-fold higher amount of camptothecin has been produced by changing the medium composition and plant growth regulators (Deepthi and Satheeshkumar 2016). Differentiated cultures like hairy roots and adventitious roots of *O. pumila* produce higher amounts of camptothecin (Saito et al. 2001).

22.5 Hairy Root Cultures

Camptothecin is a monoterpenoid indole alkaloid produced in the plants which belong to *Rubiaceae*, *Apocynaceae*, *Nyssaceae*, and *Loganiaceae* (Cui et al. 2015). In a study reported by Ochoa-Villarreal et al. (2016), hairy root cultures were established infecting plant explants with *A. rhizogenes*, a Gram-negative soil bacterium that transfers its plasmid DNA that carries the genes which encode enzymes responsible for auxin biosynthesis which induce hairy roots. Thus accumulated auxin at the site of *A. rhizogenes* infection influences the surrounding cells mitotically to produce hairy roots. The differentiated hairy roots exhibit high growth rate,

independent of growth hormones, high genetic stability, lateral branching, and lack of geotropism. These attributes make the hairy roots to accumulate the desired anticancer plant metabolites.

An efficient hairy root culture was established for the enhancement of camptothecin in *O. pumila* (Saito et al. 2001). Significant improvements in the camptothecin production were achieved by releasing into the adsorption medium using Diaion HP-20 (a polystyrene resin). Nearly a twofold improvement in plumbagin was observed with combined elicitation of methyl jasmonate and chitosan in *Plumbago indica* hairy root cultures (Gangopadhyay et al. 2011). Likewise there are *Ophiorrhiza pumila*, *N. foetida*, *C. acuminata*, *O. liukiuensis*, and *O. kuroiwai* (Lorence et al. 2004) are some of the few potential plant species which can be explored with genetic transformation to improve camptothecin production in the future. From these observations, it could be speculated that hairy root culture could be a sustainable strategy for the product enhancement of anticancer compounds and also useful for the understanding and elucidation of metabolic pathway and factors responsible for the metabolite synthesis.

22.6 Immobilizations

Slow growth rate and limited metabolite production can further offer the utilization of an alternative system for the product enhancement of anticancer compounds *in vitro*. In this context, immobilized plant cell and tissue cultures offer the continuous production of the desired metabolites. Immobilization is advantageous in the following contexts: (a) improved viability of cells in stationary phase, facilitating ease of maintenance and recovery, (b) simplified downstream processing (in case of secretary products), (c) cost-effective and lesser chance of contamination obtained through utilization of high cell density in small-scale bioreactors, (d) low shear stress for shaking cultures, (e) increased product accumulation, (f) improves flow rates by utilizing flow through reactors, and (g) knocks down the problems associated with mixing and aeration such as fluid viscosity in suspension cultures (Dicosmo and Misawa 1995). Matrices like agar, agarose, carrageen, polyurethane foam, alginate, and vanadium sulfate are used for the immobilization of plant cells and tissues and release of produced metabolites (anticancer compounds) into the medium (van der Heijden et al. 1989). It has been suggested that the adsorption method of immobilization is superior over cell entrapment (Dicosmo and Misawa 1995). The immobilization process ensures the sustenance of cells for a longer period of time and reduces the cost incurred on production of desired anticancer compounds (Moreno et al. 1995).

The surface immobilization of cultured cells of *Catharanthus roseus*, *Nicotiana tabacum*, and *Glycine max* has been achieved for the production of useful metabolites. Komaraiah et al. (2003) produced the plumbagin 21 times higher than control cultures by using immobilization followed by elicitation with chitosan. Increased production of baccatin and paclitaxel was reported changing alginate concentration (Bentebibel et al. 2005). Taxane production was improved with 2% calcium alginate

in shake flasks and in bioreactor cultures (Bentebibel et al. 2005). Release of taxol and baccatin III from the plant cells into the culture medium was enhanced by 2.5- and 3.6-fold, respectively, using vanadium sulfate (Cusido et al. 1999).

22.7 Bioreactor and Scale-Up Process

Bioreactors are devices or systems that are used to carry out biological processes to produce the desired products. Sudo et al. (2002) have produced camptothecin in cultures of *O. pumila* in a 3 l bioreactor. A total yield of 22 mg was obtained after 8 weeks of culture period. An approximate amount of total camptothecin released into the culture medium was found to be 17% (3.6 mg). Additionally, for the easy purification, the excreted camptothecin could be adsorbed with polystyrene resin Diaion HP-20. Taxol production by cell suspensions of *T. baccata* in bioreactors was reported by Srinivasan et al. (1995). Paclitaxel and baccatin III were enhanced in different types of bioreactors by combining with an immobilization event (Bentebibel et al. 2005). Among them, stirred bioreactor potentially induced 43.43 mg/l paclitaxel at 16 days of culture period which is equal to 2.71 mg/l per day. Likewise, Navia-Osorio et al. (2002) established an efficient culture system for the production of taxol and baccatin III in *T. baccata* var. fastigiata and *T. wallichiana* suspension cultures using 20 l airlift bioreactor. When both the cell line at their maximum growth stage of accumulating the metabolites (28 days) *T. wallichiana* (21.04 mg/l) was superior to the *T. baccata* (12.04 mg/l) in the accumulation patterns of taxol and baccatin III. Further, they observed the release of taxol (40%) and baccatin III (67%) into the release of taxol and baccatin III into the growth medium. A commercial producer, rootec GmbH, Seuzach, Switzerland, has established an efficient bioreactor for the production of camptothecin in hairy roots production at large scale (Wildi et al. 2003). In this system, culture medium is sprayed continuously to grow roots on racks, and then continuous and semi-continuous modes are used to harvest the camptothecin.

22.8 Cambial Meristematic Cells as a Source of Anticancer Compound Production

Cambial meristematic cells provide an attractive alternative to traditional dedifferentiated cells for the synthesis of anticancer compounds. For example, elicitation of *T. cuspidata* cambial meristematic cells (CMCs) with methyl jasmonate induced paclitaxel production (Lee et al. 2010). Contrarily, the production of paclitaxel in *T. cuspidata* dedifferentiated cell lines (DDCs) derived from either needle or embryos was recorded to be reduced; aggregation of plant cells in cell suspension cultures was confirmed as the negative sign for the product yield (Joshi et al. 1996). Pronounced cell aggregates are typically found in DDCs in this context, and the reduced aggregation size of CMCs is observed between two and three cells per cluster. Using a 3 l airlift bioreactor, *T. cuspidata* CMCs produced 98 mg/kg of fresh

cell weight (FCW) after 10 days of elicitation with MeJA, whereas needle- or embryo-derived DDCs produced only 11 and 13 mg/kg of FCW. In a 20 l airlift bioreactor, 268 mg/kg of paclitaxel is synthesized by CMCs. Contrast to this, no paclitaxel production was detected by either *T. cuspidata* needle- or embryo-derived DDC lines. Apart from paclitaxel, CMCs produced higher amounts of taxamarin A and C which are abietane tricyclic diterpenoids derivatives from *Taxus* species (Yang et al. 1999). Traditional DDCs which show some limitations for the synthesis of natural products such as fresh cell weight, cell aggregation, product yield, and sensitivity to shear stress represent significant obstacles. These problems associated with DDCs can be overcome by CMCs that provide a sustainable and cost-effective production of plant anticancer compounds.

22.9 Conclusions and Future Prospects

In conclusion, plant cell and tissue cultures can serve as an alternative means for the enhanced production of anticancer compounds. In this chapter, the strategies and approaches that are employed for the product enhancement of anticancer compounds are described. Every strategy and approach has got its own advantages and disadvantages over each other. Hence, there is a need for the substantial improvement for the product enhancement in lifesaving anticancer compounds on a commercial scale in order to bring down the cost of these compounds. Elucidation and understanding of the biosynthesis pathways of anticancer compounds and their regulation and seasonal influence on the production of anticancer compounds in respective plants in the wild are the prime concerns that help to overcome the difficulties experienced by various scientific groups for the overproduction of anticancer plant metabolites using plant cell and tissue cultures. Identification of suitable precursors, optimization of elicitors, medium composition growth regulators and culture conditions are the important parameters that are associated with product enhancement in plant cell cultures. A comprehensive knowledge of these factors and conditions will be of great importance for manipulating the desired metabolite production through product/metabolic engineering. Application genetic tools or advanced molecular techniques could be helpful to overproduce anticancer compounds through heterologous production of desired metabolites in lower organisms like bacteria and yeast. Paucity of information and knowledge about functions of enzymes, their regulation, respective genes sequence information and their expression associated with anticancer compounds are the setbacks in product enhancement by metabolic engineering, although there are some success stories reported pertaining to anthocyanin biosynthesis. Future studies pertaining to production of anticancer compounds using plant cell and tissue cultures should focus on addressing these aforementioned issues so as to resolve the complexities associated with plant secondary metabolites over production. With the knowledge gained, the attempts should focus on desired metabolite product enhancement through heterologous gene expression and metabolic engineering in lower organisms to develop cost-effective technologies for the large-scale production of anticancer compounds so as to ensure their availability at low prices affordable to the common man.

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Biotechnological Intervention Through Tissue Culture in *Hedychium coronarium*: A Potential Anticancer Plant

23

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Abstract

Hedychium coronarium is a valuable medicinal plant and commonly known as butterfly ginger, belonging to family Zingiberaceae. It has enormous medicinal values in traditional system of medicine and also has the potential to be used in modern medicine. A number of bioactive compounds with different pharmaceutical properties including anticancer activity have already been isolated from this plant. Realizing its medicinal potential, there is an increasing demand for this plant species. This has exposed the plant to unsustainable harvesting. Thus the species requires conservation-friendly approaches in its use. Regeneration of plants with uniform biochemical and genetic makeup through plant tissue culture is vital for conservation of such plant species and sustainable development. Till date several tissue culture-mediated plant regeneration protocols and assessment of biochemical and clonal fidelity of the regenerated plants have been reported on *H. coronarium*. The aim of this chapter is to present a comprehensive account of the tissue culture-mediated biotechnological intervention in *H. coronarium* and also to summarize the different works on genetic diversity of this plant species for its conservation strategies.

Keywords

Anticancer properties · *In vitro* propagation · Medicinal plant · Regeneration · Somatic embryogenesis

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23.1 Introduction

The member of the family Zingiberaceae comprises of 53 genera, and more than 1300 species are generally found in the tropics and subtropics (Devi et al. 2014). The plants of this family are usually annual or perennial rhizomatous herb with high economic potential and used as spices, condiments, flavouring agents, medicines, dyes, perfumes and vegetables (Rajamma et al. 2012; Devi et al. 2014). The presence of vital oils in the rhizome and flowers has made some plants of high economical values (Ravindran and Babu 2005; Sabu 2006). *Hedychium coronarium* is one of the most valuable plants of Zingiberaceae known for its pharmaceutical and ornamental properties. It is commonly known as butterfly lily or white garland lily. *H. coronarium* is under severe threat in their natural habitat due to restricted distribution, overexploitation, harvest, habitat destruction and unregulated trade (Chadha 2005; Mohanty et al. 2013). The species is among the medicinal plants of conservation concern in India. It is enlisted as a vulnerable plant in Odisha state, whereas in Kerala and Karnataka, they are near threatened. The aim of this chapter is to present a comprehensive account of the tissue culture-mediated biotechnological intervention in *H. coronarium* and also to summarize the different works on genetic diversity of this plant species for its conservation strategies.

23.2 Botany of *Hedychium coronarium*

23.2.1 Origin and Geographical Distribution

H. coronarium is supposed to be originated in the Himalayan region of India and Nepal (Kunnumakkara et al. 2008), and subsequently it has been introduced in other countries due to its medicinal and ornamental properties (Li and Fan 2007). It is found in tropical and subtropical regions of Japan, India, South China and South Asian countries including Bangladesh (Bisht et al. 2012). The plant is an invasive weed in Brazil (Santos 2004; Souza and Correia 2007). In India the plant is distributed mostly in the moist parts of Kerala, Karnataka, Odisha, Madhya Pradesh, Chhattisgarh, Sikkim, Manipur, Assam, Uttar Pradesh, Bihar and South India (Mohanty et al. 2013; Pachurekar and Dixit 2017).

23.2.2 Botanical Description

The plant is an aromatic, rhizomatous and erect herb growing up to 0.6–1.5 metre (Fig. 23.1 a, b). The rhizomes are fleshy, spreading horizontally under the soil surface. The plant has pseudostem, covered with leaf sheaths, which flowers only once and after that it is replaced by a new pseudostem. The leaves are large, oblong or lanceolate shaped. Flowers are strongly fragrant, white, borne in dense, terminal racemes (Fig. 23.1 c, d). The bracts are overlapping and ovate to obovate and measure up to 4.5–5 cm long, green and each having 2–4 flowers. Calyx is sparsely

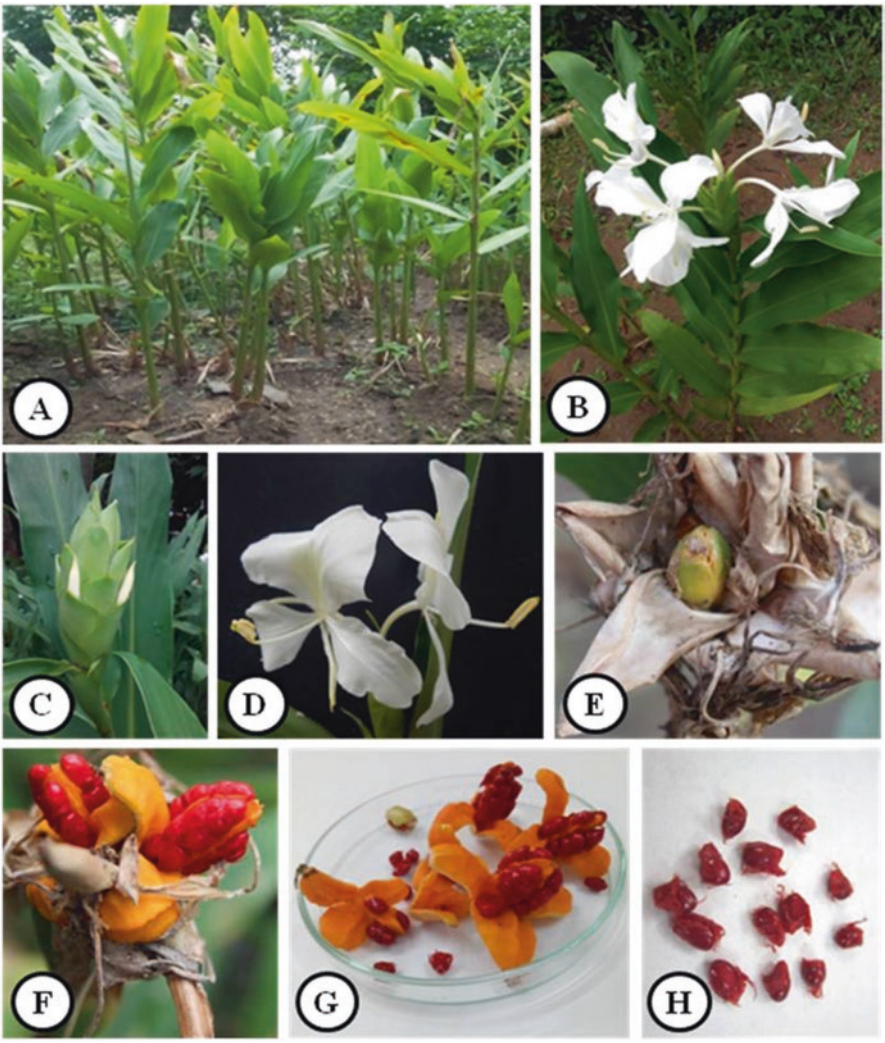

Fig. 23.1 *Hedychium coronarium* (**a**) Plants growing in experimental field. (**b**) Plants with flowers. (**c**) Inflorescence with flower buds. (**d**) Flowers. (**e**) Young capsule. (**f**, **g**) Splitted ripen capsules with seeds. (**h**) Seeds covered with aril

hairy and covers less than half of the length of corolla tube. The petals are linear-lanceolate shaped, with obcordate or obovate labellum, white with dark yellow spots at the base. The lateral staminodes are oblong, lanceolate and white in colour. The anther is strongly curved and up to 1.5 cm long (Fig. 23.1d). The fruit is oblong capsule, dehisces longitudinally by three valves and bright orange inside, has numerous seeds covered with red-coloured aril; seeds borne on axile placenta (Fig. 23.1 e–h).

23.2.3 Propagation of Plant

H. coronarium is conventionally propagated through rhizome or seeds (Chadha 2005), of which the plant regeneration by vegetative means is very common (Verma and Bansal 2010). Usually rhizomes are divided/separated into segments with growing shoots. The larger segments can be directly planted in the field, but the smaller ones preferred the pot culture grown under the greenhouse followed by transfer to permanent field in summer or late in the spring (Chadha 2005). Annual separation of rhizomes is necessary for the better growth of the plants. After fading of flowers, the removal of old stems is also essential to promote new growth of shoots. Seed germination is found to be very poor and usually not a preferred method of propagation (Mishra 2013).

23.3 Applications of *H. coronarium*

23.3.1 In Traditional Medicine

H. coronarium is known in both Indian as well as Chinese system of traditional medicine (Chan and Wong 2015). Almost all parts are used for the treatment of different ailments including diabetes, tonsillitis, infected nostrils, fever, tumour, headache, hypertension, inflammation, stomach disorders and pain due to rheumatism (Suresh et al. 2010; Bisht et al. 2012; Kiem et al. 2012; Donipati and Sreeramulu 2015). In India the rhizome is used to treat fever, rheumatism and throat-associated problems by some tribal community in Bihar and Manipur (Kunnumakkara et al. 2008; Singh et al. 2014; Devi et al. 2015). The rhizome powder is used to cure diarrhoea by tribal groups of Uttar Pradesh (Shukla et al. 2013), and rhizome paste is applied against snake bite (Ray et al. 2011). Flower extract is marketed as eye drop in Amarkantak region of Madhya Pradesh, India (Mishra 2013), and the juice obtained from the base of the areal plant part is applied to reduce swelling (Kunnumakkara et al. 2008). Furthermore, the rhizome is used by the local community in Bandarban District (Bangladesh) to kill worms (Uddin et al. 2015). Rhizomes are also known for the treatment of rheumatic pain, skin diseases and headache in Vietnam (Kiem et al. 2011; Chan and Wong 2015). Leaves are boiled and administered orally to treat indigestion in Malaysia (Ibrahim 2001), whereas it is applied to cure stiff and sore joint in Thailand (Chan and Wong 2015). Hot water infusion of leaves is consumed to cure hypertension in Brazil (Ribeiro et al. 1986; Chan and Wong 2015). The juice derived from mature seeds of the plant is used for skin treatment by the locals of Hawaii (Kunnumakkara et al. 2008; Parida et al. 2013; Behera et al. 2014).

23.3.2 Other Uses

The plant is widely used for ornamental purposes due to its beautiful, scented flowers (Chiba et al. 2013). The fragrance of the flower is due to mixture of monoterpenoid and benzenoid compounds. L-Linalool, 1,8-cineole and 1,3,7-octatriene-3,7-dimethyl

are the main components of petals (Li and Fan 2007; Li and Fan 2011). The flowers of the plants have been mainly used in perfume industries (Chadha 2005; Chan and Wong 2015). The stem has 42–48% cellulose and thus has potential to be used in the paper industries as raw materials (Chadha 2005; Bisht et al. 2012). *H. coronarium* has also been used as vegetables in different parts of the world. In India the rhizomes, tender shoots and flowers are consumed as raw vegetables in Manipur (Devi et al. 2010; Sarangthem et al. 2012; Singh et al. 2013) and Odisha (Misra et al. 2013). The flower is also consumed as vegetable in Hawaii and Japan (Chan and Wong 2015).

23.4 Anticancer Properties of *H. coronarium*

A few studies have already been carried out in *H. coronarium* to test the pharmacological activities of the plant and to substantiate its traditional uses. Different extracts from the rhizome of the plant have exhibited anti-inflammatory, analgesic, leishmanicidal (Endringer et al. 2014), antibacterial (Aziz et al. 2009) and cytotoxic activity (Dash and Sheikh 2015). Studies related to anticancer activities of *H. coronarium* have been reviewed here. Endringer et al. (2014) have reported cancer chemoprevention activity of labdane diterpenes from the ethanol extract of rhizome of *H. coronarium* using a variety of *in vitro* tests like inhibition of NF-kB, COX-1and -2, ARE induction and cell proliferation. ARE induction, promoted by isocoronarin D (EC_{50} 57.6 ± 2.4 μM), decreased significantly by introducing a substitute at C-15 and by removing the hydroxyl group at C-14, as observed with methoxycoronarin D and ethoxycoronarin D (EC_{50} >60.2 and >57.8 μM, respectively). On the other hand, these features dramatically increased the NF-kB inhibitory potency of methoxycoronarin D and ethoxycoronarin D (IC_{50} 7.3 ± 0.3 and 3.2 ± 0.3 μM, respectively) in comparison to isocoronarin D. Weak anti-proliferative activity was observed for isocoronarin D and methoxycoronarin D against HepG2 cell lines, and similar weak anti-proliferative activity has been reported for isocoronarin D and ethoxycoronarin D against LNCaP cell line (Endringer et al. 2014). Methoxycoronarin D exhibited low cytotoxicity in cancer cells MCF-7, A549 and SK-N-SH (Suresh et al. 2010) and HeLa and HUMEC (Zhan et al. 2012). Coronarin D was found to be more potent than its analogue coronarin D acid (Kunnumakkara et al. 2008). Coronarin D inhibited NF-kB activation pathway, which leads to inhibition of inflammation, proliferation, invasion, and osteoclastogenesis, as well as potentiating of apoptosis (Kunnumakkara et al. 2008). *In vitro* anticancer activity of hexane, chloroform and methanol extract of rhizome of *H. coronarium* was assessed against the human breast cancer cell line (MCF-7). Maximum inhibition of cell growth was observed in methanol extracts (Donipati and Sreeramulu 2015).

Labdane-type diterpenes; coronarins A, B, C and D; and one known labdane-type diterpene, (*E*)-labda-8(17),12-diene-15,16-dial, were isolated from rhizomes of *H. coronarium* and investigated for their cytotoxic activity against V-79 cells (Itokawa et al. 1988). Coronarins A and B showed particularly significant cytotoxic activity against V-79 cells (Itokawa et al. 1988). Isocoronarin D was reported to be most active against HuCCA-1, A549, MOLT-3, KB, HeLA, MDA-MB231, T-47D, HL-60, P388 and HepG2 cancer cell lines, with the IC_{50} at about 4 μg/ml, except for

a moderate activity on S102 cell line (Chimnoi et al. 2009). Compounds, isolated from hexane extract of rhizome of *H. coronarium*, were tested for their cytotoxic properties against the A-549 (lung cancer), SK-N-SH (human neuroblastoma), MCF-7 (breast cancer) and HeLa (cervical cancer) cell lines by using sulforhodamine B (SRB) assay. The 4-hydroxy-3-methoxy ethyl cinnamate; 4-hydroxy-3-methoxycinnamaldehyde; hedy-chenone; coronarin C and coronarin D exhibited potent cytotoxic activity against A-549 cell line with the LC_{50} value ranging from 1.26 to 8.0 μM. Two new compounds, 6-oxo-7,11,13-labdatrien-17-al-16,15-olide and 7,17-dihydroxy-6-oxo-7,11,13-labdatrien-16,15-olide, showed moderate cytotoxicity on the above tested cell lines (Suresh et al. 2010). Bioactive compounds isolated from rhizome of *H. coronarium* were assayed for their cytotoxic activities against B16 (murine melanoma), HT-29 (human colon), HeLa (cervical carcinoma) and HepG2 (hepatoblastoma) cell lines and showed that all isolated compounds have potent cytotoxic activities against all the four cancer cell lines (Zhan et al. 2012). Recently, Dash and Sheikh (2015) reported that methanolic extract of rhizome of this plant displayed cytotoxicity against brine shrimp nauplii (*Artemia salina*). The LC_{50} value of the methanolic rhizome extract was 0.39 μg/ml, whereas the LC_{50} of the reference anticancer drug vincristine sulphate was 0.52 μg/ml (Dash and Sheikh 2015).

23.5 Tissue Culture-Mediated Biotechnological Intervention

Conventional propagation of *H. coronarium* through rhizomatic bud is a season-dependent process (Chadha 2005). Plant tissue culture has already been established as an alternative method to circumvent such problems by producing large number of plants season independently and useful for conservation of such threatened plant species. A report on somatic embryogenesis by Huang and Tsai (2002) was probably the first tissue culture study on *H. coronarium*. Since then, a number of *in vitro* studies have already been reported on *H. coronarium* including micropropagation through shoot proliferation from different explants and somatic embryogenesis.

23.5.1 *In Vitro* Plant Regeneration

23.5.1.1 Explants

The success of *in vitro* plant regeneration through micropropagation depends upon a number of factors including composition of basal medium, concentration and combinations of plant growth regulators, culture conditions and the choice of explants to initiate cultures (Naik and Chand 2011). Usually explants with pre-existing meristems are preferred for clonal propagation of plants, as they are less susceptible to genetic instability compared to callus-mediated organogenesis (Shenoy and Vasil 1992). In the plants belonging to Zingiberaceae including *H. coronarium*, rhizome buds are meristematic. As expected, the sprouted rhizome bud/axillary buds have been the choice of explants for micropropagation of the species (Table 23.1). Micropropagation of *H. coronarium* using shoot tips derived

Table 23.1 *In vitro* plant regeneration studies in *H. coronarium*

Explants	Basal culture medium + Plant growth regulator(s)		Remarks	References
	Shooting	Rooting		
Axenic shoot tip	MS + 1.0 mg/l BA + 0.5 mg/l NAA	½ MS + 0.5 mg/l NAA	–	Bisht et al. (2012)
Axillary bud of rhizome	MS + 3.0 mg/l BA + 3.0 mg/l Kin + 0.2 mg/l TDZ	MS + 3.0 mg/l Kin + 0.5 mg/l IAA	Assessment of clonal fidelity of the mother and micropropagated plants using RAPD and ISSR markers	Parida et al. (2013)
Rhizome bud	MS + 2.0 mg/l BA + 0.5 mg/l NAA		–	Mohanty et al. (2013)
Rhizome bud/eye	MS + 1.0 mg/l BA + 1.0 mg/l PG	½ MS (liquid) + 1.0 mg/l NAA	–	Verma and Bansal (2013)
Rhizome bud	MS + 1.0 mg/l TDZ ↓ MS + 1.0 mg/l BA	½ MS (liquid) + 1.0 mg/l NAA	–	Verma and Bansal (2014)
Axillary bud of rhizome	MS + 3.0 mg/l BA + 3.0 mg/l Kin + 0.2 mg/l TDZ	MS + 3.0 mg/l Kin + 0.5 mg/l IAA	Analysis of essential oil compositions of rhizome and leaves of the micropropagated and conventionally grown plants and assessment of antioxidant activity by DPPH assay	Parida et al. (2015a, b)

from *in vitro* raised seedlings as explants has also been reported (Bisht et al. 2012). Size of explants, i.e. rhizome bud, also plays a major role in shoot proliferation of *H. coronarium*. Mohanty et al. (2013) reported the bud size of 0.5–1.0 cm as optimum for multiple shoot regeneration. At the same time, rhizome explants with bud those measured more than 1.5 cm (failed to produce multiple shoots) and less than 0.5 cm (failed to induce any shoot) were found to be unsuitable for shoot proliferation (Mohanty et al. 2013).

23.5.1.2 Shoot Proliferation

Full-strength Murashige and Skoog (1962) (MS) was used as basal medium in all the reported micropropagation protocol of *H. coronarium*. Different workers found different growth regulators, either alone or in combination, suitable for shoot proliferation of *H. coronarium* (Table 23.1). Bisht et al. (2012) used MS medium supplemented with 1.0 mg/l benzylaminopurine (BA) in combination with 0.5 mg/l 1-naphthaleneacetic acid (NAA) for shoot proliferation from axenic shoot tip explants. They recorded 7.9 shoots/explant with average shoot length of 6.0 cm after 50 days of culture. Parida et al. (2013) recorded maximum 13.2 shoots/explant using axillary bud of rhizome as explants on MS medium supplemented with

3.0 mg/l BA, 3.0 mg/l kinetin (Kin) and 0.2 mg/l thidiazuron (TDZ) within 8–12 weeks. In the same year, MS medium augmented with 2.0 mg/l BA and 0.5 mg/l NAA was found to be effective for one-step plant regeneration of *H. coronarium* (Mohanty et al. 2013). They for the first time reported a one-step plant regeneration, i.e. simultaneous shoot and root regeneration in a single medium. Highest numbers of simultaneous shoots (3.6) with average shoot length of 4.7 cm and roots (4.0) with average root length of 4.2 cm were recorded at 45 days of culture. The protocol developed by Mohanty et al. (2013) was a rapid and cost-effective protocol as it did not required a different rooting stage and thus saved the additional cost of rooting medium and time.

Verma and Bansal (2013) studied the influence of different additives including activated charcoal (AC), casein hydrolysate (CH), phloroglucinol (PG), silver nitrate ($AgNO_3$) and coconut milk (CM) on shoot multiplication of *H. coronarium* from rhizome bud explants on MS medium supplemented with 1.0 mg/l BA. Of these additives, AC and PG showed positive response to shoot proliferation; PG has been the most effective. Highest number of shoot regeneration (6.56 shoots/explant) was observed on MS + 1.0 mg/l BA supplemented with 1.0 mg/l PG. They also found that a comparatively higher concentration (5.0 mg/l) of CH was also beneficial for shoot proliferation. Verma and Bansal (2014) tested three cytokinins, viz. TDZ, BA and Kin, for shoot proliferation. In contrast to other reports, they suggested augmentation of TDZ at 1.0 mg/l to MS medium for highest number (14.21) of shoot regeneration. However, it was found necessary to transfer the cultures to 1.0 mg/l BA-supplemented MS medium for further elongation of shoots. They found Kin as the least effective plant growth regulators.

23.5.1.3 Rooting of *In Vitro* Regenerated Shoots

MS medium supplemented with NAA (0.5–1.0 mg/l) has been commonly used for rooting of shoots of *H. coronarium* (Table 23.1). Bisht et al. (2012) reported that semi-solid ½ MS supplemented with 0.5 mg/l NAA favoured best rooting (12.2 roots/shoot) with average root length of 4.68 cm. In contrast, Verma and Bansal (2013, 2014) observed best rooting on liquid half-strength MS medium supplemented with 1.0 mg/l NAA. Verma and Bansal (2013) recorded highest number of roots (14.6) after 42 days of culture, whereas maximum 7.6 roots were reported by Verma and Bansal (2014) after 28 days of culture. Parida et al. (2013) could induce best rooting (6.3 roots after 90 days) on MS medium supplemented with 3.0 mg/l Kin and 0.5 mg/l IAA.

23.5.1.4 Acclimatization of In Vitro Generated Plantlets

Successful acclimatization of the *in vitro* regenerated plantlets has been reported in *H. coronarium* using different potting mixtures consisting of forest soil/organic compost/sand (1:1:1) (Bisht et al. 2012), sand/soil/farmyard manure (1:1:1) (Verma and Bansal 2012, 2013, 2014), soil/cow dung/sand (1:1:1) (Parida et al. 2013) and garden soil/sand (1:1) (Mohanty et al. 2013), followed by transfer to soil with varying degree (65–100%) of success.

23.5.2 Somatic Embryogenesis

Sheath and leaves (Huang and Tsai 2002), meristematic rhizome (Verma and Bansal 2012) and immature filament (Wang et al. 2016) have been used for induction of somatic embryo in *H. coronarium*. Embryogenic callus was induced by Huang and Tsai (2002) on VW medium containing 5.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l BA and 200 ml/l coconut water. A large number of somatic embryos were produced directly from the surface of the calli when cultured on MS medium containing 4.0 mg/l BA and 0.05 mg/l NAA. Subsequently Verma and Bansal (2012) reported that MS medium augmented with 0.5 mg/l 2,4-D was best for callus induction. Somatic embryos were induced and matured on growth regulator-free basal medium. Culture of the somatic embryos on a cytokinin-supplemented medium was necessary for the germination of the embryos. Maximum conversion rate was observed on MS medium supplemented with 0.5 mg/l BA (Verma and Bansal 2012). Recently, Wang et al. (2016) reported a method of *in vitro* plant regeneration by somatic embryogenesis. Most of the regenerated plants were diploid, but they also observed triploid, tetraploid and hexaploid plants. Callus was induced on MS supplemented with 4.0 mg/l 2,4-D, 4.0 mg/l NAA and 1.0 mg/l BA. The callus was subsequently cultured on MS augmented with 1.0 mg/l 2,4-D, 0.25 mg/l NAA and 0.25 mg/l BA for proliferation of embryogenic callus. Somatic embryos were produced by culturing the callus on medium composed of basal salts of MS medium, vitamins of Gamborg's B_5 (1968) medium, 100 mg/l glutamine, 230 mg/l proline, 100 mg/l malt extract, 0.25 mg/l NAA, 0.5 mg/l TDZ and 45 g/l sucrose for 20 days followed by transfer to growth regulator-free MS medium for 30 days. Germination (85%) of these somatic embryos required MS medium supplemented with 0.2 mg/l NAA and 0.5 mg/l BA. However these germinated somatic embryos were subsequently transferred to ½ MS medium containing 1.0 g/l AC for proper growth of shoot and roots (Wang et al. 2016).

23.5.3 Assessment of Clonal and Biochemical Fidelity of the *In Vitro* Regenerated Plants

23.5.3.1 Clonal Fidelity Using Molecular Markers

Assessment of clonal fidelity of the *in vitro* regenerated plants using molecular markers in *H. coronarium* has been carried out by Parida et al. (2013). Till date this is the only such study on this plant. They used two DNA-based molecular markers, i.e. random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR), to check the genetic stability of the regenerants up to 2 years with that of the mother plant. They could not find any polymorphic bands in the mother plant and micropropagated plants and thus suggested that the plants are true to type. Initially 25 RAPD primers were screened but final analysis was carried out with 14 primers. These 14 primers generated 57 distinct scorable bands with average of 4.1 bands per primer. The number of bands for each primer was found to be from 2 to 7 and ranging from 225 to 2000 bp. The primer OPD20 exhibited highest number (7), whereas

OPA18 and OPD18 generated lowest (2) number of monomorphic bands (Parida et al. 2013). During ISSR analysis study, Parida et al. (2013) found that 9 primers yielded a total of 77 bands with an average of 8.5 bands. The sizes of bands were found to be ranging from 200 to 2000 bp. The primers (GAC)5 and (CAA)5 produced the highest (13) and the lowest number of bands (5), respectively. After 2 years, with confirmed genetic fidelity, the micropropagated plants were transferred to field condition, and evaluation of morphological characters was carried out (Parida et al. 2013).

23.5.3.2 Biochemical Fidelity

Essential oils of *H. coronarium* have medicinal properties (Joy et al. 2007; Lu et al. 2009) and are also useful in making perfumes (Chan et al. 2008). The plants regenerated through tissue culture techniques should be assessed for its biochemical fidelity with the mother plants prior to recommending them for commercial utilization. Keeping this in view, Parida et al. (2015a, b) analysed the chemical composition of the essential oil isolated from leaves and rhizomes of micropropagated plants with that of the mother/conventionally grown plants by GC-MS. Parida et al. (2015a) could identify about 30 and 16 compounds from leaf oil of the conventionally grown plant and tissue culture raised plants, respectively. At the same time, a total of seven and three compounds were identified from essential oil of rhizome of the mother plant and micropropagated plant. All the major compounds, viz. β-pinene, eucalyptol and linalool, were found to be present in both micropropagated and conventionally grown plant. Subsequently, Parida et al. (2015b) revalidate the presence of β-pinene, eucalyptol and linalool in the rhizome oil of both the micropropagated and conventional plants. Thus according to them, the micropropagated plants were true chemical clones of the mother plant. They also assessed and found similar antioxidant activities (2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay) of the rhizome-derived essential oils of both types of plants as compared to standard ascorbic acid. A comparative qualitative phytochemical analysis was carried between the leaf, rhizome and root extract of normal field grown (*in vivo*) plant and tissue culture-generated (*in vitro*) plant (Behera et al. 2014). Results revealed the presence of saponins, flavonoids, tannins and phenolics in different solvent extracts of root, leaf and rhizome of the plant (Behera et al. 2014).

23.6 Genetic Diversity Studies

The assessment of genetic diversity of a threatened plant species is essential for the development of its conservation strategies (Gaafar et al. 2014). Recently, the molecular characterization of *H. coronarium*, a vulnerable species of Odisha state (India), has been reported by Parida et al. (2017) using RAPD and ISSR markers. They studied four different populations, i.e. Malkangiri, Phulbani, Khurda and Angul districts of Odisha (India), using 15 RAPD and 9 ISSR markers. Both markers together generated a total of 140 bands, of which 131 were monomorphic, 3 were polymorphic and 6 unique bands. The band size was ranging from 180 to 3000 bp. Two

major clusters were found in the dendrogram. The lowest and highest similarities detected were 0.978 and 1.000, respectively (Parida et al. 2017).

23.7 Conclusions and Future Prospects

Due to threat status of *H. coronarium*, especially in India, a number of *in vitro* plant regeneration protocols have been developed recently for the production of plant clones. Among the various methods used, the micropropagation of rhizome is the best one for the mass propagation of *H. coronarium*. However, further refinements of these protocols are desired for their commercial uses in pharmaceutical industries and germplasm conservation. Efforts should also be made to develop plant regeneration protocol through organogenesis. Neither direct adventitious nor did callus-mediated organogenesis systems have been developed for the plant species. Efficient direct organogenesis and refined micropropagation techniques through rhizome buds are required for development of genetic transformation protocol for *H. coronarium*. Establishment of callus and cell suspension culture, with potential to scale up through bioreactors, could be useful in both the ways for large-scale plant regeneration and secondary metabolite production. Overall *in vitro* plant regeneration of *H. coronarium* with bioactive principles equivalent to the mother plant and cell culture methods for production of selective secondary metabolites could be useful for commercial production of pharmaceutically important compounds. In addition, efforts should also be made for the development of synthetic seed and slow-growth technology for germplasm conservation. Successful development of cryopreservation method could also be effectively used for the long-term conservation of the species. Till date, only a single genetic diversity study has been carried out in *H. coronarium*. It is imperative to study the genetic diversity between and among the different populations of *H. coronarium* in India, so that proper conservation strategies could be developed.

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