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Anwar Shahzad
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Aastha Sahai *Editors*

Recent Trends in Biotechnology and Therapeutic Applications of Medicinal Plants

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Foreword

Since ancient ages, medicinal plants have been used as a source of drugs in all cultures. Remedies and healthcare preparations relied on herbs and plants. The use of traditional medicine and medicinal plants in most developed and developing countries, as a normative basis for the maintenance of good health, has been widely observed. Moreover, the use of medicinal plants in the industrialized societies has been showing trends of development of several drugs and chemotherapeutics from these plants as well as biotechnological applications. Herbal remedies have become more popular in the treatment of minor ailments, considering the increasing costs of personal health maintenance. The concerns in this book initially begin with a chapter introducing the different types of phytoactive compounds: substances which represent the activity of plant extracts. Several biotechnological approaches used to reveal and manipulate these treasury compounds at commercial level are discussed in greater details. In this publication, the editors concentrated on the biotechnological applications of the plants and trends of development of several novel approaches, vaccination, and potential sources for newer antimicrobial agents, without ignoring *in vitro* established protocols held by workers and researchers in this field. The book is a reference text with essential information for various scientists, including advance students, teachers as well as research scientists in different medical fields and biotechnology applications.

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Preface

MEDICINAL plants are moving from fringe to mainstream with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilize eco-friendly and bio-friendly plant-based products for the prevention and cure of different human diseases. Even Western world is now looking for natural remedies which are safe and effective. Natural products isolated from higher plants and microorganisms have been providing novel, clinically active drugs since long time. Biotechnology plays a major role in the production of natural products through biosynthesis and bioengineering which reduce the dependence on large amount of plant samples thus relieving pressure on natural resources. Development of medicinal plants into therapeutic drugs involves skills, techniques, tools and knowledge from areas of botany, biotechnology, phytochemistry and medical science.

This book gives an insight into how these diverse areas of science are playing their role in realizing the therapeutic potential of medicinal plants. Various themes in the book concentrate on the role of an individual area and its application in medicinal plant sciences. The book starts with the illustration of phytoactive compounds which are responsible for therapeutic activity of medicinal plants. The identification of biologically active compounds is an essential requirement for quality control and dose determination of plant-based drugs. A medicinal plant can be viewed as a synthetic laboratory as it produces and contains a number of chemical compounds. These phytoactive compounds are generally the secondary metabolites produced by plants in response to any stress and infection. Chapters describing various biotechnological tools and techniques (bioreactors, hairy root culture and elicitation) which are required for the production and enhancement of these secondary alkaloids are included in this book.

Screening of extracts of natural products is a preliminary step for identifying active agents in different plant species. Studies, and an overview on recent trends, on screening of antimicrobial compounds in plant extracts from different geographical regions are included. Therapeutic effect of natural plant products against common human disorders like dental caries and periodontal diseases have been discussed in a separate chapter.

An informative and enriching theme, in brief, has been framed, discussing novel applications of plants as neutraceuticals and edible vaccines. These new approaches in plant science give an idea of the vast unutilized potential of these natural resources. An important part of the therapeutic plant science is the conservation of these fast depleting medicinal plant resources. Tissue culture is useful for multiplying and conserving the plant species, which are at the verge of extinction due to high commercial demand. The last theme of the book provides exquisite compilation of in vitro regeneration protocols for different commercially important, rare, threatened and endangered medicinal plant species.

Thus the book aims to provide comprehensive information on basic tools and technologies involved in the therapeutic applications of medicinal plants together with recent ongoing trends and advances in this field. This book will be beneficial to students studying biotechnology, phytochemistry, medical science and botany. Also, it would prove a great source of information on recent studies and trends in medicinal plant science for researchers involved in medicinal plant and phyto-pharmaceutical researches.

During preparation of this book, we did our sincere efforts to provide good scientific contents and hope that the readers, including students, teachers and researchers will find it useful.

We thank our learned contributors for their timely submissions and cooperation during compilation of the book. The editors deeply appreciate the scientific advice provided by the reviewers and the time to time assistance provided by the Springer book editorial team, especially from Machado Elisabete and Zuzana Bernhart. We also wish to acknowledge the support provided by our family members and dear friends that provided us the boost to complete this energetic task.

And finally, we sincerely acknowledge the blessings from the Almighty God, for making this task easy for us.

Manama, Bahrain
Aligarh, India
26 January 2013

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Contents

Chapter 1
Phytoactive Compounds from *In Vitro* Derived Tissues

Anwar Shahzad, Arjumend Shaheen, M. Imran Kozgar, Aastha Sahai, and Shiwali Sharma

1.1 Introduction

Plant tissue culture is an important area of research in plant biotechnology which eventually lead to the production of a vast number of phytoactive compounds in laboratory conditions. The *in vitro* derived phytoactive compounds have many advantages over the other sources of production viz synthetic sources etc. The phytoactive compounds can be obtained from a wide variety of plant sources viz algae, bryophytes, pteridophytes, gymnosperms and angiosperms. Medicinal plants play a key role in world heath care systems (Bajaj and Williams 1995). Because of the many scientific, economic and ecological advantages of plant tissue culture, it is nowadays considered an important stategy for *in vitro* production of bioactive compounds for drug industries (Srivastava and Srivastava 2007). Tissue culture techniques are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Sauerwein et al. 1992). Tissue culture is an *in vitro* propagation technique of a wide range of excised plant parts, through which a mass of cells (callus) is produced from an explant tissue. The callus produced, can be utilized directly to regenerate plantlets or to extract or manipulate some primary and secondary metabolites (Singh 2004). In order to obtain high yields suitable for commercial exploitation, efforts have been focused on the isolation of biosynthetic activities of cultured cells, achieved by optimizing the cultural conditions, selecting high-producing strains, and employing precursor feeding, transformation methods, immobilization techniques and elicitation (Premjet et al. 2002; Collin 1987). With the

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advent of modern drug discovery, traditional sources were greatly receded in modern societies. During the past decade, interest in polyphenols, including isoflavonoids has increased considerably because of its beneficial effects in cardiovascular diseases, postmenopausal symptoms and cancer (Dixon and Ferreira 2002; Nastel 2004). In the last few years promising findings have been reported for a variety of medicinally valuable substances, some of which may be produced on an industrial scale in the near future (Nuutila and Oksman 2003). Recently considerable attention has been paid to utilize eco-friendly and bio-friendly plant based products for the prevention and cure of different human diseases including microbial infections (Dubey et al. 2004). The potential of higher plants as source for new drug is thus still largely unexplored (Hostettaman and Walfender 1997). In view of the commercial importance given to the secondary metabolites in recent times, efficient production of bioactive compounds by tissue culture technology has gained popularity (Vaniserce et al. 2004). Moreover the continuous and non-organized exploitation has resulted in many plants becoming rare and some even became extinct. To overcome this limitation, biotechnologists suggested the "use of cell and tissue culture technology rather than to use the whole plant" for the extraction of certain secondary metabolites (Rajendra and D'souza 2000). Moreover there are a number of cultured cells producing metabolites not observed in the plant, e.g., *Lithospermum erythrorhizon* cultures have been observed to synthesize rosmarinic acid (Fukui et al. 1984). It has become apparent that the choice of original plant material having high yields of the desired phytochemical may be important in establishing high-yielding cultures (Dues and Zenk 1982). Furthermore, the need to repeatedly screen for high- producing lines (due to inherent instability of cell lines) has been emphasized, although the nutritional composition of the medium is also important (Whitaker et al. 1986). Several products were found to be accumulated in cultured cells at a higher level than those in native plants through optimization of cultural conditions. For example, ginsenosides by *Panax gingseng* (Ushiyama et al. 1991), rosmarinic acid by *Coleus blumei* (Ulbrich et al. 1985), shikonin by *Lithospermum erythrorhizon* (Takahashi and Fujita 1991), diosgenin by *Dioscorea* (Rokem et al. 1984), were accumulated in much higher levels in cultured cells than in the intact plants. In this chapter several important *in vitro* derived phytoactive compounds and their potent sources of derivation have been discussed.

1.2 Phytoactive Compounds and Their *In Vitro* Sources of Derivation

Work has been done on a large number of plant species through *in vitro* studies and subsequently a handsome amount of phytoactive compounds have been derived so far. Phytoactive compounds have been classified by various authors in different categories and the pattern of classification varies. The structural organization of some of the different types of phytoactive compounds, as such or of their backbone

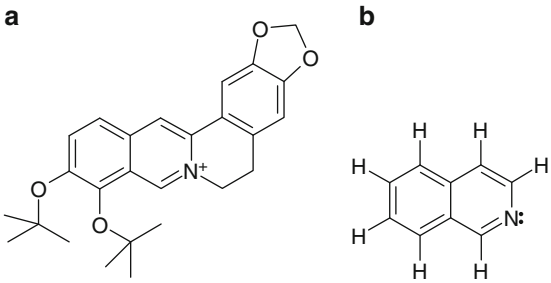

Fig. 1.1 Berberine (**a**) and Isoquinoline (**b**)

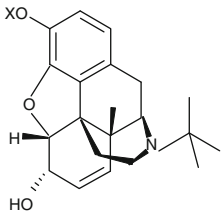

Fig. 1.2 Morphine (X = −H) and Codien (X = $-CH_3$)

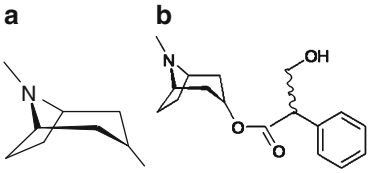

Fig. 1.3 Tropane (**a**) and Atropine (**b**)

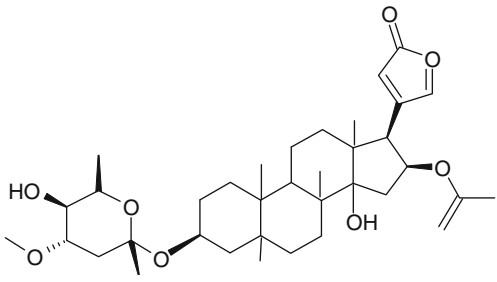

Fig. 1.4 Glucosides skeleton

outlook, have been given in Figs. 1.1, 1.2, 1.3, 1.4, 1.5, and 1.6 which have been drawn using MDL ISIS Draw 2.5 Software programmes (developed by MDL Information Systems, Hayward, California). Some of the basic and important phytoactive compounds are categorized as follows along with their plant sources and *in vitro* studies of their derivation.

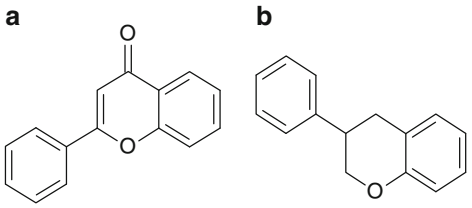

Fig. 1.5 Flavones (**a**) and Isoflavanes (**b**)

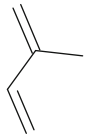

Fig. 1.6 Isoprene backbone

1.2.1 Alkaloids

Alkaloids occupy the prime position in the secondary plant metabolites of which several thousand are known. According to Harborne (1998) "there is no one definition of the term 'alkaloid' which is satisfactory, but alkaloids generally include 'those basic substances which contain one or more nitrogen atoms, usually in combination as part of a cyclic system". Alkaloids can further be classified into a number of sub categories based on their structural differences and their mode of action.

1.2.1.1 Occurrence

Alkaloids are found in about 15 % of vascular plants belonging to more than 150 families and are widely distributed in higher plants particularly in the dicotyledon families like Apocynaceae, Berberidaceae, Rubiaceae, Asteraceae, Papaveraceae, Papilionaceae, Ranunculaceae, Rutaceae, Leguminoseae, Menispermaceae, Lauraceae and Solanaceae and is present less frequently in lower plants. Armaryllidaceae and Liliaceae are important alkaloid bearing plants.

1.2.1.2 Functions of Alkaloids in Plants

Some of the possible functions of alkaloids in plants are as follows:

1. These are the poisonous agents which protect plants against insects and herbivores,
2. These serve as regulatory growth factors similar to hormones,
3. These are byproducts of plant metabolism,
4. These serve as reserve substances capable of supplying nitrogen or other necessary fragments to the plant development

5. Alkaloids are the end products of detoxification reactions representing a metabolic locking-up of compounds otherwise harmful to the plant.

1.2.1.3 Few Examples of Alkaloids

Ajmalicine
Morphine, Codiene, Thebaine or Morphine Alkaloids
Berberine
Tropane alkaloids or Atropine
Vinca alkaloids

1.2.1.4 Alkaloids Derived Through Tissue Culture and Their Plant Sources

Berberine

Berberine a quaternary ammonium salt derived from the protoberberine group of isoquinoline alkaloids can be obtained from plants like *Berberis aquifolium*, *Berberis vulgaris* and *Berberis aistata*, *Hydrastis candensis*, *Phellodenderon amurense*, *Coptis chinensis*, *Tinospora cordifolia*, *Argemone mexicana* and many more. Berberine is a useful antibacterial agent and stomachic and its anti-inflamatory effect has been reported by Otsuka et al. (1981).

Coptis japonica: It takes 5–6 years to produce coptis roots, the raw material for berberine production. In contrast, cultured coptis cells have been shown to produce moderate amounts of berberine (2–4 % on a dry wt. basis) in a short period (3 weeks) (Yamada and Sato 1981). Furuya et al. (1972) have investigated the production of berberine by *Coptis japonica* cell cultures since 1970s and Sato and Yamada (1984) selected a high berberine producing cell lines of *Coptis japonica*.

Morphine Alkaloids (Morphine, Codeine and Thebaine)

Morphine is a potent opiate analgesic drug. Most abundant alkaloid found in opium has a high potential for addiction and tolerance. Its uses in medical science are marvelous and is widely used to treat chronic severe pain, myocardial infraction, acute pulmonary edema, etc.

Codeine a natural isomer of methylated morphine, is an opiate used for its analgesic, antitusive and anti-diarrheal properties. Being the second most abundant alkaloid in opium it is used to cure a number of diseases viz diarrhea, irritable bowelsyndrome and to relieve cough.

Thebaine an opiate alkaloid chemically similar to both morphine and codeine cause convulsions similar to strychnine poisoning at higher doses. It is not used therapeutically but can be converted into a variety of compounds viz oxycodone, oxymorphone, nalbuphine, naloxone, naltrexone, buprenorphine and etorphine.

***Papaver somniferum* L.**: Production of opium alkaloids have been reported from callus tissue cultures of *Papaver somniferum* Lin. (Khanna and Khannna 1976; Staba et al. 1982). Tetraploid, tracheid containing callus of this plant reportedly produce codeine, morphine and thebaine (Erdelsky 1978). The high yielding tissues having large cells containing amorphous alkaloid contents were observed in *P. somniferum* and *P. rhoeas* (Sarin and Khanna 1989).

Ajmalicine

It is an alkaloid found naturally in various plants such as *Rauwolfia spp*., *Catharanthus roseus* and *Mitragyna speciosa* (Wink and Roberts 1998; Kurz et al. 1981; León et al. 2009). It is an antihypertensive drug used in the treatment of high blood pressure (Wink and Roberts 1998).

Rauwolfia spp: Ajmaline and ajmalicine are pharmacologically important monomeric indole alkaloids having antihypertensive activities (Wilkins and Judson 1953). They are root-specific and are found in most of the *Rauwolfia* spp. Ajmalicine production through biotechnological means has been confined mainly to *Catharanthus roseus*. Development of hairy root cultures of *R. micrantha* using suitable strains of *Agrobacterium rhizogenes* and the studies on the production of ajmaline and ajmalicine under the influence of auxins were conducted by Sudha et al. (2003).

***Cathranthus roseus* (L.) G. Don**: Distribution and accumulation of alkaloid content in plant parts of *Catharanthus* viz. roots, stems, and leaves (Misra and Kumar 2000). Increased alkaloid content was also reported in *Catharanthus* plants by the application of 2,4-D, kinetin and IAA (Amit et al. 2005; Srivastava and Srivastava 2007). In cell suspension culture of *Catharanthus*, if the glucose concentration is increases in the medium, the secologanin increases simultaneously with increase in the terpenoidindole alkaloid production (Contin et al. 1998). There are reports on improvement of indole alkaloid production in cell cultures of *C. roseus* treated by various elicitors (Zhao et al. 2005).

Tropane Alkaloids or Atropine

This are a class of alkaloids which possess a tropane ring in their chemical structure and occurs naturally in many members of family Solanaceae.

Shizanthus: Previous chemical studies on *Schizanthus* have shown that this genus accumulates a number of tropane-derived alkaloids such as hydroxytropane esters, dimeric- tropane-diesters, cyclo-butane-tricarboxylic acid triesters and pyrrolidine alkaloids (San-Martín et al. 1980, 1987; Gambaro et al. 1982, 1983; De la Fuente et al. 1988; Muñoz et al. 1991, 1994; Muñoz 1992; Muñoz and Cortés 1998; Griffin and Lin 2000). The well known effects of tropane alkaloids are as anticholinergic, antiemetic, para-sympatholytic and anesthesic agents (Hashimoto and Yamada

1992; Fodor and Dharanipragada 1994) have stimulated considerable interest during the last decades in the biosynthetic pathway leading to tropane alkaloids formation (Lounasmaa and Tamminen 1993). *Schizanthus hookeri*, *S. grahamii* and S. *tricolor* (formerly *S. litoralis*) are three Andean species that accumulate several tropane bases, in particular schizanthines (San-Martín et al. 1987; De la Fuente et al. 1988; Muñoz et al. 1991; Muñoz and Cortés 1998), and grahamine isolated from *S. grahamii* (Hartmann et al. 1990). Many efforts have been made to develop economically feasible methods for the production of tropane alkaloids by applying cell culture techniques (Zarate et al. 1997b; Christen 2000; Khanam et al. 2001). The main efforts toward the industrial preparation of tropane alkaloids by cell culture methods have concentrated on hyoscyamine and scopolamine (Lounasmaa and Tamminen 1993; Oksman-Caldentey and Hiltunen 1996; Oksman-Caldentey and Arroo 2000). The biosynthesis of tropane alkaloids has been extensively studied over the last decades (Robins et al. 1994). In particular, it has been demonstrated that the site of biosynthesis is the root, the alkaloids being translocated from the roots to the aerial parts of the plants (Hashimoto et al. 1991). Despite considerable efforts to produce secondary metabolites by undifferentiated plant cell cultures, it has become increasingly apparent that having a degree of morphological organization present greatly enhances the likelihood of successful product formation *in vitro* (Robins and Walton 1993).

Atropine and scopolamine are pharmaceutically important tropane alkaloids, exclusively produced by plants, and the industrial demand for them is strong (Oksman-Caldentey and Arroo 2000). Owing to their characteristic effects on the central nervous system and anticholinergic activities, they have well-established diversified therapeutic uses in the field of ophthalmology, cardiology, gastroenterology, etc. (Kursinszki et al. 2005).

***Atropa belladonna* L. and *Atropa acuminate* Royle ex Miers (Solanaceae)**: *A. belladona* is an endangered medicinal plant species native to Europe and Asia, normally serves as one of the main sources of these tropane alkaloids (Anonymous 1948) while *Atropa acuminate* Royle ex Lindl. is a native Asian species, which is also listed as an endangered medicinal plant due to its indiscriminate exploitation from the natural resources coupled with little or no attention towards any focused cultivation practice. *A. acuminate* closely resembles *A. belladonna* in its chemical profile and morphology, except in the flower colour, and official recognition of the use of its alkaloids was given in the fifth addendum to the *British Pharmacopia* (Anonymous 1948). Most importantly, the major alkaloids in the two species are the same; they are synthesized in the roots and take 3–4 years of field maturation of the plant for generation of extractable root mass (Khan and Harborne 1991). The prolonged gestation phase between planting and harvesting coupled with unpredictability in the supply of raw materials due to environmental and pathological hindrances and plant-to-plant discrepancies in the content of the active principles have led to the employment of different biotechnological tools for revealing an effectual substitute source of these important therapeutic molecules. Modern biotechnological techniques have been employed most extensively involving *Atropa belladonna* as a model system (Banerjee

et al. 2002; Bonhomme et al. 2000). *A. baetica*, another species of this genus, has also gained ample attention in terms of biotechnological intervention (Zarate et al. 1997a, b; Zarate 1999; Jaber-Vazdekisi et al. 2006).

However, in spite of the great resemblance of *A. acuminate* to *A. belladonna*, no biotechnological intervention has so far been directed towards the former. Amongst the different biotechnological tools implemented so far concerning both *A. baetica* and more particularly *A. belladonna*, *Agrobacterium rhizogenes* mediated 'hairy root' cultures have attracted major research attention the world over in the area of *in vitro* production of plant-derived secondary products (Guillon et al. 2006; Milen et al. 2007). The increased importance of hairy root cultures as advantageous alternative sources of plant-based compounds is mainly due to their faster growth rate, genetic and biochemical stability over long periods in culture and accumulation of products of a type and quantity equivalent to or even higher than those of the parent plant (Hu and Du 2006).

Vinca Alkaloids

***Catharanthus roseus* (L.) G. Don.**: is well known for vinblastine and vincristine, which are widely employed as chemotherapeutic agents against cancer. Various studies show the presence of other antineoplastic alkaloids in the plant (Piovan and Filippini 2000; Schiel and Berlin 1987). In the intact plant, vindoline is localized in the leaves and catharanthine is found in both the leaves and roots. Vinblastine is used to cure leukemia since 1950s and has increased the survival rate of childhood leukemias by 80 %. Another antileukemic drug, vincristine is also widely used. For alkaloid extraction, *in vitro* plant multiplication is an ideal approach to produce leaf material in large quantity (Debnath et al. 2006). Large scale fermentation and alkaloid production of cell suspension cultures has also been reported (Eilert et al. 1987; Schiel and Berlin 1987; Zhao and Zhu 2000). Reports on various factors on the release of secondary metabolites from roots of *Datura stramonium*, *Catharanthus roseus*, and *Tagetes patula* cultured *in vitro*, like effect of the medium pH (Sayavedra and Krikorian 2000), sucrose (Merillon et al. 1984) have been reported. Enhanced alkaloid production by transformed root cultures (Parr and Peerless 1988) and use of elicitors (Eilert et al. 1987) has also been performed. Quantitative analysis of various alkaloids produced *in vitro* and *in vivo has* been extensively done (Atta-ur-Rahman et al. 1998; Chu and Bodnar 1997; Datta and Srivastava 1997; Drapeau et al. 1987) in *Catharanthus roseus*.

1.2.2 Glycosides

According to IUPAC glycoside is any molecule in which a sugar group is bonded through its anomeric carbon to another group via a glycosidic bond. Glycosides can be linked by an O- (an *O-glycoside*), N- (a *glycosylamine*), S-(a *thioglycoside*), or C- (a *C-glycoside*) glycosidic bond. Glycosides play numerous important roles in

living organisms. Many plants store chemicals in the form of inactive glycosides. These can be activated by enzyme hydrolysis, which causes the sugar part to be broken off, making the chemical available for use. Many such plant glycosides are used as medications.

1.2.2.1 Glycosides Derived Through Tissue Culture and Their Plant Sources

Aloe saponaria var. saponaria: Akira et al. (1983) reported that a pale yellow callus tissue (370 g) cultured in the dark and showing a strong yellow fluorescence was harvested after 9 weeks. The MeOH extract (10 g) was subjected to CC on Amberlite XAD-2 using HO and MeOH as solvents, successively. The MeOH eluate (0.3 g) was chromatographed on Sephadex LH-20 eluted with Me, CO followed by Si gel eluted with 10 % MeOH-EtOAc to afford a mixture of glucosides (0.06 g, yield 0.016 7). The glucoside mixture was separated by prep. TLC to give glucosides of aloesaponol I-III. Each glucoside was identified by: (1) co-chromatography with a standard sample on TLC; and (2) hydrolysis with crude hesperidinase to give the aglycone which was identified by co-chromatography (TLC) with an authentic specimen.

***Aloe barbadensis* Mill.**: Akira et al. (1983) reported that callus tissue of *Aloe barbadensis* grown in the dark produced two new tetra-hydro-anthracene-glucosides, 3,4-dihydro-2,4,8,9-tetrahydroxy-6-methyl-1(2H)-anthracenone-4-O_P-D-glucopyranoside and 3,4-dihydro-2-methoxy-4,8,9-trihydroxy-6-methyl-l(2H)-anthracenone-4-O_B-D-glucopyranoside.

***Uncarla tomentosa* (Willd.) DC. and *Guettarda platypoda* DC.**: Aquino et al. (1989a, b) have isolated three new quinovic acid glycosides 1–3 from *U. tomentosa* (Cerri et al. 1988) and seven quinovic acid glycosides from *Guettarda platypoda* DC. (Aquino et al. 1988, 1989a, b). All these glycosides showed varied structures among which four groups can be discerned, including glycosides having a c-3, aC-28, a c-3,28, or a c-3,27 glycosylation pattern.

***Scrophularia nodosa* L.**: Characteristic for the genus *Scrophularia* is the accumulation of iridoid glycosides like aucubin, catalpol, and harpagoside (Pauli et al. 1995). Grabias et al. (1995) have reported the presence of the iridoidsharpagide, harpagoside, harpagide acetate in the seeds of *S. nodosa*. From a pharmacological point of view the iridoids are among the most interesting compounds in these plants. They show a variety of properties such as choleretic, vasoconstrictory, hepatoprotective, antiinflammatory, antiviral, and antimicrobial effects. The antibacterial and further biological activities of aucubin are probably attributed to the aglycone aucubigenin, which is formed after cleavage by β-glucosidase (Rischer et al. 1998; Wichtl 1989).

Drosera: Marczak et al. (2005) reported that from the extracts of *D. binata*, flavonoid glycosides and free aglycones were identified. On the basis of UV spectra and MS obtained from HPLC UV and from HPLC-MS analyses (in the positive and negative ion modes), four flavonol 3-*O*-monoglycosidesof methylated myricetin, quercetin, isorahmnetin orrhamnetin and kaemferol or fisetin, respectively, were identified.

A 3-*O*-diglycoside of the tetrahydroxy flavonol (containing hexose and deoxyhexose moieties) was identified, and the presence of the free aglycones was also established. The flavonoid aglycones and their conjugates have been reported as important components of extracts from other species of the genus *Drosera*, among them being myricetin, quercetinand kaempferol (Budzianowski 2000; Kolodziej et al. 2002; Kukulczanka and Budzianowski 2002).

***Stevia rebaudiana* Bertoni**: *Stevia rebaudiana* belonging to the family Asteraceae, is a perennial shrub which grows up to 1 m. The leaves of Stevia are the source of the diterpene glycosides, viz. stevioside and rebaudioside, which are estimated to be 100–300 times sweeter than sucrose (Ishima and Katayama 1976; Tanaka 1982).

***Carica papaya* L.**: Very little has been published on the chemical composition or biochemistry of *Carica papaya* (pawpaw) (Flath and Florrey 1977; Macleod and Pieris 1983; Marfo et al. 1986a, b). The glucosinolates, distributed among a number of plant families (Fenwick et al. 1983; Daxenbichler et al. 1991), come into contact with cytosolic thioglucosidases (myrosinases) on tissue disruption, leading to the formation of isothiocyanates, nitriles and thiocyanates with fungitoxic and insect behaviour-modifying properties (Wallsgrove and Bennett 1995). Benzylglucosinolate (glucotropaeolin) and its breakdown product benzylisothiocyanate, which are major metabolites of the Caricaceae (Gmelin and Kjaer 1970; Tang 1971; Tang et al. 1972) and may be useful in the chemotaxonomy of related species (Tang et al. 1972). Secondary metabolites in dried leaves of *C. papaya* included the expected benzylglucosinolate and also the cyanogenic glucoside sprunasin (derived from L-Phe) and tetraphyllin B (Spencer and Seigler 1984). *C. papaya* is thus one of the few examples known of a plant containing both glucosinolates and cyanogenic glucosides.

Drypetes: species from the Euphorbiaceae are also reported to contain glucosinolates and cyanogenic glucosides (Saupe 1981). The biosynthetic pathway(s) for glucosinolates and cyanogenic glucosides are probably dissimilar (Wallsgrove and Bennett 1995; Poulton and Moller 1993), though both pathways involve conversion of aminoacids to aldoximes. Formation of aldoximes for the biosynthesis of aliphatic/alkenylglucosinolates is catalysedin *Brassica* species by flavoprotein NADPH dependent monooxygenases (MO) (Bennett et al. 1993, 1995a, b, 1996). The enzymes involved in the formation of aldoximes for aromatic glucosinolates appears to be determined by the plant species. All *Brassica* species studied contain lavoprotein MOs (Bennett et al. 1996), but *Tropaeolum majus* and *Sinapis* species have a cyt P450 MO (Bennett et al. 1996; Du et al. 1995; Du and Halkier 1996). In initial studies of *C. papaya* an L-Phe-dependent MO was found (Bennett et al. 1996).

1.2.3 Flavones, Flavonoids, and Flavonols

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). The addition of a 3-hydroxyl group yields a flavonol (Fessenden and Fessenden 1982). Flavonoids are also hydroxylated phenolic

substances but occur as a C6-C3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection (Dixon et al. 1983), it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as for quinones. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya et al. 1996).

Catechins, the most reduced form of the C3 unit in flavonoid compounds, deserve special mention. These flavonoids have been extensively researched due to their occurrence in oolong green teas. It was noticed some time ago that teas exerted antimicrobial activity (Toda et al. 1989) and that they contain a mixture of catechin compounds. These compounds inhibited *in vitro Vibrio cholera* O1 (Borris 1996), *Streptococcus mutans* (Batista et al. 1994; Sakanaka et al. 1989; Sakanaka et al. 1992; Tsuchiya et al. 1994), *Shigella* (Vijaya et al. 1995), and other bacteria and microorganisms (Sakanaka et al. 1992, Thomson 1978). The catechins inactivated cholera toxin in *Vibrio* (Borris 1996) and inhibited isolated bacterial glucosyltransferases in *S. mutans* (Nakahara et al. 1993), possibly due to complexing activities as for quinones. Flavonoid compounds exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids such as swertifrancheside (Pengsuparp et al. 1995), glycyrrhizin (from licorice) (Watanbe et al. 1996), and chrysin (Critchfield et al. 1996) against HIV. More than one study has found that flavone derivatives are inhibitory to respiratory syncytial virus (RSV) (Barnard et al. 1993; Kaul et al. 1985). Kaul et al. (1985) provide a summary of the activities and modes of action of quercetin, naringin, hesperetin, and catechin in *in vitro* cell culture monolayers. While naringin was not inhibitory to herpes simplex virus type 1 (HSV-1), poliovirus type 1, parainfluenza virus type 3, or RSV, the other three flavonoids were effective in various ways. Hesperetin reduced intracellular replication of all four viruses; catechin inhibited infectivity but not intracellular replication of RSV and HSV-1; and quercetin was universally effective in reducing infectivity. The authors propose that small structural differences in the compounds are critical to their activity and pointed out another advantage of many plant derivatives: their low toxic potential. The average Western daily diet contains approximately 1 g of mixed flavonoids (Kuhnau 1976); pharmacologically active concentrations are not likely to be harmful to human hosts. An isoflavone found in a West African legume, alpinumiso-flavone, prevents schistosomal infection when applied topically (Perrett et al. 1995). Phloretin, found in certain serovars of apples, may have activity against a variety of microorganisms (Hunter and Hull 1993). Galangin (3,5,7-trihydroxyflavone), derived from the perennial herb *Helichrysum aureonitens*, seems to be a particularly useful compound, since it has shown activity against a wide range of gram-positive bacteria as well as fungi (Afolayan and Meyer 1997) and viruses, in particular HSV-1 and coxsackie B virus type 1 (Meyer et al. 1997). Delineation of the possible mechanism of action of flavones and flavonoids is hampered by conflicting findings. Flavonoids lacking hydroxyl groups on their b-rings are more active against microorganisms than are those with the 2OH groups (Chabot et al. 1992); this finding supports the idea that their microbial target is the membrane. Lipophilic compounds would be more

disruptive of this structure. However, several authors have also found the opposite effect; i.e., the more hydroxylation, the greater the antimicrobial activity (Sato et al. 1996). This latter finding reflects the similar result for simple phenolics. It is safe to say that there is no clear predictability for the degree of hydroxylation and toxicity to microorganisms.

1.2.3.1 Flavonoids Derived Through Tissue Culture and Their Plant Sources

Eucalyptus gunnii **Hook**: The flavonoids were identified on the bases of UV absorption spectra in MeOH alone or in the presence of diagnostic reagents (MeOH + sodium methoxide; MeOH + $AlCl_3$; MeOH + $AlCl_3$ + HCI; MeOH + sodium acetate; and MeOH + NaOAc + H_3BO_3) (Mabry et al. 1970) and chromatographic behavior on reverse phase HPLC. The concentration of the flavonoid glycosides was quantitatively determined by means of a Hitachi double beam spectrophotometer (model 150–20). For the latter purpose the molar extinction coefficient of each substance (e) In MeOH was employed (Mabry et al. 1970). The molar extinction coefficient of the standards purchased from Sarsyntex (France), expressed as X max (log e) in MeOH (Mabry et al. 1970) was used during the quantitative analysis of the plant flavonoids after identification; the values obtained were the following: for quercitrin, 349.2 (4.13) and for isoquercitrin, 357.6 (4.01).

Vaccinium: The tissues of this plant were adapted for continuous cell cultures producing high volumes of flavonoids, including anthocyanin pigments (Fang et al. 1998, 1999). Several members of the genus *Vaccinium*, including cranberries, blueberries, bilberries, and lingonberries, have been established as functional food sources of bioactive flavonoid compounds (Bomser et al. 1996; Prior et al. 1998), and the ability of cell culture extracts to inhibit the initiation stage of carcinogenesis was established (Smith et al. 1997; Madhavi et al. 1998).

Scutellaria baicalensis **Georgi**: a laminaceous perennial herb native to the region of north China and Siberia, contains a variety of flavone glycosides; most of them are glucuronides (Takido 1987). Two new flavone glucosides, 5,2′,6′trihydroxy-6,7 - dimethoxyflavone 2′-O-ß-D-glucopyranoside and 5,2′,6′-trihydroxy-6,7,8-trimethoxyflavone 2′-O-ß-D-glucopyranoside, which were structurally interesting chemicals having the glucose moiety at the B-ring position, were isolated from the roots (Ishimaru et al. 1995). In spite of some biosynthetic studies on glucuronide flavones of callus cultures (Yamamoto 1991) and experiments on flavonoid production in hairy root cultures (Zhou et al. 1997) of this plant, an attempt on the modification of flavonoid metabolism using transformed tissue cultures has not been done. In this study, the adventitious root and hairy root [transformed by *Agrobacterium rhizogenes* A13 strain, which integrates ß-glucuronidase (GUS) genes into the plant genome] cultures of *S. baicalensis* were established and their flavonoid production (both S. and glucosides) in various culture conditions was investigated.

Dried roots of *Scutellaria baicalensis* are a very old and well known drug in traditional Chinese medicine for the treatment of bronchitis, hepatitis, diarrhoea and tumours (Chiang Su New Medical College 1977; Tang and Eisenbrand 1992). Many papers reported that flavonoids from the roots of this species have an inhibitory action against human immunodeficiency virus (HIV-1) (Li et al. 1993), human T cell leukemia virus type I (HTLV-I) (Baylor et al. 1992) and mouse skin tumour promotion. In this context, the production of bioactive flavonoids by plant tissue and organ cultures is an important and promising aspect of modern biotechnology. Studies on calli of *S. baicalensis* have been reported (Yamamoto et al. 1987; Seo et al. 1993). Hairy root cultures are considered to offer better prospects for the commercial production of secondary metabolites than undifferentiated cell cultures (Flores et al. 1987; Toivonen 1993). Moreover, there are a few reports that hairy roots have been induced from species of Labiatae (Tanaka and Matsumoto 1993); hairy root cultures have been used to produce flavonoids (Toivonen and Rosenqvist 1995).

Zhou et al. (1997) reported the establishment of hairy root cultures of *S. baicalensis* and transformation that was proved by PCR (Williams and Ronald 1994). Growth of *S. baicalensis* hairy root cultures was best in B5 liquid medium. They also reported the structure of a new flavone glucoside5,7,2′,6′-tetrahydroxyflavone 2′-O-fl-D-ghicopyranoside determined by UV, LH and 13C NMR spectral evidence. Fifteen known flavanoids and five known pheny-lethanoids were also isolated.

1.2.4 Terpenes

Terpenes, also known as terpenoids or isoprenoids, represent the oldest group of small molecular products and are probably the most widespread group of naturally occurring organic chemicals comprising around 30,000 substances (Degenhardt and Gershenzon 2003), most abundant as compared to other metabolites (Fig. 1.7) and have many functions in the plant kingdom and in human health and nutrition (Roberts 2007).

The terpenoids play different types of functional roles as plant hormones (e.g., gibberellins, abscisic acid), photosynthetic pigments (e.g., phytol, carotenoids), electron carriers (e.g. plastoquinone), mediators of polysaccharide assembly (e.g., polyprenyl phosphates) and structural components of membranes (e.g. phytosterols) (MacGarvey and Croteau 1995). In addition many specific terpenoid compounds serve in communication and defense (Harborne 1991; MacGarvey and Croteau 1995).

Terpenoids are available in relatively large amounts in the form of essential oils, resins, waxes, camphor, menthol, pyrethrins (insecticides), cleaners, antiallergenic agents, and solvents, rosin (a diterpene), limonene, carvone, nepetalactone (in catnip), hecogenin (a detergent), and digitoxigenin are also important terpenes (Croteau et al. 2000; Zwenger and Basu 2008). These are important renewable resources which

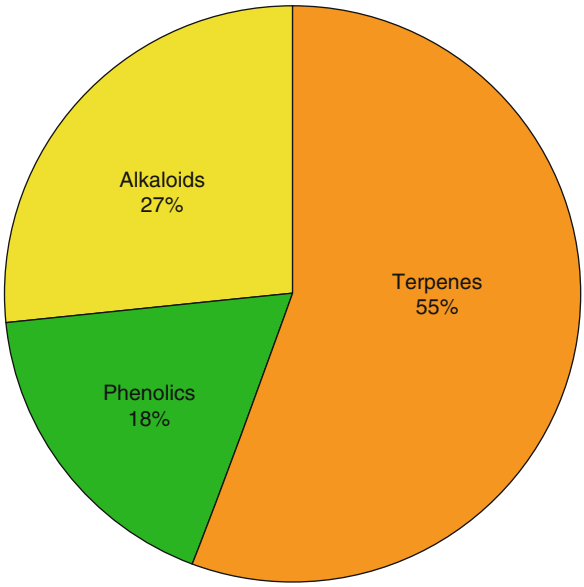

Fig. 1.7 Pie chart representing the major groups of plant secondary metabolites according to Croteau et al. (2000) and reproduced from Zwenger and Basu (2008)

provide a range of commercially useful products, including compounds of pharmaceutical companies as drug components, solvents, flavorings and fragrances, adhesives, coatings and synthetic intermediates (Dawson 1994; MacGarvey and Croteau 1995).

Although, all organisms naturally produce some terpenoids but most of terpenoids are of plant origin, and hundreds of new structures are reported every year (Sacchettini and Poulter 1997; Penuelas and Munne-Bosch 2005; Withers and Keasling 2007). Some terpenoids are, as such a part of primary metabolism, but many produce terpenoids via secondary metabolism (Goto et al. 2010). Plants have an enormous capacity to synthesize huge amounts of diverse terpenoids, particularly via the combination of the terpenoid biosynthetic route and other secondary metabolic pathways (Goto et al. 2010). Terpenoids are contained in many herbal plants and several terpenoids have been shown to be available for pharmaceutical applications, (e.g., artemisinin used for malaria medications and taxol for cancer) and for dietary use also (Mo and Elson 1999).

1.2.4.1 Terpenes Derived Through Tissue Culture and Their Plant Sources

Secondary metabolites, as terpenes, mostly include compounds produced by plants in response to stress (Keeling and Bohlmann 2006). Subsequently these compounds were exploited by humans for their beneficial role in a diverse array of applications (Balandrin et al. 1985; Zwenger and Basu 2008). Despite the fact of such economic importance of terpenoids the production rate is not upto the mark as to be used in present era of human populations alarming growth, henceforth, the demands are

not fulfilled as various terpenoid compounds are found in low yield from natural sources. In this backdrop, plant cell cultures have been investigated as an alternate production strategy (Roberts 2007). Production of secondary metabolite like terpenes and terpenoids through plant tissue culture, also known as phytoproduction, can be developed profitably for the production of these chemicals (Linden 2006).

Plant tissue culture being an *in vitro* technique allows clonal propagation of transformed clones and its applications includes inserting genes for plant secondary metabolites, including taxol (a diterpene alkaloid), a well-known anticancer agent have been well elaborated by Vaniserce et al. (2004) and Zwenger and Basu (2008). They pointed that *in vitro* cell culture methods provide systematic advantages like the ability to manipulate plant environment, regulation and extraction of metabolic products and control of cell growth. For producing terpenes in the laboratory, others have suggested to employ transgenic crops for terpenes synthesis and production, as reported by Zwenger and Basu (2008). Henceforth, genetic modification of *Arabidopsis* has been performed to study the production of different terpenoids by up regulating terpene synthase genes (Aharoni et al. 2003), which led to an increase and in depth understanding of how terpenes might function (Zwenger and Basu 2008).

United States US patent for the production of terpenes, terpenoids and/or small molecules in the transgenic glandular trichome-bearing plants have been reported which are being stored in the form of essential oils therein (Lange et al. 2012) and the role of tissue culture in this context would be golden opportunity for the increasing of such trichome-bearing plants via micropropagation and to be used for transgenic experiments. It has been reported that biotechnological cultivation, through cell culture studies and clonal propagation techniques, of plant cells and tissues can be used to increase the production of secondary metabolites (Yesil-Celiktas et al. 2010) and the terpenes and related compounds are not exceptions. Large scale production of these compounds, for commercial purposes, is usually done in bioreactors. But the major opportunity identified for this technique is that a wide variety of chemical compounds including aromatics, steroids, alkaloids, coumarins and terpenoids can undergo biotransformations using plant cells, organ cultures and enzymes (Giri et al. 2001). The metabolite pattern found in hairy roots is similar, if not always identical to that of plant roots. Plant cell cultures, associated with metabolic engineering of whole plants, is an effective tool to both increase terpenoid yield and alter terpenoid distribution for desired properties such as enhanced flavor, fragrance or colour (Roberts 2007). The production of terpenes (as secondary metabolite) is often associated with cell differentiation and therefore a possible strategy to incite transgenic differentiated organ culture and produce specific metabolites which are normally biosynthesized in these differentiated cells (Saito 1993).

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Chapter 2
Bioreactors: A Rapid Approach for Secondary Metabolite Production

Shiwali Sharma and Anwar Shahzad

2.1 Introduction

Plant secondary metabolites are important for plant interactions with their environments. These metabolites possess important properties such as antibiotic, antifungal, antiviral, UV protection, and pest deterrents. These chemicals have also been used by man for thousands of years as medicines. Today plants are responsible for the production of over 30,000 types of chemicals including pharmaceuticals, aromas, pigments, cosmetics, nutraceuticals and other fine chemicals. However, the quality and quantity of active substance from wild collected and field grown plants is often fluctuating and heterogeneous depending on environmental conditions. Infestations, diseases and the application of pesticides additionally decrease the quality of the plant materials. In this situation, plant cell and hairy root cultures are cited as the possible alternative source of secondary metabolite, since the environmental conditions those affect plant metabolism can be strictly controlled (Zhong 2001; Mulbagal and Tsay 2004). Plant cell cultures allow fully controlled production and downstream processing, without any risk of foreign gene dissemination in the environment or contamination by mammalian pathogens and a good public acceptance. They have also been used in several recent studies as a possible mean for the production of various recombinant proteins such as antibiotics, enzymes, vaccines and blood factors (Doran 2000; Choi et al. 2003; Gao et al. 2004; Hellwig et al. 2004). To date, about 20-plant made proteins (PMPs) have been produced in proof-of-principle studies (Marshall 2006). A landmark success in the manufacturing of plant cell culture based protein was the first registration of a PMP in February 2006, which was for the vaccine against "Newcastle Disease Virus" (Evans 2006).

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However, between 2.5 and 25-fold lower protein levels are achieved nowadays if plant cell and tissue cultures are used as productive organisms instead of the usual mammalian cells (Hellwig et al. 2004; Wurm 2005). Since, plant cell suspension and hairy root culture are used as "natural bioactive factories" to produce plant-derived compounds, thus standardization of an advanced bioreactor system for these two natural bioactive factories is a key step towards commercial production of secondary metabolites by plant biotechnology.

Traditionally, microbiologists have played the dominant role in bioreactor development with assistance from those in multiple disciplines, including biochemists, geneticists and chemical engineers. While the fermentation process-the precursor to modern bioreactors-has been used since prehistoric days, the major advancements of the last half century had as much to do with technology as with biology. The present chapter will discuss the advancements in bioreactor design for scale-up of plant cell and hairy root cultures for enhanced production of secondary metabolites.

2.2 Bioreactor Versus Chemical Reactor

The discoveries of Louis Pasteur soon led to the development of bioreactors for the large scale culture of bacteria and yeasts. 'Bioreactors' referred as mechanical vessels in which organisms or tissues are cultivated in liquid nutrient media under controlled environment. By definition, a bioreactor is a system in which a biological conversion is affected. This definition can apply to any conversion involving enzymes, microorganisms, and animal or plant cells. The bioreactors referred to here include only mechanical vessels in which (a) organisms are cultivated in a controlled manner and/or (b) materials are converted or transformed via specific reactions. Quite similar to conventional chemical reactors, bioreactors differ in that they are specifically designed to influence metabolic pathways. Traditional chemical reactor models and designs that may be used for bioreaction as well include: continuous stirred-tank reactors, continuous flow stirred-tank reactors, and plug-flow reactors, singularly or in series; ebullized-bed (*i.e.*, "bubbling and boiling") reactors; and fluidized bed reactors. The term bioreactor is often used synonymously with 'fermenter', however in the strictest definition; a fermenter is a system that provides an anaerobic process as for producing alcohol from sugar. In addition, bioreactors are designed to provide a higher degree of control over process upsets and contamination, since the organisms are more sensitive and less stable than chemicals.

Bioreactors differ from conventional chemical reactors in that they support and control biological entities. As such, bioreactor systems must be designed to provide a higher degree of control over process upsets and contaminations, since the organisms are more sensitive and less stable than chemicals. Biological organisms, by their nature, will mutate, which may alter the biochemistry of the bioreaction or the physical properties of the organism. Analogous to heterogeneous catalysis, deactivation or mortality occur and promoters or coenzymes influence the kinetics

of the bioreaction. Although the majority of fundamental bioreactor engineering and design issues are similar, maintaining the desired biological activity and eliminating or minimizing undesired activities often presents a greater challenge than traditional chemical reactors typically require.

Bioreactors and bacterial fermentation have played a vital role in the production of many medicinal products including the majority of antibiotics. The first large scale manufacture of antibiotics occurred during World War II with the production of penicillin. It was discovered early on that the most efficient production of penicillin occurred through fermentation processes rather than using direct chemical synthesis. Since this time many other antibiotics have been characterized and mass-produced using fermentation.

2.2.1 Mode of Bioreactor Operation: Batch, Fed-Batch, Chemostat and Continuous Perfusion

The bioreactor can be operated either in batch, fed-batch, chemostat or in continuous perfusion mode. In batch system all nutrients are supplied in the beginning of the culture. While, the fed-batch is started at a low volume and the culture is later supplied with concentrated feed solution to maximum volume and no medium is removed. In chemostat the culture is constantly supplied with fresh medium and used medium and cells are removed simultaneously. In perfusion culture, fresh medium is supplied at the same rate than spent culture is withdrawn (biomass is returned or retained in the vessel).

In batch the cells grow until essential nutrients becomes limiting and the cells and product are harvested. The cell densities are generally about 5×10^6 cells/ml. In fed-batch process the culture time may be longer (even 10–15 days) as nutrients are added during the cultivation. Cell densities of 10^7 cells/ml are achieved. In perfusion the nutrients are constantly added and also possibly inhibiting products are removed. Cell densities are significantly higher on perfusion processed (even $2–4 \times 10^7$ cells/ml) and culture time can be 15–75 days (Dalm 2007).

Theoretically speaking, continuous culture is most promising for obtaining high bioreactor productivity. Seki et al. (1997) and Phisalaphong and Linden (1999) demonstrated the enhancement of paclitaxel production by continuous cell culture of *Taxus cuspidata* and by using a semi-continuous culture of *Taxus canadensis* with total cell recycle, respectively. However, due to the practical problems of cell line stability and long-term sterile operation of bioreactors, such work is still limited in the laboratory. On the other hand, as shown by Van Gulik et al. (1992), a chemostat culture technique is useful to obtain reliable data on the stoichiometry of the growth of plant cells in a bioreactor. Several other groups worked with suspension-cultured plant cells by using semi-continuous or fed-batch cultures to achieve steady-state growth. In addition, Westgate et al. (1991) presented fed-batch cultivation kinetics for continuous approximation in *Cephalotaxus harringtonia* cultures.

For the production of therapeutic products, batch and fed-batch processes were used mainly, whereas for diagnostic products, the perfusion was preferred (Farid 2006). This might be because the therapeutic products are produced in larger scale than the diagnostic products and as Bibila and Robinson (1995) suggested that the fed-batch is simpler to operate than the perfusion in large scale and can still produce over ten times higher titers than the batch process. The perfusion systems are considered more difficult because of the continuous operation, requirement of a cell-retention device and long run time. In small to medium scales, the perfusion is more efficient way of production than the fed-batch.

2.2.2 Key Issues in Bioreactor Design and Operation

The goal of an effective bioreactor is to control and positively influence biological reaction. To accomplish this, the chemical engineer must take into consideration two areas. One is the suitable reactor parameters for the desired biological, chemical and physical (macrokinetic) system. The macrokinetic system includes microbial growth and metabolite production. Microbes can include bacteria, yeast, fungi, and animal, plant, fish and insect cells, as well as other biological materials.

The other area of major importance in bioreactor design involves the bioreaction parameters, including:

- Controlled temperature
- Optimum pH
- Sufficient substrate (usually a carbon source), such as sugars, protein and fats
- Water availability
- Salts for nutrition
- Vitamins
- Oxygen (for aerobic process)
- Gas evolution
- Product and byproduct removal

In addition to controlling these, the bioreactor must be designed to both promote formation of the optimal morphology of the organism and to eliminate or reduce contamination by unwanted organisms or mutation of the organism.

2.2.3 Advantages of Bioreactors

Bioreactors act as a biological factory for the production of high-quality products and provide many advantages listed as follows (Fulzele 2000; Su 2006):

- Controlled supply independent of plant availability
- Increased working volumes
- Homogeneous culture due to mechanical or pneumatic stirring mechanism

- Better control of culture due to mechanical or pneumatic stirring mechanism
- Better control of cultural and physical environment, therefore easy optimization of growth parameters such as pH, nutrient media, temperature, etc. for achieving metabolite production
- Reproducible yield of end product under controlled growth conditions
- Enhanced nutrient uptake stimulating multiplication rates and yielding a higher concentration of yield of bioactive compounds
- Simpler and faster harvest of cells
- The opportunity to perform biosynthetic and/or biotransformation experiments related to metabolite production with enzyme availability
- Easier separation of target compounds because of lower complexity of extract
- Better control for scale-up

2.3 Conventional Bioreactors

On the basis of three main classes of culture systems, bioreactors can be distinguished as follows;

- Microbial bioreactors
- Mammalian cell culture bioreactors
- Plant cell/organ culture bioreactors

The present chapter deals with bioreactors used especially for plant cell/organ culture system.

However, shake flasks (SFs) are considered the simplest and smallest type of bioreactor, but due to structural features and metabolite biosynthesis characteristics inherent to hairy root culture, different bioreactors have been designed different to those used for plant cell cultures (Shanks and Morgan 1999). In other words, bioreactor configurations have been designed according to the idiosyncrasies of the particular hairy root cultures.

Based on the continuous phase, reactors used to culture hairy roots can be roughly classified as liquid-phase, gas-phase and hybrid bioreactors. Mckelvey et al. (1993) were the first to study the fundamental differences between liquid and gas-phase reactors in delivering nutrients, including oxygen, to roots. They evaluated the effect of the reactor environment on hairy root growth and demonstrated that different culture environment affected biomass accumulation. Some of the modifications of these bioreactors are as follows:

2.3.1 Liquid-Phase Reactor

In liquid-phase reactors, roots are submerged in the medium. Some of reactors of this category are as follows:

2.3.1.1 STR (Stirred Tank Reactor)

STR is a mechanically agitated liquid-phase bioreactor. To supply oxygen for aerobic processes conducted in STRs, bubbles of compressed air are spared into the bioreactor. An aeration device is placed in the impeller region to produce a well-dispersed gas phase in the continuous liquid phase (Mishra and Ranjan 2008). Most of the large scale biosynthetic processes based on plant cell suspension cultures are carried out in STR, at different volumes, and their well-defined engineering parameters like heat and mass transfer with the means of impeller or turbine blades. Generally, STRs preferable for culturing plant cells rather than animal. As proof, the world's largest plant cell culture facility, situated in Ahrensburg, Germany, has a battery of STR, of up to 75,000 l in volume.

The plant cells are perhaps not as sensitive as animal cells to hydrodynamic forces but are more sensitive than microbial cells because they have a large volume (with a size even larger than animal cells) and rigid (inflexible) cell wall. They also tend to form even larger aggregates up to a few mm in diameter. The cell morphology and aggregate size are very dependent on the shear forces in the surrounding fluid. Therefore, plant cells are also considered to be shearing sensitive. In addition, plant cells in suspension culture tend to adhere to the walls of the culture vessels and accumulate at the head space. At high cell densities, the biomass volume can take up 50–90 % of the culture volume because of the high water content of plant cells. Moreover, it is not feasible for hairy root culture due to wounding and callusing as a result of shear stress caused by the impeller rotation (Taya et al. 1989; Hilton and Rhodes 1990).

To reduce the shear stress, recently some modified STRs have been developed. Modified STRs have flat blade turbines or large impeller and baffles that are agitated at a very low speed; alternatively hairy roots can be grown in a steel cage or mesh to isolate roots from the impeller inside the STR (Kondo et al. 1989). The need for protecting HRs (hairy roots) against the shear stress also depends greatly on the sensitivity of the particular root system which may vary from plant to plant. For the HRC (hairy root culture) of *Catharanthus roseus*, the application of a STR with a mesh-isolated impeller system was essential (Nuutila et al. 1994), whereas the non-fragile HRs of another *Catharanthus* species, namely C. *trichophyllus* were successfully cultivated in a simpler STR. A total of 10 g FW (fresh weight) of *C. tricophyllus* HRs was inoculated into the bioreactor. After a period of 9.5 weeks of culture, the yield of biomass was found to be about 2,500 g FW (Davioud et al. 1989).

Chattopadhyay et al. (2002) carried out submerged cultivation of *Podophyllum hexandrum* in a 3 l STR fitted with a low-shear setric impeller for the production of podophyllotoxin. They found that the substitution of sucrose by glucose resulted in higher growth and alkaloid production in the dark. 100 rpm was sufficient to mix the culture broth in the bioreactor without causing any significant cell damage. Biomass and podophyllotoxin accumulation in 3 l bioreactor under batch growth conditions were 6.5 g/l and 4.26 mg/l respectively in 22 days. This resulted in all overall podophyllotoxin productivity of 0.19 mg/l day, which represented an increase of 27 % in combined to its productivity in a SF. Successful scale-up of hairy root culture of *Glycyrrhiza glabra* achieved by Mehrotra et al. (2008) using 5 l capacity

mechanically agitated bioreactor provided with a nylon mesh septum. After 30 days of sterile run, 310 g of root biomass was harvested from bioreactor culture vessel, recording about 20 times increase over initial inoculums (16.0 g).

2.3.1.2 ALR (Air-Lift Reactor)

Also known as a tower reactor, an airlift bioreactor can be described as a bubble column containing a draught tube in which the reaction medium is agitated and aerated by the introduction of air or another gas mixture and the circulation is enhanced by internal draught tubes or external loops. Thus, the reactor volume is separated into gaseous and un-gaseous regions, generating a vertically circulating flow (IUPAC 1997). These are similar to STRs but lack impeller. Air is typically fed through a sparger ring into the bottom of a central draught tube that controls the circulation of air and the medium. Air flows up the tube, forming bubbles, and exhaust gas disengages at the top of the column. The degassed liquid then flows downward and the product is drained from the tank. The tube can be designed to serve as an internal heat exchanger, or a heat exchanger can be added to an internal circulation loop. Compared to the STRs, ALRs typically require less power for a given performance. ALRs are useful with relatively less viscous fluids and when there is a need for gentle agitation and low-cost oxygen transfer. Oxygen mass transfer acts as a major limiting factor in most liquid-phase reactors, since mechanical agitation and shear stress cause disorganization and callus formation which reduce the biomass productivity. Though, conventional ALRs have been widely used for scale-up cultures of HR lines (Buitelaar et al. 1991; Oka et al. 1992; Uozumi et al. 1995). Such reactors could not yield an optimum biomass of HRs mainly on account of root factors; first uneven distribution of root tissue at certain regions and secondly excessive gas-phase channeling, leading to blockage of liquid flow due to clumping of root tissue (Kim and Yoo 1993). Further, it has also been reported in the literature that HR biomass yields increased dramatically in ALRs if the roots were immobilized (Taya et al. 1989). However, the BCRs, even with a support matrix, failed to produce an optimum HR biomass of *Hyoscymus muticus* (McKelvey et al. 1993).

Airlift systems provide some advantages like:

- Simple design with no moving parts or agitator shaft seals, for less maintenance, less risk of defects and easier sterilization.
- Lower shear rate, for greater flexibility-the system can be used for growing both plant and animal cells.
- Efficient gas-phase disengagement.
- Large, specific interfacial contact-area with low energy input.
- Well-controlled flow and efficient mixing.
- Well-defined residence time for all phases.
- Increased mass-transfer due to enhanced oxygen solubility achieved in large tanks with greater pressures.
- Large-volume tanks possible, increasing the output.
- Greater heat-removal vs. conventional stirred tanks.

The main disadvantages are:

- Higher initial capital investments due to large scale processes.
- Greater air throughput and higher pressures needed, particularly for large-scale operation.
- Low friction with an optimal hydraulic diameter for the riser and down comer.
- Lower efficiency of gas compression.
- Inherently impossible to maintain consistent levels of substrate, nutrients and oxygen with the organisms circulating through the bioreactor and conditions changing.
- Inefficient gas/liquid separation when foaming occurs.

Plant cells have large vacuoles and slow growth, so hairy roots require comparatively low oxygen supply of about 0.05–0.04 vol of air/vol of liquid/min. ALR can supply the low O_2 demands with low shear effects. Humified air is passed through glass grid that functions as aerators. These have been found to be more successful for hairy roots than STR. ALR have several advantages such as combining high loading of solid particles, providing good mass transfer, relatively low shear rate, low energy requirements, and simple design (Jolicoeur et al. 1999). The major drawback is their unsuitability for high density plant cultures. Tikhomiroff et al. (2002) designed a two-liquid-phase bioreactor to extract indole alkaloids from *Catharanthus roseus* hairy roots with silicon oil. Partition studies between silicon oil and culture medium showed that the silicon oil did not alter the availability of nutrients. In their experimental setup, ALR with a closed loop configuration allowing the use of a small quantity of silicon oil was used. The affinity of tabersonine and löchnericine for silicon oil is nine times higher than for the aqueous phase. The growth of the hairy roots was not significantly modified by the presence of silicon oil. The overall specific yields of tabersonine and löchnericine were increased by 100–400 % and 14–200 %, respectively, with the use of silicon oil in non-elicited control cultures (Tikhomiroff et al. 2002).

A successful comparative study was performed in different types of ALRs-cone, balloon, bulb, drum and column-of 5 l capacity each. It has been reported (Shin et al. 2002) that the cone-type ALR gave the highest biomass of 27 mg DW/g of red beet (*Beta vulgaris*) HRs and the highest betacyanin accumulation. The production of betacyanin in balloon, bulb, drum and column bioreactors was reported as 18, 15, 9 and 7 mg DW/g respectively. The production of artemisinin was also investigated by Liu et al. (1998) in four different culture systems of 3 l capacity each, namely SF, BCR, modified BCR and modified ALR (inner-loop). The artemisinin contents of HRCs in the modified ALR (inner-loop), BCR and modified BCR bioreactor were found to be 0.536, 0.368 and 0.446 mg/l respectively. Thus, the artemisinin content was higher in a modified ALR and SF was 0.536 and 0.548 g DW/l respectively. The results show that optimizing the design of the ALR can accelerate growth and artemisinin accumulation.

Homogenous distribution of *Echinacea purpurea* hairy roots and high cichoric acid production was observed in a modified ALR by installing a stainless steel mesh draught tube with an average pore size 700 μm, slightly larger than hairy roots,

about 500 μm (Abbasi et al. 2009). Improved root growth and cichoric acid production were improved by increasing the aeration rate from 0.002 m^3 h^{-1}. The hairy root cultures in the modified bioreactor exposed once to 6 min of ultrasound treatment at day 20 gave the highest biomass accumulation of 12.8 g l^{-1}, which resulted in the maximum cichoric acid production of 178.2 at 30 days.

These results show the practical feasibility of ALRs for the large-scale production of HRs.

2.3.1.3 BCR (Bubble Column Reactor)

It is a column shaped reactor in which the roots are submerged in the medium. Liquid mixing is obtained by the up flow of air bubbles generated from an air distributor situated at the bottom of the column. Thus, like an ALR, in a bubble column the bubbles create less shear stress, so that it is useful for organized structures such as shoots, bulbs, corms, tubers and hairy roots. In this case, the bubbling rate needs to be gradually increased with the growth of hairy roots. Moreover, the division of a bubble column into segments, and installation of multiple spargers increases the mass transfer (Buitelaar et al. 1991).

Kwok and Doran (1995) cultured *Atropa belladonna* hairy roots in a modified 2.5 l multi-compartment BCR for analysis of growth kinetics, stoichiometry and atropine production. Average biomass density reached 9.9 g l^{-1} DW after 43 days of batch culture; local root densities in some parts of the vessel were considerably higher, up to 17 g l^{-1}. Specific atropine content in the roots varied from 4.1 mg g^{-1} DW at the beginning of the culture to 1.4 mg g^{-1} after 28 days; 35 mg or 14 mg l^{-1} atropine was produced over the 43-days culture period.

The main advantages of BCRs are the low capital costs, uncomplicated mechanical configurations and not to high operational costs due to low energy requirements. However, common they are less suitable for the processes where highly viscous liquids exist.

Recently, Deng et al. (2009) successfully used an illuminated BCR (300 ml) to propagate the transgenic line of *Laminaria japonica* (kelp) gametophyte in which a human acidic fibroblast growth factor gene (*hafgf*) was successfully transferred via biolistic gene gun. Using a batch culture having an initial dry cell density of 129.75 mg l^{-1}, a maximum of 1,695 mg l^{-1} was obtained. This was achieved using an aeration rate of 1.08 air min^{-1} l^{-1}culture in a medium containing 1.5 mM inorganic nitrate and 0.15 mM phosphate.

2.3.1.4 BTBR (Balloon Type Bubble Reactor)

The disadvantages of ALRs and BCRs are foaming induced by large volumes of air, and growth of cells in the head space. The phenomenon of foaming and cell growth on the wall of the vessel is due to the diameter of the vessel and the top of the vessel being the same (Paek et al. 2001). To overcome this problem ALRs was modified in

such a way that it has a larger top-section diameter. By using a concentric tube for cell lifting at the riser part of the vessel base, foaming was much reduced. This bioreactor was found to be reliable for cell, tissue and organ culture. Pilot scales BTBR of 300, 500 and 1,000 l have been utilized for the production of biomass of various valuable plant species (Paek et al. 2001). Choi et al. (2000) were the first who successfully achieved 150-fold enhanced growth when ginseng adventitious roots were grown in 500 l BTBR for 7 weeks. The adventitious roots grown in bioreactors contained 1 % of dry root weight, which corresponds half of the content for the field grown plants.

Cui et al. (2010) observed enhanced production phenols, flavonoids, hypericins and chlorogenic acid through biomass enhancement of hairy roots of *Hypericum perforatum* in a 3 l balloon type bubble bioreactor (BTBB), a modified ALR. They investigated the effects of different concentrations of plant growth regulator combinations, inoculums sizes and Murashige and Skoog (MS 1962) medium dilutions on biomass and accumulation of total and flavonoids in hairy roots. By inoculating 6 g/l of hairy roots into BTBB containing half-strength MS supplemented with 0.1 mg/l kinetin with 1 mg/l IBA, 104.2 g/l adventitious roots were harvested containing 56.47 mg/g DW total phenolics, 35.01 mg/g DW flavonoids, 0.97 mg/g DW chlorogenic acid and 1.389 mg/g DW hypericin. Similar results were obtained with ginseng, *Eleutherococcus sessiliflorus* and *Echinacea purpurea* (Paek et al. 2005; Abdullah et al. 2005; Wu et al. 2007).

2.3.1.5 EFR (Ebb and Flood Reactor)

A novel type ebb and flood reactor system (a periodic immersion system) was developed for the mass propagation of several plant species (Paek et al. 2005). In this type of bioreactor, a supporting net was use to hold the plant material in order to avoid the complete submersion of expants in the liquid medium. In this system, medium is pumped from a storage tank into the culture vessel. A series of channels helps to supply nutrient solution evenly to the plant material, resulting in uniform growth. The medium remains in the vessel for a few minutes, after which it drained back to the storage tank for re-use. The drainage process is controlled by a solenoid valve at intervals of between 4 and 8 h, depending on plant species and explants type.

2.3.1.6 CFR (Convective Flow Reactor)

This reactor was developed by Carvalho and Curtis (1998) consisted of a stirred tank, a peristaltic pump and a tubular culture chamber. In the stirred tank, the medium was oxygenated and a positive displacement pump re-circulated the liquid between the stirred tank and tubular reactor. After 30 days of culture, 550 g fresh weight per liter (FW l^{-1}) of *Hyoscymas muticus* hairy roots were harvested, this is more than a 79 % increase in biomass compared to BCR. Although CFR showed improved performance compared to a BCR, it may not be a realistic large-scale

system due to pressure required to circulate the culture medium at a high enough velocity to overcome the flow resistance of the root bed (Carvalho and Curtis 1998).

2.3.1.7 TBR (Turbine Blade Reactor)

This is a combination of ALR and STR. Here cultivation space is separated from agitation space by stainless steel mesh, so that hairy roots do not come in contact with impeller and air is introduced from the bottom and dispersed by an eight-blade impeller that stirs the medium. This is efficient for hairy roots of carrot (Kondo et al. 1989). Amongst various bioreactors, Kondo et al. (1989) found that TBR as an advantageous due to high oxygen transfer ability. After 30 days of culture 10 g/l of hairy root cell were obtained in each tested reactor but the maximum growth rate was found (0.63 g/l per day) for TBR.

2.3.1.8 RDR (Rotating Drum Reactor)

RDRs have significantly higher surface area to volume ratios than other reactor types. As a consequence, mass transfer is achieved with comparably less power consumption, according to Danckwert's surface renewal theory (Danckwert's 1951). These features are favorable for bioprocesses utilizing shear-sensitive tissues, as well as for photo bioreactors (Sajc et al. 2000). This consists of a drum-shaped container mounted on rollers for support and rotation. The drum is rotated at only 2–6 rpm to minimize the shear pressure on hairy roots. Kondo et al. (1989) used this system for hairy root of carrot. In preliminary experiments, they observed that hairy root cells adhering to the wall of the reactor were lifted above the liquid medium as the drum rotated. Cell disruption occurred due to these repeated drops and growth was slower. However, most of cells did not become detached from wall of the reactor as the hairy roots grew and the cell growth become active, but overall growth was slow. To improve the slow growth, a polyurethane foam sheet was fixed onto the surface of the drum, to which hairy roots get attached. With this modification, the growth rate was significantly improved and an improved growth rate of 0.61 g/l per day was recorded.

Draw backs of liquid-phase reactor: Following are the main draw backs of liquid-phase reactor:

- In liquid-phase reactor, it has been shown many times that oxygen deficiency due to mass transport limitation is a growth-limiting factor (Yu and Doron 1994; Yu et al. 1997) the problem increases as the scale of the reactor increases.
- Sparged air bubbles can be captured enlarged and remain entrapped in root clumps, resulted in gas flow channeling depletion of oxygen (Flores and Curtis 1992; Singh and Curtis 1994).

Direct evidence for oxygen deficiency in SF and BCR has been observed by Weathers et al. (1999), who measured mRNA transcripts of alcohol dehydrogenase,

an indicator of oxygen deprivation. They showed that in both liquid systems, oxygen was limiting. Thus, Kwok and Doran (1995) modified the simple BCR for improving the supply of oxygen to roots. Gas was introduced at multiple points to a BCR that was divided into three segments with wire mesh with each segment containing a sparger. Although 9.9 g dry weight per liter (g DW l^{-1}) of *Atropa belladonna* hairy roots were harvested after 43 days bulk mixing was poor even as early as day 14. Later, Kwok and Doran (1995) investigated the use of micro- porous polypropylene membrane tubing that was inserted into a gas-driver reactor along with a sparger to provide supplementary oxygen to the root bed.

2.3.2 Gas-Phase Reactor

In such reactors roots are exposed to air or other gas mixture. Nutrients (medium) are usually delivered to roots as droplets. However, there is considerable variation in the size of droplets. Since the continuous phase is gas, the roots must be immobilized in the reactor. DiIorio et al. (1992), Liu et al. (1999) and Woo et al. (1996) used horizontal sheets of mesh and at inoculation roots were spread onto mesh. Alternatively, Williams and Doran (2000) used vertical sheets. However, Chatterjee et al. (1997) cultured roots on packing rings made of nylon mesh in a shake flask for a week so that roots became anchored to the mesh ring. Few gas-phase reactors are as follows:

2.3.2.1 NMR (Nutrient Mist Reactor)

The concept of nutrient mist culture system was first suggested by Weathers et al. (1988). Mist bioreactor is a possible alternative to submerged culture in airlift system. NMRs are gas-phase reactors in which plant organ culture is dispersed in an air phase by immobilization on a mesh supported and the liquid medium is introduced in to the bioreactor as a mist-phase using ultrasonic transducers producing very size droplets of a few micrometers (0.5–30.0 μM). Due to continuous gas-phase in spray and mist reactors, internal root anchor matrices (often horizontal mesh trays and cylindrical stainless steel meshes) are required in all circumstances. NMR offers definite advantages for hairy root culture such as easy operation, low shear stress wherein root receive rapid replenishment of nutrients and removal of toxic metabolites and ease of scaling up. In a NMR, gas exchange can be enhanced. Thus, the limitation of gas exchange is no longer as serious as those in other conventional bioreactor.

Roots grow well when mist is supplied on an intermittent basis and a misting period lasting from 1 to 5 min is followed by a period when both transducer and the gas flow are shut off. Because the continuous phase is air rather than a liquid, the availability of oxygen is not likely to limit root growth until beds are extremely dense. Instead, droplet transport and deposition in the root bed during the mist

supply phase may limit growth if an adequate supply of nutrients does not reach the surface of all roots. Thus, mist deposition is a key step in the mass transfer of nutrients to the roots. Some researchers have demonstrated the advantages of mist culture system in plant tissue culture. Weathers and Giles (1988) reported that various plants were successfully regenerated and efficiently increased in number by using mist culture. Woo and park (1993) also obtained good results from culture of *Dianthus caryophyllus* multiple shoots in a mist bioreactor system. Regarding the mist culture of potato, Kurata et al. (1991) reported the better growth of potato shoots in a simplified mist culture system. Later on, Hao et al. (1998) provided the protocol for propagation of potato tubers in a newly designed NMR by using two culture methods. Such modified NMR provided an environment supporting multiple-layer culture. The percentage of the inocula eventually forming tubers in a two-step method was 98 % with only 54 % being formed in a one-step method. However, NMR was first used by DiIorio et al. (1992) for hairy root culture.

Whitney (1992) compared the performance of various bioreactors for cultivating hairy roots of *Nicotiana tabacum* (tobacco) and *D. stramonium* and found the effective growth rate and yield of tobacco hairy roots in NMR than STR, TBR and ALR. Hairy roots of *Stizolobium hassjoo* were cultivated in 3 and 9 l NMR by Huang et al. (2004) to investigate the effects of intermittent medium supply, oxygen uptake rate and other growth-related parameters. The biomass concentrations achieved in an SF, a 3-l NMR and a 9-l NMR were 6.0, 9.8 and 9.1 g DW/l respectively. These results indicate that the NMR is more favorable for HRC than an SF.

In a comparative study by Kim (2001), *Artemisia annua* hairy roots grown in NMR produced nearly three times as much artemisinin as roots grown in BCR, 2.64 and 0.98 μg/g DW respectively. In the study of Suresh et al. (2005), the final root densities of *Tagetes patula* was compared in various bioreactors: an AMR (acoustic mist reactor), a BCR, a NMR and an SF. The biomass concentrations achieved in these reactors were 5.4, 1.47, 1.12 and 6.9 g DW/l respectively. The design of AMR enables efficient mist generation with high energy efficiency.

LCMRs (low-cost mist reactor), such as that marketed by ROOTek Bioactives AG (Witterswil, Basel, Switzerland) are a recent development in the field of biotechnology in which hairy root cultures can be cultured under ideal conditions. LCMR having a cultivation chamber of plastic film (Wink et al. 2005). Unlike the conventional bioreactor, the roots in the LCMR are grown under sterile conditions. There is no need for herbicides and insecticides. Its modular construction renders the LCMR system very flexible (Mishra and Ranjan 2008).

Advantages of gas-phase reactors: Following are few merits of liquid-phase reactor;

- The oxygen transfer limitation can be reduced or eliminated for growing hairy roots (Weathers et al. 1999).
- Low hydrodynamics stress (Towler et al. 2006).

Disadvantages of gas-phase reactors:

- There is no way to uniformly distribute the roots in the growth chamber without manual loading (Kim et al. 2002).

2.3.3 Hybrid Reactor

In hybrid reactors, the roots are exposed to liquid phase and then grown in a gas-phase environment. Ramakrishnan et al. (1994) proposed the solution for the above mentioned disadvantage of gas-phase reactor by using a hybrid reactor. Initially the reactor works as a BCR in order to suspend, distribute and attach roots to the packing rings in the reactor. After 2 weeks growth, root clumps were dense and the reactor was switched to a trickle-bed operation, thus exposing roots to a gas environment.

The most cited and largest hybrid bioreactor (bubble column-spray reactor) to grow hairy roots (*Datura stramonium*) is the 500 l Wilson-Bioreactor (Wilson 1997). Later on, Wilson (1997) followed the suggestion given by Ramakrishnan et al. which was the exploitation of both liquid and gas-phase reactors for hairy root culture of *Datura stramonium*. He cultured *D. stramonium* hairy roots in a 500 l hybrid reactor that used submerged culture for 21 days followed by a droplet-phase for 40 days. It is noteworthy that in this experiment the root inoculation step was completed mechanically and required no manual labor. In a seed vessel placed on the top of the 500 l reactor, the roots were grown as inoculums for the large-scale reactor. A helical screw with a blade located in the bottom of the seed vessel was used to cut and transfer the roots to the reactor. The main reactor contained assemblies of wire chains and bars to immobilize the roots. The distance between each assembly line (the assembly ran from top to bottom) was determined by effective volume, i.e., the volume that can be filled by roots from a single inoculation point. This parameter depends on the species and must be determined empirically. At the end of a run a total of 39.8 kg fresh weight of hairy roots was harvested, yielding 79.6 g FWl^{-1} packing density. This is not the highest packing density obtained for hairy roots, but it demonstrates that with appropriate design, a large-scale hairy root culture is possible.

2.4 Advancement in Bioreactor Technology: Disposable Bioreactors

"Disposable bioreactors" mean single-used sterile plastic bags that are inoculated, fed and aerated through plastic vents and that are generally mixed by keeping them on a swing. Cultivation containers of disposables are typically made of Food and Drug Administration-approved biocompatible plastics (e.g., polyethylene, polystyrene, polyetrafluorethylene, polypropylene). Sometime, plastic cultivation container which are reusable and intended for multiple usages [e.g., CIRAD's RITA bioreactor (Alvard et al. 1993) or Rootec's Low Cost Mist Bioreactor (Wink et al. 2005)] are not recognized as disposable.

Although disposable bioreactors are mainly used for cell expansions, glycoprotein secretions and virus generations realized with mammalian and insect cell lines, there are several reports delineating their suitability for the cultivation of plant cell and organs (hairy roots, meristematic clusters, somatic embryos).

Generally disposable bioreactors are used for small-medium scale production e.g., Wave bioreactor (Wave Biotech, USA) can be used for 500 l production (Weber et al. 2002), disposable stirred tank bioreactors (Hyclone and Baxter, USA) are for use for 250 l cultivations may be even 1,000 l (Aldridge 2005). XDR (Xcellerex, Marlborouh, USA) single-use bioreactors are today available at 200–2,000 l scale (Anonymous 2008). For plant cell cultures flexible bags up to 100 l culture volume prevail as single-use cultivation containers (Eibl and Eibl 2009a, b). For optimum mass transfer and cell growth, these bags are usually mechanically or pneumatically driven. In mechanically driven bioreactors mass and heat transfer is controlled by internal and external mechanical devices, while in pneumatically driven bag bioreactor mass and heat transfer is achieved by direct sparging with air or gas. Few disposable bioreactors and their modifications can be categories under following classes:

2.4.1 Wave-Mixed Bag Bioreactors

Due to the movement of their one-dimensional platform inducing a wave in the disposable bags (Cellbags®), these bioreactors are referred as wave-mixed bag bioreactors. The first prototype of a bag bioreactor is the Wave Bioreactor® (BioWave) introduced by Wave Biotech limited company in 1988. It is the most widely used in cultivations aimed at rapid production of undifferentiated bioactive cells, secondary metabolites and recombinant proteins (Eibl and Eibl 2006, 2008). This can be explained by the availability of special bags which take into account the physiology and morphology of plant cell and tissue cultures, namely their frequent growth and product formation.

In this system, culture bags are inflated and rocked to provide oxygen transfer and mixing. These culture bags have an enlarged port and a screw cap allowing trouble-free inoculation as well as sampling of liquid cultures with non-Newtonian behavior. Moreover, bags with additionally integrated nylon mesh (which acts as a matrix supporting the self-immobilization of the roots and thereby improving their growth) have been found to be suitable for hairy root-based cultivations (Eibl and Eibl 2006). Specific power input and shear stress greatly depended on the filling level of the culture bag. This means that shear stress is highest in the BioWave when the culture bag is rocking with minimum filling level at maximum rocking angle and rocking rate. Eibl et al. (2003) observed the superiority of BioWave to surface or bubble-free aerated cell culture bioreactors made of glass or stainless steel. They firstly used BioWave for MTA cell suspension (suspension of genetically modified BY-2 cell secreting a full length antibody at 26 °C and 0.5 vvm air in the dark). Thereafter, plant suspension experiments aimed at the expansion of bioactive grape and apple cells for cosmetics (Schürch et al. 2008; Cuperus et al. 2007), secretary accumulation of paclitaxel and baccatin III with European yew cells (Eibl and Eibl 2008; Bentebibel et al. 2005; Bonfill et al. 2007) and barley cell-derived expression of human collagen (Ritala et al. 2008) were realized in the BioWave.

Terrier et al. (2007) modified this BioWave rector into another flexible disposable bioreactor, the Wave and Undertow (WU) bioreactor for the cultivation of soya

cells up to 100 l working volume. It consists of a large flexible plastic container partly filled with medium and inflated with air. The system is located on a horizontal table of variable length and equipped on one side or each side with a platform rose periodically. The intermittent rising movement of the platform to the rest point and down/descending movement back to initial position enable continuous mixing and aeration through the wave/undertow motion; the platform ascension leads to the wave formation; the wave propagates through the bags, bounces off at the extremity creating an undertow which returns to the initial point. This action is repeated, creating a new impulse to ensure persistence of flow within the WU bioreactor. Wave/undertow induction provides liquid culture mixing and bubble-free aeration. The oxygen transfer is accomplished by transport from the headspace air to the liquid culture and enables growth without apparent shear stress. Platform movements are simply achieved by pneumatic jacks located under the platform.

2.4.2 Stirred Bag Bioreactors

Although stirred bioreactors made of glass or stainless steel are the most frequently used types for submerged cell cultures and in particular plant suspension cells (Eibl and Eibl 2002; Su 2006), only difference to their plastic counterparts in animal cell derived productions (Card and Smith 2006; Thermo Fisher Scientific 2007; Collignon et al. 2007; Eibl and Eibl 2009a, b) were found. All four commercially available systems; the XDR-Disposable Stirred Tank Bioreactor (Xcellerex), the Thermo scientific Hyclone Single-Use Bioreactor (SUB), the BIOSTAT CultiBag STR (Sartorious Stedim) and the Pad-Drive disposable Bioreactor (Artelis ATMI Life Sciences) are basically equipped with aeration devices (micorsparger or sparger ring) and rotating or tumbling impellers. The cylindrical or cubical culture bag, which in addition has gas filters and ports for integration of sensor probes and line sets, is always shaped and fixed in a customized steel support container with cooling and heater jacket. Unfortunately, only mixing characterization results and scale-up parameters of the 50 and 250 l SUB has been published to date (see Eibl et al. 2009).

2.4.3 Pneumatically-Driven Bag Bioreactors

They were the first disposable bioreactors for plant liquid cultures at Liter-Scale. These are simpler in design and more scalable than the described stirred bag system. Osmotek has already introduced the LifeReactor in the early 1990s (Ziv 1999). In the LifeReactor, a bubble column bioreactor for micropropagation, mass and heat transfer is achieved by direct sparing of the conically shaped disposable culture bag. Organ cultures such as meristematic clusters and somatic embryos were grown in this bioreactor to produce propagules of ornamental, vegetable and woody plant species for subsequent plant regeneration and transfer to greenhouse. These propagules include fern, Gladiolus, lilies, orchids, potatoes and bananas (Ziv et al. 1998,

Ziv 1999, 2000, 2005) within 1 month cultivation cluster biomass increased up to eightfold (Ziv 2005). Ziv points out that, in comparison with glass bubble columns, which are often used in organogenic micropropagations (Takayama and Akita 2006; Paek et al. 2005; Prasad 2007), the LifeReactor has increased proliferation and reduced shearing and foaming (Ziv 2005). Bearing in mind the problem of hyperhydricity (Etienne et al. 1997) for submerged organ cultures, the LifeReactor bags, ebb-and-flow mode could be easily implemented. The temporary immersion version of the LifeReactor, named the Ebb-and-Flow Bioreactor, allows hyperhydricty control by manipulating the frequency and duration of immersion.

2.4.4 Box-In-Bag Bioreactors

These bioreactors are regarded as special case because it can be both pneumatically and mechanically agitated. The horizontally used Box-In-Bag bioreactor is a two-compartment system. It is composed of one disposable bag (with two ports for inoculation, and air/medium inlet and outlet) into which a plastic box with a perforated bottom has been introduced. The inoculated propagules are proliferated on polyurethane foam disks by periodic ebbing and flowing in this box. For this, overpressure of sterile air is applied to push the culture medium from the storage container into the bioreactor, as well as to return it. First experiments confirmed Box-In-Bag bioreactor's suitability for pilot-scale production of pre-germinated Robusta somatic embryos. Biomass and the number of embryos were higher than in 10 l glass jar systems (Ducos et al. 2007a, b).

Advantages of the disposable bioreactor: The main advantage of the disposable bioreactor is that the cleaning and sterilizing issues are removed and that the investment costs are minimized (Farid et al. 2005). There is no doubt that the use of disposable bioreactors guarantees high flexibility and contributes to both time (set-up times, development times, and production turnaround times) and 30–40 % cost saving (Eibl and Eibl 2006, 2007; DePalma 2006; Flanagan 2007; Morrow 2007). Consequently, the process economics can be improved by disposable bioreactors. In addition, there is low contamination risk for cross-contamination which results in higher process safety. Most of the biopharmaceutical facilities are multipurpose-plants, where the potential cross contaminations are a great concern, and the lower contamination risk is really an advantage (Carson 2005).

Disadvantages of the disposable bioreactor: However, the bioreactor bags fulfill the basic criteria of the bioreactor, but it does face limitations such as:

- On the other hand, the main disadvantages are that are unavailable and the operating costs can be increased significantly because of constant buying of new bags (Farid et al. 2005).
- The other disadvantages include the scale-up complications, reliance on suppliers and the increased expenses as the operating costs and waste costs are increased as also more solid waste is created in the process. Also more warehouse storage space is needed (Carson 2005; Farid et al. 2005; Fox 2005).

- Mixing is the major problem with the disposable bioreactor bags. Since the mixing only provided by the 'rocking mixing'.
- Sensors are expensive and could not be regarded as disposable item thrown away after every use. These sensors could be used again after sterilization in the disposable plastic bags.
- Oxygen requirement is generally the limiting factor in lager scale, as the disposable bag reactors are usually operated through surface aeration. Singh (1999) studied that the $k_L a$ values for disposable (20 and 200 l) were about 4 l/h, whereas for similar size stirred tank bioreactors are usually around 50 l/h. The $k_L a$ the oxygen delivery to the cells is limited. The oxygen mass-transfer can be improved for example using pure oxygen instead of air or increasing the air flow or impeller speed (Soderberg 2002).

2.5 A Versatile Miniature Bioreactor: A Novel Bioreactor Design for Bio-Electrochemistry Studies

Recent reproducible investigations on bio-microsystems such as nanoparticles essentially require a flexible, but well-defined experimental setup, which in its features corresponds to a bioreactor. In this regards, Kloke et al. (2010) developed a novel miniature bioreactor with a volume in the range of a few milliliter that is assembled by alternate stacking of individual polycarbonate elements and silicon gaskets. All the necessary supply pipes are incorporated as bore holes or cavities within the individual elements. Their combination allows for a bioreactor assembly that is easily adaptable in size and functionally to experimental demands. It allows for controlling oxygen transfer as well as the monitoring of dissolved oxygen concentration and pH-value. A mass transfer coefficient for oxygen of 4.3×10^{-3} s^{-1} at a flow rate of only 15 ml min^{-1} and a mixing time of 1.5 s at a flow rate of 11 ml min^{-1} were observed for the modular bioreactor. Single reactor chambers can be interconnected via ion-conductive membranes to form a two-chamber test setup for investigations on electrochemical system such as fuel cells or sensors. The versatile applicability of this modular and flexible bioreactor was demonstrated using *Escherichia coli* and *Shewanella oneidensis* cultures (Kloke et al. 2010).

2.6 Conclusion and Future Prospects

Plant cell, tissue and organ culture is often an effective system to study the biological significance of bioactive metabolites under in vitro conditions, as well as for producing natural products for bio-processing applications (Paek et al. 2005; Saifullah et al. 2008). Moreover, establishing an adventitious root suspension culture system would accelerate large-scale multiplication, strain improvement and

species conservation (Holobiuc and Blindu 2006), but optimization and scale-up process from shake flasks to production scale bioreactors is required to increase root biomass and secondary metabolites (Choi et al. 2000; Yu et al. 2001; Lee and Natesan 2006). Several bioreactors have been designed for the large scale production of plant cell and hairy roots. Bioreactors used for hairy root cultures tend to be relatively complex, due to the necessity for unique configurations, which can compensate for the heterogeneous, cohesive, structured and entangled nature of fibrous roots. Though roots have been grown in various bioreactors-STR, BCR, ALR, TBR and NMR-the question as to which of these alternatives can be successfully and economically scaled-up has yet to be definitively answered. Despite the many recent improvements in our understanding of plant tissue cultures in bioreactors, this technology has met with even less commercial success than observed for cell suspension cultures for the production of secondary metabolites. However, plant tissue cultures remain a promising goal, and many such projects are currently under deployment. It is believe that the introduction of commercial ginsenoside production by culturing adventitious roots in 10,000 and 20,000 l balloon-type bioreactors in Korea will also encourage the international development of hairy root-based manufacturing processes (Choi et al. 2006). Furthermore, many pharmaceutical and biotechnology companies have introduced disposable bag technology in order to increase process efficiency over the last 10 years. Because this technology also greatly reduces the risk of cross-contamination, disposable bags are preferred in applications in which an absolute or improved process safety is a necessity, namely the production of functional tissue for implantation (tissue engineering), the production of human cells for the treatment of cancer and immune system diseases (cellular therapy), the production of viruses for gene therapies, the production of therapeutic proteins, and veterinary as well as human vaccines. To date, disposable bioreactors have primarily been used in cell cultures. The shift in their usage in microbial or yeast systems will not be easy and will require significant technical advances. Going forward, bioreactor volume will probably not increase much above 2,000 l; both Xcellerex and Thermo Fisher have stated that this is the limit for the foreseeable future. The maximum working volume for the Wave is 500 l. Technical improvements are required in mixing, sparging, and heating and cooling of the current models. These are essential to meet the demands of the high-titer processes, especially at larger volumes. At present, the instrumentation used is based on conventional probes. It is expected that these will be replaced by disposable sensors. There will be a large number of new entrants into this market followed by a consolidation. Disposable bioreactors will become the dominant technology in bio-manufacturing operations. Their adoption is being driven by two trends-reduced manufacturing costs and the development of high-titer processes. Given the conservative nature of our industry and the requirements of scale-up and scale-down, disposable technologies that mimic the conventional stainless-steel bioreactor will be most readily adopted. However, further developments in bioreactor cultivation processes and in metabolic engineering of plant cells for metabolite production are expected in the near future.

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Chapter 3
Hairy Root Culture: An Efficient System for Secondary Metabolite Production

Shiwali Sharma, Anwar Shahzad, and Aastha Sahai

3.1 Introduction

Plants are a potential source for many important drugs because they are able to produce various chemical entities and bioactive molecules through the process known as metabolism. Plant cell carries out both primary and secondary metabolism. Primary metabolism involves the synthesis of polysaccharides, proteins, lipids, RNA and DNA through utilization of sugars, amino acids, common fatty acids and nucleotides whereas secondary metabolism is activated during particular stages of growth and development or during periods of stress limitation of nutrients or attack by microorganisms (Yazaki et al. 2008). Secondary metabolites generally derived from primary metabolites through modifications, such as methylation, hydroxylation and glycosidation. Therefore, secondary metabolites are naturally more complex than primary metabolites and are classified on the basis of chemical structure (e.g., aromatic rings, sugars), composition (containing nitrogen or not), their solubility in various solvents or the pathway by which they are synthesized. They have been categorized into Terpenes (composed entirely of carbon and hydrogen), phenolics (composed of simple sugars, benzene ring, hydrogen and oxygen) and nitrogen and/or sulphur containing compounds (Chinou 2008) (Table 3.1). It has been observed that each plant family, genus and species produces a characteristic mixture of these metabolites.

These compounds usually have very complicated structures and/or exhibit chirality. Consequently, in many cases organic synthesis is not cost effective. Many of these natural products can be obtained by direct extraction from plants. However, this method is known to cause serious ecological problems. The large-scale production of valuable materials, by virtue of field grown plants and original habitats has been

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Table 3.1 Classification of secondary metabolites

Terpenes (composed of C and H)	Phenols (composed of sugars, benzene ring, H and O)	Nitrogen and/or sulphur containing compound
Monoterpenes: Limonene	Phenolic acids: Caffeic	Alkaloids: nicotine
Sesquoiterpenes: Farnesol	Coumarins: umbellifereone	Glucosinolates: Sinigrin
Diterpenes: Taxol	Lignas: podophyllin	
Triterpenes, cardiac glycosides: Digitogenin	Flavonoids: anthocyanin	
Tetraterpenoids: Carotene	Tannins: gallotannin	
Sterols: Spinasterol	Lignin	

limited, primarily by a variety of environmental factors, including low growth rates, restricted cultivation areas, climate dependency, pests, plant diseases, intense labor requirement and the overall time-consuming nature of the tasks inherent to the pursuit. Furthermore, the volume and range of phytochemicals used by modern society are continuously expanding due to explosive population rise. These challenges demand to develop new ways for the production of plant derived metabolites at commercial level.

Plant cell suspension culture has been considered an alternative source to agricultural process for producing valuable secondary metabolites, totally independent of geographical and climatic conditions. Although in vitro culture of plant cell is now a mature technology with successful applications in agricultural crop improvement, germplasm storage, and micropropagation, but the application for commercial production is limited. Notable exceptions include the commercial production of shikonin, berberine and ginseng cells in Japan and pilot-scale trials for the production of sanguinarine, rosmaric acid, digoxin, geraniol and immunologically active polysaccharides, which are currently underway in the USA, Canada, and Germany (Mavituna 1992; Giri and Narasu 2000; Lee et al. 2004). The biggest challenge for producing secondary metabolites from plant cell suspension culture is that secondary metabolites are usually produced by specialized cells and/or at distinct developmental stages (Balandrin et al. 1985). Some compounds are not synthesized if the cells remain undifferentiated (Berlin et al. 1985). Therefore, undifferentiated plant cell cultures often lose, partially to accumulate secondary products (Rokem and Goldberg 1985; Charlwood and Charlwood 1991). Thus, in vitro culture of differentiated and organized tissues (particularly the roots) was focused as their behavior have been claimed to be much more predictable when compared with that of cell suspension cultures (Parr 1989). The plant roots seem to be the most suitable for large scale cultivation since the roots are the site of synthesis and/or storage of certain chemicals of pharmaceutical importance. However, there are some reports of co-cultured differentiated tissues (e.g. shoots + roots) being used to produce secondary metabolites (Subruto et al. 1996; Mahagamasekera and Doran 1998). Slow growth rate due to highly organized nature of normal roots posed another serious limitation in commercialization of technology using root biomass as a source for secondary metabolite production. Recently root culture has been re-developed as an experimental tool making use of

natural ability of a soil bacterium *Agrobacterium rhizogenes* to transfer genes into the host plant genome. *A. rhizogenes*, a gram negative bacterium infects a wide range of plant species and causes the neoplastic plant disease syndrome known as 'hairy root disease'. Attentions are now being focused on genetic transformation of plants using this natural vector as an important alternative to intact plants as well as cell suspension cultures for the production of secondary metabolites.

3.2 Hairy Root Culture

3.2.1 Mechanism of Hairy Root Induction

The term 'hairy root' was first mentioned in the literature by Stewart et al. (1900) (see Srivastava and Srivastava 2007). The identity of the hairy root-causing organism remains uncertain for a long time. Riker et al. (1930) described and named the hairy root-causing organism as *Phytomonas rhizogenes* which was later renamed as *Agrobacterium rhizogenes* by the same group. A large number of small roots protrude as fine hairs directly from the infection site in response to *A. rhizogenes* attack, a phenomenon that gave rise to term 'hairy root'. The first directed transformation of higher plants using *A. rhizogenes* was made by Ackermann (1977).

The interaction between *A. rhizogenes* and plants involves a complex series of events, the temporal sequence of which is defined by cellular activities of the interacting partners like that of processes involved with related species *A. tumefaciens*.

Agrobacterium recognizes some signal molecules in the form of various phenolic compounds released by wounded plant cells such as acetosyringone and α-hydroxy acetosyringone and become attached to them (chemotactic response). After bacterial colonization and attachment to plant cells at or near wound site, the infection leads to insertion of T-DNA fragments of the T_i-plasmid (tumor inducing plasmid of *A. tumefaciens*) or R_i-plasmid (root inducing plasmid of *A. rhizogenes*) to the plant cells. Genes of T-DNA fragment mediate the formation of neoplastic crown gall tumor and hairy root tissues, followed by the synthesis of sugar and amino acid conjugates known as 'opines' which are used by the invading bacteria as a source of carbon and nitrogen (Binns and Thomashow 1988). Genes encoded in T-DNA have eukaryotic regulatory sequences, enabling their expression in infected plant cells. The transformation events are triggered by *vir* genes located in a 40-kbp region of Ri-plasmid called the virulence (vir) region. The *vir* genes are only expressed in the presence of acetosyringone. Various sugars also act synergistically with acetosyringone to induce high level of *vir* gene expression. Finally the expression of T-DNA genes coding for auxin synthesis and other rhizogenic functions results in root formation at the infection site of host plant.

Most *Agrobacterium* strains contain only one type T-DNA, but some (like those carrying agropine type R_i-plasmids) transfer two independent T-DNA denoted as T_L-DNA (left handed T-DNA) and T_R (right handed T-DNA). T_R-DNA has high homology to the T-DNA of the T_i-plasmid of *A. tumefaciens* while, T_L-DNA is

strikingly different and has homology to the T-DNA carried by the R_i-plasmid of mannopine *A. rhizogenes* strains (Nilsson and Olsson 1997). Both T_L-DNA and T_R-DNA are transferred and integrated independently into the host plant genome. Previously, it was assumed that the synthesis of auxin can be ascribed to the T_R-DNA, but the genes of T_L-DNA direct the synthesis of a substance that induces the cells to differentiate into roots under the influence of endogenous auxin synthesis (Ooms et al. 1986; Shen et al. 1988). But now it is clear that, the transfer of T_L-DNA is essential for induction of hairy root syndrome, and transfer of T_R-DNA does not provoke formation of roots from transformed cultures (Nilsson and Olsson 1997; Sevon and Oksman-Caldentey 2002). T_R-DNA contains two genes (*iaa*M and *iaa*H) responsible for the biosynthesis of auxin and genes are responsible for the synthesis of theopines, mannopine (*mas1′* and *mas2′*) and agropine (*ags*). T_L-DNA carries 18 open reading frames (ORF), four of which are essential for hairy root formation; ORF10, ORF11, ORF12 and ORF15 are corresponding to gene *rol*A, *rol*B, *rol*C and *rol*D respectively. The *rol*B gene is absolutely essential for hairy root induction. Even when expressed alone, the *rol*B gene can induce significant hairy root production (Nilsson and Olsson 1997). Conformation that a plant cell is transformed can be obtained by transformed root morphology exhibited by hairy root cultures and their transformed regenerants. The hairy roots have altered phenotype and these roots show high degree of lateral branching, profusion of root hairs and lack of geotropism.

Different strains of *A. rhizogenes* vary in their transforming ability (Kumar et al. 1991; Giri et al. 1997). Hairy roots obtained by various infections with different bacterial strains exhibit different morphologies. The differences in virulence and morphology can be explained by the different plasmid harbored by the strain (Nguyen et al. 1992). [(*Detailed information regarding the mechanism involved in gene transfer can be found in the report of* Gelvin (2000))].

According to opine synthesized by hairy roots and utilized by bacterium, *A. rhizogenes* strains were grouped into two main classes (Petit et al. 1983):

- **Agropine-type strains** (**e.g.**, **A4**, **15834**, **HR1**, **LBA 9402**)-which induce roots to produce agropine, mannopine and corresponding acids.
- **Mannopine-type strains** (**e.g.**, **8196**, **TR7**, **TR101**)-which elicit roots containing only mannopine, mannopinic acid and agropinic acid.

While, Zhou et al. (1997) classified the strains of *A. rhizogenes* into five classes: octopine, agropine, nopaline, mannopine, cucumopine.

3.2.2 Establishment of Hairy Root Culture

For successful establishment of hairy root culture system for a certain plant species, several essential conditions should be taken into consideration. These conditions include the selection of best bacterial strain of *A. rhizogenes*, an appropriate explants, a proper antibiotic to eliminate redundant bacteria after co-cultivation and a suitable culture medium. Amongst, Agropine are most often used strains owing to their

strongest virulence. Most plant materials such as hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root or tuber can be used to induce hairy roots (Giri et al. 2001; Krolicka et al. 2001; Azlan et al. 2002; Sevon and Oksman-Caldentey 2002). However, for different species, the proper explants material may vary and the age of the explants is most critical, generally juvenile material being optimal.

To induce hairy roots, explants are separately wounded and infected with *A. rhizogenes* strain either by direct inoculation with a thick, viable bacterial suspensions and incubation on a solid medium or by co-cultivation in liquid medium. Two or three days later, the infected explants are subsequently transferred to a solid medium with antibiotics, such as cefotaxime sodium, carbencilin disodium, vanocomycin, ampicilicin sodium, claforan, streptomycin sulphate or tetracycline, ranging in concentration from 100 to 500 µg/mL, generally for 3 days to kill or eliminate redundant bacteria (Giri et al. 2001; Krolicka et al. 2001; Pavlov et al. 2002a, b; Rahman et al. 2004). The neoplastic hairy roots will be emerged at the site of infection within a short period of time, which varies from 1 week to over a month depending on different plant species. Thereafter, roots are individually cut off and subculture to a hormone-free nutrient medium e.g., MS (Murashige and Skoog 1962) or B_5 (Gamborg et al. 1968) where they grow in a profusely branch manner with abundant lateral branches. The whole process of hairy root induction can be explained by Fig. 3.1. Successful genetic transformation can be demonstrated in either of two ways, directly or indirectly detecting T-DNA or opine respectively. The direct way is preferred, as in some cases opine production is not stable and may even cease (Sevon and Oksman-Caldentey 2002). To detect T-DNA, either polymerase chain reactions (PCR) (Le Flem-Bonhomme et al. 2004; Palazon et al. 2003) or Southern blot hybridization (Nin et al. 1997; Xie et al. 2001) can be used.

3.2.3 SAAT: A New Approach of Transformation

Recently, a new technique named sonication-assisted *Agrobacterium*-mediated transformation (SAAT) has also been developed to induce hairy roots in those plant species which are difficult to transform. Trick and Finer (1997) observed that SAAT treatment produces small, uniform fissures and channels in tissues of various plants which facilitate the access of *A. rhizogenes* to the internal plant tissues. Recently, this technique was successfully used by Le Flem-Bonhomme et al. (2004) for transforming the hypocotyls of *Papaver*.

3.2.4 Characteristics of Hairy Root Culture

Hairy roots have various attractive properties for secondary metabolite production like:

- Roots are plagiotropic (grow away from the vertical) and neoplastic (cancerous) in nature, therefore do not require external supply of growth hormones. The plagiotropic characteristic is advantageous; as it increases the aeration in liquid medium and thereby leading an elevated accumulation of biomass.

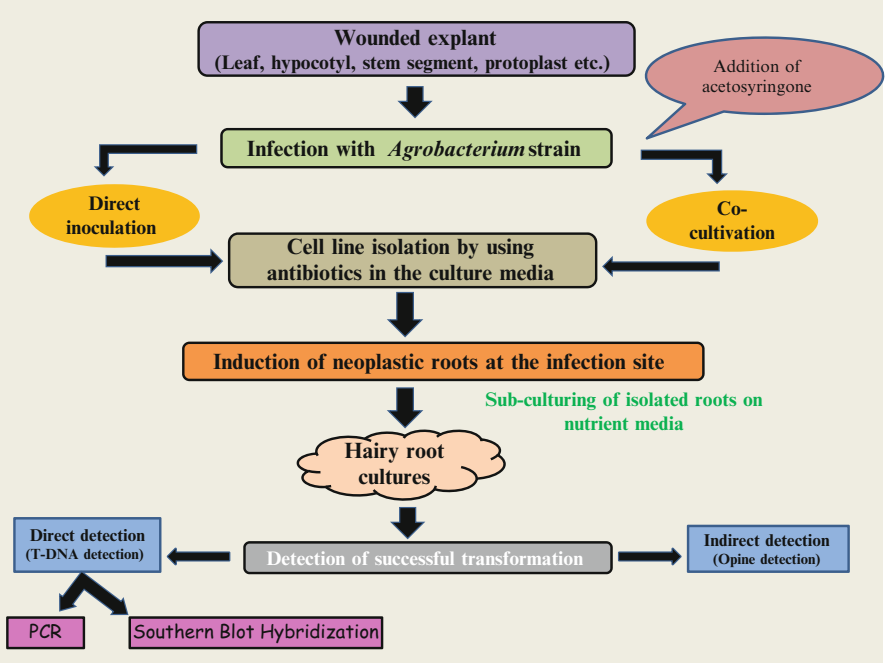

Fig. 3.1 Establishment of hairy root culture

- They often exhibit about the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Banerjee et al. 1998; Kittipongpatana et al. 1998).
- Genetic stability is another important characteristic of hairy roots. For example, hairy root culture of *Hyoscyamus muticus* showed equal or higher levels of hyoscyamine synthesis compared to the roots of a whole plant (Flores and Filner 1985) and maintained the same biosynthetic capacity for more than 15 years (Flores et al. 1999).
- Various useful products are only synthesized and accumulated in organized in vitro tissue (root), but not formed in suspension or callus culture of shoots and leaves; in such a critical situation hairy root culture is the only approach to obtain these useful chemicals at commercial level.
- In nature certain metabolites are only produced in aerial parts not in roots. Hairy root culture is found to be fruitful for extraction of such chemicals. For example, accumulation of lawsone, a napthoquinone derivative, is restricted to the aerial parts of wild-type henna (*Lawsonia inermis*); however, in hairy roots cultures, lawsone has been found in significant quantities in (Bakkali et al. 1997). Similarly, Artemisinin was successfully produced in hairy root culture (Weathers et al. 1997; Jaziri et al. 1995; Liu et al. 1999) which was previously thought to accumulate only in the aerial parts of *Artemisia annua* plant (Wallaart et al. 1999).

3.3 Hairy Root Culture: 'Productive Vehicle' for Secondary Metabolites

The high biosynthetic potential of hairy root culture was largely neglected for years and the investigations that were performed mainly focused on the mechanism of hairy root syndrome. However, the investigations of mid-1980s and early-1990s on the production of biologically active substances, especially alkaloids revealed the potential of transformed root systems. Now, hairy root culture technique is being interestingly adapted as a new research line for the production of bioactive compound. With several attractive features, the production of more than a specific compound synthesis, it acquires a great commercial importance (Fig. 3.2). Many medicinal plants have been transformed successfully by *A. rhizogenes* for enhanced production of secondary metabolites (Table 3.2). Following approaches are being adapted for enhanced secondary metabolite production through hairy root culture:

3.3.1 Increased Biomass Yield

Some secondary metabolites are growth-associated (Bhadra and Shanks 1997; Bhadra et al. 1998). Thus, the manipulation of extracellular environment to improve the growth has been used as a strategy to increase the productivity of valuable metabolites. The culture medium, particularly its nutrient content has a major impact on hairy root growth; it has become a target for maximizing phytochemical accumulation in hairy root cultures (Condori et al. 2010; Shinde et al. 2010). In a study on *Plumbago zeylanica*, MS was found to be best for hairy root growth in dark condition as compared to other nutrient media (B5 and SH) tested (Sivanesan and Jeong 2009). However, the medium composition being modified often with respect to its concentration of carbon, nitrogen and phosphorous sources (Wilhelmson et al. 2006) and other macronutrients (Sivakumar et al. 2005). A general approach for media optimization is to use statistical analysis (Sung and Huang 2000; Wilhelmson et al. 2006). In the first stage of this approach, the components of the nutrient media (independent variables) are varied, and the resulting culture growth and biosynthesis parameters of the desired metabolites are determined. Multivariate analysis is then applied to explore the interactive 'nutrient media-biomass-product' relationships between compounds in the biological systems. In parallel to experimental testing, interdisciplinary computational approaches were also adopted to predict the optimal growth conditions for high biomass and phytochemical production. Two artificial neural network models (regression and back propagation) were applied to estimate biomass yield in a licorice (*G. glabra*) hairy root cultures derived from *A. rhizogenes* transformed leaf explants (Prakash et al. 2010). Variables including volume, pH, sugar content of the culture medium, and the density of the inoculums were tested by both models for fresh biomass production. The robustness of the models was verified by experimentally obtained data. More accurate results were found using the regression neural network as compared to the back propagation

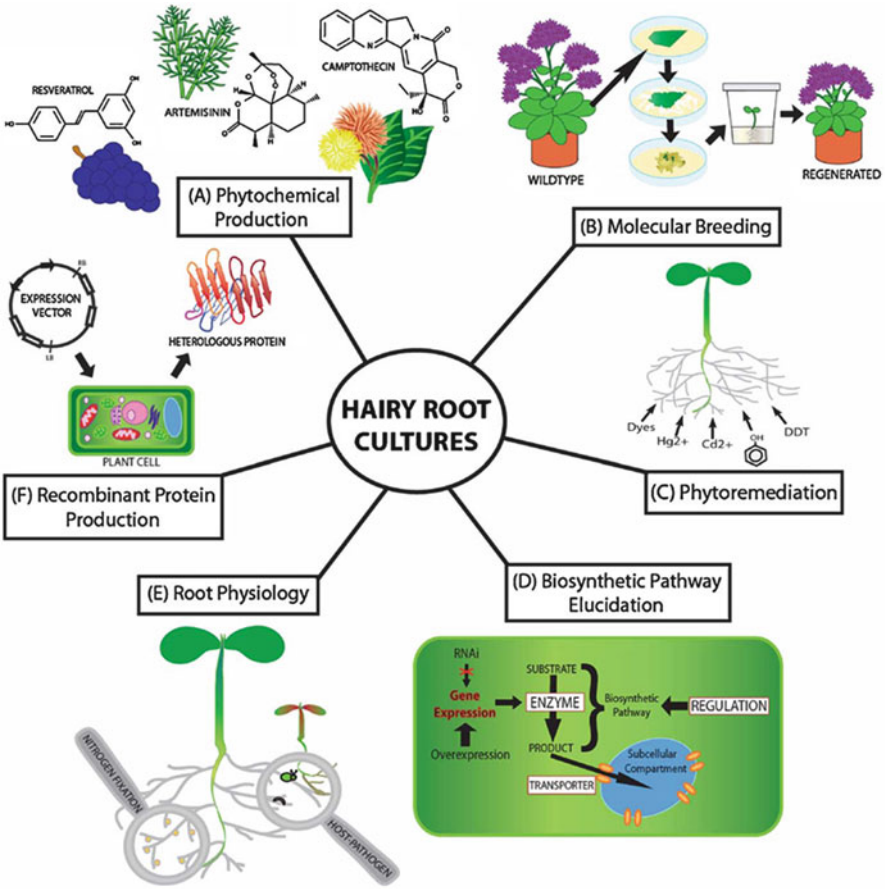

Fig. 3.2 The diverse and abundant uses of hairy root cultures. (**a**) Phytochemical production in hairy roots is a major topic of study that spans several classes of phytochemicals, including alkaloids, terpenoids, and phenolics. (**b**) Molecular breeding by infection of ornamental plants with *Agrobacterium rhizogenes* and regeneration of whole plants from hairy roots yields plants with desirable phenotypes, such as compact size, for horticultural purposes. (**c**) Hairy root culture has been used as a model system for studying Phytoremediation of toxic substances and reactive dyes. (**d**) Molecular, biochemical and genetic studies in hairy roots have accelerated Biosynthetic pathway elucidation for phytochemicals, which, in turn, facilitates metabolic engineering in hairy root cultures. (**e**) Root physiology studies ranging from nitrogen fixation, iron-deficiency, aluminum toxicity, to host–pathogen interactions have been conducted in hairy root cultures. (**f**) Recombinant protein production in this system has been explored as a rapid, contained, low-cost, genetically stable means of producing human antibodies, cytokines, and other protein therapeutics (Taken from the report of Ono and Tian (2011). With permission)

neural network, presumably due to the better learning potential of the regression neural network (Prakash et al. 2010). In another modeling study, mathematical equations were proposed to predict the growth of hairy roots in relation to nutrient distribution in the medium and within dense hairy root networks (Bastian et al. 2008).

Table 3.2 List of secondary metabolites production through hairy root culture

Plant	Family	Metabolite	Medicinal importance/effective against	References
Ammi majus	Apiaceae	Xanthotoxin (furococumarin)	Leucoderma	Krolicka et al. (2001)
Ophiorrhiza pumila	Rubiaceae	Camptothecin	Antitumor, AIDS, falciparum malaria	Sato et al. (2001)
Solanum aviculare	Solanaceae	Solasodine	Used as a base material for the production of steroid contraceptives	Koehle et al. (2002)
Glycyrrhiza pallidiflora	Fabaceae	Flavonoids	Meant for the treatment of gastric ulcers, anti-inflammatory and anti-tussive	Li et al. (2002)
Beta vulgaris	Chenopidiaceae	Betalains	Strong aphrodisiac, laxative	Pavlov et al. (2002a, b)
Catharanthus roseus	Apocynaceae	Indole alkaloids (vinblastine, vincristine (in aerial part)) Ajmalicine, serpentine and reserpine (roots)	Anti-cancerous	Ayora-Talavera et al. (2002)
Physalis minima	Solanaceae	Physalins	Diuretic, febrifuge, vermifuge	Azlan et al. (2002)
Datura metel; *Hyoscyamus muticus*	Solanaceae	Tropane alkaloids	Nacrotic, anti-cholinergic and anti-spasmodic activity	Moyano et al. (2003)
Papaver somniferum	Papaveraceae	Benzylisoquinoline alkaloids (Morphinan, codeine and sanguinarine)	Analgesic; antibiotic	Park and Facchini (2000), Le Bonhomme et al. (2004)
Rauvolfia micrantha	Apocyanaceae	Ajmalicine, ajmaline	Antihypertensive	Sudha et al. (2003)
Hyoscyamus niger	Solanaceae	Tropane alkaloids	Nacrotic, anti-cholinergic and anti-spasmodic activity	Zhang et al. (2004)

(continued)

Table 3.2 (continued)

Plant	Family	Metabolite	Medicinal importance/effective against	References
Gmelina arborea	Lamiaceae	Verbascoside	Claimed to be stomachic, galactagogue laxative and anthelmintic; improve appetite, useful in hallucination, piles, abdominal pains, burning sensations	Dhakulkar et al. (2005)
Datura innoxia	Solanaceae	Tropane alkaloids (Scopolamine and hyoscyamine)	Nacrotic, anti-cholinergic and anti-spasmodic activity	Dechaux and Boitel-Conti (2005)
Atropa belladonna	Solanaceae	Tropane alkaloid (hyoscyamine, atropine and hyoscine)	Used against Parkinson's disease	Richter et al. (2005)
Gmelina arborea	Verbenaceae	Verbascoside	Anti-inflammatory, wound healing, inhibit platelet aggregation	Dhakulkar et al. (2005)
Linum album; *Linum persicum*	Linaceae	6-methoxy-podophyllotoxin	Anticancer	Wink et al. (2005)
Artemisia annua	Asteraceae	Artemisinin	Antimalarial	Weathers et al. (2005)
Saussurea involucrata	Asteraceae	Rutin, hispidulin and syringin	Anti-inflammatory; antifungal	Fu et al. (2005)
Stizolobium hassjoo	Leguminoceae	3,4-Dihydroxyl-L-phenylalanine	Therapeutic agent against Parkinson's disease	Sung and Huang (2006)
Harpagophytum procumbens	Pedaliceae	Iridoid glycosides	Anti-inflammatory; analgesic; antidiabetic	Georgiev et al. (2006)
Arachis hypogaea	Papilionaceae	Resveratrol	Anti-inflammatory, antioxidant, anti-infective, anti-cancerous	Kim et al. (2008)
Datura stramonium	Solanaceae	Hyoscyamine	Nacrotic and anti-spasmodic activity , Used against Parkinson's disease	Pavlov et al. (2009)
Glycyrrhiza uralensis	Fabaceae	Flavonoids	Anti-mutagenic, anti-ulcer, anti-tumor, anti-microbial	Zhang et al. (2009)

Plumbago zeylanica	Plumbaginaceae	Plumbagin	Diuretic, antibacterial and used against leprosy	Sivanesan and Jeong (2009)
Fagopyrum esculentum	Polygonaceae	Rutin	Antioxidant, anti-carcinogenic, antithromobotic, cytoprotective, vasoprotective	Kim et al. (2010)
Abrus precatorious	Fabaceae	Glycyrrhizin	Diuretic, tonic, alexitric, anti-fertility	Dixit and Vaidya (2010)
Przewalskia tangutica	Solanaceae	Scopolamine and hyoscyamine (tropane alkaloids)	Parasympatholytic	Lan and Quan (2010)
Nepeta cataria	Labiatae	Rosmarinic acid	Astringent, antioxidant, anti-inflammatory, antimutagenic, antimicrobial, antiviral	Yang (2010)

Effect of Cytokinins and auxins on growth and morphogenesis of hairy roots indicates that auxins play an important role in hairy root growth. The sensitivity of hairy root tips to exogenous auxin was found to be 100–1,000 times higher than that of untransformed material (Ohkawa et al. 1989). To enhance growth and rosmarinic acid production in *Nepeta cataria*, hairy root cultures were grown for 15 days in media supplemented with various concentrations of auxins (IAA, IBA, and NAA). Cultures treated with IBA induced maximum biomass enhancement (13.5 g/L) and production of rosmarinic acid (19.2 mg/L) (Yang 2010).

While, recent studies have shown that inoculum size and age strongly influenced the growth of *Panax ginseng* hairy root cultures (Jeong et al. 2004) and betalains production form *Beta vulgaris* hairy root cultures (Pavlov et al. 2003). Jeong et al. (2004) found that growth rate of *P. ginseng* hairy roots was maximum when a 0.7 % (w/v) inoculum was used and significantly lowered with 0.4 % (w/v) inoculum. The optimal duration of subculture cycle was found to be 10 days for *P. ginseng* (Jeong et al. 2004) and 14 days for hairy roots of *B. vulgaris* (Pavlov et al. 2003).

In general, artificial polyploidy enhances the vigor of determinate plant parts and may be favorable where vegetative organs and biomass constitute the economic product. Recently, artificial polyploidy has been considered as a potential method for increased secondary metabolite production (Lavania 2005). When analyzing the alkaloid profiles, similar types of major metabolites were detected in hairy root cultures induced from diploid and tetraploid *Datura stramonium* plants (Pavlov et al. 2009). However, the concentration of compounds significantly enhanced in tetraploid-derived hairy root cultures as compared to diploid-derived hairy root cultures. In this study, the hairy root cells underwent endo-reduplication and a large fraction of the nuclei contained double the number of chromosomes of the parental cells (Pavlov et al. 2009). Similarly, colchicine induced stable tetraploid hairy root clones of *Artemisia annua* showed major differences in growth and development compared to diploid clones. Artimisinin yields of these tetraploid clones were 2–5 times higher than that of the diploids (De Jesus-Gonzalez and Weathers 2003).

There are so many reports showing increased metabolite production through biomass enhancement of hairy root cultures. In a preliminary study on hairy root induction in *Gmelina arborea*, about sevenfold biomass increment was achieved at the end of 4 weeks as compared to non-transformed seedling roots and suggesting the potential ability of hairy roots to synthesize verbascoside, a phenylpropanoid glycoside of medicinal value (Dhakulkar et al. 2005). In *Saussurea involucrata*, increased biomass yield of hairy roots (66.7 g/L fresh weight) and flavonoid (102.3 mg/g dry weight) were achieved after 20 days of incubation (Fu et al. 2005). Enhanced scopolamine (0.68 mg/g dry weight) and hyoscyamine (1.13 mg/g dry weight) production as compared to wild type roots was reported through hairy root culture of *Przewalskia tangutica*. It was the first time that hairy root cultures of *P. tangutica* were established to produce tropane alkaloids (Lan and Quan 2010). Another study on *A. rhizogenes*-mediated transformation of *Abrus precatorious* showed significant increment (5.25 times) in fresh weight of hairy roots from initial fresh weight. A maximum of 700 mg of glycyrrhizin was obtained from 20 g roots of field grown plant of *A. precatorious* giving 35 mg/g dry weight concentration

of glycyrrizin (Dixit and Vaidya 2010). Kim et al. (2010) propagated *Fagopyrum esculentum* hairy roots for enhanced production of rutin, an important flavonol glycoside. The biomass of hairy roots (12.6 g dry weight L^{-1}) was around 2.4 times more than that of wild-type roots (5.3 g dry weight L^{-1}). The content of rutin in hairy roots was found to 1.3 mg/g dry weight which was 2.6 times more than that of wild-type roots where the amount of rutin was 0.5 mg/g dry weight.

Le Flem-Bonhomme et al. (2004) have been established hairy root cultures of *Papaver somniferum*, a natural source of morphinan, codeine and sanguinarine alkaloids. The total alkaloid content was higher in transformed roots (0.46 % dry weight) than non-transformed roots (0.32 % dry weight). The transformed roots accumulated 3-times more codeine (0.18 % dry weight) than intact roots (0.05 % dry weight). Morphine (0.255 % dry weight) and sanguinarine (0.014 % dry weight) were found in the liquid culture medium. While, Kim et al. (2008) studied five different strains differing in their ability to induce peanut (*Arachis hypogaea*) hairy roots and also showed varying effects on the growth and resveratrol production in hairy root cultures. *A. rhizogenes* R1601 is the most effective strain for the induction (75.8 %), growth (7.6 g/L) and resveratrol production (1.5 mg/g) in hairy root of peanut.

3.3.2 Elicitation and Precursor Feeding

Stimulation of biosynthetic activity using elicitation and precursor feeding is the most studied approach to optimize product accumulation in plant cell cultures. Elicitation strategies are compounds or treatments that induce plants to synthesize phytoalexins at elevated levels. Since little is known of the biosynthetic pathways of most secondary metabolites in plants, the effect of elicitation on a plant cell/tissue culture cannot be easily predicted. Therefore, elicitation approaches are performed by trial and error. The effect of elicitors depends on many factors, such as, the concentration of elicitor, the growth stage of culture at the time of elicitation and contact time of elicitation. Both biotic and abiotic elicitors can be used to stimulate secondary metabolite biosynthesis in plant cell/tissue culture, thereby reducing the processing time necessary for high product yields. Elicitors of non-biological origin (abiotic elicitors), such as heavy metals and ultraviolet light, which induce phytoalexin synthesis, are actually designated as abiotic stresses (Lu et al. 2001; Ramachandra and Ravishankar 2002). Secondary metabolite production through elicitation will be discussed in detail in following chapter of '**Elicitation**'.

3.3.3 Over-Expression of Foreign Gene

Over-expression of multiple biosynthetic genes or transcription factors that control the expression of enzymes in pathways targeted by bioengineering is a promising

strategy to improve accumulation of certain secondary metabolites by enhancing rate-limiting steps or by blocking competitive pathways. In this regard, *Agrobacterium*-mediated transformation provides a rapid and simple means. Several studies reveal the key role of *pmt* (putrescine *N*-methyl transferase) gene in tropane alkaloid biosynthesis and there have many attempts to increase the scopolamine production by over-expressing *pmt* gene. In most cases, the plant material has been transformed with this heterlogous gene (*pmt*) from tobacco, under the control of CaMV 35-S promoter, with the advantages of no feedback inhibition by downstream products and a high affinity for the substrate (Zhang et al. 2005). The *pmt* over-expressing plants of *Atropa belladonna* and *Nicotiana sylvestris* have already been produced by Sato et al. (2001). No changes were observed in *Atropa* alkaloid content, while the nicotine content in *N. sylvestris* leaves increased significantly. Similar behavior has been expressed by engineered roots of *Hyoscyamus muticus* and *Datura metel* (Moyano et al. 2003). However, in both species the over-expression of *pmt* gene from tobacco increased the hyoscyamine content, but the production of scopolamine improved significantly only in *D. metel*, in *Hyoscyamus* tropane alkaloid level remained similar to that of wild type hairy roots. As already mentioned, in *Atropa belladonna* over-expression of *pmt* gene only increased the accumulation of the direct metabolite *N*-methyl putrescine (Rothe et al. 2001) while the effect on total alkaloid level was marginal. Similar to *pmt* gene, engineered *A. belladonna* root lines with strong over-expression of *tr* gene (tropane reductase) from *D. stramonium* showed more enzyme activity of the respective reductase and a high level of the enzyme products, tropane and pseudotropine (Richter et al. 2005).

Researchers have worked on genetic engineering of pharmaceutically important tropane alkaloids, in which the conversion of hyoscyamine to much more valuable alkaloid, scopolamine is the major goal. A rough correlation has been found between H6H (hyoscyamine 6-β-hydroxylase) activity and the ratio of scopolamine to hyoscyamine in scopolamine producing cultured roots (Oksman-Caldentey and Strauss 1986). H6H, therefore, is a promising target enzyme that, if over expressed in hyoscyamine accumulating tissues, would result in increased scopolamine levels in transgenic plants or roots. In this way, several unattractive hyoscyamine-rich, but scopolamine-poor plants may now become promising candidates for large scale scopolamine production by means of cultured roots.

The first successfully altered plant by metabolic engineering was *Atropa belladonna* (Yun et al. 1992). The *h6h* gene from *H. niger* was over-expressed in the target plant and the progenies of single primary transformed plant showed elevated scopolamine contents, resulting in near to complete conversion of hyoscyamine to scopolamine in the mature plants. Similar results were achieved with *H. muticus* hairy roots over-expressing the same gene, in which the best transgenic clone had a 100-fold enhancement of scopolamine, while hyoscyamine content remained same (Jouhikainen et al. 1999). Zhang et al. (2004) reported the simultaneous introduction and over-expression of genes encoding the rate-limiting upstream enzyme PMT and H6H of scoplolamine biosynthesis. The best line produced 411 mg/L scopolamine which was over nine times more than that in the wild-type (43 mg/L) and more than twice the amount in the highest scopolamine producing *h6h* single-gene transgene line (184 mg/L).

They concluded that the pulling force of the downstream enzyme, H6H plays a more important role in stimulating scopolamine accumulation in *H. niger*, whereas the functioning of the upstream enzyme PMT increased proportionally.

Similar to tropane alkaloids, the regulation of flavonoid biosynthesis together with indole alkaloids is quite well understood in comparison with other secondary metabolites and a lot of transgenic materials over-expressing *chi* (chalcone isomerase) and *chs* (chalcone synthase) were constructed (Cain et al. 1997; Shul'ts et al. 2000; Li et al. 2002; Ralston et al. 2005). Zhang et al. (2009) observed enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* by combining the over-expression of *chi* gene with the elicitation treatment and reported a maximum of 2.838/100 g dry weight of total flavonoids than 0.842/100 g dry weight in wild-type hairy roots.

3.4 Other Applications

3.4.1 Hairy Root Culture for Molecular Farming: Expression of Foreign Proteins

However, plants are the potential source of industrial and therapeutic proteins, but the extraction and purification of complex proteins from plant tissues require a laborious and costly method. Thus, the possibility to express functional animal proteins in hairy roots makes this plant material attractive for molecular farming, with several advantages over field-cultured plants. These advantages are mainly based on the fact that hairy roots are cultured in confined recipient, avoiding transgene or pharmacologically active protein dissemination in the environment, and in controlled conditions of growth, avoiding pollution of the produced proteins. Moreover, animal proteins expressed in hairy roots are often secreted in the culture medium which provides an easy way of their extraction than intact plant cells. The proteins, those can be produced by hairy roots include medicinally important enzymes such as superoxide dismutase, peroxidase, phytase (Kim and Yoo 1996; Hyon and Yoo 2002; Jin et al. 2005), foreign proteins such as monoclonal antibodies (Sharp and Doarn 2001), the human secreted alkaline phosphatase (Gaume et al. 2003) and ribosome-inactivating proteins (Thorup et al. 1994). Three genes from *Ralstonia eutropha*, a type of bacteria necessary for polyhydroxybutyrate (PHB) synthesis, were introduced into the hairy roots of sugar beet (Menzel et al. 2003). It was observed that the 20 transgenic hairy root clones produced up to 55 mg high molecular PHB per gram dry weight. Wongsamuth and Doran (1997) reported the production of monoclonal antibodies by hairy roots. They initiated hairy roots from transgenic tobacco plants expressing a full-length I_g G monoclonal antibody and tested the long-term stability of antibody expression in hairy roots, variation between clones, the time course of antibody accumulation in batch culture and the effect of different factors on antibody accumulation and secretion. Antibody degradation in the medium was

a significant problem however, affecting the final titers and this remains to be resolved. Later on, Sharp and Doran (2001) reported that murine I_g G1 production in hairy roots of tobacco and improved the accumulation of the antibody by increasing the dissolved oxygen tension to 150 % air saturation. While, the non-toxic lectin subunit ricin B, fused to GFP, was expressed in tobacco hairy roots and secreted in the culture medium (Medina-Bolivar et al. 2003). This fusion protein was tested in mouse as an antigen, showing that protein fused to ricin B (a mucosal adjuvant in mammalian immune responses) can be efficiently produced by hairy roots. Recently, Doran (2006) reviewed the current status and problems associated with the production of foreign protein by hairy root cultures (such as low accumulation levels, instability in the culture medium etc.) and outlined strategies to minimize their degradation and losses. Although some of these problems have not yet been fully resolved, there is a little doubt that these application will continue to expand in the future.

3.4.2 Functional Analysis of Genes

Several recent reports highlight the important contribution of hairy root cultures to identification of biosynthesis and regulatory genes as well as transporter. For example, the stress hormone methyl jasmonate (MeJa) which often as a role as secondary messenger in elicitor transduction pathways, is also efficient in inducing or increasing the production of valuable secondary metabolites in hairy root cultures (Palazon et al. 2003; Yaoya et al. 2004; Komaraiah et al. 2003; Nakanishi et al. 2005). This inducible system was also used to discover unsown genes involved in the metabolite pathways. Such a strategy was also recently applied to *Catharnathus roseus* cell cultures and made possible the definition of a gene-to-metabolite network (Rischer et al. 2006). Similarly, the treatment of ginseng hairy roots with MeJa enabled the identification and the study of 3134 expressed sequence tags (ESTs) (Choi et al. 2005). By this means, it was possible to characterize several genes encoding enzymes such as squalene synthatse, squalene epoxydase, oxidosqualene cyclase, cytochrome P450 and glycosyltransferase, all of which are involved in the biosynthesis of the triterpene glycoside ginsenosides (Choi et al. 2005). Such studies are needed to gain information and new tools to design metabolite engineering strategies. Owing to their capacity for fast growth in vitro and the ease of being elicited, the hairy root cultures will be increasingly used for such studies in non-model plants, including medicinal plants.

Another powerful technique for identifying new gene functions is the T-DNA activation tagging. It consists of random integration of a T-DNA carrying a constitutive enhancer promoter element (often the cauliflower mosaic virus 35S enhancer element) into the plant genome. When this enhancer element integrates near to a gene, it will increase the expression of this gene and give rise to a gain of functional mutant. This strategy has been successfully applied to a number of plant species such as, *Arabidopsis thalina*, *Solanum tuberosum* and *Nicotiana tobaccum* (Seki et al. 2005). To facilitate the application of a forward genetcics

approach for gene discovery in hairy root cultures, binary vectors were designed and constructed specifically for activation tagging in hairy roots. T-DNA activation-tag technology can be applied to plant recalcitrant for regeneration (e.g., tree species), to characterize new genes important for the root biology, including those involved in biotic and abiotic stress resistance, developed or in the regulation of biosynthesis pathways. AtTT2 is a MYB transcription factor that controls expression of several proanthocyanidins and flavonoid biosynthesis genes (Nesi et al. 2001). Microarray analysis of genes induced in *Medicago truncatula*. Hairy root cultures over-expressing AtTT2 led to cloning of UGT72L1, a glycosyltransferase with specificity for (−)-epicatechin (a proanthocyanidin biosynthesis precursor) (Pang et al. 2008). The expression of UGT72L1 was associated with the accumulation of proanthocyanidins and epicatechin glycosides in developing *M. truncatula* seeds (Pang et al. 2008).

Over-expression studies in hairy root cultures can be complimented by employing RNA interference (RNAi) to knockdown gens of interest. Two back-to-back publications described the use of an RNAi approach in tobacco hairy root cultures (*Nicotiana tobaccum* and *N. glauca* respectively) to elucidate the role of NgA662, a NADPH-dependent reductase, in pyridine alkaloid biosynthesis in tobacco (Deboer et al. 2009; Kajikawa et al. 2009).

While, Kumagi and Kouchi (2003) studied transgenic lines of *Lotus japonicus* that express GUS by constitutive or nodule-specific promoters. *L. japonicus* were super transformed by infection with *A. rhizogenes* containing gene constructs for the expression of hairpin RNAs (hp RNAs) with sequences complementary to the GUS coding region. The results indicated that the GUS activity in those lines decreased more than 60 %. This suggests that transient RNA silencing by hairy roots transformation provides a powerful tool for loss-of-function analysis of genes that are expressed in roots (Kumagi and Kouchi 2003).

3.4.3 Regeneration of Whole Plants

Hairy roots are able to whole plant regeneration in several plant species. Generally, these transgenic plants are genetically stable. However, in some cases, transgenic plants have shown an altered phenotype compared to controls. These plants display 'hairy root syndrome' due to combined expression of the *rol*A, B and C loci of the R_i plasmid. Each locus is responsible for a typical phenotypic alternation, i.e., *rol*A is associated with internodes shortening and leaf wrinkling, *rol*B is responsible for protruding stigmas and reduced length of stamens, *rol*C responsible for internode shortening and reduced apical dominance. Some of the altered phenotypes have proven to be useful in plant breeding programs. Dwarfing, altered flowering, wrinkled leaves, increased branching due to reduced apical dominance may also be useful for ornamentals. Dwarf phenotype is an important characteristic for flower crops such as *Eustoma grandiflorum* and *Dianthus* (Giovanni et al. 1997). Transformed roots can also regenerate somatic embryos following the addition of the appropriate

phytohormone. Cho and Wildholm (2002) reported that when cultured in medium with 7.5–10.0 mg 2, 4-dichlorophenoxy acetic acid (2, 4-D), the hairy roots of *Astragalus siniensis* developed somatic embryos.

3.4.4 Phytoremediation

Phytoremediation is an emerging technology that uses green plants to remove, accumulate or otherwise render metals or organic contaminants present in soils and ground-waters benign. In this technology, plant roots are central to the remediation action. Hairy root cultures have been a valuable model root system to elucidate the transformation processes and fate of the contaminants without interference from microbes. Hairy root cultures of plant hyper accumulators are being used to study heavy metal uptake. Removal of polychlorinated biphenyls from the culture medium has been monitored in *Solanum nigrum* hairy roots (Khas et al. 1997). Significant progress on the transformation processes and fate of the nitro aromatic explosive 2, 4, 6-trinitrotoluene (TNT) in plants has been made in mass balance and fate experiments using *Catharanthus roseus* hairy roots as a model system. TNT was shown to be reduced to mono aminio dinitrotoluenes, and then conjugated at the amine groups with at least a six-carbon unit, and finally incorporated into un-extractable 'bound' residues in the cell wall material (Bhadra et al. 1999). The occurrence of the same conjugate in a diversity of plant species (aquatic versus terrestrial) reiterated the importance of conjugation as a fate process in TNT metabolism.

3.4.5 Production of Novel Compounds

The use of hairy root cultures in biotransformation processes for the production of novel compounds has also been reported (Wilson et al. 1987; Ushiyana and Furuya 1989; Kawaguchi et al. 1990; Asada et al. 1993; Flores et al. 1994). For example, transformed hairy roots of *Scutellaria baicalensis* accumulated glucoside conjugates of flavonoids instead of the glucose conjugates accumulated in untransformed roots (Nishikawa and Ishimaru 1997). Li et al. (1998) isolated a new compound named licoagrodione from *Glycyrrhiza glabra*, which was shown to possess strong antimicrobial activities. However, other two novel isoprenylated flavonoid compounds with anti-microbial and anti-oxidant activities were detected by Asada et al. (1998) in hairy root cultures of *G. galbra*. In another interesting study, a novel benzoquinone, hydorxyechinofuran B, was found to be secreted from *Lithospermum erythrorhizon* hairy root cultures when media conditions were altered (Fukui et al. 1998). This species produces the well known red dye and antibacterial compound, shikonin, when cultivated in ammonium-ion-free liquid medium. In this study, the addition of low levels of ammonium was found to induce the synthesis of the brown compound benzoquinone. Berkov et al. (2003) reported the biosynthesis of a new tropane alkaloid ester in tetraploid hairy roots of *Datura stramonium*.

3.4.6 Germplasm Conservation

Hairy root culture can also be used for the production of artificial seeds thus, providing an effective tool for ex vitro germplasm conservation. Hairy roots in the form of artificial seeds are a reliable delivery system for clonal propagation of elite plants with genetic uniformity, high yield and low production cost. Nakashimada et al. (1995) produced artificial seeds of horseradish. In *Ajuga reptans* GUS-transformed hairy roots been used for producing artificial seeds (Uozumi 2004). While, root tips of hairy roots of *Panax ginseng* (Yoshimatsu et al. 1996) and shoot tips of hairy roots regenerants have been cryopreserved in horseradish (Phunchindawan et al. 1997).

3.5 Problems of Hairy Root Culture

Although, hairy root culture is an emerging technique of genetic transformation with a wide range of applications, but it also has some major problems still remain to be solved such as;

- Different regulation of secondary metabolism in related species (Moyano et al. 2003).
- Over-expression of key enzymes does not always improve secondary metabolism (Koehle et al. 2002).
- Co-suppression of endogenous and foreign genes (Ayora-Talavera et al. 2002).
- Silencing of transgenes (Sivakumar 2006).
- Morphological alteration of regenerated plants (Han et al. 1993).
- Possible reduction of chromosome numbers during sub-culture (Xu and Jia 1996).
- Hairy roots usually produce opine-like substances which are lethal to mammalian cells (Yoshikawa and Furuya 1987) probably this is one of the reason that GMOs are not always accepted in several countries, specially in regards no medicines containing live genetically-modified organisms have been approved for use.

3.6 Conclusion and Future Prospects

Hairy root culture is a potential approach for the production of secondary metabolites, especially pharmaceuticals because it has many good traits, such as rapid growth rate, easy culture and genetic manipulation, and most importantly an efficient ability of enhanced production of secondary metabolites than unorganized cells and wild-type root. Hairy root cultures induced from rare medicinal plant species can be used for regenerating whole plants, making it an alternative and complementary ex situ biodiversity conservation method to seed banks. Long-term preservation of hairy root

cultures is critical for germplasm conservation and maintaining clonal lines for high-level phytochemical or recombinant protein production. In this regard, hairy root cultures have been stored at ambient, low and sub-zero temperatures with success. Stable alkaloid production was also observed in transgenic *Catharanthus roseus* hairy roots after 5-year maintenance in liquid cultures (Peebles et al. 2009). Still, there are many serious problems in metabolite production through hairy root culture which require further attempts for their resolution. One of the biggest challenges for commercial phytochemical and recombinant therapeutic protein production in hairy root culture is the production bottleneck. Further exploration into inexpensive novel elicitors and bioreactors will aid their industrial implementation by increasing yields and driving down production costs. Further, enhancement of knowledge regarding plant metabolic pathways and the mechanisms of their regulation in the near future should give us powerful tools for exploiting the biosynthetic potential of hairy roots.

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Glossary

Agrobacterium *Agrobacterium* is a genus of Gram-negative bacteria established by H. J. Conn that uses horizontal gene transfer to cause tumors in plants. *Agrobacterium* is well known for its ability to transfer DNA between itself and plants, and for this reason it has become an important tool for genetic engineering.

Plasmid An extra-chromosomal, autonomous circular DNA molecule found in certain bacteria, capable of autonomous replication. Plasmids can transfer genes between bacteria and are important tools of transformation.

T-DNA Transferred DNA of the tumor-inducing (Ti) plasmid of some species of bacteria such as *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. It derives its name from the fact that the bacterium transfers this DNA fragment into the host plant's nuclear DNA genome.

Hairy root A phase of crown gall (especially in apples) during which there is abnormal development of fine fibrous roots.

Secondary metabolite Organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism. Secondary metabolites often play an important role in plant defense against herbivory and other interspecies defenses. Humans use secondary metabolites as medicines, flavorings, and recreational drugs.

Phytoremediation The treatment of environmental problems (bioremediation) through the use of plants that mitigate the environmental problem without the need to excavate the contaminant material and dispose of it elsewhere.

Opines Low molecular weight compounds found in plant crown gall tumors or hairy root tumors produced by parasitic bacteria of the genus *Agrobacterium*. Opine biosynthesis is catalyzed by specific enzymes encoded by genes contained in a small segment of DNA (known as the T-DNA, for 'transfer DNA'), which is part of the Ti plasmid, inserted by the bacterium into the plant genome. The opines are used by the bacterium as an important source of nitrogen and energy. Each strain of *Agrobacterium* induces and catabolizes a specific set of opines.

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Chapter 4
Elicitation: An Alternative Approach Towards Commercialization of Secondary Metabolite Production

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4.1 Introduction

Plants are known for the production of a large array of natural products, also referred to as secondary metabolites. Plant secondary metabolites represent a huge number of natural compounds with a wide diversity in chemical structure. They are economically important to man due to their multiple applications, such as pharmaceuticals, flavors, fragrances, insecticides, dyes, food additives, toxins, etc. However, it is well known that their production is frequently low and depends on the physiological and developmental stage of the plant. The majority of pharmaceutically important secondary metabolites are obtained from wild or cultivated plants, although some attempts have been made, but their chemical synthesis in most cases has not been economically feasible. Therefore, production of plant secondary metabolites by cultivation of plants and chemical synthesis are important agronomic and industrial objectives. As a promising alternative to produce plant secondary metabolites, plant cell culture technology has many advantages over traditional field cultivation and chemical synthesis, particularly for many natural compounds that are either derived from slow growing plants or difficult to be synthesized with chemical methods (Zhao and Verpoorte 2007; Zarate and Verpoorte 2007).

Plants exhibit a wide array of defense strategies against pathogen attack. The resistance against pathogen is performed by both pre-existing (constitutive) and induced defense systems. Inducible defense responses are triggered following recognition of a range of chemical factors termed 'elicitors' (Hammond-Kosack and Jones 1997). Originally the term elicitor was used for molecules capable of

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inducing the production of phytoalexins, but it is now commonly used for compounds stimulating any type of plant defense (Ebel and Cosio 1994; Ramachandra and Ravishankar 2002). Eventually, the induction of defence responses may lead to enhanced resistance. They might be both of biotic and abiotic origin.

The first biotic elicitors were described in the early 1970 (Keen 1975). Since then, numerous publications have accumulated evidence for pathogen-derived compounds that induce defense responses in intact plants or plant cell cultures. They comprise distinct compounds among either oligosaccharides or lipo and glycol-proteins. Such biotic elicitors often originate from the pathogen (exogenous elicitors) but in some cases are liberated from the attacked plant by the action of enzymes of the pathogen (endogenous elicitors) (Boller 1995; Ebel and Cosio 1994).

Elicitors act as signal compounds at low concentrations, providing information for the plant to trigger defence, distinguishing elicitors from toxins, which may act only at higher concentrations and/or affect the plant detrimentally without active plant metabolism (Boller 1995). The terms are overlapping, however, as exemplified by certain fungal compounds like fumonisin B1. While fumonisin B1 can be seen as a phytotoxin in the interaction of the necrotrophic Pathogen *Fusarium verticillioides* with its host maize (Desjardins and Plattner 2000), it acts as a elicitor switching on active plant defence and cell death programmes in the model plant *Arabidopsis* (Stone et al. 2000).

Elicitors are usually capable to induce various modes of plant defense including the production of ROS (reactive oxygen species), the hypersensitive response and the production of phytoalexins i.e., antimicrobial secondary compounds (Montesano et al. 2003). The induction of phytoalexin biosynthesis has gained special importance in biotechnological approaches to improve the production of secondary metabolites. Many of these compounds are of high value as therapeutics or otherwise biologically active agents. An example is the bio-production of taxol, a diterpenoid found in the bark of *Taxus* trees. This compound is approved by the Food and Drug administration (FDA) for the treatment of ovarian and breast cancer. There is a high demand for taxol, but its synthesis production is extremely costly, so biosynthesis in *Taxus* spp. Cell culture has become the focus of extensive research (Heinrich 2002). In general, plant cell cultures are rich sources of valuable pharmaceuticals and other biologically active compounds (Chen et al. 2002), however, relatively few cultivars and derived cell cultures synthesize secondary metabolites over extended and in amounts suitable for commercial exploitation. Elicitation studies have shown promise in increasing yields and cutting production costs (Miao et al. 2000; Zhang and Wu 2003).

In recent research into in vitro culture systems, a wide variety of elicitors have been employed in order to modify cell metabolism. These modifications are designated to enhance the productivity of useful metabolites in the cultures of the plant cells/tissues. The cultivation period, in particular can be reduced by the application of elicitors, although maintaining high concentrations of product (Ramachandra and Ravishankar 2002).

4.2 Classification of Elicitors

According to Radman et al. (2003) elicitors are classified as physical or chemical, biotic or abiotic and complex or defined depending on their origin and molecular structure.

4.2.1 Biotic Elicitors

Biotic elicitors are molecules of either pathogen or host origin that can induce defense responses in plant tissue. Often complex biological preparations have been used as elicitors, where the molecular structure of the active ingredients is unknown. Examples of such elicitors are yeast extract and microbial cell-wall preparations.

In recent years, the exact molecular structure of an increasing number of elicitors has been elucidated, including various polysaccharide, oligosaccharides, proteins, glycoproteins and fatty acids (Anderson 1989; Hahn et al. 1989).

4.2.1.1 Proteins and Glycoproteins as Elicitor

Protein elicitors have been used to elucidate the role of ion channels in plant cell membranes in the signal transfer triggered by external stimuli. Proteins and enzymes are another class of elicitors that triggers defense reactions. e.g., in plant cell cultures. Cellulase cause rapid accumulation of phytoalexins in *Nicotiana tabacum* cell cultures, an increase in the production of capsidol and debneyol and production of two previously unknown phytoalexins (Threlfall and Whithed 1988).

The pathogenic fungus *Phytophtora drechleri* secretes elicitins (protein elicitors) that induce necrosis in tobacco leaves: holo-proteins (proteins involved in the phototropic signal perception) cause concentration-dependent leaf necrosis. At least three isoforms of this elicitor are produced by *P. drechleri* (Huet et al. 1992).

Glycoproteins have also been shown to elicit phytoalexins in plant cell cultures. A glycoprotein of molecular mass 46 kDa isolated from *Phytophthora nicotianae* was shown to elicit phytoalexin accumulation in tobacco callus (Farmer and Helgeson 1987). A 42 kDa glycoprotein purified from *P. megasperma* f. sp. *glycinea* was shown to stimulate various defense responses in cultured parsley cells including ion fluxes, oxidative burst, expression of defense-related genes and phytoalexin accumulation (Nürnberger et al. 1994a). However, parsley leaves did not respond to the pure glycoprotein (Parker et al. 1991). A protein of molecular mass 60 kDa isolated from the bacteria *Pseudomonas solanacearum* was described as an inducer of the hypersensitive response in potato based on its capacity to elicit callus browning when applied at very low concentrations (Huang et al. 1989). Harpins and PopA1 are proteins isolated from *Pseudomonas* species which induce necrotic zones when infiltrated into tobacco leaves, similar to the hypersensitive necrosis induced by the incompatible bacteria from which they are secreted

(Arlat et al. 1994; He et al. 1993). Elicitins are small extracellular holoproteins produced by many species of *Phytophthora*. Upon application to tobacco plants they were shown to induce tissue necrosis (Billard et al. 1988; Ricci et al. 1989), as well as production of ethylene and the phytoalexin capsidiol (Milat et al. 1991).

4.2.1.2 Oligosaccharides as Elicitor

Oligosaccharides derived from cell walls of fungi and plants, including β-glucans, chitin, chitosan, and pectin, are inducers of the synthesis of a wide spectrum of defensive chemicals in plant tissues. These oligosaccharides are generated at infection or wound sites and may be early signals to activate genes whose products, such as antibiotic phytoalexins, extensin, proteinase inhibitors, pathogenesis-related proteins (PR proteins), and lignin, enhance the plants' defenses against pathogens and herbivores. Unlike the N-linked oligosaccharides that are involved in recognition systems in animals and yeast, the well-characterized carbohydrates that activate plant defensive genes are not covalently attached to proteins. They are relatively small oligomers that are hydrolytic fragments derived from cell walls of attacking pathogens or pests or from the cell walls of the plant itself (Darvill and Albersheim 1984; Ryan 1987). The ability of these oligosaccharides to alter gene expression patterns has stimulated renewed interest in intracellular and intercellular signaling processes in plants.

Chitosan is a mostly acetylated β-1, 4-linked D-glucosamine polymer, which acts as a structural component of the cell wall of several plant fungal pathogens, such as *Fusarium* species. Orlita et al. (2008) used chitin and chitosan as elicitor of coumarins and furoquinolone alkaloids in *Ruta graveolens* (common rue). There was a significant increase in the growth rate of *R. graveolens* shoots in the presence of either chitin or chitosan. Moreover, the results of the elicitation of coumarins and alkaloids accumulated by *R. graveolens* shoots in the presence of chitin and chitosan show that both compounds induced a significant increase in the concentrations of nearly all the metabolites. Adding 0.01 % chitin caused the increase in the quantity (μg/g dry weight) of coumarins (pinnarin up to 116.7, rutacultin up to 287.0, bergapten up to 904.3, isopimpinelin up to 490.0, psoralen up to 522.2, xanhotoxin up to 1531.5 and rutamarin up to 133.7). The higher concentration of chitosan (0.1 %) induced production of simple coumarins (pinnarin up to 116.7 and rutacultin up to 287.0), furanocoumarins (bergapten up to 904.3, isopimpinelin up to 490.0, psoralen up to 522.2, xanhotoxin up to 1531.5) and dihydrofuranocoumarins (chalepin up to 18 and rutamarin up to 133.7). Such a dramatic increase in the production of nearly all metabolites suggests that these compounds may be participating in the natural resistance mechanisms of *R. graveolens*. Whereas, Putalum et al. (2007) used chitosan in hairy root cultures of *Artemisia annua*. Artemisinin production by hairy roots of *A. annua* was increased six-fold to 1.8 μg mg^{-1} dry weight over 6 days by adding 150 mg chitosan l^{-1}.

Elicitor-like substances are involved in the symbiosis between leguminous plants and rhizobia (Cullimore et al. 2001). The bacterial partner produces

chemical signals-nodulation (Nod) factors-that are responsible for appropriate recognition of the bacteria by the plant partner and subsequent nodulation. The Nod factors are lipo-chito-oligosaccharides and there are pieces of evidence suggesting that the perception of Nod factors has evolved from recognition of more general elicitors of plant defence such as chitin fragments or LPS (Boller 1995; Cullimore et al. 2001). Interestingly, LPS from plant growth-promoting rhizobacteria trigger induced systemic resistance (ISR) to subsequent infections of plant pathogens without eliciting the accumulation of PR proteins or phytoalexin (van Loon et al. 1998).

Cline and Coscia (1988) reported the stimulation of Sanguinarine production by combined fungal elicitation and hormonal deprivation in cell suspension cultures of *Papaver bracteatum*. The fungal elicitor preparations from either homogenized mycelia of *Dendryphion penicilatum*, a specific pathogen of *Papaver* species or conidia of *Verticillium dakliae*, a general pathogen, were added to 14-day-old suspension cultures of *Papaver bracteatum*. Dendryphion extracts elicited an accumulation of the benzophenanthridine alkaloid, sanguinarie, which was not greatly influenced by hormone deprivation. Millimolar concentrations of dopamine were detected under all conditions. The baine was found when cells were cultured in hormone-free media, but it was not elicitor dose dependent. Verticillium-elicited cultures accumulated sanguinarine in an elicitor dose-dependent manner only under conditions of hormonal deprivation, resulting in an elevation of sangirine levels 5- to 500-fold greater than controls (2–10 % dry weight).

Wang et al. (2001) reported a protocol for the enhancement of Taxol production and excretion in *Taxus chinensis* cell culture by fungal elicitation and medium renewal. An endophytic fungus, *Aspergillus niger*, isolated from the inner bark of a *Taxus chinensis* tree, was used as an elicitor to stimulate the Taxol (paclitaxel) production in a *Taxus chinensis* cell suspension culture. Different elicitor doses and elicitation times were tested in a batch culture; and the highest volumetric Taxol yield was achieved when 40 mg/l of the fungal elicitor (carbohydrate equivalent) was added to the culture during the late exponential-growth phase. The elicitation resulted in a more than two-fold increase in the Taxol yield and about a six-fold increase in total secretion. The Taxol yield was further improved substantially by applying medium renewal and re-elicitation to the culture. In particular, with repeated medium renewal (in a way similar to medium perfusion) and a second elicitation of the culture, the volumetric Taxol yield was increased to 67.1 mg/l, which was about seven times the amount obtained in the non-elicited batch culture.

4.2.2 Abiotic Elicitors

Abiotic elicitors or stress agents, on the other hand, include ultraviolet radiation, heavy metal salts, and other chemical compounds with diverse mechanisms of action (Eilert 1987). The use of abiotic elicitors in plant cell cultures has received less attention compared with the biotic elicitors (Radman et al. 2003).

4.2.2.1 Heavy Metal Salts as Elicitor

Some heavy metal salts are often found to trigger phytoalexin production. For example, $AgNO_3$ increased significantly scopolamine release (three-fold) and scopolamine and hyoscyamine accumulation (5- to 8-fold) in hairy root culture of *Brugmansia candida*. While, $CaCl_2$ had little effect on accumulation or release of either alkaloid (scopolamine and hyoscyamine). $CdCl_2$ acted positively on the release of both alkaloids (3- to 24-fold), but was highly detrimental to growth. (Pitta-Alvarez et al. 2000). Zhao et al. (2010) examined the effects of biotic and abiotic elicitors on the production of diterpenoid tanshinones in *Salvia miltiorrhiza* cell culture. Four classes of elicitors were tested, heavy metal ions (Co^{++}, Ag^{+}, Cd^{++}), polysaccharides (yeast extract and chitosan), plant response-signaling compounds (salicylic acid and methyl jasmonate), and hyperosmotic stress (with sorbitol). Of these, Ag (silver nitrate), Cd (cadmium chloride), and polysaccharide from yeast extract (YE) were most effective to stimulate the tanshinone production, increasing the total tanshinone content of cell by more than ten-fold (2.3 mg/g versus 0.2 mg/g in control). The stimulating effect was concentration-dependent, most significant at 25 μM of Ag and Cd and 100 mg/l (carbohydrate content) of YE. Of the three tanshinones detected, cryptotanshinone was stimulated most dramatically by about 30-fold and tanshinones I and IIA by no more than five-fold. Meanwhile, most of the elicitors suppressed cell growth, decreasing the biomass yield by about 50 % (5.1–5.5 g/l versus 8.9 g/l in control) (Zhao et al. 2010).

4.2.2.2 Ultrasound as Elicitor

The low-energy ultrasound (US) can act as an abiotic elicitor to induce plant defense responses and stimulate secondary metabolite production in plant cell cultures (Wu and Lin 2002). In addition, US can induce cell membrane permeabilization so as to enhance intracellular product release. This cell-permeabilizing effect may be complementary to the two-phase culture to accomplish product release from the cells and removal from the medium. In the study of Lin and Wu (2002), the combination of US stimulation and in situ solvent extraction in a *Lithospermum erythrorhizon* cell culture led to 2- to 3-fold increases in the yield of shikonin. While, in *Taxus chinensis* Wu and Lin (2003) achieved 1.5- to 1.8-fold increase in taxol yield with 2 min US treatment once or twice during a week-culture period. Ramani and Jayabaskaran (2008) reported enhanced catharanthine and vindoline production in suspension cultures of *Catharanthus roseus* by ultraviolet-B light. A dispersed cell suspension culture from *C. roseus* leaves in late exponential phase and stationary phase were irradiated with UV-B for 5 min. The stationary phase cultures were more responsive to UV-B irradiation than late exponential phase cultures. Catharanthine and vindoline increased 3-fold and 12-fold, respectively, on treatment with a 5-min UV-B irradiation.

4.2.2.3 Light as Elicitor

Light plays a role in both growth and secondary metabolite production. Many hairy root lines when exposed to light, turn green, and develop mature chloroplasts fully capable of photosynthesis (Flores et al. 1993). Green roots have metabolic capabilities distinct from their non-green counterparts. For example, *Ipomoea aquatica* hairy roots grown in the light produce twice as much biomass and four times as much peroxidase as roots grown in the dark (Taya et al. 1994).

Roots do not have to turn green, however, to show profound alterations in secondary metabolism in response to light. For example, increasing light intensity to about 200 mmol m^{-2} s^{-1} doubles the growth yield of *A. annua* hairy roots. In contrast, light inhibited the growth of *Tagetes patula* hairy roots and significantly altered the type of thiophenes produced compared to dark-grown cultures (Mukundan and Hjortsø 1991). Sauerwein et al. (1992) also found that the alkaloid content of both normal and hairy roots of *Hyoscyamus albus* was greater in roots grown in the light compared to roots grown in the dark. Bhadra et al. (1998) studied the effect of light on growth as well as indole alkaloid accumulations of *Catharanthus roseus* hairy root cultures. The total yield of alkaloids was significantly different between light-adapted and dark-grown roots. More interestingly, they demonstrated that production of some alkaloids during a specific growth phase shifts when light conditions are altered. Light-adapted roots show growth-associated production of serpentine, and non-growth-associated production of tabersonine. When roots are shifted from dark to light, production of ajmalicine shifts from being growth-associated to non-growth-associated. Taken together these responses are not surprising since considerable evidence now shows that many of the enzymes in the terpenoid and other alkaloid pathways are regulated by light.

4.3 Active Mechanism of Elicitation in Plant Cells

The active mechanisms employed by elicitors are complex and distinctive. As little is known regarding the biosynthetic pathways of most secondary plant metabolites, the effects of elicitation on a plant cell/tissue cultures are difficult to predict. Therefore, elicitation approaches tend to be empirical steps. The effects of elicitors rely on a host of factors, including the concentration of the elicitor, the growth stage of the culture at the time of elicitation and the contact duration of elicitation (Lu et al. 2001).

Plant cells respond to various biotic and abiotic elicitors by activating a wide array of reactions viz., ion fluxes across the plasma membrane, synthesis of reactive oxygen species (ROS) and phosphorylation and dephosphoryaltion of proteins. These are all putative components of signal transduction pathways that lead to elicitor-induced defense response, e.g. the activation of defense genes and hypersensitive cell death (Dietrich et al. 1990; Nürnberger et al. 1994b). It has been suggested that ROS alone cannot mediate a sufficient disease resistance response

in plants, but in combination with Nitric oxide (NO) can function synergistically to activate a stronger response (Wang and Wu 2005). NO is a diffusible, bioactive signaling molecule (Beligni and Lamattina 2000; Neill et al. 2003; Romero-Puertas et al. 2004). In cell suspension cultures, NO plays a crucial role in the synthesis of secondary meatbolites via chemical (e.g., methyl jasmonate, Wang and Wu 2005), physical ultrasound (Wang et al. 2006), or microbial elicitors (Wang and Wu 2005), physical ultrasound (Wang and Wu 2004; Xu et al. 2005).

Wu et al. (2007) reported the involvement of NO in elicitation, accumulation of secondary metabolites and antioxidant defence in adventitious roots of *Echinacea purpurea*. When roots were treated with 100 mM sodium nitropruside (SNP), an exogenous NO producer, the accumulation of phenolics, flavonoids, and caffeic acid derivatives was enhanced.

Intensive research has been devoted to establishing the mechanism of elicitation in plants. Research was focused mainly on the biotic, particularly carbohydrate elicitors and the effects of abiotic elicitors on the over production of secondary metabolites in plants is poorly understood.

A general mechanism for biotic elicitation in plants may be summarized on the basis of elicitor-receptor interaction. When a plant or plant cell culture is challenged by the elicitor an array of biochemical activities occurs which are as follows;

- Binding of the elicitor to a plasma membrane receptor (Cheong and Hahn 1991; Nürnberger et al. 1994b; Leburun-Garcia et al. 1999).
- Altered ion fluxes across the plant cell membrane i.e., Cl^- and K^+ efflux, Ca^{++} influx (Zimmermann et al. 1999; Ivashkina et al. 2001).
- Increased activity of the plant phospholipases was found in some plant tissues and cultured cells after elicitor contact (Munnik 2001; Wang 2001; Laxalt and Munnik 2002); synthesis of secondary messengers Ins (Hammond-Kosack and Jones 2000) P_3 and diacylglycerol (DAG) (Mahady et al. 1998) mediating intracellular Ca^{++} release, nitric oxide (Delledone et al. 2002; Huang et al. 2002) and octadecanoid signaling pathway (Piel et al. 1997).
- Rapid changes in protein phosphorylation patterns have been observed upon elicitor treatment of a variety of cell cultures (Boller 1995; Siegrist et al. 1998)
- G-protein activation (Mahady et al. 1998; Luan 1998) which are also involved in the early responses to elicitors.
- Activation of NADPH oxidase responsible for AOS and cytosol acidification (Leburun-Garcia et al. 1999).
- Cytoskeleton reorganization (Kobayashi et al. 1995).
- Generation of active oxygen species (Apostol et al. 1989; Levine et al. 1994).
- Accumulation of pathogenesis-related proteins such as chitinases and glucanases, endo-polygalacturonases, hydroxyproline-rich glycoproteins, protease inhibitors (Stintzi et al. 1993; Benhamou 1996).
- Cell death at the infection sit (hypersensitive response), (Luan 1998; Mahady et al. 1998).
- Structural changes in the cell wall (lignifications of the cell wall, callus deposition), (Kauss et al. 1989).

- Transcriptional activation of the corresponding defense response genes (Memelnik et al. 2001; Cormack et al. 2002).
- Plant defence molecules such as tannins and phytoalexins are detected 2–4 h after stimulation with the elicitor (Ito and Shibuya 2000; Pedras et al. 2002).
- Synthesis of jasmonic and salicylic acids as secondary messenger (Katz et al. 2002).
- Systematic acquired resistance (Leburun-Garcia et al. 1999).

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Glossary

Elicitor An elicitor, in biology, is a molecule that enhances the production of another.

Phytoalexins Any of various antimicrobial chemical substances produced by plants to combat infection by a pathogen (as a fungus).

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Chapter 5
Plant Natural Products as a Potential Source of Antimicrobial Agents: An Overview and a Glimpse on Recent Developments

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5.1 Introduction

Looking back to the literature, anyone would realize that human beings were utilizing the potential of natural products of plant origin to cure infections, for instance, ancient cultures in India, Greece, Serbia etc. were using moulds and other plants as a remedy to treat infections (Anonymous 2012a).

In 1877, Pasteur and Joubert recognized the potential of "microbial" products as therapeutic agents that could inhibit the growth of *Anthrax* bacilli. However, the milestone in the field of antimicrobial agents is believed to be the advent of

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penicillin by Alexander Fleming from the fungus *Penicillium* (Shahid et al. 2009a, b). Subsequently the potential for antibacterial and antifungal properties was searched in other fungi and higher plants.

However due to selection pressure, as a result of increasing and irrational usage of existing antimicrobial agents, the microorganisms have started expressing resistance to these compounds. Therefore, scientists are continuously trying to search for newer compounds, harboring antimicrobial properties, derived from the plant species. In this chapter, the potential of plant species to yield newer antibacterial agents will be illustrated with an emphasis on compounds exclusively isolated in very recent years.

5.2 Antibacterial Potential in Plant Natural Products

We previously published an exhaustive literature review on antibacterial potential of plant natural products, and the readers are encouraged to read the same for a descriptive list of plant species harboring antibacterial potential and the chemical compounds, reported by various workers (Shahid et al. 2009a). In this section some of the plant species recently reported (2011–2012) to bear the antibacterial potential will briefly be reviewed.

5.2.1 Quercus dilatata *L.*

Quercus dilatata L., known as "Holly Oak", is commonly found in Afghanistan, Pakistan, and temperate Himalayas from Kashmir to Nepal (Anonymous 2012b).

The tree is up to 20 m in height. The leaves are elliptic-ovate to broadly lanceolate (4–12×1.6–5.5 cm), entire to spiny toothed, and containing nerves 9–12 pairs, forked at the extremities (Anonymous 2012b). Few other species of *Quercus* were reported to possess antibacterial activity (Jamil et al. 2012) and used to treat infected wounds and burns (Jamil et al. 2012). However, in a recent study by Jamil et al. (2012), the crude methanolic extract of aerial parts of *Quercus dilatata* L. was described, as a first report, to possess antibacterial activity against all of the bacterial species tested, namely, *Escherichia coli*, *Bacillus subtilis*, *Salmonella setuball*, *Bordetella bronchiseptica*, *Staphylococcus aureus* and *Micrococcus luteus* (Jamil et al. 2012). Further bioassay guided fractionation and phytochemical analysis of the extracts demonstrated that ethyl acetate, acetone, ethanol and 50 % methanol fractions contained the activity; the most active fraction was reported as ethanol extract by the authors (Jamil et al. 2012). Phytochemical analysis demonstrated the presence of alkaloids as a major component responsible for the activity and HPLC analysis of the active partitioned fraction (Ethanol) demonstrated a distinct peak not corresponding to the

peaks of other natural compounds reported from *Quercus* viz. ascorbic acid, quercitrin, gallic acid and rutin; this suggests the presence of a different compound that needs to be elucidated structurally (Jamil et al. 2012).

This recent study by Jamil et al. reports the presence of a new antibacterial compound in *Quercus dilatata* L. that needs to be characterized further for its exact identification.

5.2.2 Gnetum montanum

Gnetum montanum is a vine commonly found in China and also in Nepal, Thailand, Vietnam and India. It belongs to Family *Gnetaceae* and commonly known as Gam Nui or Sot Nui (Anonymous 2012c). It is an evergreen dioecious climber that is branching and swollen at nodes (Anonymous 2012d). Leaves are dark green, oblong-ovate and 12–30 cm in length, while the plant produces a red colored fruit of 1.5 × 1 cm diameter. Very recently Martin and colleagues described antibacterial potential of *Gnetum montatum* extract against *Pseudomonas aeruginosa* wild-type strain PAO1 (Martin et al. 2011). The authors described the presence of various new natural products such as 6a,7-didehydro-1,9,10-trihydroxy-2-methoxy-6-methylaporphinium trifluoroacetate, *N*-methyllaudanosolinium trifluoroacetate, 3′-hydroxy-*N*, *N*-dimethylcoclaurinium trifluoroacetate, and 1,9,10-trihydroxy-2-methoxy-6-methylaporphinium trifluoroacetate along with the previously known natural products such as latifolian A and magnocurarine. The anti-Pseudomonal activity was reported in the natural product latifolian A (Martin et al. 2011).

Recent years have really witnessed a much faster appearance of antibiotics resistance in bacteria as opposed to the development of antibacterial compounds. The drug-industry has tried its best to respond to the challenge of rising resistance and recently developed some novel β-lactams compounds such as ceftobiprole, ceftaroline, etc. However, the pace of antimicrobial drug development has drastically slowed during the last decade with only few newer agents available. The scientists are now looking forward towards plant natural products as an alternative to combat this threatening issue of antibiotics resistance. To that end, various research groups have recently tried to explore the antimicrobial potential of plant extracts. Table 5.1 summarizes some of the examples of the recently explored plant species for their antibacterial potential.

5.3 Antimycobacterial Potential

It is a well known fact that >40 % of the world population is infected with tubercle bacilli, however a diminutive percentage of the infective population develops tuberculosis (TB). According to a recent review, approximately eight million new

Table 5.1 Some of the very recently described plant species with antibacterial activity

Plant species	Active extract	Compound responsible	Organism	References
Abrus schimperi	Ethanol	Pendulone	*Staphylococcus aureus*; MRSA	Rahman et al. (2011)
Crotalaria retusa	Ethanol		*Pseudomonas aeruginosa*	Devendra et al. (2012)
Elephantopus scaber L.	Ethanol (root)		*S. aureus*, *E. coli*, *P. aeruginosa*	Anitha et al. (2012)
	Ethanol (leaves)		*E. faecalis*, *P. mirabilis*, *S. typhi*, *Enterobacter* spp.	
	Chloroform		*Bacillus cereus*	
Hemigraphis colorata	Benzene		*Acinetobacter* spp., *S. aureus*	Anitha et al. (2012)
Chelidonium majus L.	Ethanol	8-hydroxydihydrosanguinarine 8-hydroxydihydrochelerythrine	ESBL-producing bacteria	Guo et al. (2008)
Sida alba L.	Polyhenol-fraction		*Enterococcus faecalis*	Konate et al. (2012)
Artocarpus heterophyllus	Latex		*Pseudomonas aeruginosa*	Siritapetawee et al. (2012)

cases and two million deaths occur each year and *Mycobacterium tuberculosis* alone accounts for more mortality than any other single bacterial species (Bueno et al. 2011). The World Health Organization (WHO) embarked to reduce the global burden of TB and bring down the TB deaths and prevalence to half of the existing situation by 2015 through its Stop TB Strategy and supporting the Global Plan to Stop TB (Anonymous 2012e). Although the treatment options are available, the emergence of the Mutli-drug-resistance (MDR) and extensively drug-resistance (XDR) in *Mycobacterium tuberculosis* has worried the clinicians in recent years. Keeping in mind the emergence of drug-resistance in tubercle bacilli, especially the emergence of XDR, where we have very little options for the treatment, it is worth finding out the natural plant products as an alternate for the cure. Several plant species have been reported to possess anti-Mycobacterial activities, to name a few, *Indigofera longeracemosa* (Bueno et al. 2011), *Calophyllum lanigerum* (Bueno et al. 2011), *Engelhardia roxburghiana* (Lin et al. 2005) and *Lantana hispida* (Jimenez-Arellanes et al. 2007). Detailed reviews on the current aspect describing the plants and the compounds responsible for the antimycobacterial activity are discussed in some of the recent review articles (Bueno et al. 2011; Guzman et al. 2012). This area seems promising to search for the novel anti-Mycobacterial plant products, especially active against MDR and XDR tuberculosis.

5.4 Antifungal Potential in Plant Natural Products

Since the introduction of one of the oldest antifungal classes of antibiotics, polyene macrolides (nystatin and amphotericin B), and subsequently the other classes such as imidazoles (clotrimazole, miconazole, ketoconazole), first generation triazoles (fluconazole, itraconazole) and second generation triazoles (voriconazole, posaconazole, ravuconazole), the fungi have evolved tremendously with the development of resistance to these antifungal agents. The industry attempted to respond, on one hand, by developing liposomal formulations (liposomized amphotericin B and nystatin) in order to develop methods for targeted drug delivery to minimize the adverse effects and development of resistance, and on the other hand by developing newer classes of antifungal drugs such as echinocandins, caspofungin, micafungin, anidulafungin, pneumocandins, pradimicins and benanomicins, nikkomycins and sordarins. However, the fungi are continuously evolving and there is a need to search for newer compound to combat the emerging problem of antifungal resistance (Shahid and Tripathi 2011). To that end, scientists are continuously searching the herbal plants for their potential antifungal action. Table 5.2 summarizes the recently reported plant species to possess antifungal potential that could be used as potential candidates for the future development of antifungal compounds.

Table 5.2 Some of the very recently described plant species with antifungal activity

Plant species	Active extract	Compound responsible	Organism	References
Polygonum ferrugineum Wedd.	DCM extract	Cardamonin	*Epidermophyton floccosum*	Lopez et al. (2011)
Momordica charantia	Ethanol	–	*Candida* spp.	Santos et al. (2012)
Origanum vulgare	Essential oil	–	Contaminant molds	Chaves-Lopez et al. (2012)
Equisetum arvense	Crude extract	–	*Aspergillus flavus*	Garcia et al. (2012)
Stevia rebaudiana	Crude extract	–	*Fusarium verticilliodes*	Garcia et al. (2012)
Zizyphus jujube	Methanol, n-hexane Chloroform, ethyl acetate	–	*Penicillium notatum*	Ahmad et al. (2011)
	n-hexane	–	*Aspergillus niger*, *Fusarium oxysporum*, *Rhizopus stolonifer*	
Cassia fistula L.	Crude (seed extract)	–	*Candia albicans*	Jothy et al. (2011)

5.5 Antiparasitic Potential in Plant Natural Products

5.5.1 Abrus schimperi

The Genus *Abrus* is a flowering plant of the family Fabaceae containing around 18 species. Abrus schimperi is a flora of tropical Africa. In a recent study by Rahman et al. (2011), the authors described the anti-Leishmanial activity of the ethanolic extract of Abrus schimperi against promastigotes of *Leishmania donovani*. The authors reported the presence of two isoflavanquinones, amorphaquinone and pendulone with IC50 values of 0.63 and 0.43 μg/ml, respectively. The authors also reported the antiplasmodial activity in the two extract against *Plasmodium falciparum* D6 and W2 strains (Rahman et al. 2011).

5.5.2 Momordica charantia

Momordica charantia is commonly known as bitter melon or bitter gourd. It is a tropical and subtropical vine of the family *Cucurbitaceae* and is commonly grown in Asia, Africa, and the Caribbean for its edible fruits. In a recent study, Santos and colleagues (2012) described anti-Trypanosomal activity in the ethanolic extract from leaves of *Momordica charantia* against epimastigotes of *Trypanosoma cruzi*; the IC50 was reported as 46.06 μg/ml (Santos et al. 2012). The authors suggested its anti-Trypanosomal activity (against epimastigotes) with moderate toxicity in comparison to the available drugs (Nifurtimox and Beznidazole).

During our search of recent literature, we noticed numerous other interesting studies reporting plant species to possess anti-parasitic activities. For instance, Nibret and Wink (2011) screened 30 Ethiopian-plant species, those were used in traditional medicine, for anti-Trypanosomal activity. They found five plants namely, *Dovyalis abyssinica*, *Albizia schimperiana*, *Ocimum urticifolium*, *Acokanthera schimperi* and *Chenopodium ambrosioides* to possess activity against Trypanosoma brucei brucei. In another study, Nibret et al. (2010) screened 20 Tanzanian-plant species for anti-Tryponosomal activity and found *Entadrophragma bussei*, *Securidaca longepedunculata*, *Warburgia salutaris*, *Zanha Africana* and *Zanthoxylum chalybeum* to possess the activity. In a recent study, Okokon et al. (2012) described anti-Plasmodial activity in the leaf-extracts from *Clausena anisata*.

5.6 Utility of Plant-Secondary Metabolites (PSMs) in Reversing Multi-drug Resistance (MDR)

Bacteria evolved producing multi-drug resistance to several antibiotics as a result of selection pressure. There are several mechanisms by which bacteria can produce resistance to these antimicrobial agents (Cowan 1999; Shahid et al. 2009c).

These escaping strategies in a resistant bacterium could be by many ways, some of them are mentioned below:

(A) modification of normal drug-binding proteins such as penicillin binding proteins (PBPs) or bypassing of the normal PBPs
(B) impermeability of outer membranes of the bacteria to drugs
(C) production of enzymes that inactivates drugs, such as β-lactamases
(D) ability to pump out drugs by efflux pumps

Recent years have witnessed the role of plant-secondary metabolites (PSMs) as the inhibitors of ABC transporter system and hence play a role in reversing antimicrobial resistance. Secondary metabolites, such as terpenoids, function as substrate for P-gp (in cancer cells) and its orthologue (in parasites), and for other ABC transporter systems such as AtrB (in fungi) and NorA efflux pump (in *Staphylococcus aureus*) and thus serve as inhibitors of ABC transporters (Andrade et al. 2000; Smith et al. 2007; Wink et al. 2012). During our search of literature, we noticed an important and exhaustive recent review on the current theme published by Wink et al. (2012) describing various PSMs that were reported to reverse the resistance in cancer cells and microbes to cytotoxic and antimicrobial agents, respectively. We encourage interested readers to go through that "must read" article to get better insight into the subject. Since our present compilation deals with the antimicrobial potential in plant-natural products, we intend to summarize here, from the existing literature, the potential role of PSMs in reversing antimicrobial resistance in some of the medically important microorganisms such as Staphylococcus aureus (including methicillin-resistant *S. aureus*), *Mycobacterium tuberculosis* (including multidrug resistant (MDR) strains) and other medically important microorganisms. Table 5.3 summarizes the PSMs, their source of isolation and their activity on ABC transporters of target microorganisms.

5.7 Antiviral Potential in Plant Natural Products with Special Reference to HIV and Influenza Virus

Despite the passage of almost three decades since the discovery of Human Immunodeficiency Virus (HIV) and AIDS, the field of Medicine is still struggling to find any effective cure for this disease syndrome. Many researchers sought plant natural products for anti-HIV activity.

Several classes of the plant natural products, including terpenoids, flavanoids and alkaloids have been reported to possess anti-HIV activity (in vitro) that possibly targeted reverse transcriptase/integrase/protease/viral fusion etc. We encourage interested readers to read the articles by Tan et al. (1991), Pengsuparp et al. (1995), and Cowan (1999) for detailed description of various plant species reported to possess ant-HIV activities and also for their reported targets of action.

During our search of recent literature, we noticed numerous new reports describing various other plant species having anti-HIV potential and those can be utilized

Table 5.3 Some of the plant secondary metabolites (PSMs) reported to possess antimicrobial activities

Microorganism	PSM	Source	Activity	References
Staphylococci				
S. aureus	Calodenin B, dihydrocalodenin B, other dimeric proanthocyanidins	*Ochna macrocalyx*	Inhibit MDR	Tang et al. (2003); Wink et al. (2012)
	Chrysosplenol-D, chrysosplenetin	*Artemisia annua* L.	Synergistic inhibition of MDR	Stermitz et al. (2002); Wink et al. (2012)
	Tiliroside	Platanus orientalis, Herissantia tiubae	Inhibits NorA efflux protein	Falcao-Silva et al. (2009); Wink et al. (2012)
MRSA[a]	Emodin	*Rheum palmatum*	Synergistic activity with oxacillin in MRSA	Lee et al. (2010); Wink et al. (2012)
	Canthin-6-one, 8-hydroxy-canthin-6-one	*Allium neapolitanum*	Inhibit MDR	O'Donnell and Gibbons (2007); Wink et al. (2012)
	Chelerythrine	*Zanthoxylum clava-herculis*	Reversal of drug resistance	Gibbons et al. (2003); Wink et al. (2012)
	5-Methoxyhydnocarpine, pheophorbide A	*Hydnocarpus kurzii*, *Berberis* spp.	Inhibitor of NorA MDR pump	Stermitz et al. (2000; 2001); Wink et al. (2012)
	N-trans-feruloyl 4′-O-methyldopamine	*Mirabilis jalapa*	Inhibits *S. aureus* over expressing NorA pump	Michalet et al. (2007)
	Totarol	*Podocarpus totara*	Inhibits NorA efflux pump	Smith et al. (2007)
Mycobacterium tuberculosis	Aegicerin	*Clavija procera*	Reversal of MDR in resistant- *M. tuberculosis*	Rojas et al. (2006)
	Piperine	*Piper nigrum*	Inhibits over expression of Mycobacterial efflux protein (Rv1258c)	Sharma et al. (2010)
	Vasicine acetate, 2-acetyl benzylamine	*Adhatoda vasica*	Inhibits MDR starins	Ignacimuthu and Shanmugam (2010)
Acinetobacter baumannii	Ellagic acid, tannic acid	Several plant species	Inhibition of efflux pump	Chusri et al. (2009); Wink et al. (2012)
Candida albicans	Plagiochin	*Marchantia polymorpha*	Reversal of the efflux pump	Guo et al. (2008); Wink et al.(2012)

[a]*MRSA* Methicillin-resistant *S. aureus*

Table 5.4 Plant species reported to possess anti-HIV activity in the recent literature

Plant species	References
Aegle marmelos	Sabde et al. (2011)
Asparagus racemosus	Sabde et al. (2011)
Coleus forskohlii	Sabde et al. (2011)
Rubia cordifolia	Sabde et al. (2011)
Ocimum sanctum Linn.	Rege et al.(2010)
Tinospora cordifolia (Willd) Miers. Ex Hook.f. &Thoms	Rege et al. (2010)
Avicennia officinalis Linn.	Rege et al. (2010)
Rhizophora mucronata Lam.	Rege et al. (2010)
Azadirachta indica	Awah et al. (2011)
Bubine alooides (L.) Willd.	Klos et al. (2009)
Leonotisleonurus (L.) R.Br.	Klos et al. (2009)

in future to prepare novel anti-HIV compounds. Readers are also encouraged to read a recent review article by Filho et al. (2010) where the authors described a list of 275 species of medicinal plants that were studied for the activity on HIV-1-protease.

A recent study was done by Sabde et al. (2011) directed towards assessment of anti-HIV activity of various extracts prepared from Indian medicinal plants as immunomodulators. Ninety-two extracts were prepared from 23 plants. Anti-HIV activity was measured in a human CD4+ T-cell line, CEM-GFP cells infected with HIV-1NL4.3. Nine extracts of eight different plants significantly reduced viral production in CEM-GFP cells infected with HIV-1NL4.3. *Aegle marmelos*, *Argemone mexicana*, *Asparagus racemosus*, *Coleus forskohlii*, *and Rubia cordifolia* demonstrated promising anti-HIV potential and were investigated for their active principles.

A recent review by Singh et al. (2011) described numerous plant species possessing anti-HIV activity, their active ingredients, assay model used and the mechanism(s) of action. Table 5.4 describes few more recently reported plant species to harbor anti-HIV potential.

Recent years have witnessed a tremendous health-threat through recent H1N1 influenza pandemic. Although officially declared in August 2010 as the end of pandemic by World Health Organization (WHO), the threat has not disappeared for its revisit (Shahid 2012). Keeping in mind the global burden of such a disease, researchers are continuously searching the natural products for their action against influenza virus. During the search for recent literature, we found some published studies that reported for the anti-influenza activities in plant extracts. Some of those studies will briefly be mentioned in this section. Shin and colleagues (2010) tested various Korean medicinal plants for their potential activities against influenza viruses and reported a promising plant species, *Agrimonia pilosa*, for its activity against all three subtypes of human influenza virus, including H1N1 and H3N2 influenza A subtypes and influenza B virus. Moreover, authors reported strong inhibitory effect (in ovo) of the extract on H9N2 avian influenza virus when tested in embryonated eggs. The plant species reported by Shin et al. (2010) could be a promising candidate for drug development against the influenza viruses. Haidari et al. (2009)

reported the anti-viral activity in pomegranate polyphenol extract (PPE) against H3N2 influenza virus. Similarly, the catechins in the green tea have been reported to possess anti-viral activity against influenza virus (Song et al. 2005).

In a recent study by Sood et al. (2012), the authors described anti-viral activity in the crude extracts of leaves and bark of Eugenia jambolana Lam. against the highly pathogenic avian influenza (H5N1) virus.

In nutshell, probably the nature has provided us the cure for most of the diseases in our environment, and it is our duty to search for cure of those ailments. To that end, natural products from the medicinal plant species should extensively be searched for as to combat these life-threatening illnesses.

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Chapter 6
Recent Trends in Prevention and Treatment of Dental Caries and Periodontal Disease by Natural Plant Products

Pranay Jain and Ram Kumar Pundir

6.1 Introduction

Medicinal plants have been used as traditional treatment for numerous human diseases for thousands of years and in many parts of the world. In rural areas of the developing countries, they continue to be used as the primary source of medicine (Chitme et al. 2003). The natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals. With respect to diseases caused by microorganisms, the increasing resistance in many common pathogens to currently used therapeutic agents, such as antibiotics and antiviral agents, has led to renewed interest in the discovery of novel anti-infective compounds. As there are approximately 500,000 plant species occurring worldwide, of which only 1 % has been phytochemically investigated, there is great potential for discovering novel bioactive compounds. There have been numerous reports of the use of traditional plants and natural products for the treatment of dental diseases. Many plant-derived medicines used in traditional medicinal systems have been reported as agents used to treat infections and a number of these have been recently investigated for their efficacy against oral microbial pathogens. Dental caries and periodontal disease are most common oral diseases showing striking geographic variations, socio-economic patterns and severity of distribution all over the world (Meghashyam et al. 2007). These diseases pose challenges when it comes to determining their microbial etiology (Ruby and Goldner 2007).

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6.2 Etiology of Dental Caries and Periodontal Disease

Dental caries is a localized, progressive demineralization of the hard tissues of the crown and root surfaces of teeth. The demineralization is caused by acids produced by bacteria, particularly *Streptococcus mutans* and lactobacilli that ferment dietary carbohydrates (Fujita et al. 2007). This occurs within a bacteria-laden gelatinous material called dental plaque that adheres to tooth surfaces and becomes colonized by bacteria. Thus, caries results from the interplay of three main factors over time: dietary carbohydrates, cariogenic bacteria within dental plaque, and susceptible hard tooth surfaces (Nishikawara et al. 2007). Among the three factors stated above, bacteria have been suggested to have the strongest effect on the prevalence or incidence of dental caries (Nishikawara et al. 2007). Periodontal disease is a group of illnesses located in the gums and dental support structures (ligament and alveolar bone) and are produced by certain bacteria encountered in subgingival plaque (Giannobile 2007). The American Association of Periodontics has classified periodontal disease into two categories:- gingivitis and periodontitis, according to the area of the gum involved. Gingivitis is very common and is manifested clinically as the bleeding of the gingival or gum tissues without bone loss. Periodontitis occurs when the plaque induced inflammatory response in the tissue results in the actual loss of collagen attachment of the tooth to the bone, and to loss of bone, which in some cases, can extend the entire length of the tooth root (Saboia-Dantas et al. 2007).

Dental caries can be traced back to be as old as civilization with its evidence seen even in skeletal remnants of preshistoric humans (Oliveira et al. 2008). The prevalence of these diseases has greatly increased in modern times. The prevalence and distribution of dental caries is different in developed and developing countries. A decline in the level of dental caries has been observed over the past four decades in developed countries (Oliveira et al. 2008). This reduction has been attributed to the use of fluorides, change in lifestyles, improved oral hygiene practices, preventive dental services and changes in diagnostic criteria (Castillo et al. 2007). The prevalence of caries is high in some developing countries and low in others, when compared to developed countries. India, a developing country, has shown an inclined trend of this disease over a relatively short period of time (Dhar et al. 2007). The prevalence of dental caries in India is 80 % with five decayed teeth per child on an average at the age of 16 years Meghashyam et al. (2007). It is a fact that oral hygiene is seen as a natural part of total body cleanliness and that people desire fresh and healthy mouth with good smelling breath (Dhar et al. 2007). Increased consumption of sugar has been stated as one of the causes for the increase in caries prevalence in developing countries (Anand et al. 2007).

Epidemiological studies performed in many parts of the world and among individuals with a widely varied background indicated that destructive periodontal disease is nearly universal in children and adolescents (Minaya-Sánchez et al. 2007). The prevalence rate of periodontal disease in India approach 90–100 % (Dhar et al. 2007).

The pathogenicity of a microbe denotes its ability to cause disease. The indigenous flora usually protects against disease (Minaya-Sánchez et al. 2007). In the oral cavity, indigenous species become pathogenic when they leave microhabitats where they are harmless (tongue papilla) to reproduce in microhabitats (subgingival crevice) where their byproducts or presence causes disease (Ruby and Goldner 2007). Indigenous species also become pathogenic when they reproduce under certain conditions in certain locations, and becomes a larger proportion of the population (Saboia-Dantas et al. 2007). Dental plaque is a complex permanent community, and the presence of large number of indigenous species do not correlate with caries or periodontal disease (Kuramitsu et al. 2007). It is the balance among these species, the anatomical characteristics of the teeth and gingival crevices and the host diet and hygiene, that, when upset, can lead to disease, referred to as conditional diseases (Castillo et al. 2007).

The primary etiologic agents of dental caries are the streptococci, mainly *Streptococcus mutans* and *S. sobrinus* (Anand et al. 2007). *S. mutans*, a Gram-positive coccus, nonmotile facultative anaerobic microorganism which can metabolize carbohydrates, is one of the most important oral bacteria which plays a major role in dental caries, bacteremia and consequently bacterial endocarditis among predisposed patients (Welin-Neilands and Svensater 2007). Secondary infections are caused by the *Lactobacillus* spp., and acid tolerant streptococci such as *Streptococcus sanguis* (= *sanguinis*), *S. gordanii* and *S. ovalis* (Ledder et al. 2007). Yeast, such as *Candida albicans*, is also prevalent in the saliva and dental biofilm of caries active individuals (Ledder et al. 2007).

The most important and prevalent organisms found in the subgingival area are anaerobic Gram-negative bacteria such as *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Tannerella* (= *Bacteroides*) *forsynthensis* (Hamlet et al. 2008). These bacteria play an important role in the onset and subsequent development of periodontitis, participating in the formation of periodontal pocket, connective tissue destruction, and alveolar bone resorption by means of an immunopathogenic mechanisms (Ledder et al. 2007). Organisms of probable periodontopathic significance include *Campylobacter rectus*, *Peptostreptococcus micros*, *Dialister pneumosintes*, *Fusobacterium* spp., *Eubacterium* spp., β-hemolytic streptococci, enterococci, pseudomonads and other enteric rods and yeasts such as *Candida albicans*, *C. parapsilosis*, *Saccharomyces cerevisiae* (Castillo et al. 2007). *Treponema denticola*, *Eubacterium saphenum*, *Porphyromonas endodontalis*, *T. forsynthensis*, *Filifactor alocis*, *Prevotella denticola*, *Cryptobacterium curtum*, *Treponema medium*, *T. socranskii*, and *Actinomyces naeslundii* have also been discovered in periodontitis (Ledder et al. 2007).

Although microbes are essential for the onset and progression of the periodontal disease, they are probably not enough. This concept is based on the fact that periodontal pathogens can occur in a person without clinical signs of the disease. In order for the disease to develop, a susceptible host is required (Fujita et al. 2007). The nature of susceptibility for destructive periodontal disease has long been an enigma. Today, several factors have been linked to increased susceptibility and

these include age, poor oral hygiene, tobacco smoking, stress, systemic diseases, decreased immunocompetence and genetic traits (Ledder et al. 2007). None of these factors by themselves are probably capable to produce the disease, but it seems likely that a set of risk factors is necessary to initiate the disease process. Exposure to lead and environmental tobacco smoke, which has high concentrations of cadmium, has been linked with an increased risk of dental caries in children (Aligne et al. 2003). For this reason, dental caries and periodontal disease are considered as a multifactorial diseases (Castillo et al. 2007).

6.3 Prevention and Treatment of Dental Caries and Periodontal Disease

Dental caries can be prevented by adequate oral hygiene, reduced frequency in sugar consumption and optimal use of fluoride. Prevention of periodontal disease is mainly based on plaque control (Casas et al. 2007). Although such approaches have been proven to be effective in controlling these diseases, dental caries and periodontal disease still remain a major public health problem in the general population with substantial economic implications (Welin-Neilands and Svensater 2007).

A view to the past indicates that in ancient times, India was probably the most advanced country in dental health services in the world. In the old ayurvedic literature of India, details of gum diseases have been described and forms of treatment have also been described. The practice of oral hygiene was included in daily rituals (Amruthesh 2008). Ancient scriptures such as vedas and puranas proposed that the natural dentition could be preserved by appropriate periodontal treatment. In the present times, ayurvedic treatment is still very popular amongst rural and uneducated people in India. For the treatment of periodontitis, massaging of gums with oils and ointments prepared from herbs is still advised. It has been reported that some of the ayurvedic preparations have various ingredients which have analgesic, anti-inflammatory properties as well as being able to facilitate cleansing and mouth freshening (Amruthesh 2008).

For dental caries, treatment is usually initiated when lesions are clinically detectable (evidence of tooth substance loss, radiotranspirancy in the tooth structure) and tissue damage is irreversible. Treatment usually involves the removal of the affected tissues and placement of a filling material. A similar approach is applicable to periodontal disease (Hajishengallis and Russell 2008). Mechanical debridement is initiated when lesions (presence of periodontal pockets, loss of attachment, radiographic evidence of bone loss, bleeding) are observed. However, there are two shortcomings in this approach. First, clinicians lack diagnostic methods to detect early tissue changes when lesions are still at a reversible change and can be treated in a noninvasive way. Secondly, as in the case of dental caries, the filling approach is not necessarily synonymous with control of the disease. Hence, the attention should be to emphasize prevention and treatment should be targeted at controlling

the etiological agents. Thus, oral microbiology plays a major role not only in the understanding of the etiology but also in preventing and treating oral diseases (Nibali et al. 2007).

In the present clinical scenario globally, there is a great interest in the use of antimicrobial agents for prevention and treatment of plaque-related oral diseases due to the spread of antibiotic resistance in oral cavity microorganisms (Lynch and Robertson 2008). There has been an extensive research on the effectiveness of minimum inhibitory concentration of agents for cariogenic and periodontopathogenic microorganisms (Lynch and Robertson 2008). Because dental caries is an infectious disease of bacterial origin, antimicrobial agents constitute a reasonable approach towards attenuating not only the bacterial biofilm *in situ*, but also its transmission from host to host (Nikolaev and Plakunov 2007). These approaches, while limited, based upon certain constraints inherent in the oral cavity, have their roots in early attempts at plaque control and extend from mechanical approaches to chemical approaches. However, a global reduction of plaque biofilm mass may not lead to the desired effect of selectively eliminating or reducing the caries associated microorganisms (Lynch and Robertson 2008). Thus, the aim of antimicrobial approach for the control of caries should be aimed not towards the elimination of all the plaque microorganisms but towards effecting an ecological shift from a cariogenic to non-cariogenic biofilm (Ledder et al. 2007). While, in the case of periodontal disease, the mechanical periodontal treatment alone is adequate to eliminate or resolve the clinical conditions in most cases, but adjunctive antimicrobial agents, delivered either locally or systematically, can enhance the effect of therapy in specific situations (Sedlacek and Walker 2007). Since the organisms involved in caries and periodontal disease are in the form of biofilms which are less susceptible to antimicrobial agents, therefore, there has been no effective antimicrobial agent yet searched, which can penetrate the biofilm (Lynch and Robertson 2008).

Antibiotics are an essential part of modern medicine. The emergence of antibiotic-resistant mutants among bacteria is seemingly inevitable, and results, within a few decades, in decreased efficacy and withdrawal of the antibiotic from widespread usage. The traditional answer to this problem has been to introduce new antibiotics that kill the resistant mutants (Coates and Hu 2007). Antibiotics have had considerable impact upon our daily life, they are not only used as antimicrobial and antineoplastic agents in medicine but are also used as growth stimulant and in the control of plant diseases (Coates and Hu 2007). Antibiotics include a chemically heterogenous group of small organic molecules of microbial origin that, at low concentrations, are deleterious to the growth or metabolic activities of other microorganisms (Thomashow et al. 2008). They represent the greatest single contribution of drug therapy for the health care of increasing population of the world and provide effective control of many microbial pathogens that have been the cause of death of humans and animals (Takahashi et al. 2007). Currently, the most commonly used antibiotics/drugs used in dental caries and periodontal diseases include amoxycillin, penicillin and metronidazole (Darouiche et al. 2008). These drugs have the potential to select for resistant bacteria within the commensal flora, but there have been reports of microbial resistance to these drugs (Zadik and Levin 2008). The process

of new drug discovery is driven largely by the desire to identify a structurally novel compound that possesses novel and potentially useful biological activity (Luzhetskyy et al. 2007).

6.4 Treatment of Dental Diseases by Natural Plant Products

To cope with the wide-spread problem of antibiotic resistance, a number of strategies such as reduced antibiotic use and antibiotic alternatives have been proposed (Prashant et al. 2007). Among antibiotic alternatives are therapies derived from complementary and alternative medicine. In fact, there are an overwhelming number of studies on the antibacterial activities of plant and natural products derivatives (Hamilton-Miller 2004). Agar diffusion methods (disc diffusion or well diffusion) are popular and have been used in a number of studies (Prabu et al. 2006). These methods involve inoculation of the surface of an agar plate with the test microorganism or pouring molten agar inoculated with the test organism into a Petri dish. The compound to be evaluated can be applied on a paper disc or into a well made in the agar. After appropriate incubation, the appearance of zones of growth inhibition around the disc or well indicates antimicrobial activity. The viable count method can be used as an alternative to agar plate diffusion methods. Cultures of microbes are centrifuged, washed and resuspended in an appropriate buffer to give a standard concentration of cells. The test compound is added to the cells, the mixture is incubated and the number of viable cells is determined by the pour plate method (Park et al. 2003). Time-kill assays are used to determine the time required for a compound or extract under investigation to eliminate the growth of the test bacteria (Bakri and Douglas 2005). Bacteria are incubated in an appropriate medium in the presence of test material at the minimum bactericidal concentration (MBC). At timed intervals, samples are removed, serially diluted and the number of surviving bacteria is determined by plating on agar media. These data are then used to plot time–kill curves. Bioautography has been used to carry out preliminary determinations of the number of active constituents in plant extracts. This method involves separation of the extract using thin layer chromatography (TLC) and overlaying the TLC plate with agar inoculated with test bacteria. After appropriate incubation, the presence of the zones of inhibition identifies fractions that contain antimicrobial components. The use of an indicator such as tetrazolium, is metabolized into a coloured product by growing bacteria can help to visualize the zones of inhibition (Tichy and Novak 1998).

6.5 Antimicrobial Activity of Crude Plant Extracts

Many studies investigating the activity of traditional medicinal plants against oral microbial pathogens have been limited to examination of crude aqueous or organic solvent extracts. For instance, it was validated in one study that chloroform extracts

of the aerial plant parts of *Drosera peltata* (Droseraceae) showed broad spectrum activity against numerous bacteria causing dental caries, with greatest activity against *S. mutans* and *S. sobrinus*. The active component was found to be plumbagin (Didry et al. 1998). Bioautography of the extracts from *Abies canadensis* (Pinaceae), *Albizia julibrissin* (Fabaceae), *Chelidonium majus* (Papaveraceae), *Ginkgo biloba* (Ginkgoaceae), *Juniperus virginiana* (Cupressaceae), *Pinus virginiana* (Pinaceae), *Rosmarinus officinalis* (Lamiaceae), *Sassafras albidum* (Lauraceae), *Tanacetum vulgare* (Asteraceae) and *Thuja plicata* (Cupressaceae) has shown to contain common antimicrobial components, while other extracts possessed chemically different constituents (Tichy and Novak 1998). A boiling water extract of *Coptidis* rhizome (Ranunculacea), a traditional Chinese medicinal plant, has been reported to exhibit bactericidal activity against oral bacteria with particularly good activity against periodontopathogenic bacteria (Hu et al. 2000). Garlic, *Allium sativum* (Liliaceae), has been used as a medicine since ancient times because of its antimicrobial properties. While garlic has been shown to have activity against a wide range of bacteria, the specific activity against the Gram-negative oral pathogens, including *P. gingivalis* has only recently been demonstrated (Bakri and Douglas 2005).

Harungana madagascariensis (Hypericaceae), a native African plant, has been reported to inhibit numerous oral pathogens such as *Actinomyces*, *Fusobacterium*, *Lactobacillus*, *Prevotella*, *Propionibacterium* and *Streptococcus* species and shows enhanced activity when used alongwith poly (D, L-lactide-co-glycolide) nanoparticles. It has been suggested that this may have been due to the bioadhesive properties of the polymer resulting in the extract being in contact with the bacteria for prolonged periods (Moulari et al. 2006). The resin exuded by the *Pistacia lentiscus* (Anacardiaceae) tree, known as mastic gum, has been often used in the remedy for oral malodour and has been shown to have antimicrobial activity against *P. gingivalis* but its low solubility limits its use for local application rather than as a mouthrinse (Sterer 2006).

Helichrysum italicum (Compositae), widely found in the Mediterranean region, has been shown to exert antimicrobial activity against *S. mutans*, *S. sanguis* and *S. sobrinus* (Nostro et al. 2004).

Propolis has been shown to exhibit antimicrobial activity against a range of oral bacteria due to the presence of numerous flavonoids and inhibit the adherence of *S. mutans* and *S. sobrinus* to glass. It has also shown to be a potent inhibitor of water-soluble glucan synthesis (Koo et al. 2000). Propolis has shown antimicrobial activity similar to chlorhexidine and greater than clove or sage extracts in a study investigating the ability of these chemicals to inhibit the growth of microbes obtained from the saliva of periodontally healthy patients and those with chronic perodontitis (Feres et al. 2005). Several plants have been used historically for care of the teeth and oral hygiene. In rural India, the mango is a commonly used in both urban and rural locations. Mango leaves are folded up and covered with tea dust (finely ground, dried *Camellia sinensis* leaves) and rubbed against the teeth. One clinical investigation of this practice investigated the antimicrobial ability of mango leaves in oral hygiene. Mango leaves were shown to inhibit two types of bacteria

(*Prevotella intermedia* and *Porphyromonas gingivalis*) in greater proportion than those using a toothbrush (Bairy et al. 2002). Chewing twigs of the mango or neem tree is a common way of cleaning the teeth in the rural and semi-urban population. These twigs are also believed to possess medicinal properties. Prashant et al. (2007) conducted a study to evaluate the antimicrobial effects of these chewing sticks on *S. mutans*, *S. salivarius*, *S. mitis*, and *S. sanguinis* which are involved in the development of dental caries. Mango extract, at 50 % concentration, showed maximum zone of inhibition on *S. mitis*. Neem extract produced the maximum zone of inhibition on *S. mutans* at 50 % concentration. Even at 5 % concentration neem extract showed some inhibition of growth for all the four species of organisms. It was recommended from the study that a combination of neem and mango chewing sticks may provide the maximum benefit.

Several studies have been reported on the antibacterial effects of chewing sticks of neem (*Azadirachta indica*) and kikar (*Acacia arabica*) on cariogenic bacteria, such as *S. mutans* and on periodontopathogens particularly *Bacteriodes* species (Akpata and Akinremisi 1997) and inhibitory action on dental plaque formation (Wolinsky et al. 1996).

Melaleuca alternifolia (tea tree oil) is well known for its antiseptic, bactericidal and antifungal effects. And while exhibiting considerably toxicity to several pathogenic bacteria and fungi, tea tree oil appears to leave normal skin flora unmolested. Applied topically, it may have negative effects including dermatitis and burning of the oral mucosa (Jandourek et al. 1998). A clinical trial using tea tree oil as an oral mouthwash observed effectiveness at controlling oral bacteria and decreasing plaque development during the time of the trial (Saxer et al. 2003). Another trial using a tea tree oil-containing gel led to a reduction in gingival inflammation and bleeding scores; plaque scores were however not decreased (Soukoulis and Hirsch 2004). The patients reported no adverse effects while using the gel; this form of application may provide a less caustic effect on the mucosa.

The miswak (*Salvadora persica*) is a chewing stick obtained from the persica plant. A traditional form of medicine, the miswak is rooted in religious and cultural custom and its use is widespread in Middle Eastern Muslim countries. One recent trial utilized a persica mouthwash and measured its effectiveness on gingival health and pathogenic bacteria (Khalessi et al. 2004). Use of persica mouthwash led to decreased levels of the cavity-causing bacterium mutans streptococci and improved indices of gingival health. Persica did not, however, reduce dental plaque amounts. Another large-scale trial evaluated several aspects of oral hygiene in Saudi Arabia including effectiveness of the miswak stick in oral health, among other aspects of dental care (al-Otaibi 2004). Investigators revealed that use of the miswak stick was at least as effective as regular toothbrushing for reduction of plaque and control of gingivitis. They also noted that persica was an effective treatment and preventive medicine for periodontal disease.

Combination treatments with botanical medicines for oral health are quite common; ingredients are variable depending on practitioner and patient variables. While the mixing of various botanicals for treatment of oral conditions can be a highly successful approach, these therapies are rarely evaluated on a larger scale,

and for good reason as treatment utilizing herbal medicines are typically highly individualized treatments. However, a combination of sage oil, peppermint oil, menthol, chamomile, echinacea, myrrh, clove oil and caraway oil were applied in a clinical trial with good results (Serfaty and Itic 1988). Investigators were able to treat gingivitis using this combination; the herbs were added to a small amount of water and rinsed three times per day. In addition, a toothpaste containing sage and peppermint oils, chamomile, *Echinacea purpurea*, myrrh and rhatany was evaluated over a period of three and six months for its role in promoting gingival health. This treatment was effective in its ability to decrease plaque size, bleeding and gingival inflammation (Yamnkell and Emling 1988).

From the native American plant *Ceanothus americanus*, ceanothic acid and ceanothetric acid demonstrated growth inhibitory effects against *S. mutans*, *Actinomyces viscosus* and *P. gingivalis* (Li et al. 1997).

The antimicrobial activity of garlic crude extract against oral microbiota was evaluated *in vitro* and *in vivo* in two separate studies by Groppo et al. (2007). The evaluation of antimicrobial activity was carried out in two phases. Study 1 consisted of the evaluation of minimum inhibitory (MIC) and bactericidal (MBC) concentrations against nine streptococci strains. In study 2, a 2.5 % garlic solution was used as a mouthwash in a 5-week study by 30 subjects. Blood agar and Mitis Salivarius Bacitracin agar were inoculated with patients' saliva to quantify oral microorganisms and *mutans* streptococci. Study 1 showed MIC ranging from 0.5 to 32.0 mg/ml for clone 2 and from 8 to 64.0 mg/ml for one extract. MBC ranged from 1.0 to 128.0 mg/ml and from 8.0 to 128.0 mg/ml regarding two extracts respectively. Study 2 showed that 2.5 % garlic mouthwash solution had good antimicrobial activity against *mutans* streptococci and oral microorganisms. Maintenance of reduced salivary levels of streptococci was observed after 2 weeks at the end of mouthwash use. Unpleasant taste (100 %), halitosis (90 %) and nausea (30 %) were reported by patients after the end of the study. It was concluded that the garlic clones have antimicrobial properties *in vitro* against streptococci and anticariogenic properties against oral microorganism in spite of its adverse effects.

Lippia sidoides (*Verbenaceae*), popularly known as "*Alecrim pimenta*" is a typical shrub commonly found in the Northeast of Brazil. Many plant species belonging to the genus *Lippia* yield very fragrant essential oils of potential economic value which are used by the industry for the commercial production of perfumes, creams, lotions, and deodorants (Botelho et al. 2007). The antimicrobial activity of the oil and the major components was tested against cariogenic bacterial species of the genus *Streptococcus* as well as *C. albicans* using the broth dilution and disk diffusion assays. The essential oil and its major components thymol and carvacrol exhibited potent antimicrobial activity against the organisms tested with minimum inhibitory concentrations ranging from 0.625 to 10.0 mg/ml. The most sensitive microorganisms were *C. albicans* and *S. mutans*. The essential oil of *L. sidoides* and its major components exerted promising antimicrobial effects against oral pathogens and is useful to combat oral microbial growth (Botelho et al. 2007).

Joshi et al. (2005) carried out a study among school children of Kulasekharan village of Kanyakumari and found that the prevalence of dental caries was low

compared to the WHO recommended values. It was found to be associated with the consumption of paddy husk powder for brushing, which implicates that the paddy husk powder might be having the antiplaque properties. Aneja et al. (2010a) reported the potent antifungal activity of *Barleria prionotis* L. bark on two *Candida albicans* strains and *Saccharomyces cerevisiae*, involved in oral diseases of human. Acetone, methanol, ethanol, aqueous (hot and cold) extracts of *Barleria prionitis* bark were screened for *in vitro* activity against four oral bacteria *Streptococcus mutans*, *Staphylococcus aureus*, *Pseudomonas* sp., *Bacillus* sp. and three oral fungi *C. albicans* strain 1, *C. albicans* strain 2 and *S. cerevisiae*. This plant was selected due to its traditional use for the treatment of oral infections. Three clinical strains namely *Pseudomonas* sp., *Bacillus* sp. and *Candida albicans* strain 2 were isolated from dental caries affected patients. The antimicrobial activity of *B. prionitis* extracts on the agar plates varied in different solvents. The methanolic bark extract of *B. prionitis* was the most effective against all the four oral bacteria and the three oral fungi. *Bacillus* sp. was found to be the most sensitive pathogen which survived upto 12.5 mg/ml, thus having an MIC of 25 mg/ml. The antimicrobial potential of *B. prionitis* bark against *Bacillus* sp. was comparable with the standard antibiotic drug, the positive control, ciprofloxacin which produced a 29.65 mm inhibition zone. Interestingly the methanolic extract of *B. prionitis* bark showed much more potent activity against all the tested oral fungi namely *S. cerevisiae*, *C. albicans* strain 1 and *C. albicans* strain 2, than the standard drug amphotericin–B thus having a great potential to control candidiasis and other oral fungal infections.

Hebber et al. (2004) reported the results of a survey carried out in western ghat region of Dharwad district of Karnataka (India). These workers suggested the use of some plants in the treatment of dental caries for example dried whole plant of *Calotropis gigantea* (*Asclepiadaceae*) is burnt to get ash which is used to massage the tooth and gum after brushing in the morning and evening for curing of plaque, caries and pyorrhea. Powdered seeds of *Cassia hirsute* (*Caesalpiniaceae*) are used to massage the teeth and gums to protect from plaque and caries. Dried seeds of *Cassia tora* are fried and powdered. It is also used to massage the teeth and gums to protect from plaque and caries. *Leucas aspera* (*Lamiaceae*) whole plant is powdered and used to massage on the teeth and gums for plaque and caries.

Aneja et al. (2010b) evaluated the *in vitro* antimicrobial activity of *Sapindus mukorossi* and *Emblica officinalis* fruit extracts were studied against *Streptococcus mutans*, *Staphylococcus aureus*, *Lactobacillus acidophilus*, *Candida albicans* and *Saccharomyces cerevisiae*. The acetone, ethanol, methanol, hot water and cold water extracts of *S. mukorossi* exhibited antimicrobial activity against one of the tested microorganisms i.e. *S. cerevisiae*. All the five extracts of *E. officinalis* showed inhibitory activity against *S. mutans* while the acetonic, hot and cold aqueous extracts showed inhibitory activity against *S. aureus* also. The largest zone of inhibition was obtained with the acetonic extract of *S. mukorossi* against *S. cerevisiae* (29.65 mm) and hot water extract of *E. officinalis* against *S. aureus* (40.32 mm). Minimum inhibitory concentrations (MIC) of the extracts were also determined against the selected microorganisms showing zones of inhibition ≥8 mm. This study

depicts that the fruits of *Sapindus mukorossi* and *Emblica officinalis* possess very good antifungal and antibacterial activities respectively and can be used as a potential source of novel antimicrobial agents used to cure dental caries.

6.6 Antimicrobial Activity of Purified Phytochemicals

6.6.1 Terpenes

Xanthorrhizol, isolated from methanolic extract of *Curcuma xanthorrhiza* (Zingiberaceae) has been shown to have high levels of antibacterial activity against oral pathogens, in some cases equal or similar to that of chlorhexidine (Hwang et al. 2000). Bakuchiol isolated from the Chinese medicinal plant, *Psoralea corylifolia* (Fabaceae), has shown activity against numerous Gram-positive and Gram-negative oral pathogens. It has been able to inhibit the growth of *S. mutans* under a range of sucrose concentrations, pH values and in the presence of organic acids in a temperature-dependent manner and also inhibits the growth of cells adhered to a glass surface (Katsura et al. 2001). Liu et al. (2006) have purified seven new ent-rosane diterpenoids and a new labdane diterpene from the Chinese medicinal plant, *Sagittaria sagittifolia* (Alismaceae). Four of these compounds (sagittine A–D) exhibit antibacterial activity against *S. mutans* and *Actinomyces naeslundii* (with MIC values of between 62.5 and 125 mg/ml) while another (Sagittine E) has been only active against *A. naeslundii* (MIC of 62.5 mg/ml).

6.6.2 Alkaloids

The alkaloid berberine isolated from *Coptidis rhizome* (Ranunculacea) exhibits bactericidal activity against oral bacteria, with greatest activity against *A. actinomycetemcomitans* and *P. gingivalis*, followed by *Lactobacillus* and *Streptococcus* species (Hu et al. 2000).

6.6.3 Sugar Alcohols

Xylitol, a sugar alcohol naturally found in plants, used as an artificial sweetener in many foods has been shown to exert anticariogenic properties against *S. mutans*, *S. saliviarius* and *S. sanguis*. *S. mutans* is significantly inhibited by xylitol at 1.56 %, while all bacteria show statistically significant inhibition at levels above 1.56 % (Sahni et al. 2002).

6.6.4 Flavonoids and Other Polyphenols

Artocarpin and artocarpesin, two active isoprenylflavones, have been reported from *Artocarpus heterophyllus* (Moraceae), which inhibit the growth of numerous cariogenic and oral bacteria, including mutans and other oral streptococci, actinomyces and lactobacilli, at MIC values of 3.13–12.5 mg/ml (Sato et al. 1996). Flavonone phytoalexins from *Sophora exigua* (Leguminosae) have been shown to inhibit the growth of numerous cariogenic bacteria, with 5,7,20,40-tetrahydroxy-8-lavandulylflavanone being the most active (Tsuchiya et al. 1994). *Erythrina variegata* (Leguminosae), used in folk medicine in tropical and subtropical regions, displays a number of biological properties, including antibacterial activity mainly due to isoflavonoid erycristagallin which interferes with bacterial uptake of metabolites (Sato et al. 2002).

A methanol extract of tender leaves of *Psidium guajava* (Myrtaceae) has been shown to exhibit inhibitory activity against *S. mutans* and the active component has been identified as guaijaverin, which have MIC values of 2–4 mg/ml (Prabu et al. 2006).

The methanolic extract of root bark of *Morus alba* (Moraceae) has also been shown to inhibit *S. mutans*, the active antibacterial constituent being identified as kuwanon G. The compound displays an MIC of 8 mg/ml against *S. mutans*, which is comparable to chlorhexidine and vancomycin (1 mg/ml). Time-kill assays indicates that *S. mutans* gets completely inactivated by 20 mg/ml of kuwanon G within 1 min. The mode of action of kuwanon G is inhibition or blocking of cell growth, as treated cells shows a disintegrated surface and an unclear cell margin (Hwang et al. 2004).

A number of components of tea, *Camelia sinensis* (Theaceae), such as monomeric polyphenols, in particular simple catechins such as epicatechin, epicatechin gallate, and epigallocatechin gallate are believed to exhibit anticariogenic effects through various modes of action, including bactericidal effects on oral bacteria, prevention of adherence of bacteria to tooth surfaces, inhibition of glucan production and inhibition of amylases (Hamilton-Miller 2004).

6.6.5 Other Phytochemicals

Humulus lupulus (Cannabaceae), commonly known as hops, has been found to display antibacterial activity against *S. mutans*, *S. saliviarius* and *S. sanguis* (Bhattacharya et al. 2003). A few recent studies have demonstrated antimicrobial activity against selected oral pathogens from natural sources. Coenzyme Q-10 (CoQ10) is widely held as a treatment for periodontal disease based on some older studies. CoQ10 is found throughout the body and in higher amounts in heart, kidney, liver and pancreatic tissues. CoQ10 plays an important role in the production of adenosine triphosphate, or ATP – the body's energy currency. Hanioka and coworkers (1994) in a Japanese study (utilizing topically-applied CoQ10) found "significant improvements" in various measures of periodontal disease progression; these investigators concluded that CoQ10 is a useful treatment for periodontal

disease as a singular treatment or in combination with other nonsurgical therapies. These limited, yet enticing findings regarding the use of CoQ10 and periodontal disease certainly provide plausible reason for its use.

Sanguinaria (bloodroot) may be found in some natural toothpaste preparations and other products for oral hygiene due to the herb's ability to inhibit the growth of certain bacteria. In one study, sanguinarine, an alkaloid constituent of sanguinaria was found to inhibit the growth of 98 % of bacterial isolates from human dental plaque (Dzink and Socransky 1985). Another clinical trial that utilized a sanguinaria-containing toothpaste and oral rinse protocol over a 6-month period demonstrated a reduction in plaque and gingival inflammation in the patient population (Hannah et al. 1989). Investigators noted a plaque reduction of 57 %, bleeding reduction of 45 %, and a decrease in gingival inflammation by 60 % in comparison to the placebo group in which plaque was reduced by 27 % and inflammation by 21 %, while bleeding increased by 30 %. In a similar study, researchers noted another significant reduction in gingivitis symptoms in comparison to placebo after a 6-month treatment period (Harper et al. 1990).

Porto et al. (2009) demonstrated that kaurenoic acid, a diterpene isolated from *Aspilia foliacea*, could be used as a prototype for the discovery of new effective anti-infection agents against pathogens responsible for caries and periodontal diseases. The study showed that kauranetype diterpenes can be potentially useful in the development of natural anti-caries and anti-periodontal agents. In another study carried out by the same research group, they demonstrated the effective antimicrobial activity displayed by the dichloromethane root extract from *Viguiera arenaria* (Asteraceae), as well as the activity of the mainly isolated and two semisynthetic pimarane-type diterpenes against some oral microorganisms, including *Streptococcus mutans*, *Streptococcus salivarius*, *S. sobrinus*, *S. mutans*, *S. mitis*, *S. sanguinis* and *Lactobacillus casei*. The compounds *ent*-pimara- 8(14),15-dien-19-oic acid (PA); *ent*-8(14),15-pimaradien-3β-ol; *ent*-15-pimarene-8β,19-diol; *ent-8*(14), 15-pimaradien-3β-acetoxy and the sodium salt derivative of PA were the most active compounds, displaying MIC values ranging from 2 to 8 μg/ml. Thus, this class of compounds seems promising as a class of new effective anticariogenic agents.

Loo et al. (2010) evaluated the effect of Ellagic acid a natural herb extract from Galla chinensis on the growth of oral bacteria as well as their generation of water-insoluble glucan and adhesion to saliva-coated hydroxyapatite (S-HA) beads. *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Actinomyces naeslundii*, *Actinomyces viscosus*, *Lactobacillus rhamnosus*, *Porphyromonas gingivalis* and *Bacteroides forsythus* were the bacterial cell lines used in this study. Antibacterial activity of *Ellagic acid* was determined by using adenosine triphosphate (ATP) bioluminescence assay at various concentrations from 0.125 to 8 mg/ml. Anthrone method was used to evaluate the level of water-insoluble glucan generated by oral bacteria. The numbers of 3H-thymidine labeled bacteria attached to S-HA was counted by scintillation counting method. Sprague Dawley rats were orally fed with 0.5 mg/ml ellagic acid for 28 days and their behaviours and excretions were monitored. Ellagic acid reduced bacterial metabolic rates and inhibited the growth of the tested bacterial strains. The waterinsoluble glucan generated by *S. mutans* and its adhesion to S-HA were reduced. Ellagic acid demonstrated no toxicity in animals

fed for 28 days. Ellagic acid might be a promising compound for the development of antimicrobial agents against oral pathogens in human, thereby reducing the incidence of dental caries.

Namba et al. (1982) isolated two compounds magnold and honokiol from the ether and methanol extract of bark of *Magnoliae cortex*. These workers reported potent antibacterial activity of these compounds against cariogenic bacterium, *S. mutans* (MIC 6.3 mg/ml). Kohda et al. (1986) isolated and identified oleonolic acid and ursolic acid as active principles from chloroform extract of *Zizyphi fructus*. These workers demonstrated inhibitory activity of these compounds against insoluble glucan formation by glucosyltransferase from cariogenic bacterium *S. mutans*. Toukairin et al. (1991) isolated polyphenolic 5′-nucleotidase inhibitors from the seed and skin of the wine grape "Koshu" designated as NPF-88 BU-IA, NPF-88 BU IB, NPF-88 BU IIA and NPF-88 BU IIB. These compounds displayed inhibitory effects on the growth of *S. mutans*, a primary cariogenic bacterium. Furthermore, they inhibited the glucan formation from sucrose. Therefore, 5-nucleotidase inhibitors can prevent cause of caries of tooth. Chewing sticks (Miswak) which are roots of *Salvadora persica* plant have been used for centuries as oral hygiene tool in many parts of the world particularly in south Arabia.

In several animal experiments and human trials, green tea and black tea have been shown to reduce plaque score and caries index. Catechins and theaflavins, polyphenolic compounds derived from tea (*Camellia sinensis*, family *Theaceae*) have been reported to prevent tooth decay and oral cancer (Lee et al. 2004). These workers suggested possible use of tea in prevention of dental caries. Xiao et al. (2004) reported the anticaries effects of five natural chinese medicine such as Radix et *Rhizoma* rhei, *Semen areca*, *Rhizoma* ligustici chuanxiong and Catechu. These products prevented the adherence of *S. mutans* and suggested to be beneficial in preventing dental caries. Oil of *Syzygium aromaticum* (clove) and *Zanthoxylum limonella* (makaen) were widely used essential oils for dental caries or flavoring of food in Thailand and other countries (Trongtokit et al. 2004).

6.7 Plant Extracts Which Inhibit the Adhesion of Oral Bacteria

Cranberry, *Vaccinium macrocarpon* (Ericaceae), has been recognized for its beneficial effects on human health, including the prevention urinary tract infections by interfering with adhesion of *Escherichia coli* to cells and preventing adhesion of *Helicobactor pylori* to gastric mucosa (Koo et al. 2006). Numerous studies have investigated the ability of cranberry juice or cranberry constituents to prevent adhesion of oral pathogens to surfaces and related phenomena, such as the production of glucans and fructans, and the formation of biofilms. *In vitro* experiments have shown that cacao bean husk extract markedly reduce the growth rate (69–72 % reduction) and inhibit insoluble glucan synthesis of *S. mutans* and sucrose-dependent adhesion of *S. mutans* and *S. sobrinus* to a glass surface (85 % inhibition at 45 mg/ml)

(Ooshima et al. 2000). In addition, *in vivo* experiments in pathogen-free rats infected with these bacteria indicate that the extract exhibit significant cariostatic activity.

Methanolic extracts of the roots of *Polygonum cuspidatum* (Polygonaceae), traditionally used in Korea to maintain oral health, has been shown to reduce the viability of *S. mutans* and *S. sobrinus*, as well as inhibit sucrose-dependent adherence, waterinsoluble glucan formation, glycolytic acid production and acid tolerance (Song et al. 2006). It has been revealed that inhibitory effects may be mediated by the presence of alkaloids, phenolics and sterol/terpenes in the extract. Aqueous and methanol extracts of cloves from *Syzygium aromaticum* (Myrtceae) have been shown to affect the cariogenic properties of *S. mutans*, as exhibited by the ability of the extracts to inhibit adhesion of the bacteria to glass, reduce cell surface hydrophobicity and inhibit the production of glucosyltransferase (Rahim and Khan 2006).

Crude aqueous extracts of *Piper betle*, a plant used traditionally in the control of dental and oral diseases in South East Asia, have been shown to inhibit the growth, adherence and glucan production of *S. mutans* (Razak and Rahim 2003).

6.8 Antimicrobial Activity of Essential Oils

The antimicrobial properties of essential oils are well known and activity against bacteria found in the oral cavity, including pathogens, has been documented (Kalemba and Kunicka 2003). Indeed, there is evidence that commercial mouthwashes containing essential oils are useful in the long-term control of plaque and mild-to-moderate gingivitis and are preferred to those containing chlorhexidine for longterm daily use (Santos 2003). A number of recent studies add to the evidence that essential oils may be suitable additives in products used for the maintenance of oral hygiene or prevention of dental disease. The essential oil of *Melaleuca alternifolia* (Myrtaceae), known as tea tree oil, has been used medicinally for many years. Tea Tree Oil has antimicrobial properties and is used in the superficial treatment of skin infections. The activity of Tea Tree Oil against an extensive collection of oral bacterial isolates has been investigated by Hammer et al. (2003) who determined MIC and MBC values in the range 0.003–2.0 % (v/v). The oil was able to inhibit the adhesion of *S. mutans* and *P. gingivalis*.

Essential oils are also capable of enhancing the activity of chlorhexidine. When used in combination, the essential oils of cinnamon and manuka were able to significantly reduce the amount of chlorhexidine required to inhibit the growth of oral pathogens (Filoche et al. 2005). This enhanced activity was also seen against bacterial cultures grown as biofilms. Between 4- and 10-fold reductions of the amount of chlorhexidine required to inhibit biofilm bacteria was observed when used in combination with cinnamon, manuka and Leptospermum morrisonii oils. The essential oils of *Artemisia lavandulaefolia* (Asteraceae), *A. capillaries*, *A. scoparia* and *A. feddei* have been shown to inhibit the growth of oral bacteria (Cha et al. 2005), with the greatest activity generally observed against obligate anaerobes. However,

the oils also showed strong activity against other groups, including facultative anaerobes and microaerophilic bacteria.

It has been reported that the essential oil of *Cryptomeria japonica* (Taxodiaceae) exhibit strong activity against all oral bacteria, with MIC of 0.025–0.5 mg/ml (Cha et al. 2007). While these in vitro results are very encouraging, the known toxicity of TTO when ingested suggests that further studies of the safety of this and other essential oils for use in the oral cavity need to be addressed (Hammer et al. 2003).

Aneja and Joshi (2010) investigated the antimicrobial activity of clove and clove bud oil by agar well diffusion method against five dental caries causing microorganisms namely *Streptococcus mutans*, *Staphylococcus aureus*, *Lactobacillus acidophilus* (bacteria), *Candida albicans* and *Saccharomyces cerevisiae* (yeast). The results indicated that clove and clove oil have a potent antimicrobial activity against the tested dental caries causing microorganisms. The highest antimicrobial activity of clove was found against *Saccharomyces cerevisiae* (25.32 mm) in methanolic extract and an MIC of 50 mg/ml and that of clove oil was found against *Streptococcus mutans* (34.32 mm) with a MIC value of 3.125 mg/ml. This study has shown the importance of clove and clove oil and indicated that clove and clove bud oil can be used as an antimicrobial agent to cure dental caries.

6.9 Conclusions

There is considerable evidence that plant extracts, essential oils and purified phytochemicals have the potential to be developed into agents that can be used as preventative or treatment therapies for dental caries and periodontal disease. While it is encouraging to see a number of clinical trials of such products, further studies of the safety and efficacy of these agents are necessary to establish whether they offer therapeutic benefits, that can help to reduce the overall burden of oral diseases worldwide.

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Chapter 7
Antimicrobial Activity of Plants Used in México for Gastrointestinal and Respiratory Disorders

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7.1 Introduction

The plant kingdom has historically been the best source of remedies for a variety of diseases and pain, with medicinal plants playing a key role in the maintenance of global health. Plants still form the basis of health care systems in many societies and are increasingly attracting attention among pharmaceutical and scientific communities. The use of and search for drugs and dietary supplements derived from plants have accelerated in recent years. Not only are medicinal plants important to the millions of people for whom traditional medicine offers the only form of health care or who use the plants for various purposes in their daily lives, they are also a source of new pharmaceuticals. Natural products, either as pure compounds or as standardized plant extracts, provide opportunities for new drug leads.

In the past decade, a worldwide increase in the incidence of infections and a rise in the resistance of some species of microorganisms to different antibiotics used in medicinal practice have been observed. Hence, there is a great demand for novel antimicrobials, across a wide range of structural classes that can act on new targets with fewer side effects.

Long before humans discovered the existence of microbes, the idea that certain plants had healing potential was widely accepted. Since antiquity, humans have used plants to treat common infectious diseases; indeed, some traditional medicines are still part of customary treatment of these diseases in some societies. Plants are rich in a wide variety of secondary metabolites, which have been found to have diverse biological properties. Some plants have also shown the ability to overcome resistance to some microorganisms (mo), which has prompted researchers to investigate their mechanisms of action and isolate the active compounds.

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Mexico has an extensive variety of plants; it is the world's fourth richest country in this respect. Some 25,000 species are registered, more than 3,000 of which are used as medicinal plants. Surprisingly, the potential medicinal properties have been studied in depth in only a small proportion (1–2 %) of these medicinal plants. However, for Mexico's numerous ethnic groups, knowledge of the local flora represents one of the main links to their traditional culture. Local medicinal plants are important to them for alleviating many common diseases, principally gastrointestinal and respiratory disorders. Traditional herbal medicine is intimately related to Mexican popular culture; its use is based on ancestral knowledge. About 25 % of the population still depends on the use of medicinal plants. As natural products of higher plants are an important source of therapeutic agents, many research groups are currently testing the antimicrobial activities of Mexican plants.

This review aims to examine recent efforts (since 2000) toward advancing knowledge about the activity of some Mexican plants and isolated compounds against the principal microorganisms that cause gastrointestinal and respiratory diseases. The information has been organized into easily accessible and comparable sections, with reference to type of microorganism studied: Gram positive, *Mycobacterium tuberculosis*, Gram negative and fungi. *M. tuberculosis* was considered separately because of the great concern worldwide for finding new antiphthisic treatments. Pure compounds isolated from these screenings are considered in a separate section.

Through our review, we found that 297 species belonging to 224 genera and 102 families had been tested; from the plants screened, 103 extracts were active against Gram positive bacteria, 47 extracts against *M. tuberculosis*, 106 extracts were active against Gram negative bacteria, and 64 extracts were active against fungi, (Table 7.1). Different solvents have been used for extraction, including hexane, chloroform, ether, ethyl acetate, acetone, ethanol, methanol, water, and acidified water, in addition to hydrodistillation of essential oils.

7.2 Bioassays

Research on medicinal plants requires adequate bioassays to monitor their effects. Assays are ideally simple, reproducible, rapid, and cheap. If active principles are present at very low concentrations, the bioassays have to be sensitive enough to detect them. On the other hand, false positives must be minimized. Currently, there are many methods to evaluate the antibacterial and antifungal activity of plant extracts. The best-known assays are based on diffusion in agar, and micro and macro dilution methods. We describe here the different methods commonly used for the determination of new antimicrobial agents from plant extracts.

Comparability of results depends largely on the techniques employed in the investigations and conclusive results can only be obtained if methods are standardized and universal. Our review revealed one of the major problems with this type of research, namely the lack of uniformity in the criteria used to study the selected activity; this

Table 7.1 Plants screened from 2000 to 2011

	Scientific name	Family	MO	Bioassay	References
1	*Acacia farnesiana* (L) Willd.	Fabaceae	Vc, F	DAM, BDT	Sánchez et al. (2010), Alanís et al. (2007), Heredia et al. (2005)
2	*Acalypha communis* Müll. Arg.	Euphorbiaceae	G+	DAM	Gutierrez et al. (2002)
3	*Acalypha hederacea* Torrey	Euphorbiaceae	B	DAM, BDT	Hernández et al. (2003), Canales et al. (2005)
4	*Achillea millefolium*	Compositae	Mt	MABA	Jimenez et al. (2003)
5	*Actinidia chinensis* (Planch.)	Actinidiaceae	Vc	DAM	Sánchez et al. (2010)
6	*Aeschynomene fascicularis* (Cham et Schlech.)	Fabaceae	B, F	DAM	Rosado et al. (2000)
7	*Ageratina pichinchensis* var. bustamenta	Asteraceae	F	ADA	Aguilar et al. (2009)
8	*Ageratum corimbosum*	Compositae	Mt	MABA	Jimenez et al. (2003)
9	*Allium sativum* (L)	Liliaceae	G−	ADA	Alanís et al. (2005)
10	*Aloysia triphylla* Britton	Verbenaceae	G−	ADA	Alanís et al. (2005)
11	*Aloysia triphylla* Royle	Verbenaceae	B	BDT	Yasunaka et al. (2005)
12	*Amaranthus hypochondriacus* (L)	Amaranthaceae	Pa	Biochemistry assay	Velasco et al. (2003)
13	*Ambrosia confertiflora*	Asteraceae	Hp	BDT	Robles et al. (2011)
14	*Amphipterygium adstringens* (Schltdl.) Standl.	Anacardiaceae	Eco, Hp, Mt	ADA, BDT, MMC, BACTEC	Castillo et al. (2007, 2009); Rivero et al. (2005); Robles et al. (2011)
15	*Ananas comosus* (L) Merr.	Bromeliaceae	Vc	DAM	Sánchez et al. (2010)
16	*Anemopsis californica* (Nutt.) Hook. and Arn.	Saururaceae	B, F	BDT	Medina et al. (2005)
17	*Annona cherimola* Miller	Annonaceae	B, F	ADA, BDT	Navarro et al. (2003); Alanís et al. (2005); Yasunaka et al. (2005); Castillo et al. (2009); Ríos et al. (2003b)
18	*Annona diversifolia* Safford	Annonaceae	G−	DAM	Luna and González (2008)
19	*Annona muricata* (L)	Annonaceae	B	BDT	Yasunaka et al. (2005)
20	*Annona purpurea* Mociño &Sessé ex Dunal	Annonaceae	G−	DAM	Luna and González (2008)
21	*Anoda cristata* (L) Schltdl.	Malvaceae	Hp, Mt	ADA, BDT, MABA	Jimenez et al. (2003); Castillo et al. (2009)

(continued)

Table 7.1 (continued)

	Scientific name	Family	MO	Bioassay	References
22	*Ariocarpus kotschoubeyanus* (Lemaire)	Cactaceae	B, F	DAM	Rodriguez et al. (2010)
23	*Ariocarpus retusus* (Scheidweiler)	Cactaceae	B, F	DAM	Rodriguez et al. (2010)
24	*Arabidopsis thaliana* (L) Heynh.	Brassicaceae	B, F	BDT, Biochemistry assay	Ochoa et al. (2008); Loeza et al. (2008)
25	*Arachis hypogaea* (L)	Fabaceae	Vc	DAM	Sánchez et al. (2010)
26	*Araucaria araucana* (Mol.) K. Koch	Araucanaceae	B, F	DAM, ADA, MMC	Céspedes et al. (2006a)
27	*Aristolochia brevipes*	Asteraceae	Mt	MABA	Navarro et al. (2011a)
28	*Aristolochia taliscana* Hook. et Arn	Aristolochiaceae	B	BDT	Yasunaka et al. (2005)
29	*Artemisa ludoviciana* Nutt.	Asteraceae	Vc, F	DAM	Sánchez et al. (2010); Damian et al. (2008)
30	*Artemisa mexicana* Wild.	Compositae	B, F	DAM	Navarro et al. (1996)
31	*Artemisia absinthium* (L)	Asteraceae	B	DAM, ADA, BDT	Canales et al. (2005); Alanís et al. (2005)
32	*Artemisia laciniata* subsp. parryi (A. Gray) W.A. Weber	Asteraceae	Eco	DAM	Heredia et al. (2005)
33	*Artemisia ludoviciana* Nutt.	Compositae	G–, Hp Mt	DAM, ADA, BDT, MABA, MMC	Heredia et al. (2005); Jimenez et al. (2003); Alanís et al. (2005); Castillo et al. (2009); Molina et al. (2006)
34	*Asclepia curassavica* (L)	Asclepiadaceae	F	ADA	Navarro et al. (2003)
35	*Asparagus officinalis* (L)	Asparagaceae	Vc	DAM	Sánchez et al. (2010)
36	*Atriplex canescens* (Pursh) Nutt.	Chenopodiaceae	Eco	DAM	Heredia et al. (2005)
37	*Baccharis glutinosa* Pers.	Asteraceae	Eco	DAM	Heredia et al. (2005)
38	*Bambusa guada*	Gramineae	Mt	MABA	Jimenez et al. (2003)
39	*Bixa orellana* (L)	Bixaceae	B, F	ADA, BDT	Navarro et al. (2003); Yasunaka et al. (2005)
40	*Bocconia arborea* watt.	Papaveraceae	B, F	ADA, DAM	Navarro et al. (1996); Julian and Delgado (2001)
41	*Bocconia frutescens* (L)	Papaveraceae	B, F, Mt	ADA, MABA	Rojas et al. (2001); Alanís et al. (2005); Cruz et al. (2008)
42	*Bougainvillea glabra* Choisy	Nyctaginaceae	F	BDT	Alanís et al. (2007)
43	*Bouvardia temifolia*	Rubiaceae	Mt	MABA	Jimenez et al. (2003)

44	*Brassica napus* (L)	Brassicaceae	Vc	DAM	Sánchez et al. (2010)
45	*Bromelia pimguin* (L)	Bromeliaceae	B	BDT, MMC	Pío et al. (2009)
46	*Bryophyllum pinnatum* (Lam.)	Crassulaceae	Hp	BDT	Robles et al. (2011)
47	*Buddleja perfoliata* Kunth	Loganiaceae	Hp	BDT, ADA	Castillo et al. (2009)
48	*Bursera arida* (Rose) Standley	Burseraceae	B	DAM, BDT	Canales et al. (2005)
49	*Bursera simaruba* (L) Sarg.	Burseraceae	B	BDT	Yasunaka et al. (2005)
50	*Byrsonima crassifolia* (*L*) *Kunth*	Malpighiaceae	B, F	DAM, BDT	Navarro et al. (1996); Rivero et al. (2009)
51	*Caesalpinia pulcherrima* (L)	Leguminoceae	G−	ADA	Alanís et al. (2005)
52	*Calandrinia micrantha* Schltdl.	Portulacaceae	Hp	BDT, ADA	Castillo et al. (2009)
53	*Calia secundiflora* (Ortega)	Fabaceae	G−, F	DAM	Pérez et al. (2008b)
54	*Calliandra californica* Benth.	Fabaceae	B, F, Mt	MABA, DAM	Encarnación et al. (2006)
55	*Calliandra houstoniana* Mill.	Fabaceae	Mt	MABA	Jimenez et al. (2003)
56	*Calophyllum brasiliense* Cambess.	Clusiaceae	B	DAM, BDT	Yasunaka et al. (2005); Cottiglia et al. (2004); Reyes et al. (2004)
57	*Campyloneurum amphostenon* (Kunze ex Klotzsch) Fée	Polypodaceae	Hp	BDT, ADA	Castillo et al. (2009)
58	*Capsella bursa-pastoris* (L) Medik.	Brassicaceae	Hp	BDT, ADA	Castillo et al. (2009)
59	*Capsicum annuun* (L)	Solanaceae	Vc	DAM	Sánchez et al. (2010)
60	*Carica papaya* (L)	Caricaceae	G−	ADA	Alanís et al. (2005)
61	*Carya illinoensis* (Wangenh) K. Koch	Juglandacea	Mt	MABA	Cruz et al. (2008)
62	*Cassia fistula* (L)	Fabaceae	Mt	MABA	Jimenez et al. (2003)
63	*Castella tortuosa*	Simaroubaceae	Hp	BDT	Robles et al. (2011)
64	*Ceanothus coeruleus* Lag.	Rhamnaceae	B, F, Mt	BDT, MABA	Molina et al. (2007); Salazar et al. (2009)
65	*Ceiba parvifolia* Rose	Bombacaceae	B	DAM, BDT	Canales et al. (2005)
66	*Chamaedora tepejilote*	Palmae	Mt	MABA, BACTEC	Jimenez et al. (2003), (2005)
67	*Chenopodium ambrosioides* (L)	Chenopodiaceae	B, Mt, Hp	ADA, BDT, MABA, MMC	Alanís et al. (2005); Yasunaka et al. (2005); Molina et al. (2006); Robles et al. (2011)
68	*Chenopodium graveolens* Willd.	Chenopodiaceae	B	BDT	Yasunaka et al. (2005)
69	*Chenopodium murale* (L)	Chenopodiaceae	G−	ADA	Alanís et al. (2005)

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Table 7.1 (continued)

	Scientific name	Family	MO	Bioassay	References
70	*Chiranthodendron pentadactylon* Larreat	Sterculiaceae	G–	ADA	Alanís et al. (2005)
71	*Chrysactinia mexicana* Gray	Asteraceae	B, F, Mt	ADA, BDT, MABA	Alanís et al. (2005); Molina et al. (2007); Salazar et al. (2009); Guevara et al. (2011)
72	*Cirsium conspicum*	Compositae	Mt	MABA	Jimenez et al. (2003)
73	*Citrus aurantifolia* (Christm) Swingle	Rutaceae	B, Mt	BDT, MABA	Camacho et al. (2008); Bocanegra et al. (2009)
74	*Citrus aurantium* (L)	Rutaceae	Eco	DAM	Heredia et al. (2005)
75	*Citrus sinensis* (L) Osbeck	Rutaceae	B, Mt	BDT, MABA	Camacho et al. (2008); Bocanegra et al. (2009)
76	*Clematis drummondii* T and G.	Ranunculaceae	B, F, Mt	BDT, MABA	Alanís et al. (2007); Molina et al. (2007); Salazar et al. (2009)
77	*Clusia salvinii* Donn. Sm.	Clusiaceae	B	BDT	Yasunaka et al. (2005)
78	*Cnidoscolus urens* (L.) Arthur	Euphorbiaceae	Eco	DAM	Heredia et al. (2005)
79	*Cochlospermum vitifolium* Willd. Ex Sprengel	Bixaceae	Mt	MABA	Estrada et al. (2009)
80	*Cocos nucifera* (L)	Palmae	G–, Vc	ADA, DAM	Alanís et al. (2005); Sánchez et al. (2010)
81	*Colubrina greggii* Wats.	Rhamnaceae	B, F, Mt	BDT, MABA	Alanís et al. (2007); Molina et al. (2007); Salazar et al. (2009)
82	*Cordia boissieri* A. DC	Boraginaceae	B, F, Mt	BA, BDT, MABA	Pérez et al. (2008b); Alanís et al. (2007); Jimenez et al. (2003); Molina et al. (2007); Salazar et al. (2009)
83	*Cordia curassavica* (Jacq.) Roem. et Schult.	Boraginaceae	B, F	DAM, ADA, BDT	Hernández et al. (2003), (2007)
84	*Cordia morelosana* Standley	Boraginaceae	Mt	MABA	Estrada et al. (2009)
85	*Coutarea latiflora* DC.	Rubiaceae	Hp	BDT	Robles et al. (2011)
86	*Crataegus pubescens*	Rosacea	Mt	MABA	Jimenez et al. (2003)
87	*Cresentia alata* Kunth.	Bignoniaceae	B, F	ADA	Rojas et al. (2001)
88	*Croton draco* Schltdl.	Euphorbiaceae	B	BDT	Yasunaka et al. (2005)
89	*Cucumis melo* (L)	Cucurbitaceae	Vc	DAM	Sánchez et al. (2010)
90	*Cucurbita pepo* (L)	Cucurbitaceae	Vc	DAM	Sánchez et al. (2010)
91	*Cunila lythrifolia* Benth.	Lamiaceae	B, F	ADA	Rojas et al. (2001)

92	*Cuphea aequipetala* Cav.	Lythraceae	Hp	BDT, ADA	Castillo et al. (2009)
93	*Cymbopogon citratus* (DC.) Stapf	Poaceae	Hp, Vc	BDT, ADA, DAM	Castillo et al. (2009); Sánchez et al. (2010)
94	*Cynara scolymus* (L)	Asteraceae	Vc	DAM	Sánchez et al. (2010)
95	*Cyperus alternifolius* (L)	Cyperaceae	B, F	BA, BDT	Pérez et al. (2008b); Salazar et al. (2009)
96	*Cyrtocarpa procera* Kunth	Anacardiaceae	B, Hp	DAM, ADA, BDT	Canales et al. (2005); Castillo et al. (2009)
97	*Dalbergia glabra* (Miller)	Fabaceae	B, F	DAM	Rosado et al. (2000)
98	*Decachaeta incompta* (DC) R.M. King & H. Rob.	Asteraceae	G−	ADA	Calzada et al. (2009)
99	*Dianthus caryophyllus* (L)	Caryophyllaceae	Hp	BDT, ADA	Castillo et al. (2009)
100	*Dichondra argentea* Humb & Bonpl	Convolulaceae	G−	ADA	Alanís et al. (2005)
101	*Diospyros anisandra*	Ebenaceae	B, F, Mt	BDT, MABA	Borges et al. (2007)
102	*Diphysa carthagenesis* (Jacq.)	Fabaceae	B, F	DAM	Rosado et al. (2000)
103	*Distictis buccinatoña* DC.	Bignoniaceae	B, F	ADA	Rojas et al. (2007)
104	*Dorstenia contrajerva* (L)	Moraceae	G−	ADA	Alanís et al. (2005)
105	*Equisetum giganteum* (L)	Equisetaceae	Hp	BDT	Robles et al. (2011)
106	*Equisetum hyemale* (L)	Equisetaceae	B, F	DAM, BDT	Canales et al. (2005); Navarro et al. (1996)
107	*Equisetum myriochaetum* Schlecht. & Cham.	Equisetaceae	Hp	BDT, ADA	Castillo et al. (2009)
108	*Eryngium carlinae* F. Delaroche	Apiaceae	Hp	BDT, ADA	Castillo et al. (2009)
109	*Esenbckia yaaxhokob*	Rutaceae	B	BA, ADA	Aguilar and Rios (2004)
110	*Eucalyptus globolus* Labill.	Myrtaceae	B, F	DAM	Navarro et al. (1996)
111	*Eupatorium aschenbornianum* Schauer	Asteraceae	F	ADA	Navarro et al. (2003); Rios et al. (2003a)
112	*Eupatorium odoratum* (L)	Compositae	B, F	BDT, MABA	Molina et al. (2007); Salazar et al. (2009)
113	*Eupatorium petiolare* Moc. ex DC.	Asteraceae	Hp	BDT, ADA	Castillo *et al.* (2009)
114	*Euphorbia prostrata* Ait.	Euphorbiaceae	Eco, F	DAM, BDT	Alanís et al. (2007); Heredia et al. (2005)
115	*Flourensia cernua* DC	Asteraceae	Eco, Mt	DAM, MABA, MMC	Heredia et al. (2005); Molina et al. (2006, 2011a, b)
116	*Foeniculum vulgare* P. Mill	Umbelliferae	B, Mt	BDT, MABA	Camacho et al. (2008); Bocanegra et al. (2009)
117	*Galium mexicanum*	Rubiaceae	B, F	BDT	Bolivar et al. (2011)
118	*Galphimia glauca* Cav.	Malpighiaceae	F	ADA	Navarro et al. (2003)

(continued)

Table 7.1 (continued)

	Scientific name	Family	MO	Bioassay	References
119	*Geranium mexicanum* H.B.K.	Geraniaceae	G–	ADA	Alanís et al. (2005)
120	*Gymnosperma glutinosum* (Spreng.) Less	Asteraceae	B	DAM, BDT	Canales et al. (2005)
121	*Gliricidia sepium* (Jacq.) Kunth ex Walp.	Fabaceae	B	BDT	Yasunaka et al. (2005)
122	*Gnaphalium americanum* Mill.	Compositae	B, F	ADA	Rojas et al. (2001)
123	*Gnaphalium canescens* DC	Asteraceae	Hp	BDT, ADA	Castillo et al. (2009)
124	*Gnaphalium oxyphyllum* DC.	Compositae	B, F	ADA	Rojas et al. (2001)
125	*Gnaphalium semiamplexicaule*	Compositae	Mt	MABA	Jimenez et al. (2003)
126	*Gossypium hirsutum* (L)	Malvaceae	B, F	ADA	Rojas et al. (2001)
127	*Grindelia inuloides* Willd.	Asteraceae	Hp	BDT, ADA	Castillo et al. (2009)
128	*Guaiacum coulteri* A. Gray	Zygophyllaceae	Hp	BDT, ADA	Castillo et al. (2009)
129	*Guazuma ulmifolia* Lam.	Sterculiaceae	B, F	DAM	Navarro et al. (1996)
130	*Gymnolaena oaxacana* (Greenm.) Rydb.	Asteraceae	B	DAM, BDT	Hernández et al. (2003)
131	*Gymnosperma glutinosum* (Spreng.) Less	Asteraceae	B, F	DAM, ADA, BDT	Canales et al. (2007); Serrano et al. (2009)
132	*Haematoxylon brasiletto* H. Karst.	Fabaceae	B	DAM, BDT	Heredia et al. (2005); Yasunaka et al. (2005)
133	*Hamelia patens* Jacq.	Rubiaceae	B	BDT	Yasunaka et al. (2005)
134	*Haplopappus spinulosus* (Pursh) DC.	Asteraceae	Hp	BDT, ADA	Castillo et al. (2009)
135	*Hedeoma drummondii*	Lamiaceae	B, F	BDT	Alanís et al. (2007); Viveros et al. (2011)
136	*Helietta parvifolia* Benth.	Rutaceae	Eco	DAM	Heredia et al. (2005)
137	*Heliopsis longipes* A. Gray Blake.	Asteraceae	B, F	DAM, ADA, BDT, in vivo	Damian et al. (2008); Molina et al. (2004); Morales et al. (2007)
138	*Heliotropium angiospermum* Murr	Boraginaceae	B, F	BDT, MABA	Alanís et al. (2007); Molina et al. (2007); Salazar et al. (2009)
139	*Hemiangium excelsum* (Kunth) A.C. Sm.	Hippocrateaceae	Hp	BDT, ADA	Castillo et al. (2009)
140	*Hesperozygis marifolia* Epling	Lamiaceae	Hp	BDT, ADA	Castillo et al. (2009)
141	*Heterotheca inuloides* Cass.	Asteraceae	Hp	BDT, ADA	Castillo et al. (2009)
142	*Hibiscus sabdariffa* (L)	Malvaceae	Hp	BDT, ADA	Castillo et al. (2009)
143	*Hippocratea excelsa* H.B.K.	Hippocrateaceae	G–	ADA	Alanís et al. (2005)

144	*Hofmeisteria schaffneri* (A. Gray)	Asteraceae	B, F	BDT	Pérez et al. (2011)
145	*Hyptis pectinata* (L) Poit	Lamiaceae	Sa	BDT	Fragoso et al. (2005)
146	*Hyptis verticillata* Jacq.	Lamiaceae	Eco	DAM	Heredia et al. (2005)
147	*Ibervillea sonorae* Green	Cucurbitaceae	Hp	BDT	Robles et al. (2011)
148	*Iostephane heterophylla* (Cav.) Hemsl.	Asteraceae	B, F	DAM, BDT	Mata et al. (2001)
149	*Ipomea batotas* (L)	Convolulaceae	Vc	DAM	Sánchez et al. (2010)
150	*Ipomoea murucoides* Roem. et Schult	Convolvulaceae	Sa	BDT	Chérigo et al. (2008, 2009)
151	*Ipomoea orizabensis* (Pelletan)	Convolvulaceae	Sa	DAM	Pereda et al. (2006)
152	*Ipomoea tricolor* Cav.	Convolvulaceae	Sa	DAM	Pereda et al. (2006)
153	*Ipomoea tyrianthina* Lindley	Convolvulaceae	Mt	MABA	León et al. (2008), (2009)
154	*Jacquinia flammea* Millsp. Ex Mez	Theophrastaceae	B, F	DAM	Sánchez et al. (2010); Garcia et al. (2011)
155	*Jatropha cordata* Müll. Arg	Euphorbiaceae	Eco	DAM	Heredia et al. (2005)
156	*Jatropha cuneata* Wiggins & Rollins	Euphorbiaceae	Hp	BDT	Robles et al. (2011)
157	*Jatropha doica*	Euphorbiaceae	F	BDT	Alanís et al. (2007)
158	*Jatropha neopauciflora* Pax.	Euphorbiaceae	B	DAM, BDT	Canales et al. (2005)
159	*Juglans mollis* Engelm.	Juglandacea	Mt	MABA	Cruz et al. (2008)
160	*Juglans regia* (L)	Juglandacea	Mt	MABA	Cruz et al. (2008)
161	*Juliania adstringens* (Schldl.) Schldl.	Julianaceae	B	DAM, BDT	Heredia et al. (2005); Canales et al. (2005)
162	*Juniperus communis*	Cupressacae	Mt	MABA	Jimenez et al. (2003)
163	*Karwinskia humboldtiana*	Rhamnaceae	B, F	BDT	Salazar et al. (2006)
164	*Karwinskia parvifolia* Rose	Rhamnaceae	B, F	BDT	Salazar et al. (2006)
165	*Kohleria deppeana* Schl. et Cham	Gesneriaceae	Hp	BDT	Robles et al. (2011)
166	*Krameria erecta*	Krameriaceae	Hp	BDT	Robles et al. (2011)
167	*Krameria secundiflora* ex DC.	Krameriaceae	Eco	DAM	Heredia et al. (2005)
168	*Laelia autumnalis* (Lex.) Lindley	Orchidaceae	Mt	MABA	Estrada et al. (2009)
169	*Laennecia schiedeana*	Asteraceae	B	DAM, BDT	Arciniegas et al. (2011)
170	*Lantana achyranthifolia* Desf.	Verbenaceae	B	DAM, BDT, MMC	Hernández et al. (2003, 2005)
171	*Lantana camara* (L)	Verbenaceae	B	DAM, BDT	Hernández et al. (2003)
172	*Lantana hispida*	Verbenaceae	Mt	MABA	Jimenez et al. (2003)

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Table 7.1 (continued)

	Scientific name	Family	MO	Bioassay	References
173	*Lantana hispida*	Verbenaceae	Mt	MABA	Jiménez et al. (2007)
174	*Lantana trifolia* (L)	Verbenaceae	Eco	DAM	Heredia et al. (2005)
175	*Larrea divaricata*	Zygophyllaceae	Mt	BACTEC	Rivero et al. (2005)
176	*Larrea tridentata* DC.	Zygophyllaceae	B, F, Hp, Mt	DAM, ADA, BDT, MABA	Heredia et al. (2005); Castillo et al. (2009); Navarro et al. (1996); Camacho et al. (2008); Bocanegra et al. (2009)
177	*Lepechinia caulescens* (Ort) Epl	Labiatae	Vc, Mt	BDT, MMC, MABA	Acevedo et al. (2005); Estrada et al. (2009)
178	*Lepidium virginicum* (L)	Brassicaceae	Mt	MABA	Estrada et al. (2009)
179	*Leucophyllum frutescens* (Berl.) I.M. Johnst.	Escrophulariaceae	B, F, Mt	BA. BDT, MABA	Pérez et al. (2008b); Alanís et al. (2007); Molina et al. (2011b); Salazar et al. (2009)
180	*Lippia alba* N.E.Br.	Verbenaceae	G–	DAM, ADA	Heredia et al. (2005); Alanís et al. (2005)
181	*Lippia berlandieri* Schauer	Verbenaceae	Hp	BDT, ADA	Castillo et al. (2009)
182	*Lippia dulcis*	Verbenaceae	Mt	MABA	Jimenez et al. (2003)
183	*Lippia graveolens* H.B. et K.	Verbenaceae	B	DAM, BDT	Hernández et al. (2003); Rivero et al. (2011)
184	*Lippia oaxacana* Rob. et Greenm.	Verbenaceae	B	DAM, BDT	Hernández et al. (2003)
185	*Loeselia mexicana* Lamb	Polemoniaceae	F	ADA	Navarro et al. (2011b)
186	*Ludwigia repens* J. R. Forst.	Onagraceae	Hp	BDT, ADA	Castillo et al. (2009)
187	*Lygodium venustum* Sw	Schizaeaceae	G–	ADA	Alanís et al. (2005)
188	*Lysiloma acapulcensis* (Kunth) Benth.	Fabaceae	F	ADA	Navarro et al. (2003)
189	*Machaeranthera cf.* parviflora A. Gray	Asteraceae	Hp	BDT, ADA	Castillo et al. (2009)
190	*Machaeranthera riparia* (Kunth) A.G. Jones	Asteraceae	Hp	BDT, ADA	Castillo et al. (2009)
191	*Machaeranthera tanacetifolia* (Kunth) Nees	Asteraceae	Hp	BDT, ADA	Castillo et al. (2009)
192	*Magnolia dealbata*	Magnoliaceae	B, F	DAM	Jacobo et al. (2011)
193	*Malva parviflora* (L)	Malvaceae	Eco, Mt, F	DAM, ADA, MABA	Heredia et al. (2005); Jimenez et al. (2003); Navarro et al. (2003)
194	*Malvaviscus arboreus* Cav.	Malvaceae	B	BDT	Yasunaka et al. (2005)
195	*Mammea americana* (L)	Clusiaceae	B	BDT	Yasunaka et al. (2005)

196	*Mangifera indica* (L)	Anacardiaceae	Vc	DAM	Sánchez et al. (2010)
197	*Manikara zapota*	Sapotaceae	Vc, Mt	DAM, MABA	Sánchez et al. (2010); Jimenez et al. (2003)
198	*Marrubium vulgare* (L)	Labiatae	Hp, Mt	ADA, BDT, MABA, MMC	Castillo et al. (2009); Molina et al. (2006); Robles et al. (2011)
199	*Matricaria recutita* (L)	Asteraceae	G−	ADA	Alanís et al. (2005)
200	*Mentha pulegium* (L)	Labiatae	B, Mt	BDT, MABA	Camacho et al. (2008); Bocanegra et al. (2009)
201	*Mentha spicata* (L)	Labiatae	Vc, Mt	DAM, MABA, MMC	Sánchez et al. (2010); Molina et al. (2006)
202	*Mentha*×*piperita* (L)	Lamiaceae	Hp	BDT, ADA	Castillo et al. (2009)
203	*Miconia mexicana* (Bonpl.)	Melastomataceae	B, F	ADA	Navarro et al. (2006)
204	*Mimosa pigra* (L)	Fabaceae	B, F	DAM	Rosado et al. (2000)
205	*Mirabilis jalapa* (L)	Nyctaginaceae	Hp	BDT, ADA	Castillo et al. (2009)
206	*Monarda austromontana* Epling	Lamiaceae	Hp	BDT, ADA	Castillo et al. (2009)
207	*Morus alba* (L)	Moraceae	Eco	DAM	Heredia et al. (2005)
208	*Moussonia deppeana* (Schltdl. & Cham.) Klotzsch ex Hanst.	Gesneriaceae	Hp	BDT, ADA	Castillo et al. (2009)
209	*Muntingia calabura* (L)	Elaeocarpaceae	B	BDT	Yasunaka et al. (2005)
210	*Musa acuminata* Colla	Musaceae	B, Mt	BDT, MABA	Camacho et al. (2008); Bocanegra et al. (2009)
211	*Nasturtium officinale* R. Br.	Cruciferae	B, Mt	BDT, MABA	Camacho et al. (2008), Bocanegra et al. (2009)
212	*Ocimum basilicum* (L)	Labiatae	B, Hp, Vc, F	DAM, ADA, BDT	Heredia et al. (2005); Alanís et al. (2005); Castillo et al. (2009); Navarro et al. (1996); Sánchez et al. (2010)
213	*Ocimum micranthum* Willd	Labiatae	Eco	DAM	Heredia et al. (2005)
214	*Olea europaea* (L)	Oleaceae	B, Hp, Mt	ADA, BDT, MABA	Castillo et al. (2009); Camacho et al. (2008); Bocanegra et al. (2009)
215	*Opuntia ficus-indica* (L)	Cactaceae	Vc	DAM	Sánchez et al. (2010)

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Table 7.1 (continued)

	Scientific name	Family	MO	Bioassay	References
216	*Persea americana* Mill.	Lauraceae	B, Hp	DAM, BDT, ADA	Castillo et al. (2009); Rodríguez et al. (2011)
217	*Peumus boldus* Molina	Monimiaceae	Eco	DAM	Heredia et al. (2005)
218	*Phaseolus vulgaris* (L)	Leguminoceae	B	DAM	Lara et al. (2009)
219	*Phlebodium aureum* (L) J. Sm.	Polypodaceae	B	BDT	Yasunaka et al. (2005)
220	*Phoradendron robinsonii*	Loranthaceae	Mt	BACTEC	Rivero et al. (2005)
221	*Phyla nodiflora* (L) Greene.	Verbenaceae	B, F	MABA, BDT	Molina et al. (2007); Salazar et al. (2009)
222	*Pimpinella anisum* (L)	Apiaceae	Hp	BDT	Robles et al. (2011)
223	*Piper amalago* (L)	Piperaceae	B	BDT	Yasunaka et al. (2005)
224	*Piper auritum* Kunth	Piperaceae	B	BDT	Yasunaka et al. (2005)
225	*Piqueria trinervia* Cav.	Asteraceae	B	BDT	Yasunaka et al. (2005)
226	*Pittocaulon bombycophole* (Bullock) H. Rob. & Brett.	Asteraceae	B, F	BDT, DAM, ADA	Marín et al. (2008)
227	*Pittocaulon filare* (McVaugh) H. Rob. & Brett	Asteraceae	B, F	BDT, DAM, ADA	Marín et al. (2008)
228	*Pittocaulon hintonii* H. Rob. & Brett.	Asteraceae	B, F	BDT, DAM, ADA	Marín et al. (2008)
229	*Pittocaulon praecox* (Cav.) H. Rob. & Brett.	Asteraceae	B, F	BDT, DAM, ADA	Marín et al. (2008)
230	*Pittocaulon velatum* (Greenm) H. Rob. & Brett.	Asteraceae	B, F	BDT, DAM, ADA	Marín et al. (2008)
231	*Plantago major* (L)	Plantaginaceae	B, Hp, F	DAM, ADA, BDT	Castillo et al. (2009); Navarro et al. (1996)
232	*Plectranthus amboinicus* (Lour.) Spreng.	Lamiaceae	Hp	BDT, ADA	Castillo et al. (2009)
233	*Pleopeltis* sp.	Polypodaceae	Mt	MABA	Jimenez et al. (2003)
234	*Poliomintha longiflora* A. Gray	Lamiaceae	Hp, B	BDT, ADA	Castillo et al. (2009); Rivero et al. (2011)
235	*Porlieria angustifolia*	Zigophylaceae	B, F	MABA, BDT	Molina et al. (2007); Salazar et al. (2009)
236	*Priva grandiflora* (Ortega) Moldenke	Verbenaceae	Hp	BDT, ADA	Castillo et al. (2009)
237	*Prosopis glandulosa* (Torr.)	Fabaceae	Vc	DAM	Sánchez et al. (2010)
238	*Prosopis juliflora* (Sw.) DC.	Fabaceae	Eco	DAM	Heredia et al. (2005)
239	*Prunus persica* (L)	Rosacea	Vc	DAM	Sánchez et al. (2010)
240	*Prunus salicina* (Lindl.)	Rosacea	Vc	DAM	Sánchez et al. (2010)

241	*Psacalium decompositum* Gray	Asteraceae	Hp	BDT	Robles et al. (2011)
242	*Psacalium radulifolium* (HBK.) H. Rob. & Brettell	Asteraceae	B, F	ADA	Garduño et al. (2001)
243	*Psidium guajava* (L)	Myrtaceae	Eco	DAM	Heredia et al. (2005)
244	*Punica granatum* (L)	Punicaceae	B, F	DAM, ADA	Alanís et al. (2005); Navarro et al. (1996)
245	*Quercus rugosa* Née	Fagaceae	Hp	BDT, ADA	Castillo et al. (2009)
246	*Quercus* sp.	Fagaceae	Mt	MABA	Jimenez et al. (2003)
247	*Rhizophora mangle* (L)	Rhizophoraceae	Eco	DAM	Heredia et al. (2005)
248	*Rivinia humilis* (L)	Phytolacaceae	B, F	BDT	Alanís et al. (2007); Salazar et al. (2009)
249	*Rosa centifolia* (L)	Rosacea	B, Mt	DAM, BDT, MABA	Canales et al. (2005); Camacho et al. (2008); Bocanegra et al. (2009)
250	*Rosmarinus officialis* L.	Lamiaceae	Eco	DAM	Heredia et al. (2005)
251	*Rubus idaeus* (L)	Rosacea	Vc	DAM	Sánchez et al. (2010)
252	*Rumex hymenosepalus*	Polygonaceae	Mt	BACTEC	Rivero et al. (2005)
253	*Ruta chalepensis* (L)	Rutaceae	G–, Hp Mt	ADA, BDT	Alanís et al. (2005); Castillo et al. (2009)
254	*Salix taxifolia* H.B. & K.	Salicaceae	Eco	DAM	Heredia et al. (2005)
255	*Salvia chia* Fernald	Labiatae	B, F	BDT	Salazar et al. (2009)
256	*Salvia coccinea* Juss. ex Murr	Labiatae	B, F	DAM, BDT	Heredia et al. (2005); Salazar et al. (2009)
257	*Salvia reflexa* Hornem	Labiatae	B, F	BDT	Salazar et al. (2009)
258	*Salvia texana*	Labiatae	F	BDT	Alanís et al. (2007)
259	*Sambucus mexicana* Presl.	Capripholeaceae	B, F	MABA, BDT	Molina et al. (2007)
260	*Sapindus saponaria*	Sapindaceae	Mt	MABA	Jimenez et al. (2003)
261	*Satureja macrostema* Benth.	Labiatae	G–, F	DAM, ADA	Alanís et al. (2005); Damian et al. (2008)
262	*Schinus molle* (L)	Anacardiaceae	B, F	BDT, MABA	Alanís et al. (2007); Molina et al. (2007); Salazar et al. (2009)
263	*Scutellaria elliptica* Muhl	Labiatae	B, F	MABA, BDT	Molina et al. (2007); Salazar et al. (2009)
264	*Sedum oxypetalum* HBK.	Crassulaceae	F	ADA	Navarro et al. (2003)
265	*Selaginella lepidophylla* Hook. et Grev.	Selaginellaceae	Hp	BDT	Robles et al. (2011)
266	*Senecio angulifolius* DC.	Asteraceae	F	ADA	Navarro et al. (2003)

(continued)

Table 7.1 (continued)

	Scientific name	Family	MO	Bioassay	References
267	*Senna racemosa* (Benth.)	Fabaceae	B, F	DAM	Rosado et al. (2000); Sansores et al. (2000)
268	*Senna villosa* Mills	Leguminoceae	G–	ADA	Alanís et al. (2005)
269	*Serjania triquetra* Radlk.	Sapindaceae	B, F	DAM	Navarro et al. (1996)
270	*Smilax bona nox* (L)	Smilacaceae	B, F	MABA, BDT	Molina et al. (2007)
271	*Solanum chrysotrichum* Schldh	Solanaceae	F	ADA, BDT, MMC	Zamilpa et al. (2002); Herrera et al. (2007)
272	*Solanum hipidum* Pers.	Solanaceae	F	ADA	González et al. (2004)
273	*Solanum nigrum* (L)	Solanaceae	Eco	DAM	Heredia et al. (2005)
274	*Solanum rostratum*	Solanaceae	F	BDT	Alanís et al. (2007)
275	*Swietenia humillis*	Meliaceae	Mt	MABA	Jimenez et al. (2003)
276	*Tagetes filifolia* (Lag.)	Asteraceae	Vc	DAM	Sánchez et al. (2010)
277	*Tagetes lucida* Cav.	Asteraceae	B, Hp, F	DAM, ADA, BDT	Castillo et al. (2009); Damian et al. (2008); Céspedes et al. (2006b)
278	*Tanacetum parthenium* (L) Sch. Bip.	Asteraceae	B, Hp	DAM, ADA, BDT	Canales et al. (2005); Castillo et al. (2009)
279	*Taxodium mucronatum* Ten	Taxodiaceae	Eco, Hp	DAM, ADA, BDT	Heredia et al. (2005); Castillo et al. (2009); Robles et al. (2011)
280	*Tecoma stans* (L) Juss. ex Kunth	Bignoniaceae	Hp	BDT, ADA	Castillo et al. (2009); Robles et al. (2011)
281	*Teloxys ambrosioides* (L)W.A.Weber	Chenopodiaceae	Hp	BDT, ADA	Castillo et al. (2009)
282	*Teloxys graveolens* (L)W.A.Weber	Chenopodiaceae	Hp	BDT, ADA	Castillo et al. (2009)
283	*Tephrosia cinerea* (L) (Pers.)	Fabaceae	B, F	DAM	Rosado et al. (2000)
284	*Thymus vulgaris* (L).	Labiatae	B, F	DAM, ADA	Alanís et al. (2005); Navarro et al. (1996)
285	*Tillandsia brachycaulos*	Bromeliaceae	B, F	DAM	Cantillo et al. (2003)
286	*Tillandsia usneoides* (L)	Bromeliaceae	Hp	BDT, ADA	Castillo et al. (2009)
287	*Tithonia diversifolia* (Hemsl.) A.G.	Asteraceae	Hp	BDT, ADA	Castillo et al. (2009)
288	*Tournefortia hartwegiana* Steudel	Boraginaceae	Mt	MABA	Estrada et al. (2009)
289	*Turnera diffusa* (Willd.) ex Schult.	Turneraceae	B	DAM, BDT	Hernández et al. (2003)

290	*Vaccinium geminiflorum* H.B. & K.	Ericaceae	Eco	DAM	Heredia et al. (2005)
291	*Vaccinium macrocarpon* (Ait.)	Ericaceae	Vc	DAM	Sánchez et al. (2010)
292	*Valeriana edulis* ssp. procera (Kunth) Meyer	Valerianaceae	Mt	MABA	Estrada et al. (2009)
293	*Verbena carolina L.*	Verbenaceae	Hp, Mt	BDT, ADA, MABA	Castillo et al. (2009); Estrada et al. (2009)
294	*Viguiera dentata* (Cav.) Spren	Asteraceae	B	DAM, BDT	Canales et al. (2005)
295	*Vitis labrusca* (L)	Vitaceae	Vc	DAM	Sánchez et al. (2010)
296	*Yucca filifera* (Chabaud.)	Agavaceae	Vc	DAM	Sánchez et al. (2011)
297	*Zornia thymifolia* Kunth	Fabaceae	B	BDT	Yasunaka et al. (2005)

MO microorganisms tested, *B* bacteria, *F* fungi, *G+* Gram positive, *G−* Gram negative, *Mt Mycobacterium tuberculosis*, *Pa Pseudomonas aeruginosa*, *Eco Escherichia coli*, *Hp Helicobacter pillori*, *Sa Staphylococcus aureus*, *Vc Vibrio cholerae*, *BA* bioautography, *DAM* agar diffusion method, *ADA* agar Dilution assay, *BDT* Broth dilution test, *MABA* microplate Alamar blue assay, *MMC* minimum microbicidal concentration

is the principal factor sometimes impeding the comparison of results from different research groups. Although the selection of the activity test is in itself a problem, the preparation of extracts also is a very important step and full attention has yet to be given to this issue. The solvent and extraction system can modify the final results. Recently we demonstrated how the storage time of the extracts can influence the results from the susceptibility tests (Pérez et al. 2008a). Moreover, a common error in many papers is to claim positive activity for low dilutions or excessively high concentrations; this error occurs because experiments with quantities higher than 1 mg/mL for extracts, as indicated by Rios and Recio (2005), are not meaningful. Therefore, in this review, we considered as active only those extracts with reported activity at concentrations equal to or lower than 500 μg/mL for bacteria and equal to or lower than 1,000 μg/mL for fungi. The exceptions are extracts with activity against *M. tuberculosis*, which were considered active when they presented activity at concentrations lower than 200 μg/mL (Tosun et al. 2004). In all cases, extracts showing MICs (the minimum concentration that allows complete inhibition of mo) lower than 100 μg/mL were considered of special interest for further development and are set in bold type in the corresponding tables.

7.2.1 Agar Diffusion Method

This method, also known as diffusion in plate, is a physical method developed using solid culture medium, in which a microorganism is challenged against a biological active substance or extract to establish a relationship between the size of the inhibition zone and the concentration of the assayed compound (Ostrosky et al. 2008).

The agar diffusion method is one of the most frequently used tests to study antimicrobial activity, especially for plant extracts and compounds obtained from plants. This method is mostly used to detect antimicrobial activity by means of the diameter of an inhibition zone, without reporting maximum or minimum limits. Most of the studies in which MICs are reported by means of dilution methods are preceded by agar diffusion screening, as a way to determine presence or absence of antimicrobial activity.

This method is limited to fast-growing microorganisms, aerobics, and facultative aerobics. The evaluation must be by comparison with a biological reference patron, such as an antibiotic (positive control), and the inhibition zone is measured from the circumference of the disk up to the limit of growth of the mo. Considering the diameter of the inhibition zone, the mo are classified as sensitive when the inhibition zone is greater than the positive control or up to 3 mm less than it, and resistant when the size of the zone is equal to or less than 2 mm; when the inhibition zone lies between the sensitive and resistant classes, the response is considered as moderate. This test is accepted by FDA and used by the NCCLS (Ostrosky et al. 2008; NCCLS 2002).

To perform this bioassay, the species under evaluation is inoculated on a nutrient agar plate maintained at 45 °C to avoid solidification and then poured into dishes. Once

the agar is solidified, filter paper disks impregnated with a known concentration of the agent are added to the surface of the agar. During the incubation, the agent diffuses from the disk containing it into the surrounding agar, forming a concentration gradient; the activity is reached at a certain distance. Therefore, the size of the inhibition zone is determined by the capacity of diffusion of the antimicrobial agent and the susceptibility of the mo toward the compound tested (Brock et al. 1987). This method is cheap, simple, and useful for the determination of activity from crude extracts. However, the method is limited by the solubility of the compound or extract under evaluation and its ability to diffuse in the agar (Brock et al. 1987; Dimayuga et al. 1998).

The agar diffusion method was used in 30 out of 89 reports about antimicrobial activity of extracts obtained from Mexican plants (Table 7.1).

7.2.2 MIC Determination

In natural product research, after preliminary screening using the agar diffusion method, a dilution test in broth or agar is used to find the MIC of a compound or extract.

7.2.2.1 Broth Dilution Test

This method considers the relationship between the growth of a mo in a liquid medium and the concentration of a tested substance, measuring the turbidity generated. This method affords quantitative results, and the speed of mo growth does not influence the results obtained (Ostrosky et al. 2008).

The method can be assayed in two ways: in a tube or in microplate wells; in the latter case, the method is known as microdilution. To measure the relative activity of the agent, the MICs of the mo are determined under defined growth conditions. Each tube containing a known concentration of the compound or extract is incubated with a calibrated suspension of the mo for a certain time. After this period, the tubes are visually examined; turbidity implies that the substance or extract does not inhibit the growth of the mo. The MIC is considered as the concentration of the tube with the maximum dilution where inhibition can be detected. The microdilution test applies the same principle, except that the assay is performed in microtiter plates (Brock et al. 1987; Koneman et al. 1991). This technique is potentially useful for determining MICs of large samples, such as in plant extract screening or bioassay guide fractionations. Moreover, the advantages of the broth microdilution over diffusion include its enhanced sensitivity (which is important if the amount of compound or extract is scarce, as is the case with a lot of natural products), the ability to distinguish between bactericidal and bacteriostatic activity, and quantitative determination of MIC (Ncube et al. 2008).

There are some other ways to examine the microbial growth in the dilution tests.

(a) Using a turbidimetric method. Absorbance at 620 nm is measured using a negative control (no antibiotic) as a blank. In this case, the MIC is defined as the concentration at which a sharp reduction in the absorbance is seen or the lowest concentration that gives a zero absorbance reading (Ncube et al. 2008; Devienne and Raddi 2002; Salie et al. 1996). Some research groups use spectrophotometer plate readers to assess the MICs.
(b) Using colorimetric indicators (usually tetrazolium salts or resazurin dye). These are added after the incubation period; a change in color or absence of color after several hours is used to determine the MIC. The use of these colorimetric indicators avoids the ambiguity associated with visual inspection or the measuring of growth inhibition rings on agar plates (Ncube et al. 2008). Moreover, a plate reader can be used to obtain more precise results.

Tetrazolium salts are electron acceptors, used to measure the reduction state of the proliferative cells in contrast with the nonproliferative cells. The salts more frequently used are MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and XTT (sodium 3′-[1-[(phenylamino)carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate). MTT is reported in multiple assays of activity from extracts or compounds isolated from plants using a bioautographic technique.

Alamar Blue (resazurin) also measures the reduced proliferation of cells. It is soluble in culture medium, stable and nontoxic. The reduction of resazurin by bacteria can be monitored in solution or on paper disks. When this indicator is used, a nonreductive culture broth, such as trypticase soy or Mueller–Hinton broth is needed. A change in the color of the Alamar Blue solution from blue to pink confirms bacterial growth. The MIC is defined as the lowest compound or extract concentration that prevents bacterial growth (Biosource International 2010).

In 38 articles we reviewed, the broth dilution test was used, mostly as microdilution in the 96-well plate assay (Table 7.1). It is important to note that 13 of the 15 antimycobacterial reports used microdilution.

7.2.2.2 Agar Dilution Assay

This method is very similar to the broth dilution method, but different concentrations of the compound tested are prepared in agar. It is not a diffusion method, because the compound is previously dissolved in the agar. In this method, an amount of the compound or extract prepared in its extraction solvent is added to melted agar (between 40 and 45 °C) to obtain different concentrations. After solidification in petri dishes, the mo is inoculated as a drop; the concentration that completely inhibits macroscopic growth of the mo is defined as the MIC (Navarro et al. 1998).

Only 19 reports in our review used this method. This test is probably not chosen more frequently by researchers because it requires large quantities of culture medium and it uses more sample material, which sometimes is a limiting factor in natural products research.

7.2.3 Minimum Microbicidal Concentration (MMC)

Once the MIC is known, the minimum microbicidal concentration (MMC) can be obtained, subculturing the preparations that do not show evidence of growth in drug (extract)-free medium. This can be done in broth or agar. In broth, the MMC is reported as the lowest concentration of the extract or compound that produces zero absorbance at 420 nm, with respect to a negative control. In agar, the lowest concentration showing lack of growth is the MMC (Navarro et al. 1998).

7.2.4 Bioautography

The diffusion and microdilution tests can be used as a bioassay guides to the fractionation of natural products; however, bioautography has also been successfully used for that purpose recently. Fisher and Lautner was the first to report the use of this method in 1961. This assay is inexpensive, simple and fast; it is supported in diffusion and chromatographic methods and allows the localization of activity from an extract on a chromatographic plate (Navarro et al. 1998; Verastegui 2000). It can be considered as the most efficient assay for the detection of compounds in a complex matrix. This assay can be divided into three groups.

(a) Direct bioautography: the mo grows on the plate after adding culture medium.
(b) Contact bioautography: the compounds are transferred from the chromatographic plate to an inoculated agar plate through direct contact.
(c) Superimposed agar bioautography: agar inoculated with the mo is applied on the plate (Rahalison et al. 1991).

In this method, two identical TLC plates with the extracts under evaluation are developed. One of the plates is submitted to bioautography, a layer of agar inoculated with the mo is added and, after incubation in an adequate temperature and for the time required by the mo to be tested; microbial growth can be visualized by the aid of any tetrazolium salt. A metabolically active mo will convert the tetrazolium salt into the corresponding intensely colored formazan, while areas with antimicrobial compounds are seen as clear white spots on a colored background. The other plate is sprayed with an appropriate derivatizing reagent or observed under a UV lamp. The R_fs of the compounds seen are compared with the inhibition zones on the first plate. Although this technique is used for bioassay-guided fractionation of active extracts from plants, some authors suggest that TLC causes disruption of the synergism between the active constituents of an extract, thus diminishing the activity (Ncube et al. 2008).

7.3 Extracts Obtained from Mexican Plants Active Against Gram Positive Bacteria

The principal pathogens causing gastrointestinal and respiratory diseases, including ATTC strains, clinical isolates from different hospitals, and wild-type and antibiotic-resistant strains, were used as mo models for the screenings. The most common Gram positive bacteria used were *Bacillus cereus*, *Bac. subtilis*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Micrococcus luteus*, *Staphylococcus aureus*, *Sta. epidermidis*, *Sarcina lutea*, *Streptococcus pneumoniae*, and *Str. pyogenes*. Of these, the most frequently employed were *Sta. aureus*, *Str. pneumoniae*, and *Ent. faecalis*. It is important to note that the quantity and type of species used in each bioassay depend on the particular collection from each research group; therefore, there are papers reporting only one species (Yasunaka et al. 2005; Encarnación et al. 2006; Serrano et al. 2009; Fragoso et al. 2005), as well as reports including four (Rojas et al. 2001; Lara et al. 2009; Céspedes et al. 2006b) or even six (Bocanegra et al. 2009) different species.

In the past decade, 103 extracts belonging to 51 Mexican plants were reported as active against at least one Gram positive bacterial species with MIC values less than or equal to 500 μg/mL. Most of the active extracts showed activity against *Sta. aureus* (59 extracts); one-third were also active against resistant strains (35 extracts) (Table 7.2).

Of great interest are the results from Yasunaka et al. (2005), who report the results of a study of antibacterial activity of crude extracts from 22 Mexican medicinal plants against *Sta. aureus*. These plants are used in Mexican traditional medicine for the treatment of diseases presumably caused by bacteria. Many extracts examined were found to be active against *Sta. aureus* 209P (15 extracts displayed high activity and 11 extracts displayed moderate activity). Both an acetonic extract from the leaves of *Calophyllum brasiliense* and a methanolic extract from the seeds of *Mammea americana* showed the highest antibacterial activity (MIC 2 μg/mL). MIC values of 15 extracts active against methicillin-sensible *Sta. aureus* (MSSA) 209P were further evaluated against two methicillin-resistant *Sta. aureus* MRSA strains (no. 3208 and no. 80401); all the extracts examined showed nearly the same MIC values, the differences being at most in two wells, against these two MRSA strains. In general, the extracts obtained from *Bursera simaruba*, *Haematoxylum brasiletto*, *Cal. brasiliense*, *Mammea Americana*, *Aloysia triphylla*, *Croton draco*, and *Gliricidia sepium* were highly active against both MSSA and MRSA, with MIC values ranging between 2 and 64 μg/mL.

An interesting activity against *Sta. aureus* has been also reported for the hexanic extract obtained from *Psacalium radulifolium* roots (Garduno et al. 2001), the methanolic and dichloromethane extracts from *Byrsonima* crassifolia cortex (Rivero et al. 2009), the essential oil from *Annona cherimola* fruits (Navarro et al. 1996), the methanolic extracts from *Cordia boissieri*, and *Schinus molle* flowers (Salazar et al. 2009), and the aqueous infusion and essential oil from *Hofmeisteria schaffneri* (Pérez et al. 2011), all of which afford MIC values of 64 μg/mL. However, none of

Table 7.2 Extract active against Gram positive bacteria (μg/mL)

	Scientific name	ES	PP	Bs	Ef	Sa	Sa R	Se	Sl	Spn	References
1	*Aloysia triphylla*	Mt	L	–	–	**64**	**64**	–	–	–	Yasunaka et al. (2005)
2	*Anemopsis califórnica*	EO		–	–	A	–	–	–	A	Medina et al. (2005)
3	*Annona cherimola*	EO	Fl	–	500	125	–	–	–	–	Navarro et al. (1996)
4	*Annona cherimola*	EO	Fr	–	500	**60**	–	–	–		Navarro et al. (1996)
5	*Calophyllum brasiliense*	Ac	L	–	–	**2**	**8**	–	–	–	Yasunaka et al. (2005)
6	*Annona cherimola*	EO	L	–	500	250	–	–	–	–	Navarro et al. (1996)
7	*Annona cherimola*	Mt	S	–	–	512	–	–	–	–	Yasunaka et al. (2005)
8	*Bixa Orellana*	Mt	S	–	–	128	128	–	–	–	Yasunaka et al. (2005)
9	*Bursera simaruba*	Mt	Ap	–	–	**8**	**8**	–	–	–	Yasunaka et al. (2005)
10	*Bursera simaruba*	Mt	S	–	–	**64**	**64**	–	–	–	Yasunaka et al. (2005)
11	*Byrsonima crassifolia*	Mt	B	**64**	**64**	**64**	133	–	–	–	Rivero et al. (2009)
12	*Byrsonima crassifolia*	DCM	B	**64**	**64**	**64**	125	–	–	–	Rivero et al. (2009)
13	*Calophyllum brasiliense*	Mt+Ac	H	–	–	**8**	**8**	–	–	–	Yasunaka et al. (2005)
14	*Calophyllum brasiliense*	Hx	L	–	–	**32**	**32**	–	–	–	Yasunaka et al. (2005)
15	*Calophyllum brasiliense*	Mt	L	–	–	**16**	**16**	–	–	–	Yasunaka et al. (2005)
16	*Ceanothus coeruleus*	Mt	R	–	–	500[a]	500[a]	–	–	500[a]	Molina et al. (2007)
17	*Ceanothus coeruleus*	Mt	Fl	–	na	125	na	–	–	–	Salazar et al. (2009)
18	*Ceiba parvifolia*	Mt	B	na	–	250	–	na	na	–	Canales et al. (2005)
19	*Chenopodium graveolens*	Mt	G	–	–	256	–	–	–	–	Yasunaka et al. (2005)
20	*Chrysactinia mexicana*	EE	Fl	–	–	na[a]	na[a]	–	–	500[a]	Molina et al. (2007)
21	*Chrysactinia mexicana*	Mt	Fl	–	–	na[a]	na[a]	–	–	500[a]	Molina et al. (2007)
22	*Chrysactinia mexicana*	EE	R	–	–	na[a]	na[a]	–	–	250[a]	Molina et al. (2007)
23	*Chrysactinia mexicana*	Mt	R	–	–	na[a]	na[a]	–	–	500[a]	Molina et al. (2007)
24	*Chrysactinia mexicana*	Mt	L	–	–	na[a]	na[a]	–	–	500[a]	Molina et al. (2007)
25	*Chrysactinia mexicana*	EO	R	–	–	250[a]	250[a]	–	–	–	Guevara et al. (2011)

(continued)

Table 7.2 (continued)

	Scientific name	ES	PP	Bs	Ef	Sa	Sa R	Se	Sl	Spn	References
26	*Clusia salvinii*	Mt+DCM	L	–	–	128	–	–	–	–	Yasunaka et al. (2005)
27	*Colubrina greggii*	Mt	Fl	–	250	na	na	–	–	–	Salazar et al. (2009)
28	*Colubrina greggii*	Mt	L	–	250	na	na	–	–	–	Salazar et al. (2009)
29	*Colubrina greggii*	Mt	R	–	–	na[a]	na[a]	–	–	500[a]	Molina et al. (2007)
30	*Cordia boissieri*	Mt	Fl	–	na	**63**[a]	125[a]	–	–	250[a]	Molina et al. (2007); Salazar et al. (2009)
31	*Cordia boissieri*	Mt	Fr	–	–	na[a]	na[a]	–	–	250[a]	Molina et al. (2007)
32	*Cordia boissieri*	Mt	R	–	–	na[a]	na[a]	–	–	250[a]	Molina et al. (2007)
33	*Cordia boissieri*	Mt	L	–	–	250[a]	**31**[a]	–	–	125[a]	Pérez et al. (2008b); Molina et al. (2007)
34	*Cordia boissieri*	Ac	L	–	–	–	500	–	–	–	Pérez et al. (2008b)
35	*Cordia boissieri*	Aq	L	–	–	–	125	–	–	–	Pérez et al. (2008b)
36	*Cordia boissieri*	Et	L	–	–	–	**31**	–	–	–	Pérez et al. (2008b)
37	*Cordia boissieri*	Et+Aq	L	–	–	–	**31**	–	–	–	Pérez et al. (2008b)
38	*Cordia boissieri*	Mt+Aq,	L	–	–	–	**63**	–	–	–	Pérez et al. (2008b)
39	*Cordia curassavica*	EO	Ap	250	–	250	–	250	**62**	–	Hernandez et al. (2007)
40	*Cordia curassavica*	Hx	Ap	250	–	500	–	500	250	–	Hernandez et al. (2007)
41	*Cordia curassavica*	Mt	Ap	na	–	500	–	500	na	–	Hernandez et al. (2007)
42	*Cordia curassavica*	Hx	B	na	–	500	–	na	500	–	Hernández et al. (2003)
43	*Croton draco*	Mt	L	–	–	**64**	**64**	–	–	–	Yasunaka et al. (2005)
44	*Cyperus alternifolius*	Mt	Ap	–	na	125	na	–	–	–	Salazar et al. (2009)
45	*Cyperus alternifolius*	Et	R	–	–	–	500	–	–	–	Pérez et al. (2008b)
46	*Cyperus alternifolius*	Et+Aq	R	–	–	–	250	–	–	–	Pérez et al. (2008b)
47	*Cyperus alternifolius*	Mt	R	–	125	250	250	–	–	–	Pérez et al. (2008b); Molina et al. (2007)
48	*Cyperus alternifolius*	Mt+Aq,	R	–	–	–	250	–	–	–	Pérez et al. (2008b)
49	*Distictis buccinatoria*	DCM	Fl	–	–	500	–	–	–	–	Rojas et al. (2007)
50	*Eupatorium odoratum*	Mt	Fl	–	–	na[a]	na[a]	–	–	500[a]	Molina et al. (2007)

51	*Eupatorium odoratum*	Mt	L	–	–	na[a]	na[a]	–	–	250[a]	Molina et al. (2007)
52	*Eupatorium odoratum*	Mt	R	–	–	na[a]	na[a]	–	–	500[a]	Molina et al. (2007)
53	*Galium mexicanum*	Cl	Ap	na	–	na	333	–	–	–	Bolivar et al. (2011)
54	*Gliricidia sepium*	Mt	L	–	–	**64**	–	–	–	–	Yasunaka et al. (2005)
55	*Gymnolaena oaxacana*	Hx	Ap	na	–	na	–	na	500	–	Hernández et al. (2003)
56	*Gymnosperma glutinosum*	Aet	Ap	na	–	125	–	125	na	–	Canales et al. (2005)
57	*Gymnosperma glutinosum*[b]	Mt	Ap	na	–	125	–	125	125	–	Canales et al. (2007)
58	*Gymnosperma glutinosum*[b]	Mt	Ap	na	–	500	–	500	125	–	Canales et al. (2007)
59	*Haematoxylum brasiletto*	Mt	S	–	–	**16**	**16**	–	–	–	Yasunaka et al. (2005)
60	*Hamelia patens*	Mt	Ap	–	–	256	–	–	–	–	Yasunaka et al. (2005)
61	*Hedeoma drummondii*	Hx	Ap	–	–	125	–	–	–	–	Viveros, et al. (2011)
62	*Hedeoma drummondii*	Mt	Ap	–	–	250	–	–	–	–	Viveros, et al. (2011)
63	*Hibiscus sabdariffa*	Aq	Fl	–	500	500	–	–	–	–	Navarro et al. (2006)
64	*Hofmeisteria schaffneri*	EO	Ap	256	–	**48**	–	–			Pérez et al. (2011)
65	*Hofmeisteria schaffneri*	Aq	Ap	**64**	–	**64**	–	–	–	–	Pérez et al. (2011)
66	*Hofmeisteria schaffneri*	DCM:Mt	Ap	na	–	na	–	–	–	–	Pérez et al. (2011)
67	*Iostephane heterophylla*	DCM:Mt	R	–	A	A	A	–	–	–	Mata et al. (2001)
68	*Jatropha neopauciflora*	La	La	na	–	na	–	na	500	–	Canales et al. (2005)
69	*Juliania adstringens*	Mt	B	na	–	250	–	na	125	–	Canales et al. (2005)
70	*Laennecia schiedeana*	Ac	Ap	–	na	na	–	250	–	500	Arciniegas et al. (2011)
71	*Lantana achyranthifolia*	EO	Ap	500	–	250	–	500	250	–	Hernández et al. (2005)
72	*Lantana achyranthifolia*	Hx	Ap	500	–	na	–	500	250	–	Hernández et al. (2003); Canales et al. (2005)
73	*Larrea tridentata*[c]	Mt	Ap	125	125	250	250	–	–	250	Bocanegra et al. (2009)
74	*Larrea tridentata*[c]	Cl	Ap	**63**	na	na	250	–	–	na	Bocanegra et al. (2009)
75	*Leucophyllum frutescens*	Mt	Fl	–	–	na[a]	na[a]	–	–	500[a]	Molina et al. (2007)
76	*Leucophyllum frutescens*	Mt	L	–	–	na[a]	na[a]	–	–	500[a]	Molina et al. (2007)
77	*Leucophyllum frutescens*	Mt	R	–	–	na[a]	na[a]	–	–	250[a]	Molina et al. (2007)

(continued)

Table 7.2 (continued)

	Scientific name	ES	PP	Bs	Ef	Sa	Sa R	Se	Sl	Spn	References
78	*Lippia graveolens*	Hx	Ap	500	–	na	–	na	500	–	Canales et al. (2005)
79	*Lippia oaxacana*	Hx	Ap	na	–	na	–	na	500	–	Canales et al. (2005)
80	*Malvaviscus arboreus*	Mt	L	–	–	256	–	–	–	–	Yasunaka et al. (2005)
81	*Mammea americana*	Ac	P	–	–	**4**	**4**	–	–	–	Yasunaka et al. (2005)
82	*Mammea americana*	Eat	P	–	–	**16**	**16**	–	–	–	Yasunaka et al. (2005)
83	*Mammea americana*	Hx	P	–	–	**8**	**64**	–	–	–	Yasunaka et al. (2005)
84	*Mammea americana*	Mt	S	–	–	**2**	**4**	–	–	–	Yasunaka et al. (2005)
85	*Micona mexicana*	Hx	L	–	na	500	–	–	–	–	Navarro et al. (2006)
86	*Micona mexicana*	Mt	L	–	na	500	–	–	–	–	Navarro et al. (2006)
87	*Muntingia calabura*	Mt	Fr	–	–	256	–	–	–	–	Yasunaka et al. (2005)
88	*Muntingia calabura*	Mt	L	–	–	128	–	–	–	–	Yasunaka et al. (2005)
89	*Musa acuminata*	Aq	St	na	na	na	na	–	–	250	Bocanegra et al. (2009)
90	*Nasturtium officinale*	Mt	Ap	na	na	na	na	–	–	250	Bocanegra et al. (2009)
91	*Phlebodium aureum*	Mt	Ap	–	–	512	–	–	–	–	Yasunaka et al. (2005)
92	*Phyla nodiflora*	Mt	L	–	–	na	na	–	–	500	Molina et al. (2007)
93	*Psacalium radulifolium*	Hx	R	–	–	**62**	–	–	–	–	Garduño et al. (2001)
94	*Sambucus mexicana*	Mt	Fl/Fr	–	–	na	na	–	–	500	Molina et al. (2007)
95	*Schinus molle*	Mt	B	–	125	125	125	–	–	250	Molina et al. (2007); Salazar et al. (2009)
96	*Schinus molle*	Mt	Fl	–	na	**63**	125	–	–	250	Molina et al. (2007); Salazar et al. (2009)
97	*Schinus molle*	Hx	Fr	–	–	na	na	–	–	**63**	Molina et al. (2007)
98	*Schinus molle*	Mt	L	–	na	125	250	–	–	250	Molina et al. (2007); Salazar et al. (2009)
99	*Schinus molle*	Mt	R	–	–	na	na	–	–	250	Molina et al. (2007)

100	*Scutellaria elliptica*	Mt	Fl/Fr	–	–	na	na	–	–	250	Molina et al. (2007)
101	*Scutellaria elliptica*	Mt	R	–	–	na	na	–	–	500	Molina et al. (2007)
102	*Viguiera dentata*	Hx	Ap	375	–	na	–	na	375	–	Canales et al. (2005)
103	*Zornia thymifolia*	Mt	L	–	–	128	–	–	–	–	Yasunaka et al. (2005)

A active ≤ 500 μg/mL, *na* no active ≥ 500 μg/m, *ES* extraction solvent, *PP* part of plant used, *Bs Bacillus subtilis*, *Ef Enterobacter faecalis*, *Sa Staphylococcus aureus*, *SaR Staphylococcus aureus* Resistant, *Se Streptococcus epidermidis*, *Sl Sarcina lutea*, *Spn Streptococcus pneumoniae*, *Hx* hexane, *Mt* methanol, *Aq* aqueous, *DCM* dichloromethane, *Ac* acetone, *EE* ethyl ether, *Cl* chloroform, *Et* ethanol, *EO* essential oil, *Aet* ethyl acetate, *Ap* aerial part, *B* bark, *G* ground parts, *S* seeds, *H* heartwood, *L* leaves, *P* fruit peels, *La* latex, *St* stem, *Fr* fruit, *Fl* flower, *R* root, [a]Two resistant strains were used [b]collected on two places, [c]Active against *Listeria monocytogenes*

them showed activity against the resistant strains used. The methanolic and dichloromethane extracts from *Byrsonima crassifolia* cortex (Rivero et al. 2009) showed the same value against *Bac. subtilis* and *Ent. faecium*, while the hexanic extract from *Schinus molle* fruits inhibited the growth of two resistant *Str. pneumoniae* strains with a MIC of 62.5 μg/mL (Molina et al. 2004). Furthermore, the essential oil obtained from the aerial part of *Cordia curassavica* presented a MIC of 62.5 μg/mL against *Sarcina lutea* (Hernandez et al. 2007). High activity of methanolic and chloroform extracts from *Larrea tridentata* against *Bac. subtilis* and *Str. Pneumoniae* was also found (Bocanegra et al. 2009).

The analysis of the results obtained in all the reviewed articles provides evidence that the activity depends not only on the organism used, but also on the extraction solvent (Yasunaka et al. 2005; Navarro et al. 1996; Molina et al. 2007; Hernandez et al. 2007; Bocanegra et al. 2009), part of the plant used, and collection site. Recently, Pérez et al. (2008a) evaluated the effects of the solvent and extraction conditions on the antimicrobial activity of three plants growing in Mexico, *Leucophyllum frutescens*, *Cordia boissieri* and *Cyperus alternifolius*, using a drug-resistant strain of *Sta. aureus*. The results indicated that storage time significantly affects the MIC; in those experiments, the methanolic, ethanolic, or ethanol:water (90:10) extracts obtained from *Cordia boissieri* afforded MIC values of 31.5 μg/mL.

7.4 Extracts Obtained from Mexican Plants Active Against *M. tuberculosis*

In the past decade, more than 50 Mexican plants have been evaluated against sensitive and resistant *M. tuberculosis* strains. This has resulted in 47 extracts obtained from 28 different plants with a MIC less than or equal to 200 μg/mL, which was considered generally as the cut off to justify a bioassay fractionation aiming to obtain possible active compounds that could compete with the available antiphthisic drugs (Table 7.3).

Table 7.3 Extract active against *Mycobacterium tuberculosis* (μg/mL)

	Scientific name	ES	PP	Mtb	RMtb	References
1	*Amphipteryngium adstringens*	DCM:Mt	St	**A**	–	Rivero et al. (2005)
2	*Aristolochia brevipes*	DCM	R	**12.5**	–	Navarro et al. (2011a)
3	*Artemisia ludoviciana*	Hx	Ap	200	200	Jimenez et al. (2003); Castillo et al. (2007)
4	*Artemisia ludoviciana*	Mt	Ap	200	200	Jimenez et al. (2003)
5	*Bocconia frutescens*	Hx	L	125	–	Cruz et al. (2008)
6	*Bocconia frutescens*	Mt	L	125	–	Cruz et al. (2008)

(continued)

Table 7.3 (continued)

	Scientific name	ES	PP	Mtb	RMtb	References
7	*Carya illinoensis*	Et	B	125	–	Cruz et al. (2008)
8	*Carya illinoensis*	Hx	B	**31**	–	Cruz et al. (2008)
9	*Carya illinoensis*	Hx	L	125	–	Cruz et al. (2008)
10	*Carya illinoensis*	Mt	B	125	–	Cruz et al. (2008)
11	*Carya illinoensis*	Mt	L	125	–	Cruz et al. (2008)
12	*Chamaedora tepejilote*	Hx	L	200	**100**	Jimenez et al. (2003); Jiménez et al. (2005)
13	*Chrysactinia mexicana*	EE	R	**62.5**	**62.5**	Molina et al. (2007)
14	*Citrus aurantifolia*	Hx	P	200	**25**	Camacho et al. (2008)
15	*Citrus sinensis*	Hx	P	200	**25**	Camacho et al. (2008)
16	*Diospyros anisandra*	Mt	B	**100**	**100**	Borges et al. (2007)
17	*Flourensia cernua*	Ac	L	200	**100**	Molina et al. (2006)
18	*Flourensia cernua*	Hx	L	**50**	**25**	Molina et al. (2006)
19	*Flourensia cernua*	Et	L	**100**	**50**	Molina et al. (2011a)
20	*Foeniculum vulgare*	Cl	Ap	200	Na	Camacho et al. (2008)
21	*Foeniculum vulgare*	Hx	Ap	200	**100**	Camacho et al. (2008)
22	*Juglans mollis*	Et	B	**100**	–	Cruz et al. (2008)
23	*Juglans mollis*	Et	L	125	–	Cruz et al. (2008)
24	*Juglans mollis*	Hx	B	**50**	–	Cruz et al. (2008)
25	*Juglans mollis*	Hx	L	125	–	Cruz et al. (2008)
26	*Juglans mollis*	Mt	B	125	–	Cruz et al. (2008)
27	*Juglans regia*	Hx	B	**100**	–	Cruz et al. (2008)
28	*Juglans regia*	Mt	L	125	–	Cruz et al. (2008)
29	*Juniperus communis*	Hx	L	**100**	**100**	Jimenez et al. (2003)
30	*Juniperus communis*	Mt	L	**100**	**100**	Jimenez et al. (2003)
31	*Lantana hispida*	Hx	Ap	200	**25**	Jimenez et al. (2003)
32	*Larrea divaricata*	DCM:Mt	L	**A**	–	Rivero et al. (2005)
33	*Larrea tridentata*	Cl	Ap	200	**100**	Camacho et al. (2008)
34	*Lepechinia caulescens*	Hx	R	**100**	–	Estrada et al. (2009)
35	*Lepechinia caulescens*	Hx	F	**100**	–	Estrada et al. (2009)
36	*Leucophyllum frutescens*	Mt	L	na	125	Molina et al. (2007)
37	*Leucophyllum frutescens*	Mt	R	**62.5**	**62.5**	Molina et al. (2007)
38	*Malva parviflora*	Hx	Ap	200	Na	Jimenez et al. (2003)
39	*Musa acuminata*	Mt	St	200	200	Camacho et al. (2008)
40	*Nasturtium officinale*	Cl	Ap	**100**	**50**	Camacho et al. (2008)
41	*Olea europaea*	Hx	L	200	**25**	Camacho et al. (2008)
42	*Phoradendron robinsonii*	DCM:Mt	Ap	**A**	–	Rivero et al. (2005)
43	*Rumex hymenosepalus*	DCM:Mt	R	**A**	–	Rivero et al. (2005)
44	*Schinus molle*	Hx	Fr	125	Na	Molina et al. (2007)
45	*Valeriana edulis*	Hx	Rz	**3.1**	–	Estrada et al. (2009)
46	*Valeriana edulis*	DCM	Rz	**25**	–	Estrada et al. (2009)
47	*Valeriana edulis*	Mt	Rz	**50**	–	Estrada et al. (2009)

A active ≤ 50 μg/mL, *na* no active ≥ 200 μg/mL, *ES* extraction solvent, *PP* part of plant used, *Mtb* *Mycobacterium tuberculosis*, *RMtb* resistant *Mycobacterium tuberculosis*, *Hx* hexane, *Mt* methanol, *Ac* acetone, *EE* ethyl ether, *Cl* chloroform, *Et* ethanol, *DCM* dichloromethane, *Ap* aerial part, *B* bark, *S* seeds, *L* leaves, *P* fruit peels, *St* stem, *Fr* fruit, *R* root, *Rz* rhizome

The best antimycobacterial activities were found with *Valeriana edulis* rhizome hexanic extract (3.1 μg/mL, Estrada et al. 2009), and with *Aristolochia brevipes* root dichloromethane extract (12.5 μg/mL, Navarro et al. 2011a); good activities were also displayed by the hexanic extracts obtained from *Carya illinoensis* cortex (31.2 μg/mL), *Juglans mollis* cortex (50 μg/mL), and *J. regia* cortex (100 μg/mL), the ethanolic extracts obtained from *J. mollis* cortex (100 μg/mL, Cruz et al. 2008), and the hexanic and ethanolic extracts from *Flourensia cernua* leaves (50 and 100 μg/mL, respectively, Molina et al. 2011a). Four species were reported by Rivero et al. (2005) with MIC's lower than 50 μg/mL, namely: *Rumex hymenosepalus*, *Larrea divaricata*, *Phorarendron robinsonii* and *Amphipteryngium adstringens*. Interestingly, the extract from *F. cernua* presented remarkable activity against a *M. tuberculosis* strain resistant to streptomycin, isoniazid, rifampin, ethambutol, and pyrazinamide (Molina et al. 2006). Furthermore, hexanic extracts from the aerial part of *Lantana hispida* (Jimenez et al. 2003), from leaves of *Olea europaea*, and from the fruit of *Citrus sinensis* and *Cit. aurantifolia* (Camacho et al. 2008) showed the same MIC of 25 μg/mL against a strain resistant to isoniazid, although the MIC obtained against the sensitive strain was 200 μg/mL. Similarly, the chloroform extract obtained from the aerial part of *Nasturtium officinale* afforded a MIC of 50 μg/mL against three different strains resistant to rifampin, isoniazid, or ethambutol; however, the MIC against the sensitive strain was 100 μg/mL (Camacho et al. 2008). An ether extract from the roots of *Chrysactinia mexicana* and a methanolic extract from the roots of *L. frutescens* gave MICs of 62.5 μg/mL against the sensitive strain and the strain resistant to streptomycin, isoniazid, rifampin, ethambutol, and pyrazinamide (Molina et al. 2007). Moreover, six extracts showed the same activity against the sensitive and resistant strains of *M. tuberculosis*, namely: an ethereal extract from *Chry. mexicana* roots (62.5 μg/mL), a methanolic extract from *L. frutescens* roots (62.5 μg/mL) (Molina et al. 2007), hexanic and methanolic extracts from *Juniperus communis* leaves (100 μg/mL) (Jimenez et al. 2003), a hexanic extract from *Chamaedorea tepejilote* leaves (100 μg/mL) (Jimenez et al. 2003, 2005), and a methanolic extract from *Diospyros anisandra* cortex (100 μg/mL) (Borges et al. 2007).

7.5 Extracts Obtained from Mexican Plants Active Against Gram-Negative Bacteria

A great number of Gram-negative bacteria were used in different assays, the most commonly used being: *Acinetobacter baumannii*, *Citrobacter species*, *Enterobacter aerogenes*, *Ent. agglomerans*, *Escherichia coli*, *Haemophilus influenzae*, *Helicobacter pylori*, *Klebsiella oxytoca*, *Kl. pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Pr. vulgaris*, *Shigella boydii*, *Sh. flexneri*, *Sh. sonnei*, *Stenotrophomonas maltophilia*, *Salmonella typhi*, *Sal. typhimurium*, *Vibrio cholerae*, and *Yersinia enterocolitica*. Of these, the most frequently included were *E. coli*, *He. pylori*, *Pseud. aeruginosa*, and *Shigella* spp.

In 17 articles, 106 extracts (obtained from 77 plants) were reported active against at least one Gram negative bacterial species with MICs equal to or lower than 500 μg/mL. Most of them were active against *He. pylori* (73 extracts obtained from 55 plants), *E. coli* (12 extracts from 9 plants), and *V. cholerae* (11 extracts from 8 plants) (Table 7.4).

The hexane extract obtained from *Hedeoma drummondii* (Viveros et al. 2011) showed activity against *E. coli* (MIC of 250 μg/mL), *Ent. aerogenes* (MIC of 62.5 μg/mL), *Pr. vulgaris* (MIC of 500 μg/mL) and *Sh. flexneri* (MIC of 250 μg/mL).

The essential oil obtained from the aerial part of *Lepechinia caulescens* displayed MICs of 3 and 4 μL/mL against *V. cholerae* (CDC-V12 and two clinical isolates, respectively) (Acevedo et al. 2005), which was the best result for activity against a Gram-negative bacterial strain found in the present survey. The essential oil obtained from the aerial part of *Cordia curassavica* showed a MIC of 62.5 μg/mL (Hernandez et al. 2007) against a clinical isolate of *V. cholerae*.

Castillo et al. (2009) recently reported a screening against *He. pylori* using 53 Mexican plants, the most active extract being a methanolic extract obtained from the leaves of *Persea americana* with a MIC lower than 7.5 μg/mL. Activity against the same mo was reported for methanolic extracts from the aerial parts of *Moussonia deppeana* and *Annona cherimola* and from the cortex of *Guaiacum coulteri* with MICs equal to or less than 15.6 μg/mL. Eight extracts showed MICs of 32.2 μg/mL against the same mo, namely methanolic extracts from the aerial parts of *Eryngium carlinae*, *Heterotheca inuloides*, *Lippia berlandieri*, *Machaeranthera parviflora*, *Marrubium vulgare*, *Ocimum basilicum*, *Plectranthus amboinicus*, and *Cymbopogon citratus*. A methanolic extract obtained from *Campyloneurum amphostenon* leaves presented a MIC lower than 62.5 μg/mL. Methanolic extracts from the aerial parts of *Buddleja perfoliata*, *Capsella bursa-pastoris*, *Gnaphalium canescens*, *Grindelia inuloides*, *Hesperozygis marifolia*, *Larrea tridentata*, *Machaeranthera riparia*, *Ruta chalepensis*, *Tanacetum parthenium*, *Teloxys graveolens*, and *Tithonia diversifolia* were active MICs of 62.5 μg/mL.

Eleven extracts displayed activity against *E. coli*. Of these, the most active were the methanolic extract obtained from seeds of *Haematoxylon brasiletto* and the hydroalcoholic extract from the heartwood of *Cal. brasiliense*, both with a MIC of 128 μg/mL (Yasunaka et al. 2005).

None of the articles reviewed reported noticeable activity (MICs higher than or equal to 500 μg/mL) against strains of *Pseud. aeruginosa*, an important intrahospital pathogen. Methanolic and aqueous extracts obtained from *Phaseolus vulgaris* seeds presented inhibition zones between 12 and 14 mm at 5 mg/mL against *Pseud. aeruginosa*; however, no positive control for comparison is reported. Moreover, the activity of *Psidium guajava* (Gutierrez et al. 2008) against this bacterium is mentioned in a review; however, the results were obtained with plants from Nigeria. The resistance of these bacteria toward most of the available antibiotics and the absence of interesting results from natural products against this mo create an opportunity for enhanced research directed toward finding new molecules that are active agents against *Pseud. aeruginosa.*

Table 7.4 Extract active against gram negative bacteria (μg/mL)

	Scientific name	ES	PP	Eae	Eco	Hi	RHi	Hp	Sb	Vc	Ye	References
1	*Acacia farnesiana*	Mt	Ap	–	–	–	–	–	–	500	–	Sánchez et al. (2010)
2	*Ambrosia confertiflora*	Mt	Ap	–	–	–	–	<200	–	–	–	Robles et al. (2011)
3	*Amphipterygium adstringens*	Aq	B	–	–	–	–	500	–	–	–	Castillo et al. (2009)
4	*Amphipterygium adstringens*	Mt	B	–	–	–	–	250	–	–	–	Castillo et al. (2009)
5	*Amphipterygium adstringens*	Mt	Ap	–	–	–	–	200	–	–	–	Robles et al. (2011)
6	*Annona cherimola*	Aq	Ap	–	–	–	–	250	–	–	–	Castillo et al. (2009)
7	*Annona cherimola*	Mt	Ap	–	–	–	–	**<15.6**	–	–	–	Castillo et al. (2009)
8	*Anoda cristata*	Mt	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
9	*Artemisia ludoviciana*	Aq	Ap	–	–	–	–	125	–	–	–	Castillo et al. (2009)
10	*Artemisia ludoviciana*	Mt	Ap	–	–	–	–	250	–	–	–	Castillo et al. (2009)
11	*Buddleja perfoliata*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
12	*Buddleja perfoliata*	Mt	Ap	–	–	–	–	**62.5**	–	–	–	Castillo et al. (2009)
13	*Byrsonima crassifolia*	DCM	B	–	500	–	–	–	–	–	–	Rivero et al. (2009)
14	*Calandrinia micrantha*	Mt	Ap	–	–	–	–	250	–	–	–	Castillo et al. (2009)
15	*Calophyllum brasiliense*	Hx	L	–	512	–	–	–	–	–	–	Yasunaka et al. (2005)
16	*Calophyllum brasiliense*	Mt+Aq	H	–	128	–	–	–	–	–	–	Yasunaka et al. (2005)
17	*Campyloneurum amphostenon*	Mt	L	–	–	–	–	**<62.5**	–	–	–	Castillo et al. (2009)
18	*Capsella bursa-pastoris*	Mt	Ap	–	–	–	–	**62.5**	–	–	–	Castillo et al. (2009)
19	*Castella tortuosa*	Mt	Ap	–	–	–	–	<200	–	–	-	Robles et al. (2011)
20	*Ceanothus coeruleus*	Mt	R	–	–	500	500	–	–	–	–	Molina et al. (2007)
21	*Chenopodium graveolens*	Mt	G	–	512	–	–	–	–	–	–	Yasunaka et al. (2005)
22	*Chrysactinia mexicana*	EE	Fl	–	–	na	500	–	–	–	–	Molina et al. (2007)
23	*Chrysactinia mexicana*	EE	R	–	–	500	500	–	–	–	–	Molina et al. (2007)
24	*Cordia boissieri*	Mt	L	–	–	na	500	–	–	–	–	Molina et al. (2007)
25	*Cordia curassavica*	EO	Ap	–	na	–	–	–	–	**62**	na	Hernandez et al. (2007)
26	*Cordia curassavica*	Hx	Ap	–	na	–	–	–	–	125	na	Hernandez et al. (2007)
27	*Coutarea latiflora*	Mt	Ap	–	–	–	–	400	–	–	–	Robles et al. (2011)
28	*Cuphea aequipetala*	Aq	Ap	–	–	–	–	125	–	–	–	Castillo et al. (2009)

29	*Cymbopogon citratus*	Mt	L	–	–	–	–	**31.2**	–	–	–	Castillo et al. (2009)
30	*Cyrtocarpa procera*	Aq	B	–	–	–	–	250	–	–	–	Castillo et al. (2009)
31	*Cyrtocarpa procera*	Mt	B	na	na	–	–	–	125	na	na	Canales et al. (2005)
32	*Eryngium carlinae*	Mt	Ap	–	–	–	–	**31.2**	–	–	–	Castillo et al. (2009)
33	*Eupatorium petiolare*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
34	*Eupatorium petiolare*	Mt	Ap	–	–	–	–	125	–	–	–	Castillo et al. (2009)
35	*Gnaphalium canescens*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
36	*Gnaphalium canescens*	Mt	Ap	–	–	–	–	**62.5**	–	–	–	Castillo et al. (2009)
37	*Grindelia inuloides*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
38	*Grindelia inuloides*	Mt	Ap	–	–	–	–	**62.5**	–	–	–	Castillo et al. (2009)
39	*Guaiacum coulteri*	Mt	B	–	–	–	–	**<15.6**	–	–	–	Castillo et al. (2009)
40	*Gymnosperma glutinosum*[a]	Aet	Ap	na	na	–	–	–	250	na	na	Canales et al. (2005)
41	*Gymnosperma glutinosum*	Mt	Ap	**<125**	–	–	–	–	**<125**	**<125**	–	Canales et al. (2007)
42	*Gymnosperma glutinosum*[b]	Mt	Ap	500	–	–	–	–	125	250	–	Canales et al. (2007)
43	*Haematoxylon brasiletto*	Mt	S	–	128	–	–	–	–	–	–	Yasunaka et al. (2005)
44	*Haplopappus spinulosus*	Mt	Ap	–	–	–	–	125	–	–	–	Castillo et al. (2009)
45	*Hedeoma drummondii*[c]	Hx	Ap	**63**	250	–	–	–	–	–		Viveros, et al. (2011)
46	*Hesperozygis marifolia*	Mt	Ap	–	–	–	–	**62.5**	–	–	–	Castillo et al. (2009)
47	*Heterotheca inuloides*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
48	*Heterotheca inuloides*	Mt	Ap	–	–	–	–	**31.2**	–	–	–	Castillo et al. (2009)
49	*Ibervillea sonorae*	Mt	Ap	–	–	–	–	<200	–	–	–	Robles et al. (2011)
50	*Jatropha neopauciflora*[a]	La	La	na	na	–	–	–	na	500	na	Canales et al. (2005)
51	*Krameria erecta*	Mt	Ap	–	–	–	–	200	–	–	–	Robles et al. (2011)
52	*Laennecia schiedeana*	Ac	Ap	–	–	–	–	–	–	–	–	Arciniegas et al. (2011)
53	*Lantana achyranthifolia*[a]	EO	Ap	na	na	–	–	–	na	250	500	Hernández et al. (2005)
54	*Lantana achyranthifolia*[a]	Hx	Ap	na	na	–	–	–	500	250	na	Hernández et al. (2003)
55	*Larrea tridentata*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
56	*Larrea tridentata*	Mt	Ap	–	250	125	–	**62.5**	–	–	–	Castillo et al. (2009); Bocanegra et al. (2009)

(continued)

Table 7.4 (continued)

	Scientific name	ES	PP	Eae	Eco	Hi	RHi	Hp	Sb	Vc	Ye	References
57	*Lepechinia caulescens*	EO	Ap	–	–	–	–	–	–	**3**[d]	–	Acevedo et al. (2005)
58	*Lippia berlandieri*	Mt	Ap	–	–	–	–	**31.2**	–	–	–	Castillo et al. (2009)
59	*Lippia graveolens*[a]	Hx	Ap	na	na	–	–	–	na	250	na	Hernández et al. (2003)
60	*Ludwigia repens*	Aq	Ap	–	–	–	–	125	–	–	–	Castillo et al. (2009)
61	*Ludwigia repens*	Mt	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
62	*Machaeranthera cf. parviflora*	Mt	Ap	–	–	–	–	**31.2**	–	–	–	Castillo et al. (2009)
63	*Machaeranthera riparia*	Mt	Ap	–	–	–	–	**62.5**	–	–	–	Castillo et al. (2009)
64	*Machaeranthera tanacetifolia*	Mt	Ap	–	–	–	–	125	–	–	–	Castillo et al. (2009)
65	*Mammea americana*	Ac	P	–	512	–	–	–	–	–	–	Yasunaka et al. (2005)
66	*Mammea americana*	Eat	P	–	512	–	–	–	–	–	–	Yasunaka et al. (2005)
67	*Mammea americana*	Mt	S	–	256	–	–	–	–	–	–	Yasunaka et al. (2005)
68	*Marrubium vulgare*	Mt	Ap	–	–	–	–	**31.2**	–	–	–	Castillo et al. (2009)
69	*Marrubium vulgare*	Mt	Ap	–	–	–	–	200	–	–	–	Robles et al. (2011)
70	*Mentha×piperita*	Aq	Ap	–	–	–	–	<250	–	–	–	Castillo et al. (2009)
71	*Mentha×piperita*	Mt	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
72	*Mirabilis jalapa*	Aq	Ap	–	–	–	–	250	–	–	–	Castillo et al. (2009)
73	*Monarda austromontana*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
74	*Monarda austromontana*	Mt	Ap	–	–	–	–	125	–	–	–	Castillo et al. (2009)
75	*Moussonia deppeana*	Mt	Ap	–	–	–	–	**15.6**	–	–	–	Castillo et al. (2009)
76	*Muntingia calabura*	Mt	L	–	512	–	–	–	–	–	–	Yasunaka et al. (2005)
77	*Ocimum basilicum*	Mt	Ap	–	–	–	–	**31.2**	–	–	–	Castillo et al. (2009)
78	*Olea europaea*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
79	*Persea americana*	Mt	L	–	–	–	–	**<7.5**	–	–	–	Castillo et al. (2009)
80	*Phyla nodiflora*	Mt	L	–	–	na	500	–	–	–	–	Molina et al. (2007)
81	*Pimpinella anisum*	Mt	Ap	–	–	–	–	<200	–	–	–	Robles et al. (2011)
82	*Piper amalago*	Mt	L	–	512	–	–	–	–	–	–	Yasunaka et al. (2005)
83	*Pittocaulon bombycophole*	DCM	R	–	–	–	–	–	–	250	–	Marín et al. (2008)
84	*Plantago major*	Mt	Ap	–	–	–	–	250	–	–	–	Castillo et al. (2009)

85	*Plectranthus amboinicus*	Mt	Ap	–	–	–	–	**31.2**	–	–	–	Castillo et al. (2009)
86	*Poliomintha longiflora*	Mt	Ap	–	–	–	–	250	–	–	–	Castillo et al. (2009)
87	*Priva grandiflora*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
88	*Priva grandiflora*	Mt	Ap	–	–	–	–	250	–	–	–	Castillo et al. (2009)
89	*Psacalium decompositum*	Mt	Ap	–	–	–	–	<200	–	–	–	Robles et al. (2011)
90	*Quercus rugosa*	Mt	L	–	–	–	–	125	–	–	–	Castillo et al. (2009)
91	*Ruta chalepensis*	Mt	Ap	–	–	–	–	**62.5**	–	–	–	Castillo et al. (2009)
92	*Schinus molle*	Hx	Fr	–	–	na	500	–	–	–	–	Molina et al. (2007)
93	*Schinus molle*	Mt	B	–	–	na	500	–	–	–	–	Molina et al. (2007)
94	*Schinus molle*	Mt	R	–	–	500	500	–	–	–	–	Molina et al. (2007)
95	*Selaginella lepidophylla*	Mt	Ap	–	–	–	–	200	–	–	–	Robles et al. (2011)
96	*Tagetes lucida*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
97	*Tagetes lucida*	Mt	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
98	*Tanacetum parthenium*	Mt	Ap	–	–	–	–	**62.5**	–	–	–	Castillo et al. (2009)
99	*Tecoma stans*	Mt	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
100	*Teloxys ambrosioides*	Mt	Ap	–	–	–	–	250	–	–	–	Castillo et al. (2009)
101	*Teloxys graveolens*	Aq	Ap	–	–	–	–	250	–	–	–	Castillo et al. (2009)
102	*Teloxys graveolens*	Mt	Ap	–	–	–	–	**62.5**	–	–	–	Castillo et al. (2009)
103	*Tillandsia usneoides*	Mt	Ap	–	–	–	–	125	–	–	–	Castillo et al. (2009)
104	*Tithonia diversifolia*	Aq	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)
105	*Tithonia diversifolia*	Mt	Ap	–	–	–	–	**62.5**	–	–	–	Castillo et al. (2009)
106	*Verbena carolina*	Mt	Ap	–	–	–	–	500	–	–	–	Castillo et al. (2009)

na no active ≥ 500 μg/mL, *Eae Enterobacter aerogenes*, *Eco Escherichia coli*, *Hi Haemophilus influenzae*, *RHi* Resistant *Haemophilus influenzae*, *Hp Helicobacter pylori*, *Pa Pseudomonas aeruginosa*, *Sb Shigella boydii*, *Vc Vibrio cholerae*, *Ye Yersinia enterolitica*, *Hx* hexane, *Mt* methanol, *Aq* aqueous, *DCM* dichloromethane, *Ac* acetone, *EE* ethyl ether, *Cl* chloroform, *Et* ethanol, *EO* essential oil, *Aet* ethyl acetate, *Ap* aerial part, *B* bark, *G* ground parts, *S* seeds, *H* heartwood, *L* leaves, *P* fruit peels, *La* latex, *St* stem, *Fr* fruit, *Fl* flower, *R* root

[a]No active against *Enterobacter agglomerans* or *Salmonella typhi*

[b]Collected on two places

[c]Active against *Shigella flexneri*, *Proteus vulgaris*

[d]μL/mL

7.6 Extracts Obtained from Mexican Plants Active Against Pathogenic Fungi

In the past decade, a dramatic worldwide increase in the incidence of fungal infections has been observed, especially in immunocompromised patients, patients undergoing cancer chemotherapy, and organ transplant recipients. Although medical advances have made it possible to lengthen the life of these patients, they are highly susceptible to fungal infections, which are often the ultimate cause of their death. The most common genus reported to cause these infections is *Candida*. However, other pathogenic fungi belonging to the genus *Aspergillus*, *Cryptococcus*, *Histoplasma*, *Coccidioides*, as well as dermatophytes belonging to the *Trichophyton*, *Microsporum*, and *Fusarium* genera, are also reported to cause severe damage.

In our survey we found that, in the past 10 years, 94 Mexican plants have been tested for activity against at least one of the fungi mentioned (Table 7.1). There is a great variation in the breakdown that each research group uses to consider an assay as positive, ranging from 0.016 to 160 mg/mL for the extracts and from 0.002 to 8 mg/mL for pure compounds. Most of the authors report a good antifungal response, even though the MICs found are 10,000 times higher than the MIC from the antifungal used as reference. Taking this into account, we considered as valuable those extracts or compounds that have a MIC equal to or lower than 1,000 (1 mg/mL) or 200 μg/mL (0.2 mg/mL), respectively. Therefore, only 26 plants (64 extracts) were included as active in this review (Table 7.5).

Garduño et al. in (2001), reported MIC values of 0.062 and 1 mg/mL against *Candida albicans* for methanolic and hexanic extracts from *P. radulifolium* roots, respectively.

Navarro et al. in (2003) reported the *in vitro* antifungal screening of 18 extracts from nine medicinal plants against four human pathogenic fungi: *Cand. albicans*, *Aspergillus niger*, *Trichophyton mentagrophytes*, and *Tri. rubrum*. The plant species selected are used as traditional medicines in Morelos State, Mexico, for the treatment of illnesses that, according to the symptomatology described, include respiratory, genital, gastrointestinal, urinary, and skin infections: *Annona cherimola*, *Asclepia curassavica*, *Bixa orellana*, *Ageratina pichinchensis*, *Galphimia glauca*, *Lysiloma acapulcensis*, *Malva parviflora*, *Sedum oxypetalum*, and *Senecio angulifolius*. Two plant extracts were active against *Tri. mentagrophytes* and *Tri. rubrum*, with MIC values of 1 mg/mL or less, namely the hexanic and methanolic extracts from *A. pichinchensis* and the methanolic extract from *Lys. acapulcensis*.

Ríos et al. in (2003b) reported the chemical composition and the antimicrobial activity of the essential oils obtained from the leaves, flowers, and fruits of *Annona cherimola*. Only the essential oil obtained from the flowers was active against *Cand. albicans* at 0.5 mg/mL.

Gymnosperma glutinosum (Spreng.) Less (Asteraceae) is an important and effective herbal medicine that is widely used in Mexico for the treatment of diarrhea. Canales et al. in (2007) used a dilution method to examine and compare the antibacterial and antifungal activities of two samples of *G. glutinosum* from two locations

Table 7.5 Extract active against fungi (μg/mL)

	Scientific name	ES	PP	Ca	Cg	Ct	Ck	Cp	Af	Ci	Hc	Tm	Tr	References
1	*Acacia farnesiana*	Et+Aq	Ap	na	–	–	–	–	na	na	500	–	–	Alanís et al. (2007)
2	*Annona cherimolia*	EO	L	500	–	–	–	–	–	–	–	–	–	Rios et al. (2003b)
3	*Bougainvillea glabra*	Et+Aq	Ap	na	–	–	–	–	na	500	na	–	–	Alanís et al. (2007)
4	*Byrsonima crassifolia*	DCM	B	1,000	–	–	–	–	–	–	–	–	–	Rivero et al. (2009)
5	*Ceanothus coerulus*	Mt	L	**62**	**31**	250	**31**	**31**	–	–	–	–	–	Salazar et al. (2009)
6	*Ceanothus coerulus*	Mt	Fl	1,000	**31**	1,000	1,000	125	–	–	–	–	–	Salazar et al. (2009)
7	*Ceanothus coerulus*	Mt	R	**31**	**31**	250	125	**31**	–	–	–	–	–	Salazar et al. (2009)
8	*Chrysactinia mexicana*	Mt	L	na	**31**	na	na	na	–	–	–	–	–	Salazar et al. (2009)
9	*Chrysactinia mexicana*	Mt	Fl	na	**31**	1,000	na	na	–	–	–	–	–	Salazar et al. (2009)
10	*Chrysactinia mexicana*	Mt	R	na	**31**	na	na	na	–	–	–	–	–	Salazar et al. (2009)
11	*Clematis drummondii*	Et+Aq	Ap	250	–	–	–	–	500	125	250	–	–	Alanís et al. (2007)
12	*Clematis drummondii*	Hx	Ap	250	–	–	–	–	na	na	125	–	–	Alanís et al. (2007)
13	*Clematis drummondii*	Aet	Ap	125	–	–	–	–	250	na	**16**	–	–	Alanís et al. (2007)
14	*Colubrina greggii*	Et+Aq	Ap	125	–	–	–	–	na	na	125	–	–	Alanís et al. (2007)
15	*Colubrina greggii*	Hx	Ap	250	–	–	–	–	–	–	250	–	–	Alanís et al. (2007)
16	*Colubrina greggii*	Aet	Ap	**62**	–	–	–	–	–	–	na	–	–	Alanís et al. (2007)
17	*Colubrina greggii*	Bt	Ap	**62**	–	–	–	–	–	–	na	–	–	Alanís et al. (2007)
18	*Colubrina greggii*	Mt	L	125	**31**	500	125	**62**	–	–	–	–	–	Salazar et al. (2009)
19	*Colubrina greggii*	Mt	Fl	125	**31**	500	125	125	–	–	–	–	–	Salazar et al. (2009)
20	*Colubrina greggii*	Mt	R	125	**31**	500	1,000	500	–	–	–	–	–	Salazar et al. (2009)
21	*Cordia boissieri*	Mt	Fl	na	125	na	na	na	–	–	–	–	–	Salazar et al. (2009)
22	*Cordia boissieri*	Et+Aq	Ap	na	–	–	–	–	na	na	125	–	–	Alanís et al. (2007)
23	*Cordia boissieri*	Hx	Ap	–	–	–	–	–	–	–	**31**	–	–	Alanís et al. (2007)
24	*Cordia boissieri*	Aet	Ap	–	–	–	–	–	–	–	**62**	–	–	Alanís et al. (2007)
25	*Cyperus alternifolius*	Mt	L-St	500	**31**	1,000	na	1,000	–	–	–	–	–	Salazar et al. (2009)
26	*Cyperus alternifolius*	Mt	R	500	**31**	500	500	1,000	–	–	–	–	–	Salazar et al. (2009)

(continued)

Table 7.5 (continued)

	Scientific name	ES	PP	Ca	Cg	Ct	Ck	Cp	Af	Ci	Hc	Tm	Tr	References
27	*Eupatorium aschenbornianum*	Hx	Ap	na	–	–	–	–	na	–	–	**30**	200	Navarro et al. (2003); Rios et al. (2003a)
28	*Eupatorium aschenbornianum*	Mt	Ap	na	–	–	–	–	na	–	–	1,000	500	Navarro et al. (2003)
29	*Euphorbia prostrata*	Et+Aq	Ap	**62**	–	–	–	–	na	500	500	–	–	Alanís et al. (2007)
30	*Euphorbia prostrata*	Aet	Ap	**16**	–	–	–	–	–	250	–	–	–	Alanís et al. (2007)
31	*Euphorbia prostrata*	Bt	Ap	**31**	–	–	–	–	–	na	–	–	–	Alanís et al. (2007)
32	*Galium mexicanum*	Cl	Ap	na	–	–	–	–	–	–	–	–	na	Bolivar et al. (2011)
33	*Gymnosperma glutinosum*[a]	Hx	Ap	na	–	–	–	–	–	–	–	**60**	–	Canales et al. (2007); Serrano et al. (2009)
34	*Hedeoma drummondii*	Et+Aq	Ap	na	–	–	–	–	na	na	500	–	–	Alanís et al. (2007)
35	*Hedeoma drummondii*	Hx	Ap	125	–	–	–	–	–	–	–	–	–	Viveros, et al. (2011)
36	*Heliotropium angiospermum*	Et+Aq	Ap	na	–	–	–	–	na	na	500	–	–	Alanís et al. (2007)
37	*Hofmeisteria schaffneri*	EO	Ap	192	–	–	–	–	–	–	–	–	–	Pérez et al. (2011)
38	*Hofmeisteria schaffneri*	Aq	Ap	128	–	–	–	–	–	–	–	–	–	Pérez et al. (2011)
39	*Hofmeisteria schaffneri*	DCM:Mt	Ap	1,000	–	–	–	–	–	–	–	–	–	Pérez et al. (2011)
40	*Jatropha dioica*	Et+Aq	R	500	–	–	–	–	na	na	1,000	–	–	Alanís et al. (2007)
41	*Jatropha dioica*	Aet	R	250	–	–	–	–	–	–	–	–	–	Alanís et al. (2007)
42	*Leucophyllum frutescens*	Et+Aq	Ap	na	–	–	–	–	na	na	250	–	–	Alanís et al. (2007)
43	*Leucophyllum frutescens*	Hx	Ap	–	–	–	–	–	–	–	125	–	–	Alanís et al. (2007)
44	*Leucophyllum frutescens*	Aet	Ap	–	–	–	–	–	–	–	**16**	–	–	Alanís et al. (2007)
45	*Leucophyllum frutescens*	Bt	Ap	–	–	–	–	–	–	–	250	–	–	Alanís et al. (2007)
46	*Loeselia mexicana*	Hx	St	na	–	–	–	–	–	–	–	1,000	1,000	Navarro et al. (2006)
47	*Loeselia mexicana*	DCM	St	na	–	–	–	–	–	–	–	500	500	Navarro et al. (2006)
48	*Loeselia mexicana*	Mt	St	na	–	–	–	–	–	–	–	250	250	Navarro et al. (2006)
49	*Lysiloma acapulcensis*	Mt	B	na	–	–	–	–	na	–	–	1,000	1,000	Navarro et al. (2003, 2006)
50	*Psacalium radulifolium*	Hx	R	**62**	–	–	–	–	–	–	–	–	–	Garduño et al. (2001)

51	*Psacalium radulifolium*	Mt	R	1,000	–	–	–	–	–	–	–	–	Garduño et al. (2001)
52	*Rivinia humilis*	Et+Aq	Ap	na	–	–	–	–	na	na	1,000	–	–	Alanís et al. (2007)
53	*Salvia texana*	Et+Aq	Ap	125	–	–	–	–	500	**62**	**62**	–	–	Alanís et al. (2007)
54	*Salvia texana*	Hx	Ap	**62**	–	–	–	–	**62**	**31**	**31**	–	–	Alanís et al. (2007)
55	*Salvia texana*	Aet	Ap	**62**	–	–	–	–	125	**16**	**16**	–	–	Alanís et al. (2007)
56	*Salvia texana*	Bt	Ap	125	–	–	–	–	250	**62**	**31**	–	–	Alanís et al. (2007)
57	*Schinus molle*	Mt	L	125	**31**	500	500	250	–	–	–	–	–	Salazar et al. (2009)
58	*Schinus molle*	Mt	Fl	**62**	**31**	250	**62**	**62**	–	–	–	–	–	Salazar et al. (2009)
59	*Schinus molle*	Mt	B	250	**62**	125	1,000	1,000	–	–	–	–	–	Salazar et al. (2009)
60	*Schinus molle*	Et+Aq	Ap	na	–	–	–	–	na	500	250	–	–	Alanís et al. (2007)
61	*Schinus molle*	Hx	Ap	–	–	–	–	–	–	na	125	–	–	Alanís et al. (2007)
62	*Schinus molle*	Aet	Ap	–	–	–	–	–	–	na	125	–	–	Alanís et al. (2007)
63	*Schinus molle*	Bt	Ap	–	–	–	–	–	–	na	**62**	–	–	Alanís et al. (2007)
64	*Solanum rostratum*	Et+Aq	P	na	–	–	–	–	na	na	1,000	–	–	Alanís et al. (2007)

na no active ≥ 1,000 μg/mL, *ES* extraction solvent, *PP* part of plant used, *Ca Candida albicans*, *Cg Candida glabrata*, *Ct Candida tropicalis*, *Ck Candida krusei*, *Cp Candida parapsilosis*, *Af Aspergillus fumigatus*, *Ci Coccidioides immitis*, *Hc Histoplasma capsulatum*, *Tm Trichophyton mentagrophytes*, *Tr Trichophyton rubrum*, *Hx* hexane, *Mt* methanol, *Aq* aqueous, *DCM* dichloromethane, *Et* ethanol, *EO* essential oil, *Aet* ethyl acetate, *Ap* aerial part, *B* bark, *L* leaves, *St* stem, *Fl* flower, *R* root

[a]Active against *Aspergillus niger*

in Mexico: San Rafael Coxcatlan (Puebla State) and Tepeji del Rio (Hidalgo State). The hexane extract showed antifungal activity against all tested fungi. In that work, IC_{50}, which is defined as the concentration of extract that produces 50 % inhibition of the area of fungal mycelial growth, was calculated. The hexane extract from the San Rafael sample was significantly more active than that from Tepeji del Rio. *Asp. niger* (IC_{50} = 23.79 μg/mL) and *Tri. mentagrophytes* (IC_{50} = 90.25 μg/mL) were the more sensitive fungal strains.

In traditional Mexican medicine, *Cordia curassavica* (Jacq.) Roemer & Schultes is used to treat gastrointestinal, respiratory, and dermatological disorders in Zapotitlan Salinas, Puebla (Mexico). Hernández et al. in (2007), investigated the antimicrobial activity of the essential oil, and the hexanic, chloroform, and methanolic extracts obtained from the aerial part of *Cordia curassavica*. The essential oil showed antifungal activity against five fungal strains: *Asp. niger*, *Tri. mentagrophytes*, *Fu. sporotrichum*, *Fu. moniliforme*, and *Rhizoctonia solani*. *Rhi. solani* was the most sensitive species to the essential oil effect (IC_{50} = 180 μg/mL). The chloroform extract was active against *Fu. sporotrichum* and *Fu. moniliforme* (IC_{50} = 500 and IC_{50} = 430 μg/mL, respectively). *Tri. mentagrophytes* and *Fu. moniliforme* were sensitive to the hexanic extract (IC_{50} = 230 and IC_{50} = 850 μg/mL). The essential oil was examined by Gas chromatography (GC) and Gas Chromatography-Mass Spectroscopy (GC–MS).

A screening of the antifungal activity of plants of the northeast of Mexico against some of the main etiological agents inductors of pulmonary mycoses (*Cand. albicans*, *Asp. fumigatus*, *Histoplasma capsulatum*, and *Coccidioides immitis*) has been conducted *in vitro* (Alanís et al. 2007). Plants evaluated were *Rivina humilis*, *Solanum rostratum*, *Schinus molle*, *Clematis drummondii* "male", *Cl. drummondii* "female", *Cordia boissieri*, *L. frutescens*, *Hedeoma drummondii*, *Bougainvillea glabra*, *Acacia farnesiana* (L) Willd, *Heliotropium angiospermum*, *Salvia texana*, *Euphorbia prostrata*, *Colubrina greggii*, and *Jatropha dioica*. Hydroalcoholic extracts from all plants were active against at least one of these fungi with MICs in the range 63–1,000 μg/mL. Hexane, ethyl acetate, and butanolic extracts were also active against the different fungi in the range 16–250 μg/mL. *B. glabra*, used traditionally in afflictions of the respiratory system, was active only against *Coccidioides immitis* (MIC = 500 μg/mL). *Rivina humilis*, *Acacia farnesiana*, *Helio. angiospermum*, *Cl. drummondii*, *Solanum rostratum*, *Cordia boissieri*, and *L. frutescens* presented activity only against *His. capsulatum*. *Salvia texana* and *Cl. drummondii* were active against all fungi tested; *Asp. fumigatus* was sensitive only to the extracts from *Salvia texana* and *Cl. drummondii*.

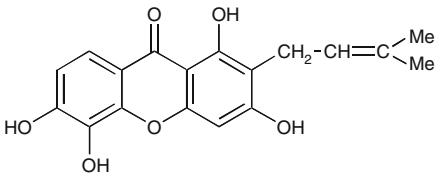
In a similar survey, Salazar et al. in (2009) evaluated methanolic extracts from 17 plants of northeast Mexico. The extracts were tested for their antimicrobial activity using seven clinically isolated yeasts belonging to (*Cand. albicans*, *Cand. krusei*, *Cand. tropicalis*, *Cand. parapsilosis*, and *Cand. glabrata*). Extracts of six plants (*Chry. mexicana*, *C. coeruleus*, *Colubrina greggii*, *Cordia boissieri*, *Cyperus alternifolius*, and *Schinus molle*) were active against *Cand. glabrata* with MIC values between 31.25 and 125 μg/mL. All plants tested, except *Chry. mexicana*, showed activity against *Can. albicans*, *Can. krusei*, *Can. tropicalis*, and *Can. parapsilosis*.

Marín et al. (2008) evaluated the effects of dichloromethane extracts obtained from the roots, stems, and flowers of different species belonging to the genus *Pittocaulon* (Asteraceae), namely *Pi. praecox* (Cav.), *Pi. hintonii*, *Pi. filare*, *Pi. velatum*, and *Pi. bombycophole*, against *Asp. niger*, *Fu. moniliforme*, *Fu. sporotrichum*, *Rhi. solani*, and *Tr. mentagrophytes*. The dichloromethane extracts from *Pi. praecox*, *Pi. bombycophole*, and *Pi. hintonii* exhibited antifungal activity against the fungal strains *Fu. sporotrichum*, *Rhi. solani*, and *Tr. mentagrophytes*: at 1,000 μg per disk, these extracts completely inhibited the mycelia growth of these fungi.

7.7 Active Isolated Compounds

Seventy four antimicrobial compounds were isolated and purified from 29 plants. From them, 54 displayed MICs ≤ 25 μg/mL against bacteria and 22, with MICs ≤ 200 μg/mL, were active against fungi.

From *Cal. brasiliense* Cambess (Clusiaceae), nine active compounds have been isolated. Those highly active against Gram-positive bacteria were: 1,3,5,6-tetrahydroxy-2-(3,3-dimethylallyl) xanthone (**1**), jacareubin (**2**), and Mammea A/BA (**3**), which were active against one sensitive and two methicillin-resistant *Sta. aureus* strains with MICs between 1 and 4 μg/mL (Yasunaka et al. 2005); brasiliensophyllic acid A (**4**), brasiliensophyllic acid B (**5**), isobrasiliensophyllic acid A (**6**), and isobrasiliensophyllic acid B (**7**), which showed activity against *Bac. cereus* with MICs between 1 and 4 μg/mL (Cottiglia et al. 2004); and brasiliensophyllic acid C (**8**) and isobrasiliensophyllic acid C (**9**), which displayed activity against *Bac. cereus*, but with a higher MIC (Cottiglia et al. 2004).



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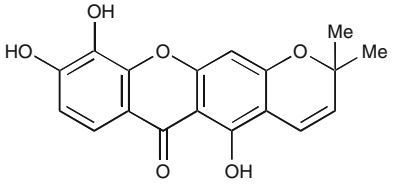

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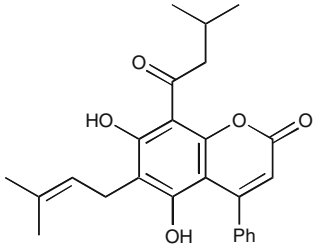

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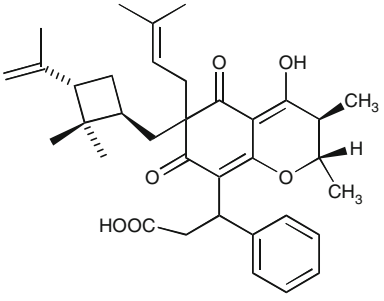

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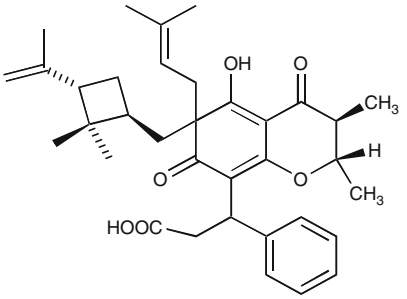

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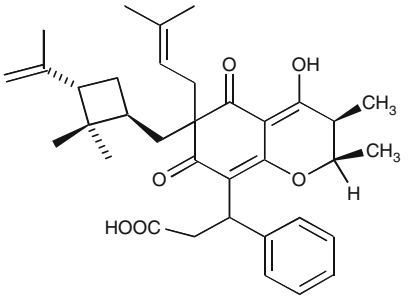

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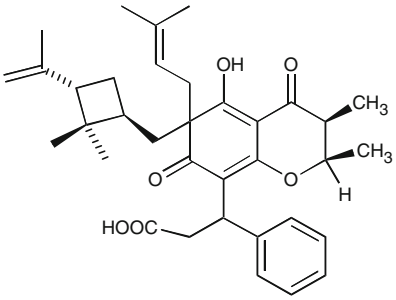

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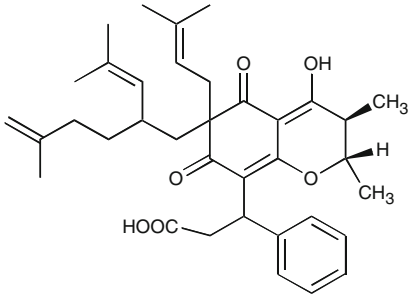

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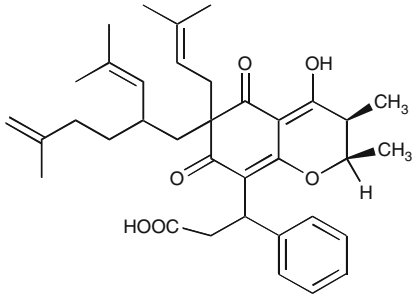
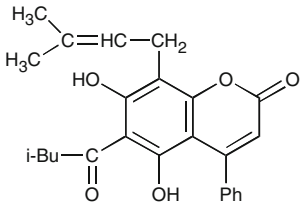


9

Furthermore, Mammea A/AA (**10**) purified from *Mammea Americana* displayed a MIC of 8 μg/mL against sensitive and resistant *Sta. aureus* strains; 16α-hydroxymollic acid (**11**) isolated from *Acalypha communis* displayed activity against *Ent. faecium* (MIC 8 μg/mL), and 7β,16β-dihydroxy-1,23-dideoxyjessic acid (**12**) isolated from the same plant showed activity against *Ent. faecium* (MIC 8 μg/mL), *Bac. cereus* (MIC 16 μg/mL), and *Sta. aureus* (MIC 16 μg/mL) (Gutierrez et al. 2002). (±)-6-Methoxydihydrochelerythrine (**13**) obtained from *Bocconia arborea* was active against *Ent. faecium* and *Sta. aureus* (MIC 25 μg/mL) (Julian and Delgado 2001). Cacalol (**14**) isolated from *P. radulifolium* and peroxisomicine A1 (**15**) from *Karwinskia parvifolia* displayed activity against *Sta. aureus* with MICs of 12 μg/mL (Garduno et al. 2001) and 16 μg/mL (Salazar et al. 2006), respectively.

An investigation of the chemical constituents from the roots of *P. radulifolium* (Garduno et al. 2001), a member of the matarique complex, which includes several members of the Asteraceae, resulted in the isolation of four new modified eremophilanes and seven related compounds. Most of the isolated compounds were tested for their antimicrobial activities against *Can. albicans*. Cacalol (**14**) was the major active compound (MIC = 0.012 mg/mL). Toxicity was determined with the *Artemia salina* test and cacalol displayed a LD_{50} of 12 ppm.



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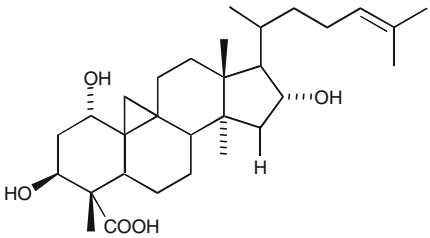

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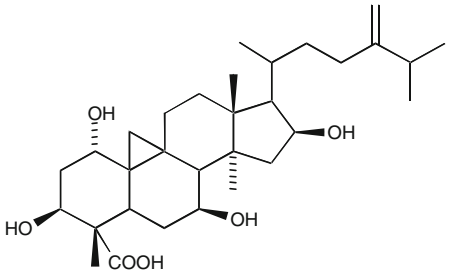

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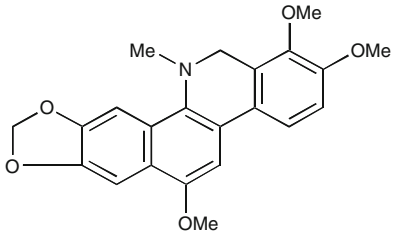

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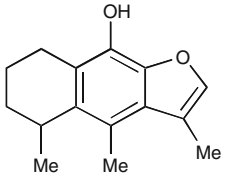

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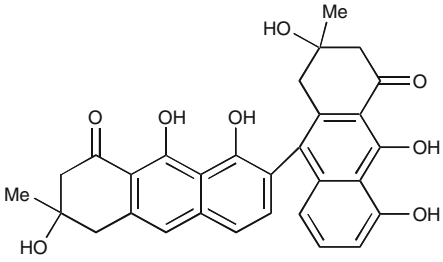
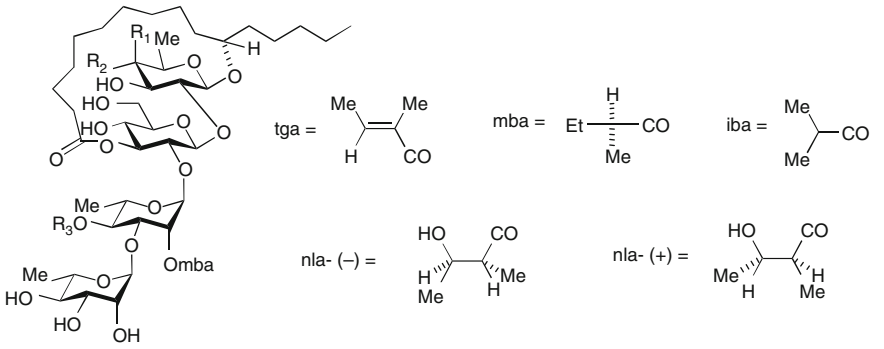


15

Five compounds named tricolorin A–E (**16**–**20**) and scammonin I (**21**), isolated from *Ipomoea tricolor*, were active against a *Sta. aureus* sensitive strain with MICs between 16 and 32 μg/mL (Pereda et al. 2006). Tricolorin A and E were the most active, with MICs between 4 and 8 μg/mL. Eight compounds isolated from *I. orizabensis* also showed activity against the same strains; orizabin XIII, XVI, XVII and XXI (**22**, **23**, **24** and **25**) were the most active against the sensitive strain with a MIC of 8 μg/mL, while orizabin X, XI, and XIV (**26**, **27**, and **28**) displayed a MIC of

16 μg/mL and orizabin XVIII (**29**) 32 μg/mL. Orizabin XX (**30**) and others did not show activity. Interestingly, all the compounds were active against a resistant strain of *Sta. aureus*, the MICs being in the range 4–16 μg/mL, except orizabin XVIII (**29**) with a MIC of 64 μg/mL.

On the other hand, the anacardic acids mixture (**31**) obtained from *Amphipterygium adstringens* presented excellent activity against *He. Pylori* (Castillo et al. 2007); cacalol (**14**) from *P. radulifolium* was the only compound reported active against two Gram negative bacteria: *E. coli* and *Proteus micracanthus*. In addition, 7,8-dihydroxy-6-methoxycoumarin (**32**) was the most active compound (25 μg/mL) against *V. cholerae* (CDC-V12), obtained from a bioassay fractionation (Céspedes et al. 2006b).

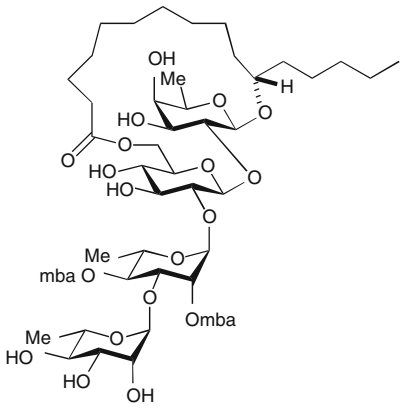


16 Tricolorin A: R_1= OH, R_2= H, R_3= mba

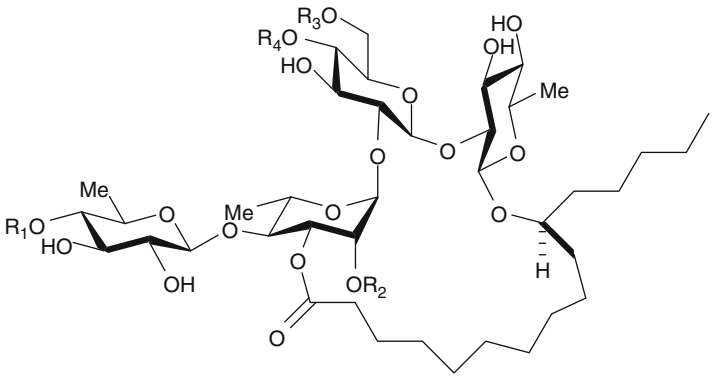
17 Tricolorin B: R_1= OH, R_2= H, R_3= iba

18 Tricolorin C: R_1= OH, R_2= H, R_3= nla-(–)

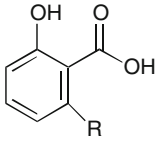
20 Tricolorin E: R_1= OH, R_2= OH, R_3= mba



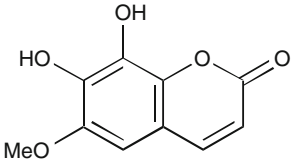
19 Tricolorin D



	R1	R2	R3	R4
21 ScammoninI	tga	mba	H	H
22 OrizabinXIII	tga	iba	nla-(+)	H
23 OrizabinXVI	tga	mba	nla-(-)	H
24 OrizabinXVII	tga	mba	nla-(+)	H
25 OrizabinXXI	mba	mba	nla-(+)	H
26 OrizabinX	tga	nla-(-)	iba	H
27 OrizabinXI	tga	nla-(+)	iba	H
28 OrizabinXIV	tga	nla-(-)	mba	H
29 OrizabinXVIII	mba	nla-(-)	mba	H
30 OrizabinXX	mba	mba	nla-(-)	H



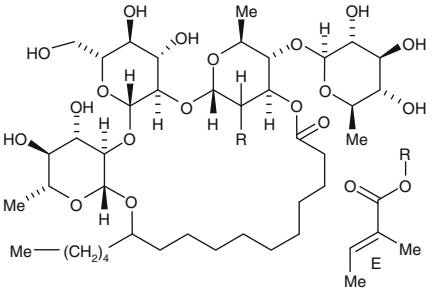
R= $C_{16;0}$, $C_{17;0}$, $C_{19;0}$



31 **32**

Six compounds were reported as active against a sensitive *M. tuberculosis* strain with a MIC of 25 μg/mL: tyrianthin 8 (**33**), tyrianthin 9 (**34**), tyrianthinic acid I (**35**), and tyrianthinic acid II (**36**), obtained from *Ipomoea tyrianthina* (León et al. 2008), and escobarine A (**37**) isolated from *Calliandra californica* (Encarnación et al. 2006) and oleanolic acid (**38**) purified from *L. hispida* (Jimenez et al. 2007). None of these compounds was active against the resistant strains tested, except escobarine A, which was active against a *M. tuberculosis* strain resistant to streptomycin, isoniazid, rifampicin, ethambutol, and pyrazinamide with a MIC of 12.5 μg/mL (Encarnación et al. 2006). From *L. hispida*, another compound, 3-acetoxy-22-(2′-methyl-2Z-butenoyloxy)-12-oleanen-28-oic acid (**39**), was isolated, which was active only

against the resistant strain to streptomycin, and isoniazid (MIC 25 μg/mL; Jimenez et al. 2007). Rivero et al. in (2005) found that six compounds previously isolated from Mexican plants, tricolorins A (**16**), B (**17**), D (**19**) and E (**20**) and two terpenes (**40** and **41**), displayed MICs of 16 μg/mL against a sensitive *M. tuberculosis* strain.



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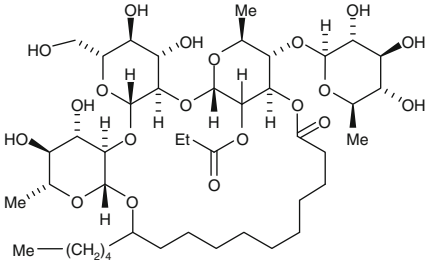

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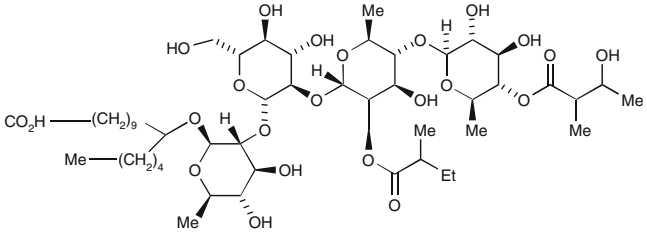

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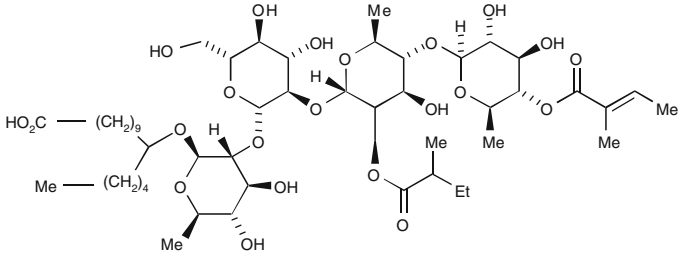

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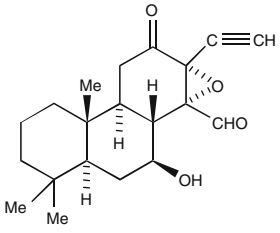

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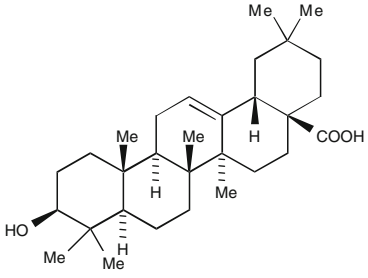

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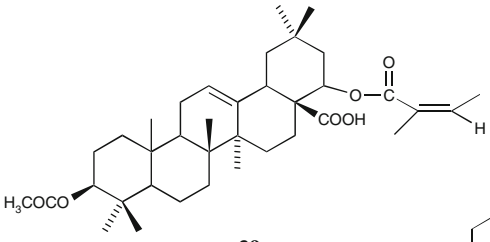

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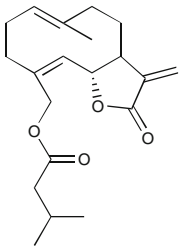

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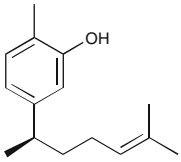
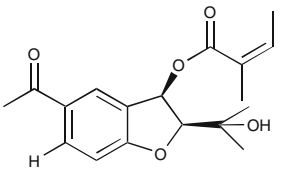


41

From the aerial part of the medicinal plant *A. pichinensis*, collected in Huitzilac Morelos (Rios et al. 2003a), two new benzofuran compounds, 5-acetyl-3β-angeloyloxy-2β-(1-hydroxyisopropyl)-2,3-dihydrobenzofuran (**42**) and 5-acetyl-3β-angeloyloxy-2β-(1-hydroxyisopropyl)-6-methoxy-2,3-dihydrobenzofuran (**43**), in addition to 4-hydroxy-3,5-diprenylacetophenone, espeletone (**44**), encecalinol (**45**), β-sitosterol, and stigmasterol, were isolated through bioassay-guided fractionation. Compound **42** showed a MIC of 200 μg/mL against *Tr. mentagrophytes* and 100 μg/mL against *Tr. rubrum*, compound **43** a MIC of 50 μg/mL against *Tr. mentagrophytes* and *Tr. rubrum*, and **44** a MIC of 100 μg/mL against *Tr. mentagrophytes* and *Tr. rubrum*, while **45** was active against all microorganisms assayed [MIC = 12.5 μg/mL (*Tr. mentagrophytes*), 12.5 μg/mL (*Tr. rubrum*), 100 μg/mL (*Can. albicans*), 200 μg/mL (*Asp. niger*)]. In further studies conducted by these investigators (Aguilar et al. 2009), 11 compounds were isolated through a bio-guided chemical analysis of the *n*-hexane extract obtained from the aerial parts of *A. pichinensis* collected in Hueyapan, Morelos. Six of these compounds had previously been isolated from the same plant collected in Huitzilac. Encecalin (**46**), taraxerol (**47**), (+)-β-eudesmol (**48**), and (+)-β-eudesmol epoxide (**49**) displayed antifungal activity against the most important dermatophytes responsible for tinea pedis infection, *Tr. rubrum* and *Tr. mentagrophytes*, and against *Cand. albicans* and *Asp. niger* (with MIC values between 6.2 and 200 μg/mL). It is important to note that the plant collected in Hueyapan, Morelos, showed effectiveness and tolerance on patients with tinea pedis in an explorative pilot investigation (Romero et al. 2006).



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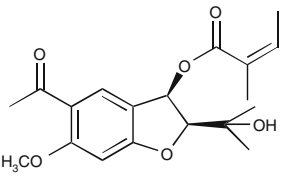

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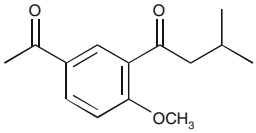

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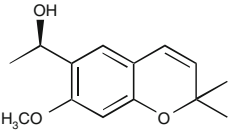

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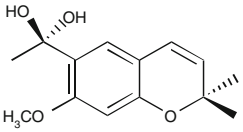

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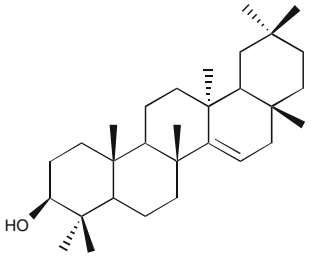

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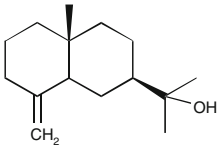

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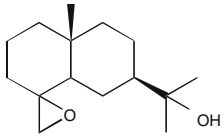
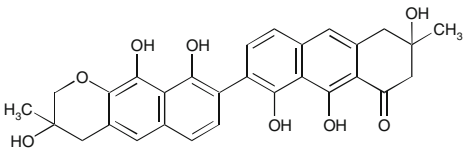


49

Salazar et al. in (2006) reported the *in vitro* activity of some hydroxyanthracenones isolated from the genus *Karwinskia* against six filamentous fungi and four yeasts. These hydroxyanthracenones (peroxisomicine A1 (**15**), isoperoxisomicine A1 (**50**), T496 (**51**), and tullidinol isomers mixture (**52**)) were found to possess antimicrobial activity, particularly against *Cand. albicans*, *Cand. boidinii*, *Cand. glabrata*, *Asp. fumigatus*, *Asp. niger*, and *Cryptococcus neoformans*, with minimal inhibitory concentrations ranging between 2 and 32 μg/mL.



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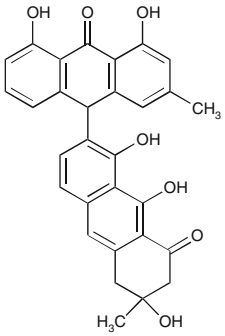

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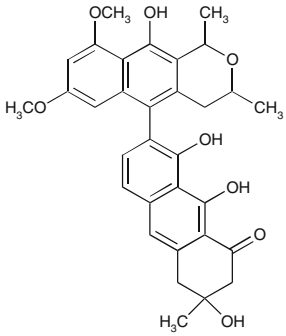
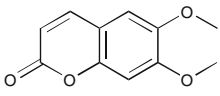


52

Mexican tarragon (*Tagetes lucida*) is an important, nutritious plant, and an effective herbal medicine. Céspedes et al. in (2006b) evaluated the antibacterial and antifungal activities of the methanolic/dichloromethane and dichloromethane extracts, coumarins, and flavonoids present in this plant. Scoparone (6,7-dimethoxycoumarin) (**53**) and 6,7-dimethoxy-4-methylcoumarin (**54**) obtained from methanolic/dichloromethane fraction, were active against *Asp. niger*, *Fu. moniliforme*, *Fu. sporotrichum*, *Rhi. solani*, and *Tr. mentagrophytes*. The Medium Fungicide Concentration (FC_{50}) values (62.5–125 μg/mL) were five and ten times higher than those observed with the positive control ketoconazole.



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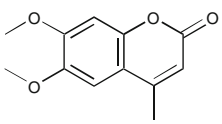
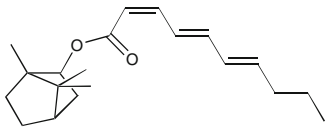


54

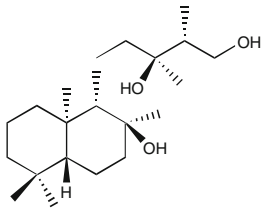
Heliopsis longipes root extract is the source of the alkamide, bornyl-*O*-deca-2E,6*Z*,8*E*-trienate (bornyl decatrienate) (**55**). This substance exerts an inhibitory effect on fungal growth (Morales et al. 2007). Furthermore, the inhibitory effect of **55** against pathogenic fungi was detected *in situ*; minimum lethal concentration (MLC) was 78 μg/mL. *Tr. rubrum*, the pathogenic agent of tinea, was unable to

cause disease in its host (Wistar rat) when the alkamide treatment was present. Interestingly, fungi growth did not occur after the bornyl decatrienate was removed from the growth medium, the rats, and the bean plants. These results strongly suggest the biocidal effect of this compound against these pathogenic fungi.



55

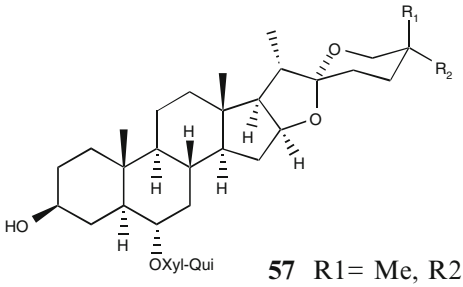
Serrano et al. in (2009) isolated and identified the antifungal compounds present in a hexanic extract of the aerial parts of *G. glutinosum*. Four of the fungi tested (*Asp. niger*, *Can. albicans*, *Fu. sporotrichum*, and *Tri. Mentagrophytes*) are of clinical importance, and two of them (*Fu. moniliforme* and *Rhi. Solani*) are of agricultural importance. The bioassay-guided purification of the methanolic partition (from hexanic extract) resulted in the isolation and identification of three antifungal compounds, an ent-labdane-type diterpene and two methoxylated flavones as the metabolites responsible for activity. The compound (+)-8*S*,13*S*,14*R*, 15-ent-labdanetetrol (**56**) was active against the five molds tested: *Rhi. solani* and *Tr. mentagrophytes* with an IC_{100} of 0.230 and 0.240 mg/mL (in both cases, respectively), and an IC_{50} of 0.100 and 0.130 mg/mL, respectively.



56

Zamilpa et al. (2002), using bioactivity-directed isolation procedures, isolated five new spirostan saponins and two sterol glycosides from *Solanum chrysotrichum* leaves. The most active compound was 6α-*O*-β-D-xylopyranosyl-(1→3)-β-D-quinovopyranosyl-25*R*-5α-spirostan-3β,23α-ol, named SC-2 (**57**) with MICs of 12.5, 12.5, 100, and 200 μg/mL against *Tri. mentagrophytes*, *Tri. rubrum*, *Asp. niger*, and *Cand. albicans*, respectively. Later, they reported the isolation of four active spirostanol saponins from *Solanum hispidum* , including an epimer of compound **57**(González et al. 2004). This epimer (**58**) is less active than compound **57**, being the only difference between both compounds the orientation of the methyl group at C-25. Furthermore, Herrera et al. in (2007), reported the biological activity of **57** against 12 *Candida* spp. of clinical significance (five strains of

Can. albicans; two strains each of *Cand. glabrata* and *Cand. parapsilosis*, and one strain each of *Cand. krusei*, *Cand. lusitaniae*, and *Cand. tropicalis*), including some fluconazole (Fluco)- and ketoconazole (Keto)-resistant clinical isolates. They found that the saponin SC-2 possesses fungicide and fungistatic activity on different *Cand. albicans* and non-*albicans* species (including some azole-resistant strains) with IC_{50} values of 200 μg/mL (in Fluco-susceptible strains) and of 400–800 μg/mL (in Fluco-resistant strains).

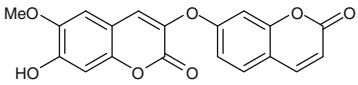


57 R1= Me, R2= H

58 R1= H, R2= Me

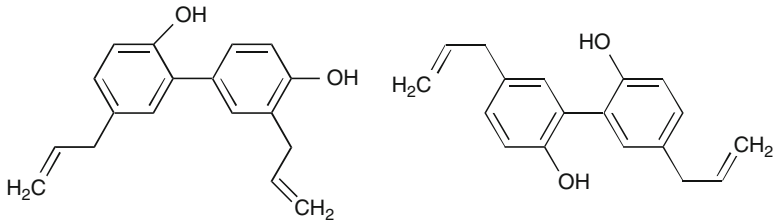
Continuing with these investigations, the authors conducted an exploratory study on the clinical and mycological effectiveness of an herbal medicinal product from *Solanum chrysotrichum* in patients with *Candida* yeast-associated vaginal infection (Herrera et al. 2009). The aim of this study was to compare the clinical (elimination of signs and symptoms) and mycological effectiveness (negative mycological studies) of a *Solanum chrysotrichum* herbal medicinal product (Sc-hmp), standardized at 1.89 mg of **57**, against ketoconazole (400 mg) in the topical treatment of cervical or vaginal infection by *Candida*. Both treatments (vaginal suppositories) were administered daily on seven consecutive nights. The study included 101 women (49 in the experimental group) with a confirmed clinical condition and positive mycological studies (direct examination and/or culture) of *Candida* infection. At the end of the administration period, both treatments demonstrated 100 % tolerability, with clinical effectiveness in 57.14 % of *S. chrysotrichum*-treated cases and in 72.5 % of ketoconazole-treated cases. The investigators concluded that, at the doses used, Sc-hmp exhibited the same clinical effectiveness as ketoconazole, but with lower percentages of mycological eradication. They believe that additional clinical studies with Sc-hmp are necessary, with increasing doses of **57**, for improving the clinical and mycological effectiveness.

Three active coumarins: daphnoretin (**59**), umbelliferone (**60**) and scopoletin (**61**) were isolated from the aerial part of *Loeselia mexicana* Brand (Navarro et al. 2011b). These compounds showed a significant antifungal activity against *Tri. mentagrophytes*, *Tri. rubrum*, *Asp. niger*, and *Cand. albicans*, showing MIC values between 12.5 and 100 μg/mL.



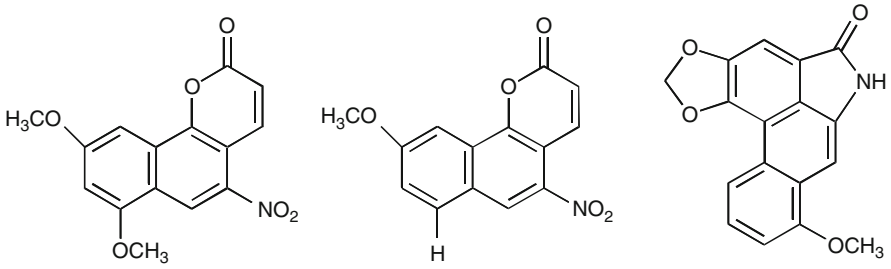
59 **60** **61**

Jacobo et al. in (2011), obtained from *Magnolia delabata* (Magnoliaceae) honokiol (**62**) and magnolol (**63**), which were active against resistant mo, including the phytopathogen bacteria *Clavibacter michiganensis* subsp. Michiganensis. Magnolol (**63**) displayed effect against *Ent. faecalis*, *Cand. albicans* and *Cand. tropicalis* at 0.4 μg/disk, and against *Acinetobacter lwoffii* at 40 μg/disk. Honokiol (**62**) was strongly active (≥12 mm de inhibition zone) against *Pseud. aeruginosa*, *Acin. baumannii*, *Acin. lwoffii*, *Cand. albicans* and *Clav. michiganensis*. The inhibition zone was greater than 12 ± 2 mm when the mixture of honokiol and magnolol was tested (0.4 μg/disk of each compound), suggesting an additive effect between the two substances.



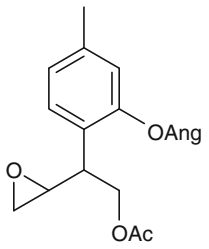
62 **63**

Eight compounds from *Aristolochia brevipes* were isolated by Navarro et al. in (2011a). Three of these compounds were active against *M. tuberculosis*: 7,9-dimethoxytariacuripyrone (**64**), 9-methoxyitariacuripyrone (**65**) and aristololactam I (**66**). Substances **64** and **65** showed activity against both sensible, monoresistant and multidrugresistant strains, with MICs ranging between 25 and 50 μg/mL. The alkaloid **66** was the most active with MICs ranging between 12.5 and 25 μg/mL against the different strains.



64 **65** **66**

The antimicrobial activities of compounds isolated from the essential oil of *Hofmeisteria schaffneri* were evaluated. 8,9-epoxy-10-acetoxythymyl angelate (**67**) and thymylisovalerate (**68**), showed the best effect against *Sta. aureus*, *Bac. subtilis* (MIC 32 μg/mL, respectively). Compounds 2′,3′-dihydroxy-2′-thymyl angelate (**69**) and thymol (**70**) showed stronger activity against yeasts (MIC = 128 μg/mL) than the other compounds (Pérez et al. 2011).



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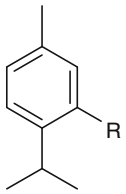
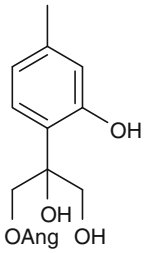


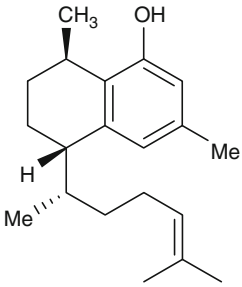
68 R = OIsoval
70 R = OH



69

Leubethanol (**71**) was isolated from methanolic root bark extract of *Leucophyllun frutescens* (Molina et al. 2011b). This new serrulatane displayed enhanced activity against a multidrug resistant strain (clinical isolate, MIC 6.3 μg/mL) compared to the sensible strain of *Mycobacterium tuberculosis* (H37Rv MIC 12.5 μg/mL).

Liriodenine (**72**), an alkaloid isolated from *Annona diversifolia* seeds, inhibited the growing of the phytopathogen fungi *Rhizopus stolonifer* and *Aspergillus glaucus*. MICs found were 200 and 100 μM respectively (De la Cruz et al. 2011).



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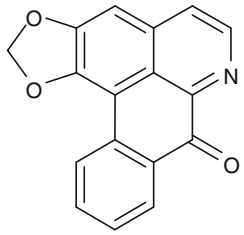
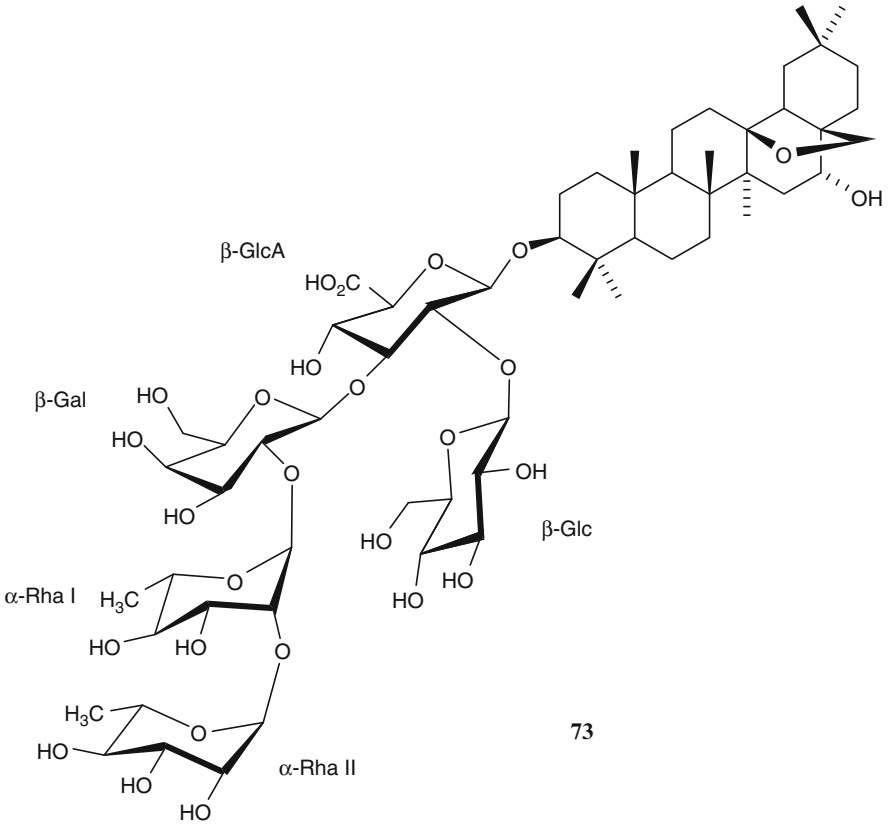


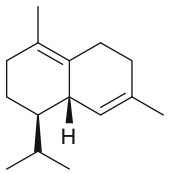
72

Sakurasosaponin (**73**) was isolated from a methanolic extract obtained from roots of *Jacquinia flammea* (García et al. 2011). The compound displayed moderate activity against dermatophytes with MICs ranging between 31.25 and 250 μg/mL; **73** also resulted high active against *Colletotrichum gloeosporioides*, a phytopagen fungus, the casual agent of antracnosis in several fruits, which is associated with postharvesting infections.



73

Pérez et al. in 2011 isolated δ-cadinene (**74**) from the essential oil obtained from the fruit of *Schinus molle*. The compound was active against two resistant *Str*. *pneumoniae* strains with a MIC value of 31.25 μg/mL.



74

7.8 Conclusion

Here, we review the literature on Mexican plants and their products as antifungal and antibacterial agents; the results strongly support the contention that traditional medicine remains a valuable resource in the potential discovery of natural product

pharmaceuticals. It has been noted that because of the emergence of drug-resistant strains of many infectious microorganisms, ethnobotany may provide new effective pharmaceutical alternatives to existing drugs.

The results obtained in recent years warrant the present review, which discusses the use of several medicinal plants against bacteria, yeast, filamentous fungi. In addition, this review aims to emphasize that it is of great importance to investigate plant species that have not been the subject of pharmacological studies, even though their popular uses have been reported.

It is important to note that the methanolic extracts resulted in the most active extracts against Gram-positive and Gram-negative bacteria. In general, the aqueous extracts did not show greater activity than previously reported, except, surprisingly, for Gram-negative bacteria. *M. tuberculosis* was more sensitive to nonpolar extracts obtained with hexane and chloroform.

Although tests may be run with collection strains, further assays with isolated pathogens would be of interest in the case of active extracts or compounds.

Finally, this review makes a contribution as a resource for future development of research into the antimicrobial activity of medicinal plants growing in Mexico.

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Chapter 8
Pharmacognosy, Phytochemistry Pharmacological and Biotechnogical approaches of *Catharanthus roseus* (L.) G. Don

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8.1 Introduction

Catharanthus roseus is well known medicinal plant, belonging to the family Apocynaceae; and is a rich source of alkaloids, which are distributed in all parts of the plant. The alkaloid content of *C. roseus* varies considerably in various parts; the maximum being in the root bark which ranges from 0.15 to 1.34 % and even up to 1.7948 in some strains (Singh and Jagdev 1996). The plant contains about 130 alkaloids of the indole group; out of which 25 are dimeric in nature. Two of the dimeric alkaloids vinblastine and vincristine mainly present in the aerial parts, have found extensive application in the treatment of human neoplasma. Among the monomeric alkaloids Ajmalicine (raubacine) found in the roots has been found to have a broad application in the treatment of circulatory diseases, especially in the relief of obstruction of normal cerebral blood flow. In combination of the *Rauvolfia's* alkaloids it has been used to lower high blood pressure.

Vinblastine sulphate (sold as Velban) is used particularly to treat Hodgkin's disease besides lymphocarcoma, choriocarcinoma, neuroblastoma, carcinoma of the breast, lungs and other organs in acute and chronic leukemia. Vincristine sulphate (sold as Oncovin) arrest mitosis in metaphase and is very effective for treating acute leukaemia in children and lymphocytic leukaemia. It is also used against Hodgkin's disease, Wilkins's tumor, neuroblastoma and reticulum cell sarcoma. Today India is the third largest manufacture of Vinblastine and Vincristine in the world and is exporting these alkaloids to European countries. High demand and low yield of these

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alkaloids in the plant has led to research for alternative means for their production. Nowadays vinblastine is converted into vincristine either chemically or via microbiological N-demethylation using *Streptomyces albogriseolus.* Vinblastine is also modified structurally to yield deacetyl vinblastine amide (Vindesine) introduced recently as Eldisine for use in the treatment of acute lymphoid leukemia in children. Biochemical coupling of alkaloids Catharanthine and Vindoline to get dimeric compounds is also achieved. Beside these, tissue culture technique is developed for the development of these dimeric alkaloids (Shah and Chauhan 1996). In the present communication a detailed application of *C. roseus* including traditional uses in various developed and developing countries, pharmacological activities and the application of various biotechnological tools viz. Optimization of Media Composition, Phytohormones, pH, Temperature, Light, Aeration, Elicitors, Mutagenesis, High Cell Density Culture, Selection of Superior cell lines, Bioreactors and Immobilization Methods, Hairy root culture, *In Vitro* Somatic embryogenesis, Biosynthesis of alkaloids in *Catharanthus*, Metabolic and Genetic Engineering in alkaloids biosynthesis, Coupling method for Alkaloids biosynthesis, Cellular Compartmentation has been applied for the enhancement of important secondary metabolites.

8.2 Taxonomy, Habit and Habitat of *Catharanthus roseus*

Catharanthus roseus is an important medicinal plant, belonging to the family Apocynaceae, commonly known as Madagascar periwinkle. It is a perennial, evergreen herb, 30–100 cm height that was originally native to the island of Madagascar. It has been widely cultivated for hundred of years and can now be found growing wild in most warm regions of the world. The leaves are glossy, dark green (1–2 in. long), oblong – elliptic, acute, rounded apex; flowers fragrant, white to pinkish purple in terminal or axillary cymose clusters; follicle hairy, many seeded, 2–3 cm long; seeds oblong, minute, black. The plant is commonly grown in gardens for beddings, borders and for mass effect. It blooms throughout the year and is propagated by seeds or cuttings. The bloom of natural wild plants are pale pink with a purple eye in the centre, but horticulturist has developed varieties (more than 100) with colour ranging from white to pink to purple.

8.3 Traditional Uses and Biological Activities of *C. roseus*

The plant has been used traditionally for the treatment of various diseases. It was used as folk remedy for diabetes in Europe for centuries (Swanston et al. 1989). In India, juice from the leaves was used to treat wasp stings. In Hawaii, the plant was boiled to make a poultice to stop bleeding. In china, it was used as an astringent, diuretic and cough remedy (Farnsworth 1961).

In central and south America, it was used as a homemade cold remedy to ease lung congestion and inflammation. Throughout the Caribbean, an extract from the

flowers was used to make a solution to treat eye irritation and infections. It also had a reputation as a magic plant, European thought it could ward off evil spirits, and the French referred to it as "violet of the sorcerers." Western researchers finally noticed the plant in 1950s when they learn of a tea Jamaican was drinking to treat diabetes. They discovered that the plant contains a motherlode of useful alkaloids (130 in all at last count). Some, such as catharanthine, leurosine sulphate, lochnerine, tetrahydroalstonine, vindoline and vindolinine lower blood sugar level, however, others act as haemostatics (arrest bleeding) and two others, vincristine and vinblastine have anticancerous properties. Periwinkle also contains the alkaloids reserpine and serpentine, which are powerful tranquilizers.

8.3.1 Antitumor Activity

Ethanol (70 %) extract of leaves was administered intraperitoneally to female mice (El-Merzabani et al. 1979), and prove to be highly active on CA-Ehrlich ascites. Alkaloid fraction of dried leaves was also used externally was actives. Nineteen patients with either flat, verruca vulgaris, plantar or genital warts were treated in this study. Six patients had all warts disappears, seven had the majority of their warts disappears, five had 50 % disappears and one showed no response (Chattopadhyay and Das 1990). Chloroform extract of leaves was active on Leuk-P388 (Cordell et al. 1974). Total alkaloids of the entire plant administered to mice intraperitoneally at a dose of 10.0 mg/kg and orally at a 75.0 mg/kg were active on Leuk-P1534 (Johnson et al. 1960).

8.3.2 Antihypertensive Activity

Total alkaloids of root administered intravenously to dogs at a dose 4.0 mg/kg were active (Chopra et al. 1959).

8.3.3 Antimutagenic Effect

Hot water extract of dried leaves was active on red blood cells. A reduction in number of micro-nucleated polychromatic red blood cells caused by various mutagens (Sharma et al. 1982).

8.3.4 Antifertility Activity

Methanol/water (1:1) extract of dried leaf and stem administered orally to male rats was active (Anon 1979).

8.3.5 Antispermatogenic Effect

Hot water extract of dried leaves administered intraperitoneally to male mice at a dose of 0.2 ml/animal produced weak activity (Murugavel et al. 1989). At 10.0 mg/animal, regressive changes in seminiferous tubules and Leyding cells, increased cholesterol in testes and degeneration of all germinal elements (Murugavel and Akbarsha 1991). Total alkaloid of entire plants administered intraperitoneally to male rats was active (Joshi and Ambaye 1968).

8.3.6 Antifungal Activity

Acetone and water extracts of dried aerial parts at a concentration (50 %) on agar plate was inactive on *Neurospora crossa* (Kubas 1972). Hot water extract of dried leaves in broth culture was active on Trichophyton mentagrophytes (Rai and Upadhyay 1988). Hot water extract of dried stem in broth culture was active on *T. mentagrophytes* and weakly active on *T. rubrum* (Chile et al. 1981). Leaves and roots on agar plate were active on *Pythium aphanidermatum* (Kulkarni and Ravindra 1988).

8.3.7 Antibacterial Activity

Benzene extract of dried flowers at a concentration of 50 % on agar plate was active on *Proteus*, *Pseudomonas*, *Shigella* and *Staphylococcus* species, however, benzene extract of leaves at a concentration of 50 % on agar plate was active on *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella* and *Staphylococcus* species (Rajas and Cuellar 1981). Ethanol (70 %) extract of dried leaves on agar plate was active on *Bacillus megaterium* and *Staphylococcus albus* and inactive on *Bacillus cereus* and *Staphylococcus aureus* (Ross et al. 1980). Total alkaloids of root at a concentration of 500 mcg/ml in broth culture were inactive on *E. coli*, *Salmonella lyphosa* and *Shigella dysenteriae* (Chopra et al. 1959). Water extract of entire plant on agar plate at a concentration of 1:4 was inactive on *Salmonella paratyphi* (Neagi and Bhatia 1956).

8.3.8 Anti-inflammatory Activity

Ethanol extract (95 %) of dried leaves was administered intraperitoneally to rats at a dose of 4000.0 mg/kg was active 65 % inhibition was noticed in Edema (Chattopadhyay et al. 1992).

8.3.9 Anti-hypercholesterollemic Activity

Hot water extract of dried leaves were taken orally to rabbits was active (Asthana and Misra 1979).

8.3.10 Antidiuretic Activity

Alkaloid fraction of the entire plant was given subcutaneously to male rats at a dose of 50.0 mg/kg was active (Neagi and Bhatia 1956).

8.3.11 Antimalarial Activity

Chloroform extract of root at a dose 400.0 mg/kg along with water extract at a dose 4.42 g/kg was administered orally to chicken produced weak activity on *Plasmodium gallinaceum* (Spencer et al. 1949).

8.3.12 Antihyperglycemic Activity

Dried leaves in the ration of male in mice at a concentration of 6.25 % of the diet for 28 days was inactive vs streptozotocin – induced hyperglycemia (M27518). Hot water extract of dried aerial parts administered intragastrically to dogs at a dose of 50.0 g/kg (dry weight of plant) was inactive, a dose of 10.0 g/kg in case of rabbits was active vs alloxan induced hyperglycemia (Swanston et al. 1989). Water extract of fresh cells administered intragastically to male rats was active vs streptozotocin induced hyperglycemia, ultimately 60 % decreased in blood sugar was observed (Benjamin et al. 1994).

8.3.13 Antimitotic Activity

Ethanol (70 %) extract of leaves was administered to female mice was active on CA-Fhrlich ascites vs induction of metaphase arrest in ascites cells. Dosing was applied 4 days after tumor cell inoculation ascetic samples removed 2, 4, 6 and 24 h post treatment (El-Merzabani et al. 1979).

8.3.14 Antiviral Activity

Water extract of callus tissue in cell culture was active on Tobacco Mosaic Virus (Misawa 1976).

8.3.15 Cardiotonic Activity

Ethanol (70 %) extract of leaf and stem administered intravenously to rats at a dose of 120.0 mg/kg was active (Chopra et al. 1959).

8.3.16 CNS Depressant Activity

Total activity of root was administered intraperitoneally to rats at a dose of 120.0 mg/kg was active (Chopra et al. 1959).

8.3.17 Cytotoxic Activity

Alkaloid fraction of dried leaves in cell culture was active on CA-9 KB,ED_{50} 0.0435 mcg/ml (Misawa 1976). Chloroform extract and culture filtrate of callus tissue in cell culture at dose of 50.0 g (dry wt of plant) were active on Leuk-L12 10 culture, water extract.

8.4 Role of Biotechnological Approaches in *Catharanthus* Micropropagation and Enhancement of Pharmaceutically Active Compounds Being Used in the Treatment of Various Diseases

Due to the pharmaceutical importance and the low content in the plant of vinblastine and vincristine *Catharanthus roseus* became an important model system for biotechnological studies on plant secondary metabolism. Researchers are focusing their attention to enhance the alkaloids yield by various ways (chemically, enzymatically, synthetically or by cell culture method). The plant cell can be cultured at large scale (Verpoorte et al. 1991) but the yield of alkaloids production is too low and limits commercial applications. In recent times, however, three strategies have been commonly used for the enhancement of alkaloids

- *In vitro* cultivation of shoot via organogenesis and somatic embryogenesis, callus or suspension by the optimization of media, phytohormones, temperature, pH, light, aeration etc. In addition, high cell density culture, elicitor's treatment, mutagenesis, bioreactors and immobilization are also practiced to improve alkaloids yield.
- Genetic engineering and over expression of biosynthetic rate limiting enzymes in alkaloid biosynthesis pathways.

8.4.1 In Vitro *Studies*

In tissue culture, the response of culture has been influenced by a number of factors which in turn regulate alkaloids yield. Some of them are discussed in brief.

8.4.1.1 Media Composition

The yield of alkaloids in suspension culture is directly influenced by the surrounding environmental conditions and genetic constitution of the concerned plant material. Over the years efforts have been made in numbers for optimization of culture media for better biomass and alkaloids production, some patents have also been filed (Van der Heijden et al. 1989; Ganapathi and Kagri 1990; Moreno et al. 1995; Mujib et al. 2002). Carbon sources and inorganic compounds play a significant role in indole alkaloid production. It was earlier reported that nitrogen and phosphate both promoted growth but had an adverse effect on alkaloids yield (Knobloch and Berlin 1980; Van Gulik et al. 1993). The inhibitory effect of nitrogen on alkaloid production has not always been observed (Drapeau et al. 1987). The effect of nitrogen on alkaloids production is dependent on carbon availability to the cells which makes the carbon-to-nitrogen ratio (C/N ratio) an important factor to be taken into account. By the determination of the cellular C/N ratio, Rho and Andre (1991) identified three distinct growth phases: an active growth phase, an accumulation phase, and a biomass decline phase (endogenous metabolism). They also noticed that phosphate (0.56 Mm), nitrate (12.97 Mm) and low concentration of ammonia were beneficial for maximum growth and increased alkaloids production. Similarly higher concentration of sucrose only enhanced biomass, the optimized glucose (500 Mm), ammonium and phosphate (0–12 Mm) were previously used for higher alkaloids yield.

Medium composition and its optimization had direct effect on induction and accumulation of indole alkaloids (Knobloch and Berlin 1980). A medium added with 6 % sucrose is favourable for both biomass and alkaloids production in *Catharanthus* (Scragg et al. 1990). Liquid medium with 3–6 % maltose was also found to be highly effective for production of somatic embryos (Junaid et al. 2006). It has been (Mujib et al. 1995) reported that agitated liquid media added with BAP (1.0 mg/l) was very productive for large-scale plant regeneration. Alteration in macro and micronutrient of MS medium (Murashige and Skoog 1962) has also been used to promote growth and subsequent alkaloid production (Smith et al. 1987b).

Similar result has also been in cell suspension culture (Schlatmann et al. 1994). Hairy root culture is a unique system, often used for root specific indole alkaloids production (Toivonen et al. 1989). Recently Batra et al. (2005) have observed an increase in growth and terpenoids indole alkaloids (ajmalicine and serpentine) yield when left and right termini-linked Ri T-DNA gene integration were made in hairy root cultures of *C. roseus*.

8.4.1.2 Phytohormones

The role of plant growth regulators in alkaloids production of *C. roseus* has been extensively studied, but the response varies with genetic make up of the used explant, type and quantity of phytohormones (Smith et al. 1987a; Ganapathi and Kagri 1990). The cytokinin applied exogenously either alone or in combination with auxins to suspension cultured cells enhanced alkaloids accumulation in tumorous and non-tumorous cell lines (Kodja et al. 1989; Decendit et al. 1992). Enzyme peroxidase play a significant role in alkaloids biosynthesis, addition of 2, 4-D to the culture medium however, reduced the peroxidase activity (Liman et al. 1998).

Hirata et al. (1990) have reported an increase in vindoline and catharanthine concentration by using 0.1 mg/l BAP and 0.1 mg/l NAA added MS medium. Exogenously supplied cytokinin increased ajmalicine and serpentine content in untransformed callus from cotyledons (Garnier et al. 1996). At the protein level it was shown that endogenously produced cytokinin did not mimic the effect of exogenously applied cytokinin in *Catharanthus* (Carpin et al. 1997a) and they also noticed that the protein pattern of Ipt transgenic callus lines were insensitive to exogenously used cytokinin. A28 KD polypeptide and simultaneous ajmalicine accumulation were noted on omission of 2, 4-D in medium and by the use of NaCl treatments (Carpin et al. 1997b; Ouelhazi et al. 1993).

8.4.1.3 pH of Culture Medium

In vitro biomass and alkaloid production are directly influenced by the pH values of the medium, values with a range of 5.5–6.5 did not have much effect on alkaloids yield. The value 5.5 was found to be optimum for serpentine production (Doller et al. 1976). Neumann et al. (1983) reported that alkaloids produced by suspension culture were stored in vacuole and simultaneously storage capacity changed as the changes of pH in the medium and vacuole take place. Low and higher values of pH were used to release intracellular alkaloids into the culture medium (Asada and Shuler 1989). It is quite known that the optimized value (5.5–5.8) occasionally fluctuates during culture time and influences *in vitro* responses including alkaloid yield.

8.4.1.4 Temperature

For *in vitro* study temperature range from 20 to 30 has been considered best for better biomass and growth of cultures, but contradictory informations have been reported about the alkaloids yield. Temperature in low range had inhibitory (Morris 1986) stimulatory (Courtosis and Guern 1980) and no effect on alkaloid yield. In the tested cell lines under different temperature range (20, 25, 30° C), highest serpentine production was recorded at 25° C (Scragg et al. 1988b) and, no effect was recorded at temperature 17, 23 and 32° C while in hairy root culture low temperature enhanced alkaloid yield (Toivonen et al. 1992).

8.4.1.5 Light

Light is an important factor for both *ex vitro* and *in vitro* morphogenetic study. Its presence, absence, time and intensity directly influence anabolic and catabolic processes, particularly secondary metabolism (Seibert and Kadkade 1980; Morris 1986). Most of the study of the effect of light was made on serpentine and ajmalicine where serpentine content was directly related to the intensity of light in *Catharanthus* (Lounasmaa and Galambos 1989), same was true for vindoline (De Luca et al. 1986) and however, another alkaloid catharanthine was decreased in the absence of light. Drapeau et al. (1987) reported that light did not affect yield but it affect the accumulation site. However, 15 h per day exposure instead of 24 improved serpentine accumulation. Although, dark-grown culture was much better in comparison to light grown, where serpentine and ajmalicine content were decreased (serpentine from 79 to 14 % and ajmalicine 78 to 18 %). Gradual transfer of dark grown culture of *Catharanthus* towards the light increased serpentine content, however, continuous exposure of light decreased serpentine level (Scragg et al. 1988b). Hirata et al. (1990) have optimized 12 h light period for better callus growth and alkaloid production and reported that dark period more than 12 h decreased alkaloid contents. Loyola-Vargas et al. (1992) found that an increased chloroplast number and enhanced chlorophyll accumulation in response to light influenced serpentine production. Besides, exposure of monochromic light such as blue (450 nm) or red (670 nm) did not effect growth and alkaloid accumulation, showed constant ajmalicine and serpentine synthesis which decreased further under white light (Hirata et al. 1990; Loyola-Vargas et al. 1992).

8.4.1.6 Aeration

Different types of gases, mainly CO_2 and ethylene, are usually evolved with in the culture. In many cases these gases reduce O_2 level in close vessels, inhibit plant culture growth and secondary metabolism. High dissolved oxygen and improved gaseous permeability at aerated condition stimulated secondary metabolism as observed by Schlatmann et al. (1994), when ajmalicine production was increased with high oxygen level. Improved oxidative metabolism at rich O_2 level is believed to be the reason for better product conversion. Aeration has been provided in culture to influence the alkaloids synthesis and to make it more efficient modern stirring devices have been employed along with traditional shake flask (Scragg et al. 1988a, b; Tom et al. 1991; Leckie et al. 1991a, b; Lee and Shuler 1991) Different types of fermenters have also been used; shikonin and ginseng, the two important secondary metabolites have been commercially produced by the use of fermenters. Paynee et al. (1988a, b) suggested the use of bioreactors in secondary metabolites production in plant cell culture of *Catharanthus*. An impeller with a speed of 100 rpm was most appropriate for the accumulation of alkaloids (Leckie et al. 1991b), however, higher impeller speed increased callus/suspension growth. The rate of ajmalicine production was studied by Hoopen et al. (1994) by using

different vessels including shake flask and bioreactors. He found that biomass was not affected by different culture vessels, however, ajmalicine production was decreased with over feeding of biomass in shake flask and fermentor.

8.4.1.7 Elicitors

New groups of triggering factors which are better known as elicitors, have been reported to stimulate the secondary metabolites (Eilert et al. 1986). The substance used as elicitors may be of biotic and abiotic in origin. Biotic elicitors include microbial filtrates (Yeast, *Pythium* and other fungal filtrate), while abiotic elicitors comprise of simple inorganic and organic molecules (vanadyl sulphate, oxalate, UV irradiation etc.).

It has been reported by Seitz et al. (1989) that addition of *Phytium aphanidermatum* filtrate increased the accumulation of phenolic compounds instead of alkaloids production. Effect of different concentrations of *Pythium vexans* extract was studied by Nef et al. (1991) who noticed that low elicitor concentration increased serpentine production but no effect was on catharanthine yield. Addition of nicotinamide (8.2 mm) in *C. roseus* cell lines was used to enhance the anthocyanin accumulation (Berglund et al. 1993). The extract of *Pythium aphanidermatum* in a hormone free cell lines responded well and induced enzymes {(TDC and anthranilate synthase (AS)} which catalyse the biosynthesis of several intermediates and subsequently accumulated tryptamine (Moreno et al. 1995). Several inorganic compounds (sodium chloride, potassium chloride and sorbitol) had also a positive effect on catharanthine accumulation (Smith et al. 1987a).

The addition of vanadyl sulphate (Tallevi and Dicosmo 1988) to cell suspension culture increased catharanthine, serpentine and tryptamine production but was concentration dependent. At 25 ppm, catharanthine and ajmalicine were primarily accumulated, and at 50–75 ppm tryptamine accumulation was only noticed. Moreover, the effect of heavy metal was studied by Backer-Royer et al. (1990) where addition of copper (200 μm) increased total indole alkaloid accumulation which was correlated with decreased tryptamine concentration.

Several stress factors (fungal elicitor, vanadyl sulphate and potassium chloride) were used and it was found that the alkaloids accumulation was concentration dependent (Kargi and Potts 1991). The optimal concentration (29,1.45 and 145 mg g-l dry weight) of fungal elicitor, vanadyl sulphate and potassium chloride into medium increased alkaloids accumulation, however, higher concentration had toxic effects and resulted in the loss of cell viability. Two fold increase in alkaloids yield was noticed added tryptophan, fungal elicitor and vanadyl sulphate to the culture production medium (Kargi and Ganapathi 1991).

Exposure of 2,2 azobis dehydrochloride (AAPH, an oxidative stress agent) and UVB irradiation to *C. roseus* increased nicotinamide and trigolline content (Berglund et al. 1996). Simultaneously phenylalanine ammonia lysate (PAL) activity was also increased. The increase in PAL activity caused by 2 μm AAPH was prevented by 0.1 mm 3-amino benzomide, which is an inhibitor of poly (ADP-ribose) polymerase.

This suggests that nicotinamide and its metabolites function as signal transmitter in response to the oxidative stress, since poly-polymerase has defensive metabolic functions. The level of vinblistine and leurosine increased in response to irradiation with near (370 nm) ultraviolet light (Hirata et al. 1991, 1992) in shoot culture of *C. roseus*, however, catharanthine and vindoline content were decreased. Leaves were more sensitive to dimeric alkaloid accumulation in comparison to shoot, however, Hirata et al. (1993) noticed that by near ultraviolet's irradiation in whole plant of *C. roseus*, accumulation of dimeric alkaloids was increased.

Yeast extract induces transcription of the biosynthetic gene encoding strictosidine (STR) in cultured *C. roseus* cells and alkalinization of the culture medium. The active principle from yeast extract was partially purified and found to be of a proteinacious in nature (Menke et al. 1999). Age of culture is very important factor for the elicitor's to be effective (Moreno et al. 1995; Ramos-Valdivia et al. 1997), addition of elicitors are preferred after a few days of inoculation of the culture when the cells are rapidly dividing.

8.4.1.8 Mutagenesis

Mutagenesis plays a potent role in the alteration of the genetic constitution which leads to produce new varieties. *Penicillium* is the most classic example, with many other successful cases. Process of mutagenesis in diploid plants is very complex. Mutagenesis enhance alkaloids yield but the route of biosynthesis and the necessary regulation procedure are not elucidated yet clearly. Therefore, mutation at target site in duplicate is really difficult. In spite of several limitations in this process, scientists in numbers have used mutagens. Berlin (1982), noticed some p-flurophynylalanine resistant cell lines of *Nicotiana tabacum* and *N. glauca* which accumulated higher level of phenolics. In case of *Catharanthus*, he noticed that a tryptophan analog resistant mutant accumulated catharanthine in both growth and production medium. Similarly several research groups used x-rays where more serpentine was produced. Beside these examples, some successful reports are available in other group of crops where mutagenesis improved metabolic accumulation.

8.4.1.9 High Cell Density Culture

In order to increase secondary metabolites production, high cell density culture feeding has been attempted with or without much success. Study on *Catharanthus* in relation to high cell density was found to be unsuccessful. Ajmalicine production was very low when inoculum potential was increased to 2:8 from 1:9 mg/g (Drapeau et al. 1987). Moreover, Moreno et al. (1993b) low-density culture increased alkaloids yields. It has also been remarked Schlatmann et al. (1994) that low oxygen level and inadequate nutrient uptake are among the possible causes for low metabolic accumulation during high cell density culture.

Selection of Superior Cell Lines

Isolation and selection of superior lines from the heterogeneous cell populations help to improve the yield of alkaloids. These cells show genetic variability which was further diversified by the use of various mutagenic agents. Ajmalicine and serpentine level were increased in *Catharanthus* by the selection of superior cell lines Zenk et al. (1977).

Bioreactor and Immobilization Methods

In tissue culture, for alkaloids production researches have been mainly focused on suspension culture which requires a rotatory shaker. For large-scale production, however, large size culture vessel fermenter/bioreactor are most important. In both types of systems a stirring devices is provided for improved aeration (Kargi and Rosenberg 1987; Drapeau et al. 1987; Scragg et al. 1988a). There are several important vessels fitted with compressors which provide filtered air. For plant culture growth and productivity, it is recommended that bioreactors with low shear stress are much more suitable than those of high shear stress. Bioreactors with improved mechanical designs are regularly introduced in bioreactors industry with innovated impeller which helps to regulate shear agitation (Joilicoer et al. 1992).

In *Catharanthus*, immobilization of plants cells has been suggested for better accumulation of terpenoids (Kutney et al. 1988; Hulst and Tramper 1989; Asada and Shuler 1989; Archambault et al. 1990). Immobilization not only maintains the cells viable for a longer period of time but also helps in extracellular alkaloids accumulation. Alginate mediated immobilized cells enhanced the accumulation of tryptamide, ajmalicine and serpentine (Zenk et al. 1977; Majerus and Pareilleux 1986). The use of agar and agarose are found to be effective for long-term maintenance of cells. In the last few years surface immobilization has been proposed using different types of matrices for large-scale production of alkaloids (Facchini et al. 1988; Facchini and Dicosmo 1990). In some other cases, negative influence of immobilization on cell was noticed (Archambault et al. 1990) gel or matrices entrapment on polysaccharide sheet is employed in many plant systems and in *Catharanthus* it is fairly successful.

Hairy Root Culture

Root contains a variety of secondary metabolites which produce alkaloids. High rooting can be induced by genetic transformation using *Agrobacterium rhizogenes*. Induced roots grew with a faster rate in hormone free medium with high accumulation of secondary metabolites in *Catharanthus* (Toivonen et al. 1992). In transgenic *Catharanthus* root, a significant increase in ajmalicine and catharanthine was noticed (Vazquez-Flota et al. 1994; Batra et al. 2005). Other groups used various types of bioreactors/fermenters to improve the growth of hairy roots and then for better production of secondary metabolites (Davidou et al. 1989; Nuutila et al. 1994).

In Vitro Somatic Embryogenesis

Although somatic embryogenesis (SE) has been reported in a wide variety of plant genera (Thorpe 1995; Mujib and Samaj 2006); in *Catharanthus roseus* it has been reported for the first time by (Junaid et al. 2006). Earlier, a preliminarily study on plant regeneration from immature zygotic embryo was reported in *Catharanthus* (Kim et al. 2004). The advantage of SE is that the initial cell populations can be used as a single cellular system and their genetic manipulation are easy and are similar to microorganisms.

8.5 Biosynthesis of Alkaloids in *Catharanthus*

Beside alkaloids, many other secondary metabolites have been isolated from *C. roseus*, which include monoterpenoids, glucosides (loganin, secologanin, deoxy-loganin, dehydrologanin) steroids (catasteron, brassinolides), phenolics, flavaonoids and anthocyanins. Metabolites are in fact the end products of a complex process comprising the involvement of several enzymes, genes, regulatory genes and (transport through) intra-and inter-cellular compartments. The TIA (terpenoids indole alkaloids) are condensation products of two biosynthetic routes which require coordination of the amount of the intermediates supplied by both pathways. The biosynthesis of vinblastine requires the participation of at least 35 intermediates, 30 enzymes, 30 biosynthetic agents, 2 regulatory genes and 7 intra and inter cellular compartments.

The first study on the biosynthesis of alkaloids was performed at the end of the 1950s for *Catharanthus*. Plants were grown in an atmosphere containing $^{14}CO_2$ and after the extraction of alkaloids; many labeled alkaloids have been detected by using column and paper chromatography. Among the isolated alkaloids vinblastine and vincristine were found only in a very low quantity. Thereafter, to increase the level of vincristine and vinblastine, cell cultures of *Catharanthus* were used. Biosynthesis of alkaloids by *in vitro* cell culture has the advantages to manipulate the physiological (rapid growth, ease of precursor feeding, etc.) and genetical process. During the biosynthesis of alkaloids of *C. roseus* various types of proteinaceous compounds have been reported in different biosynthetic pathways.

8.6 Metabolic and Genetic Engineering in Alkaloids Biosynthesis

In alkaloids biosynthesis the role of several enzymes have been discussed in *Catharanthus roseus*, a few of them have been purified, identified, characterized and their encoding genes were also cloned.

The alkaloids biosynthesis is a very complex process arise from the precursor's tryptamine and secologanin. These two precursors are derived from two different pathways. Tryptamine is formed by the enzyme tryptophan decarboxylase (TDC), which has been reviewed earlier by various workers (Bentley 1990; Poulsen and Verpoorte 1992; Singh et al. 1991) while the strictosidine synthetase (SSS) helps in the coupling of tryptamine and secologanin to produce strictosidine (Madyastha and Coscia 1979; Inouye and Uesato 1986). The other enzymes such as geraniol 10-hydroxylase (G10H), NADPH-cytochrome P-450 reductase, and anthranilate synthetase (AS) have the similar TDC activities which are involved in the biosynthesis of indole alkaloids (Poulsen et al. 1993). The TDC enzyme has been purified from cell suspension culture (Pennings et al. 1989) and ultimately the cDNA gene was established (Pasquali et al. 1992). The cytochrome P450 enzyme, geraniol-10-hydroxylase (G10H) and other enzymes have been studied extensively from intact plant of *C. roseus*. By HPLC study (Collu et al. 1999) and selection of a cell line with high G10H activity (Collu et al. 2001); the enzyme was purified to homogenity (Collu et al. 1999). Based on the internal amino acid sequences obtained from the digested protein, gene was cloned and functionally expressed in yeast. The enzyme belongs to the CYP76B subfamily and is designated as CYP76B6. The activity of this enzyme was induced by treating the cells with the cytochrome P450 inducer phenobarbital and decreased after treatment of the inhibitor ketoconazol (Contin et al. 1999).

Besides, many other enzymes have been identified and characterized that metabolize strictosidine, which after undergoing several rearrangements produced cathenamine and ajmalicine (Hemscheidt and Zenk 1985; Stevens 1994). An another important enzyme is desacetoxyvindoline-4-hydroxylase (DAVH), active during vindoline biosynthesis, purified from intact plant of *C. roseus*. The native enzyme is a monomer, has a molecular weight 45KD with three isoforms (Carolis et al. 1994).

Recently, attention has been paid on the regulation of mevalonate biosynthesis that terminates with the end product strictosidine. The encoding genes and enzymes of different steps of mevalonate pathway have been elucidated (Maldonado-Mendonza et al. 1992). After the formation of strictosidine, first step of alkaloid biosynthesis is the removal of sugar moiety from strictosidine to form an unstable aglycone. Two strictosidine β-glucosidases (SG) were partially purified and characterized from *C. roseus* cell cultures (Hemscheidt and Zenk 1980; Stevens 1994).

Feeding of terpenoids precursors to *C. roseus* cell suspension cultures increased alkaloids production (Moreno et al. 1993; Facchini and DiCosmo 1991). Addition of tryptophan (0.5 Mm) to *Catharanthus* cells resulted in high intracellular levels of tryptamine and an increase in STR activity but did not influence ajmalicine accumulation much (Bongaerts 1998). As in other feed back inhibitions, product accumulation depends upon the product degradation and this phenomenon has been reported in cell suspension culture of *Catharanthus*. It is now known that the precursor for alkaloids (tryptophan to tryptamide) was located in the cytosol whereas the enzyme SSS has been localized in the vacuole (Stevens et al. 1993).

8.7 Coupling Methods for Alkaloids Biosynthesis

The bisindoles are derived from the coupling of vindoline and catharanthine. Catharanthine is thought to be derived from strictosidine via 4,21-dehydrogeissos-chizine, stemmadenine and dehydrosecodine while vindoline is derived from strictosidine via stemmadenine and tabersonine. This pathway (transformation of tabersonine to vindoline) has got orderly six reactions (Balsevich et al. 1986; De Luca et al. 1986). The enzyme anhydrovinblastine synthase couples catharanthine and vindoline to yield AVLB which was purified and characterized from *C. roseus* leaves. This heme protein has a molecular weight of 45 KD and shows the peroxidase activity. During this enzymatic coupling both the monomers were incubated at 30° C with cultured *C. roseus* cells as enzymatic source at acidic pH (tris buffer 7.0). Only after 3 h the chemical reaction produced vinblistine and anhydrovinblastine as major products along with other dimeric alkaloids. Vindoline and catharanthine were also non-enzymatically coupled to the dihydropyridinium intermediate (DHPI) under near- UV light irradiation with a peak at 370 nm in the presence of flavin mononucleotide. Subsequently DHPI can be reduced to anhydrovinblastin (AVLB) with an overall yield of 50 %, based on initial amount of vindoline. Vinblastine content was further improved up to 50 % by using various compounds as stimulants (Bede and Dicosmo 1992).

Similarly, vincristine can be isolated from vinblastine by chemical conversion. Two routes are employed; first route is the isolation of *N*-deformyl-VCR which further converted into vincristine by formylation. The second method involves a formylation of the *C. roseus* extract in which conversion of *N*-deformyl – VLB to VCR takes place, after which the material is oxidized. In both cases vincristine was purified by column chromatography and then sulfated (Lily medicinal information service, Netherland).

It was also reported that $MnCl_2$ and FMN/FAD stimulated coupling process. However, in the absence of *C. roseus* cell suspension enzymes, ferric acid stimulated coupling process. The production of vinblastine through enzymatic coupling pathway is thought to be highly efficient and likely to be used commercially very soon.

Vindoline and bisindole alkaloids are accumulated only in green tissue and not found in root and cell suspension cultures (Endo et al. 1987). The developmental regulation of TDS, SSC and the enzymes involved in late steps of vindoline biosynthesis has been studied extensively (De Luca et al. 1986; Fernandez et al. 1989). In seedlings of *C. roseus* expression of these enzymes was not under strong developmental control where enzymes activity were modulated by tissue specific or light dependent factors. The concentration of vindoline, catharanthine and 3,'4'-anhydrovinblastine (AVLB) are age dependent (Naaranlahti et al. 1991). Vinblastine increased as seedlings matured, reaching a steady concentration when the plants become more than 3 months old. On an average, whole seedlings, young plants and mature plants contained 7,11.5 and 12-µg/g dry weight VLB, respectively. After induction of shoot formation the VLB contents increased rapidly to similar levels of *in vitro* seedlings (Datta and Srivastava 1997).

8.8 Cellular Compartmentation

Subcellular compartmentation plays an important role in alkaloids metabolism. This process of metabolism involves the participation of plant cell to separate the enzyme from their substrates and end products. In this, alkaloids biosynthesis requires three cellular compartments, namely vacuole, cytosol, and plastid (Meijer et al. 1993).

The transformation of tryptophan into tryptamine takes place in cytosol (De Luca and Cutler 1987; Stevens et al. 1993), SSS in vacuoles (Mcknight et al. 1991; Stevens et al. 1993). SG was tightly bound to the tonoplast boundary (Stevens et al. 1993). Synthesis of strictosidine takes place inside the vacuole which later transported to the cytoplasm where its glucose moiety detached. Ajmalicine has the potentiality to move freely across the cell membrane and accumulated into the vacuoles here it converted into the serpentine by use of peroxidases (Blom et al. 1991) produced serpentine stored in vacuole and cannot pass the tonoplast. In cell suspension cultures alkaloid accumulation seems to be restricted to certain cells (Neumann et al. 1983). Permeability of cell plays a potent role to release plant products. There are several permeabilizing agents like DMSO and Triton X-100 are found to be very effective in *Catharanthus* cell culture. Besides, for the release of secondary products several other agents (chitosan, alginate beads, electroportion and ultra sonication) have been used with or without cell viability in other groups of plants.

The cell membrane with active uptake mechanism has also been noticed in *Catharanthus*. Most of the secondary products are generally accumulated intercellular, however several compounds such as taxol, anthraquinones are identified in the media which filtrate itself through membrane. For this extracellular product secretion, addition of resin XAD-7 enhanced the product adsorption in *Cinchona* (Ganapathi and Kagri 1990). The media provided with amberlite type resin and XAD-7 resin adsorbed ajmalicine and catharanthine effectively in *C. roseus*.

8.9 Conclusions

Different pharmacological studies and the traditional used proved the high medicinal properties of the Catharanthus; which continuously being used in the treatments of number diseases. Various important alkaloid, mostly the monomers were successfully identified in culture media with the enhanced yields; however the commercial production is still far away. The main problem is due to the lack of optimization of cultural conditions and several strategies leading with increased accumulation of secondary metabolites. A detail studies are required to know the proper enzyme functions at various levels, product membrane permeability and adsorption for improvements towards achieving a viable economic production methodology. In addition, over-expression of enzymes and the genetic modification could be very useful via organogenesis or somatic embryogenesis for the production of desired levels of secondary metabolite.

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Chapter 9
Nutraceuticals: Recent Developments and Future Prospectives

Pranay Jain and Ram Kumar Pundir

9.1 Definition and Introduction

The term "nutraceutical" was coined from "nutrition" and "pharmaceutical" in 1989 by Stephen DeFelice. Nutraceutical is defined as, "a food or part of a food that provides medical or health benefits, including the prevention and/or treatment of a disease" (Keservani et al. 2010). When functional food aids in the prevention and/or treatment of disease(s) and/or disorder(s), it is called a nutraceutical. It should be noted that the term nutraceutical, as commonly used in marketing, has no regulatory definition (Gupta et al. 2010). Thus, nutraceuticals differ from dietary supplements in the two aspects: (i) nutraceuticals must not only supplement the diet but should also aid in the prevention and/or treatment of disease and/or disorder; and (ii) nutraceuticals are used as conventional foods or as sole items of a meal or diet (Kalra 2003). Nutraceuticals also refer to natural functional/medical foods or bioactive phytochemicals that have health promoting, disease preventing or medicinal properties. These nutraceuticals normally contain the required amount of vitamins, lipids, proteins, carbohydrates, minerals, or other necessary nutrients, depending on their emphases (Whitman 2001).

The concepts of nutraceuticals, functional or medical foods, or dietary supplements are confusing and most often they can be used interchangeably. These concepts may be distinguished by their description from different points of view, e.g. functional

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food is a more general term to emphasize foods with specific or strong purposes (Koletzko et al. 1998); Dietary supplements have more defined health roles such as vitamins, minerals, herbs or other botanicals, amino acids, and other dietary substances intended to supplement the diet by increasing the total dietary intake of these ingredients (Halsted 2003). Dietary supplements are not intended to treat or cure disease, whereas nutraceuticals more emphasize the expected results of these products, such as prevention or treatment of diseases (Ross 2000).

Phytochemicals and their metabolic products may also inhibit pathogenic bacteria while stimulate the growth of beneficial bacteria, exerting prebiotic-like effects (FAO 2007). Interactions between functional food components, such as prebiotics, probiotics, phytochemicals, and intestinal microbiota, have consequences on human health (FAO 2007).

Key definitions: There are some terminologies are defined (Camire 2003) as follows

Term	Definition
Antioxidants	Chemicals that protect against oxidation and free-radical damage to biomolecules (lipids, proteins, carbohydrates, and DNA)
Bioactivity	A given agent's effect on a living organism or on living tissue
Botanicals	Processed plants or their extracts that are believed to affect health
Designed foods	Raw, fresh agricultural products that contain supplemental nutraceuticals or phytochemicals to benefit health and decrease chronic disease risk
Dietary supplement	A product (other than tobacco) that is intended to supplement the diet and bears or contains one or more of the following dietary ingredients such as vitamin, mineral, amino acid, herb, or other botanical; is intended for ingestion in the form of a capsule, powder; and is not represented as a conventional food or as a sole item of a meal or diet
Functional foods	Foods containing physiologically active components with a health benefit beyond basic nutrition
Nutraceutical	Nutrients and non-nutrient compounds in food that have health-promoting, disease preventive, or medical properties
Phytochemicals	Plant compounds imparting a benefit to human health, depending on an individual's dietary practices, lifestyle, and genetic makeup

Dietary components play beneficial roles beyond basic nutrition, leading to the development of the functional food concept and nutraceuticals (Laparra and Sanz 2010). Several naturally derived food substances have been studied in cancer therapies. Vitamin E, selenium, vitamin D, green tea, soy, and lycopene are examples of nutraceuticals widely studied in human health (Brower 1998).

Nutraceuticals are dietary supplements that are also called functional foods. Functional foods are defined as being similar in appearance to conventional foods, are consumed as part of a usual diet, and are known to improve health status and render physiological effects beyond basic nutritional function expected of conventional foods. However, nutraceuticals are products produced from foods, but sold in the medicinal form of capsule, tablet, powder and solution (Ali et al. 2009). Nutraceuticals have been claimed to have a physiological benefit or provide protection against the diseases include cardiovascular agents, antiobese agents, antidiabetics,

Table 9.1 Various nutraceutical substances grouped by food source

Plants	Animals	Microorganisms
α-Glucan conjugated	Linoleic Acid (CLA)	Cheese
Ascorbic acid	Eicosapentaenoic acid (EPA)	Yoghurt
γ-Tocotrienol	Docosahexenoic acid (DHA) products	Other dairy
Quercetin	Spingolipids	
Luteolin	Choline	
Cellulose	Lecithin	
Lutein	Calcium	
Gallic acid	Coenzyme Q10	
Indole-3-carbonol	Creatine	
Pectin	Minerals	
Daidzein		
Glutathione		
Allicin		
δ-Limonene		
Genestein		
Lycopene		
Capsaicin		
β-Ionone		
α-Tocopherol		
β-Carotene		
Zeaxanthin		

Modified from: Keservani et al. (2010)

anticancer agents immune boosters, chronic inflammatory disorders and degenerative diseases. Table 9.1 showing various nutraceutical substances grouped according to food source.

9.2 Worldwide Status

Globally, the functional food and nutraceutical industry represents in excess of a $75.5 billion US industry (Just-food 2007) with prospects of growing to $167 billion by 2010. Japan is the second largest market in the world for nutraceutical products after the United States. Its nutraceutical market has exhibited a steady average growth rate of 9.6 % per annum for the past decade (Functional Food Japan 2006).

In India, the most common forms of functional foods and nutraceuticals are available as traditional Indian Ayurvedic Medicines (IAM), these are marketed under different brand names (Patwardhan et al. 2005). India is the home of a large number of medicinal herbs, spices and tree species that have a substantially large domestic market with no major foreign competition at present (Datta and Basu 2002). Estimated value of the industry is $10 billion per annum with exports of $1.1 billion per annum making a significant contribution to the export market (Singh et al. 2003).

India is strong and a growing force in the international health foods market (Verma 2009). Rapid urbanization, rising incomes, changing lifestyles and dietary patterns, and growing health consciousness have triggered the growth of health and wellness foods in India. The health and wellness foods market is currently estimated to be around US$ 1.6 billion and is expected to reach US$ 7.5–10 billion by 2015 growing at 25–30 % compound annual growth rate (The Health and Wellness Foods Industry in India 2009).

A similar profile for the industry can be developed for China. Both functional foods and nutraceuticals are part of the traditional Chinese diet and are also a large component in Traditional Chinese Medicine (TCM). The industry is represented by around 1,000 small to medium sized enterprises located throughout China. On the other hand, there is a growing awareness on the importance of nutrition and diet for long term good health. These have contributed to a favorable market conditions for Nutraceutical industry in India. India has a lot of advantages like qualified human resources, world class research and development facilities and varied raw material-aspects that give our country a leading edge (Patwardhan et al. 2005).

9.3 Significance

Nutraceutical have the potential to play a role in healthy eating and to contribute to the prevention and treatment of diseases so that how functional components in foods could expand the role of disease prevention and treatment. Some examples of foods of specific nutraceutical substances as shown in Table 9.2.

The nutraceuticals are preferred due to

- Since new molecule is difficult to discover and more expensive and risky then ever before, many pharmaceutical companies are now trying to nutraceuticals so that there is undoubtedly a very huge and growing market.
- The belief among consumers that these “food like substances” are either harmless or least toxic as compared to conventional pharmaceuticals.
- Increased healthcare costs with conventional pharmaceuticals, recent legislation and scientific discoveries.
- Inappropriate dietary habits are seen as contributing to the leading cause of deaths of due to coronary heart disease, certain type of cancers etc. the role of nutraceuticals in treating these conditions is thus speculated (Rishi 2006).
- The emergence of diet-disease relations have lead to search of specific constituents of plants, animals and minerals having a beneficial role for our mental and physical health (Dhami and Chopra 2000).
- Nutraceuticals are gaining popularity as people are relying on them for safeguarding their health and avoiding side effects associated with drugs as well.
- As public knowledge in this field has evolved, manufacturers have sought to fulfill their appetite for these products resulting in exploding market. There are around 5,000 established nutraceutical products, worldwide.

Table 9.2 Some examples of foods of specific nutraceutical substances

Nutraceutical substance	Foods
Allyl sulfur compounds	Onions, garlic
Isoflavones (e.g., genestein, daidzein)	Soybeans and other legumes, apios
Quercetin	Onion, red grapes, citrus fruit, broccoli, Italian, yellow squash
Capsaicinoids	Pepper fruit
EPA and DHA	Fish oils
Lycopene	Tomatoes and tomato products
Isothiocyanates	Cruciferous vegetables
β-Glucan	Oat bran
Resveratrol	Grapes (skin), red wine
β-Carotene	Rosemary
Catechins	Teas, berries
Adenosine	Garlic, onion
Indoles	Cabbage, broccoli, cauliflower, kale, Brussels, sprouts
Curcumin	Turmeric
Ellagic acid	Grapes, strawberries, raspberries, walnuts
Anthocyanates	Red wine
Cellulose	Most plants (component of cell walls)
Lutein, zeaxanthin	Kale, collards, spinach, corn, eggs, citrus
Monounsaturated fatty acids	Tree nuts, olive oil
Inulin, Fructooligosaccharides (FOS)	Whole grains, onions, garlic
Lactobacilli, Bifidobacteria	Yogurt and other dairy
Catechins	Tea, cocoa, apples, grapes
Lignans	Flax, rye

Modified from: Keservani et al. (2010)

- Long history of use and better patient tolerance as well as public acceptance.
- Renewable source, cultivation and processing environmental friendly and local availability.
- Plants are one of the most important resources of human foods and medicines. Rapidly increasing knowledge on nutrition, medicine, and plant biotechnology has dramatically changed the concepts about food, health and agriculture, and brought in a revolution on them. Nutritional therapy and phytotherapy have emerged as new concepts and healing systems have quickly and widely spread in recent years (Mani et al. 2002; Zhao 2007).

9.4 Classification of Nutraceuticals

Nutraceuticals can be classified based on the origin of the beneficial ingredient and/or the therapeutic benefit claimed (Table 9.3). Nutraceuticals can also be classified on the basis of their chemical nature. This approach allows nutraceuticals to be categorized under molecular/elemental groups. This preliminary model

Table 9.3 Classification of nutraceuticals

Ingredients	Therapeutic uses
Antioxidants	Bone and joint health
(a) Vitamins: vitamin C, vitamin E	
(b) Carotenoids: â-Carotine, lycopine	
(c) Thiols: lipoic acid, gutathione	
(d) Enzymes: dismastase	
(e) Minerals: Co-Enzyme Q-10, super oxide, selenium, copper, manganese, zinc	
Dairy-based ingredients	Cancer risk reduction
Plant extracts and Phytochemicals	Cognitive and mental function
Nutritional lipids and oils	Eye and gut health
Fibres and carbohydrates	Immune system
Probiotics	Maternal and infant health
Lactobacilli	
Lactobacillus rhamnosus	
Lactobacillus reuteri	
Lactobacillus casei	
Bifidobacterium	
Bifidobacterium lactis	
B. longum	
B. breve	
B.infantis	
Streptococcus	
Lactococcus	
Lactococcus platinum	
L. reuteri	
L. agilis	
Enterococcs	
Saccharomyces	
Bacillus	
Pediococcus	
Prebiotics	Respiratory and skin health
1. Fructo oligosaccharides	
2. Inulins	
3. Lactilol	
4. Galacto-oligosaccharides	
5. Soybean oligosaccharides	
6. Isomalto-oligosaccharides	
7. Gluco-oligosaccharides	
Amino acids and proteins	Weight management
Soy-based ingredients	Women's health
	Cardiovascular health

includes several large groups, which then provide a basis for sub classification or subgroups, and so on. One way to group nutraceuticals grossly is as follows (Rajasekaran et al. 2008).

- Isoprenoid derivatives
- Phenolic substances
- Fatty acids and structural lipids
- Carbohydrates and derivatives
- Amino acid-based substances
- Microbes
- Minerals

9.4.1 Nutrigenomics

Nutrigenomics may also be called as nutritional genomics, the junction between health, diet, and genomics, can be seen as the combination of molecular nutrition and genomics. The diverse tissue and organ-specific effects of bioactive dietary components include gene-expression patterns (transcriptome); organization of the chromatin (epigenome); protein-expression patterns, including posttranslational modifications (proteome); as well as metabolite profiles (metabolome). It will promote an increased understanding of how nutrition influences metabolic pathways and homeostatic control, how this regulation is disturbed in the early phases of diet-related disease, and the extent to which individual sensitizing genotypes contribute to such diseases. Eventually, nutrigenomics will lead to evidence-based dietary intervention strategies for restoring health and fitness and for preventing diet-related disease (Afman and Müller 2006).

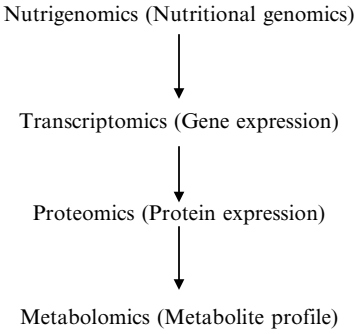
9.5 Nutrigenomics: Strategies and Future

There are two main strategies of nutrigenomics. The first strategy will provide detailed molecular data on the interaction between nutrition and the genome, whereas the second strategy might be important for human nutrition, given the difficulty of collecting tissue samples from healthy individuals. The first strategy, typically applied by smaller research groups, will reveal the identification of transcription factors that function as nutrient sensors and the genes they target; elucidation of the signaling pathways involved, and characterization of the main dietary signals; measurement and validation of cell- and organ-specific gene expression signatures of the metabolic consequences of specific micronutrients and macronutrients; elucidation of interactions between nutrient-related regulatory pathways and proinflammatory stress pathways, to understand the process of metabolic dysregulation that leads to diet-related diseases; and identification of genotypes that are risk factors

for development of diet-related human diseases (such as diabetes, hypertension, or atherosclerosis) and quantification of their impact. The second strategy is the application of nutritional systems biology to develop biomarkers of early metabolic dysregulation and susceptibility (stress signatures) that are influenced by diet. This strategy requires large consortia, considerable research funding, and excellent multidisciplinary (and possible multinational) collaboration (Afman and Müller 2006).

Different genomic technologies, namely transcriptomics, proteomics, and metabolomics, are complementary in the types of information they generate, but are at different points in their development at this time. Ultimately, parallel use of these methods will allow us to describe the phenotype of a biological system, such as a human being, in all its complexity, which is the major goal of nutritional systems biology (Desiere 2004). Transcriptomics is a relatively mature technology compared with other "-omics" technologies. At this point, it is possible to get an overview of the expression of virtually all genes in a single microarray experiment, but it is not yet possible to measure the whole proteome or metabolome (German et al. 2003; Dunn et al. 2005).

Metabolomics is also in the early stages of development. It is not known how many endogenous metabolites exist or how many exogenous food-derived metabolites can be measured in human samples (urine, plasma). Scientists must first overcome a number of hurdles, such as full recovery of all metabolites from body fluids or tissue samples and the need to develop extensive databases with the required information about the nutritionally relevant metabolome. Metabolomics produces enormous amounts of data that require sophisticated instrumentation and software to allow researchers to extract meaningful information from the data. Existing instrumentation is quite sophisticated; the present limitations appear to be with the software needed to handle metabolomic data. The potential for nutritional applications of metabolomics is considerable, and a number of research teams are addressing these limitations (German et al. 2003). Flow chart showing the concept of nutrigenomics.



9.6 Regulatory Status

The governmental administration of food and drugs in many countries such as the United States of America (http://www.cfsan.fda.gov/list.html), Canada (http://www.hc-sc.gc.ca/index_e.html), European Union (http://www.emea.eu.int), China (http://www.sfda.gov.cn/cmsweb/web portal), and India (http://mohfw.nic.in/), have strict regulations on food and drugs in terms of manufacturing, servicing, and marketing, and usage. Various countries are making corresponding laws, or complementary regulations, or addressing issues with new explanations. The legislation for the marketing of functional foods in the European Union (EU) was in legislative process. Particularly, the safety-basis of nutritional or phytotherapy products are based on risk analysis. In Europe, scientific risk assessment is performed by the European Food Safety Authority and risk management is performed by the European Commission. Canadian government provides some guidelines for evidence-based clinical practice of clinical nutrition support, enteral or parenteral nutrition for ill adults. The more detailed regulations on nutraceuticals, phytonutriton or phytotherapy, or nutritional therapy are being worked out through consultations with expert panels who can provide descriptions of regulatory hurdles for these products and practices, Good Manufacturing Practice (GMP) compliance, Generally recognized as safe (GRAS) status, analytical methods and validation. Various government-supported projects study on improvement of nutritional professional services for the public. For example, studies indicated that dietitians' ability to incorporate an evidence-based approach is largely determined by their education and training, work experience, and professional association involvement. Therefore, advanced education and training is important for dietitians to participate in nutritional services, contribute to knowledge discovery, translation, and outreach to improve the nutritional status and health of populations (Ohama et al. 2006).

9.7 Future Prospectives

Intensive growth in use of nutraceutical and functional food products has made it necessary for the industry to develop new more global supply chain relationships (Hobbs 2002). Access to "credible quality" produce is needed to deal with rising consumer skepticisms and to promote international growth within the industry. Application of modern approaches in genomics, proteomics and metabolomics to the study of genetics and the biochemistry of functional foods derived from plants and animals has potential to allow us to characterize these products better. Moreover, these approaches will enable us to modify product characteristics with precision. Using marker assisted breeding and the application of genetic engineering new products with potentially better characterized and optimized benefits to human and animal health can be developed over relatively short periods of time (Laroche 2007).

Considerable attention now is being paid to characterization of the natural organism diversity found throughout the planet. Natural diversity in growth and biochemical makeup of organisms presents us with unique opportunities to expand the product base within the functional food and nutraceutical marketplace. Plants in particular, present us with opportunities to alter their biochemical makeup to adapt to new applications and challenges, as well as providing us with a diverse array of growth responses that can be exploited to allow their use under many different regional ecotypic conditions. Moreover, by selecting plants with uniform performance over a wide range of environments, marketing agencies can label their products reliably and consumers can benefit from more predictable health and nutritional effects. Development of better characterized and research proven products will help enhance consumer confidence in functional food and nutraceutical products produced in Canada and elsewhere and this will help these products in the global marketplace. Nutraceuticals are destined to play an important role in future therapeutic developments but their success will be governed by control of purity, safety and efficacy without inhibiting innovation. Nutraceuticals will continue to appeal because they are convenient for today's lifestyle. Some are also genuinely researched and offer novel ingredients that can bring about health benefits quicker than would normally be the case through eating conventionally healthy foods alone. The present accumulated knowledge about nutraceuticals represents undoubtedly a great challenge for nutritionists, physicians, food technologists and food chemists. Public health authorities consider prevention and treatment with nutraceuticals as a powerful instrument in maintain in health and to act against nutritionally induced acute and chronic diseases, thereby promoting optimal health, longevity and quality of life. A place for nutraceuticals in clinical practice is emerging, but important pharmaceutical and clinical issues need to be addressed by further research.

9.8 Conclusions

Nutraceuticals may be beneficial to health, but we are still learning about their benefits and possible harmful effects. It is scientific area generated all over the world. In many cases nutraceuticals over an advantage over the synthetic drugs under development by the pharmaceuticals industry. It is novel pharmacological activity that are become interesting in their possible clinical use and thus helping in prevention and therapeutic in several diseases. Most of the pharmaceuticals companies often lack motivation to pursue these difficulties in obtaining the patents. Many nutraceuticals, functional foods and naturally occurring compounds that have been investigated and reported in various studies revealed that these products are extremely active, have profound effect on cell metabolism and often have little adverse effect. It is natural that people's focus is shifting to a positive approach for prevention of diseases to stay healthy. It is hope that government agencies and research centers will give support for further research in nutraceuticals.

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Chapter 10
Plant Edible Vaccines: A Revolution in Vaccination

Aastha Sahai, Anwar Shahzad, and Mohd. Shahid

10.1 Introduction

Since time immemorial, plants besides providing food and shelter have also been used for basic preventive and curative health care by human beings. Plants were the first and only source for every kind of medication in primitive times. Gradually, animal derived products were also started being used as medicines for some ailments like snake venom has been used for medical purposes for thousands of years. In Chinese medicine snake skin was used to treat superficial diseases, including skin eruptions, eye infections or opacities, sore throat, and hemorrhoids. However, plants still remained to be the primary source of medication till now for their ease of availability and safe nature.

With the advent of modern science and technology, pharmacology has shown remarkable advances with the artificial production of plant and animal based medicines in laboratories. Apart from this some novel medicinal compounds were also synthesized to combat life threatening diseases. All these advancements were aimed to increase average human life span and to decrease the child mortality rate. A significant step taken in this regard was the Universal Immunization Programme (UIP) launched by WHO in 1980s in which children all over the world are vaccinated against six deadly diseases (BCG, OPV, diphtheria, tetanus, pertussis, and measles)

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with the aim of immunizing 80 % of all children by 1990. However, even after all these efforts 20 % of infants are still left unimmunized; responsible for approximately two million unnecessary deaths every year, especially in remote and impoverished parts of the globe (Langridge 2006). Moreover, immunization remains an unfinished agenda with an estimated 19.3 million children were not reached with three doses of DTP vaccine in 2010. Those who are not immunized – about every Fifth Child – are mostly among the poorest and the most vulnerable.

Vaccination is an important tool for prevention of many diseases. It is the administration of vaccine to produce immunity to a disease. Vaccination involves the stimulation of the immune system to prepare it for the event of an invasion from a particular pathogen for which the immune system has been primed (Arntzen 1997). On recognizing the foreign substance (live/ weakened microbes, antigen or proteins) which enters the body through vaccine, the immune system activates, producing antibodies to destroy the invader. Not only this but this attack leaves behind the 'memory' T and B cells which guards body in case of any future attack by that pathogen. Some vaccines provide lifelong protection; others (such as those for cholera and tetanus) must be re-administered periodically (Langridge 2006). Classically, this has been achieved by presenting the immune system with whole viruses or bacteria that have been killed or made too weak to proliferate much. However, these conventional vaccines poses threat of actually acquiring the infection from weakened or attenuated pathogenic strains. Thus newer approach uses sub unit vaccines and recombinant vaccines. These vaccines consist of specific macromolecules that induce a protective immune response against a pathogen rather than administering the whole pathogen in the body. A "subunit vaccine" refers to a pathogen-derived protein (or even just an immunogenic domain of a protein, i.e. "an epitope") that cannot cause disease but can elicit a protective immune response against the pathogen. Very often the subunit-vaccine candidate is a recombinant protein made in transgenic production-hosts (such as cultured yeast cells), then purified, and injected as vaccines to immunize against a specific disease (Mor and Arntzen 2002). These sub-unit vaccines increases vaccine safety by circumventing the need to use live viruses or microbes and has thus made them the preferred approach for vaccine manufacturers (National Institute of Allergy and Infectious Diseases 1998).

Unfortunately, traditional subunit vaccines are expensive to produce and not heat stable which limits their availability and use in developing countries. Although these vaccines have an undue advantage over traditional conventional vaccines but they are expensive and their storage and transportation pose a problem as many of them require refrigeration. This is a disadvantage in many of the developing countries where the vaccines were needed most. Thus, to ensure a successful global immunization a whole new idea of plant edible vaccines was presented by Arntzen and co-workers in 1992, by introducing the concept of transgenic plants as a production and delivery system for subunit vaccines (Mason et al. 1992; Mason and Arntzen 1995). They envisaged that production would be as cheap as agriculture, that distribution would be as convenient as marketing fresh produce and that administration would be as simple and safe as eating (Mor et al. 1998).

10.2 Concept of Plant Edible Vaccines

Plants have long been considered an ideal expression system for many of the animal derived proteins, antibodies and pharmacologically important compounds. Factors in favor of plant systems as sources of animal derived proteins include: the potential for large-scale, low-cost biomass production using agriculture; the low risk of product contamination by mammalian viruses, blood borne pathogens, oncogenes and bacterial toxins; the capacity of plant cells to correctly fold and assemble multimeric proteins; low downstream processing requirements for proteins administered orally in plant food or feed; the ability to introduce new or multiple transgenes by sexual crossing of plants; and the avoidance of ethical problems associated with transgenic animals and the use of animal materials (Doran 2000). The first pharmaceutically relevant protein made in plants was human growth hormone, which was expressed in transgenic tobacco in 1986 (Barta et al. 1986). Since then, many other human proteins have been produced in an increasingly diverse range of crops. In 1989, the first antibody was expressed in tobacco (Hiatt et al. 1992).

The idea of suitability of plants as vaccine production and delivery system was presented by research group of Charles Arntzen in 1990s. This idea was quite promising in terms of providing a successful tool for mass immunization in developing countries where till now vaccination is hampered by high cost of production and delivery of vaccines. Basic concept of edible vaccines involves the production of plants containing antigens required to stimulate the immune response in human body. Thus by simply eating the plant product people will get vaccination against diseases. However the concept sounds quite simple but its development was equally complex. To produce edible vaccine, plants are engineered to contain desired gene of interest which codes for the antigen. This process is accomplished by transformation and the altered plant containing foreign gene is called transgenic plant. Thus, the process remains the same as with subunit vaccine preparation because it contains only desired antigen and not the whole pathogen. These vaccines basically work in the same way as the injected DNA vaccine, since a peptide sequence similar to an infectious part of a pathogen is synthesized, by itself, and is used to prime T and B cells in the body. The big difference in this case is that the protein sequences are encoded in a plant to form the desired protein. This protein is then ingested, as the plant or its fruit is eaten. One becomes immune against the ingested protein, as T and B cells become stimulated to proliferate and differentiate (Mor et al. 1998). Expression of vaccines in plant tissues eliminates the risk of contamination with animal pathogens, provides a heat-stable environment, and enables oral delivery, thus eliminating injection-related hazards (Walmsley and Arntzen 2000).

For production of edible vaccines, it is desirable to select a plant whose products are consumed raw to avoid degradation during cooking. Thus, plants like tomato, banana and cucumbers are generally the plants of choice. Vaccines made from plant material have enormous potential for use, in the developing world. Also it may be much easier to persuade people to eat protective vegetables than to accept injections or take pills. Vaccines made from mashed potato, designed to protect

against travellers' diarrhoea, have already been tested in humans. Vaccines made from dried tomatoes have also been developed. Both have been developed by the Cambridge-UK-based biotechnology company Axis Genetics, who pioneered the techniques involved.

10.3 Mechanism of Action

Edible vaccines are mucosal-targeted vaccines that stimulate both the systematic and mucosal immune network, activating the first line of defense of human body through mucosa. The mucosal surfaces are found lining the digestive tract, respiratory tract and urinoreproductive tract. Mucosal immune system (MIS) is the first line of defense and the most effective site of vaccination, nasal and oral vaccines being the most effective (Mor et al. 1998; Korban et al. 2002). Before understanding the mechanism of action of edible vaccines it is important to understand the functioning of MIS. Induction of a mucosal immune response starts with the recognition of an antigen by specialized cells called M-cells. These cells are localized in the mucosal membranes of lymphoid tissues such as Peyer's patches within the small intestines. The M-cells channel the antigen to underlying tissues where antigen-presenting cells internalize and process the antigen. The resulting antigenic epitopes are presented on the APC surface, and with the assistance of helper T cells activate B cells. The activated B cells migrate to the mesenteric lymph nodes where they mature into plasma cells and migrate to mucosal membranes to secrete immunoglobulin (Ig) A. On passing through the mucosal epithelial layer towards the lumen, the IgA molecules complex with membrane-bound secretary components to form secretary IgA (sIgA). Transported into the lumen, the sIgA interacts with specific antigenic epitopes and neutralize the invading pathogen (Walmsley and Arntzen 2000). When edible vaccines are eaten they degrade majority of the plant cells in the intestine as a result of the action of digestive and bacterial enzymes. This degradation results in the release of antigens present in the plant product. The whole process occurs near the Peyer's patches (Rudzik et al. 1975) where M cells recognize the released antigen and lead to the production of IgA antibodies in mucosal lymphoid tissues through the above mentioned mechanism. These IgA antibodies get transported across the epithelial cells into the lumen where they interact with released antigens depicting immunogenic response.

10.4 Preparation of Edible Vaccines

Plant edible vaccines are basically prepared using two strategies. Either through the introduction of antigen producing gene in plant genome via *Agrobacterium* species; the process known as transformation. This can also be done by directly inserting the target gene in plant genome without the help of any vector. In this case gene gun

eliminates the requirement of vector by directly bombarding the target gene in plant tissue, through the technique known as bolistic method. Second strategy involves the use of plant viruses as vectors which results in transient expression of the foreign gene. The effect of the added genes is to make the plant or plant virus produce antigens which are normally found only on the surfaces of the pathogen. However, both the techniques require introduction of genes taken from the pathogenic microbe that infect humans but transformation technique offers certain advantages.

10.4.1 Transformation

There are three types of plant transformation methods:

1. *Agrobacterium* mediated transformation
2. Micro projectile bombardment/Bolistics method
3. Electroporation

First method involves the use of *Agrobacterium tumefaciens* as a carrier or vector system for introduction of antigen producing gene in plant genome. *Agrobacterium tumefaciens* is a soil bacterium responsible for producing crown gall tumor in plants via its Ti (tumor inducing) plasmid. *A. tumefaciens* and *A. rhizogenes* has the property to integrate their plasmid DNA with nuclear genome of host (plants) cells. This property is exploited for introducing foreign gene in plants and the process is called 'transformation' while the plants produced through this process are 'transgenic plants'. During transformation the bacterial plasmid is first disarmed by removing tumor inducing gene and the target gene for a selected immunogen is introduced. Along with the target gene, antibiotic resistance genes were also added in the plasmid which acts as marker genes to identify the transformed plant tissues containing bacterial plasmid. The bacterium is then co cultivated with the wounded plant tissues so that the bacterial DNA penetrate the plant cells. Following this the target gene randomly incorporates in the plant nuclear genome. After selection through antibiotic resistance successfully transformed plant tissues are identified and regenerated into whole plants under in vitro conditions. These transformed plant lines show varying degree of expression for introduced gene thus producing varying amount of desired antigen (Shah et al. 2011). Plant line showing highest expression is identified and propagated on large scale to be used as edible vaccine. The drawback of this method is that it gives low yield and the process is slow. It requires about 6 weeks to18 months for whole plants to be formed through this process depending on regeneration ability of various plant species under in vitro conditions. This method is more successful in dicotyledonous plants like potato, tomato and tobacco which are easy to transform through *Agrobacterium* in comparison to monocotyledons.

Second technique is capable of directly introducing the gene in plant cells through gene gun. This method is called 'Bolistic method'. In this method selected DNA sequences are precipitated onto metal microparticles and bombarded against

the plant tissue with a particle gun at an accelerated speed. Microparticles penetrate the walls and release the exogenous DNA inside the cell where it will be integrated in the nuclear genome through mechanisms that have yet to be clearly understood (Mishra et al. 2008). This method is quite attractive because DNA can be delivered into cells of plant which makes gene transfer independent of regeneration ability of the species. But the chief limitation is the need for costly device particle gun (Shah et al. 2011).

In another technique called 'Electroporation', DNA is introduced into cells by exposing them for brief period to high voltage electrical pulse which is thought to induce transient pores in the plasma lemma (Singh 2002). The cell wall presents an effective barrier to DNA, therefore, it has to be weakened by mild enzymatic treatment so as to allow the entry of DNA into cell cytoplasm.

Transformation technique results in stable expression of the foreign gene allowing production of subsequent generations of large numbers of transgenic plants, either by vegetative or sexual means. The seeds collected from these transgenic lines could be stored, and used as and when necessary for the molecular farming (Malabadi et al. 2012). It also provides the opportunity to introduce more than one gene for possible multi-component vaccine production. In addition, judicious choice of genetic regulatory elements allows organ and tissue-specific expression of foreign antigens (Mason and Arntzen 1995). Furthermore, plant DNA is not known to interact with the animal DNA and plant viral recombinants do not invade mammalian cells (Sala et al. 2003).

10.4.2 Chimeric Plant Virus Expression Systems

Another approach of plant edible vaccine production utilizes plant viral expression systems which addresses the low yield issue of transformation approach. The rationale of this approach is based on the notion that during viral replication, gene copy number becomes amplified, resulting in a much higher level of transgene expression than observed with stable transformation. Among the potential advantages of transient viral expression of transgenes over stably transformed transgenic plants are the shorter time for cloning of foreign genes in the viral genome as compared with the time required to transform plants, the ease at which antigen production can be scaled up, and the wide host range of plant viruses that allows the use of multiple plant species as biofactories (Koprowski and Yushibov 2001). Capsid proteins of many types of viruses can assemble into virus-like particles (VLPs). Devoid of the viral genetic material, VLPs often resemble the native virions in their morphology, antigenic properties and stability. These VLPs are genetically engineered to express the desired peptides/proteins.

Two techniques namely 'Overcoat technology' and 'Epicoat technology' are used to design VLPs that contain antigen DNA on their surface. Some plant viruses like CPMV (cowpea mosaic virus), alfalfa mosaic virus, TMV (tobacco mosaic virus), CaMV (cauliflower mosaic virus), Potato virus X and tomato bushy stunt

virus can be used effectively for this purpose. Overcoat technology permits the plant to produce the entire protein, whereas epicoat technology involves the expression of only foreign protein (Lal et al. 2007). The recombinant virus is then inoculated into the host plant which produces several antigenic proteins (Mor et al. 1998). These particulate antigens generally elicit stronger mucosal immune responses than soluble antigens, which can often repress the immune response by inducing immunotolerance (Garside and Mowat 1997). However, each single antigen expressed in plants must be tested for its proper assembly and can be verified by animal studies, Western blot; and quantified by enzyme-linked immunosorbent assay (ELISA) (Haq et al. 1995). This method offers rapid onset of expression, and the systemic spread of virus so that protein is produced in every cell (Desai et al. 2010). VLPs are therefore predicted to make excellent mucosal vaccines (Estes et al. 1997).

However, the method poses certain limitations such as transient expression is less easy to initiate. Also the subsequent plant generations do not inherit the foreign gene as it is not incorporated into the plant genome. Thus the production of a plant virus-derived vaccinogen requires an extra step of inoculation of the host plants with the chimeric virus. Before administration, chimeric virus particles are often purified from host-plant tissues that are unpalatable, containing toxins or are not practical for direct consumption. Nevertheless, the high level of foreign protein expression (up to 2 g/kg of plant tissue) within a short period (1–2 weeks after inoculation) makes this an attractive alternative for vaccine production (Walmsley and Arntzen 2000).

Plants and plant viruses were not used until 1993, however, when an epitope from the foot-and-mouth disease virus (FMDV) was expressed on the surface of cowpea mosaic virus (CPMV) (Usha et al. 1993). Chimeric plant viruses were proven effective as carrier proteins for vaccinogens in 1994 after rabbits raised an immune response against purified chimeric CPMV particles expressing epitopes derived from human rhinovirus (Valenzuelz et al. 1985). Majorly TMV and CPMV were used initially as plant viral expression systems because of advantages of high yield (l–2 g of virus per kg of host tissue), thermostability and ease of virus purification, but now a days other plant viruses like alfalfa mosaic virus (AMV), CaMV (cauliflower mosaic virus) etc. are also being used. An increased interest in using plant virus vectors was witnessed for development of vaccines against many animal and human diseases (Adams et al. 1987; Clarke et al. 1990; Dedieu et al. 1992; Mastico et al. 1993; Lomonossoff and Johnson 1995).

10.5 Need and Advantages of Plant Edible Vaccines

In 1992, WHO estimated that three to five million children's lives could be saved each year if new vaccines were available to control or prevent commonly occurring infectious diseases. CVI emphasized on the need to create technologies that would make vaccine cheap and more reliable, especially for the developing world. CVI (Children's vaccine initiative) also presented idea of needle free immunization to

save millions of children from the pain of needles and to avoid risk of death due to needle carried infections. The attention was laid on oral vaccines as they fulfill all the requirements and are an effective means of vaccination by activating the mucosal immunity where most of the pathogens attack first. Development of heat stable oral vaccines were demanded which could serve the purpose of mass immunization in developing countries. Being heat stable they can eliminate the requirement of cold chains which are very costly and generally not found those parts where vaccination is needed most.

Subunit vaccines based upon recombinant cell culture expression systems are feasible but, for commercial- scale production, these systems require fermentation technology and stringent purification protocols so that sufficient amounts of recombinant protein can be obtained for oral delivery. Even with technological improvements, fermentation-based subunit vaccine production may be a prohibitively expensive technology for developing countries where novel oral vaccines are urgently needed (Mason and Arntzen 1995). Plant derived edible vaccines are the answer to all these requirements. These vaccines offer all those advantages which make them an ideal candidate to create a disease free world. Following are the advantages of plant edible vaccines:

(a) Plant derived edible vaccines are cheap as they eliminate the expenses associated with fermenters, purification, adjuvant, cold chain logistics, storage/transportation, needle free-administration, and sterile delivery (Davoodi-Semiromi et al. 2009, 2010; Walmsley and Arntzen 2003; Pascual 2007; Malabadi 2008; Daniell et al. 2009; Gomez et al. 2010).
(b) Plant derived vaccine offer higher safety because they are needle/syringe free that is why risk of infections occurring from syringes is eliminated.
(c) These vaccines do not require trained medical personnels for its administration unlike conventional vaccines which uses syringes as delivery system
(d) Plant derived vaccines are safe and have less chances of contamination with human and animal pathogenic microorganisms, because plants are not hosts for human infectious agents (Giddings et al. 2000; Ma et al. 2004).
(e) As edible vaccine can be administered orally, they elicit both mucosal and systemic immunity which is not observed in traditional vaccines.
(f) These vaccines are heat stable and can be stored at room temperature, unlike traditional vaccine which need cold chain storage which increases the yearly cost to preserve vaccines and limits their availability to areas having cold storage facility (Nochi et al. 2007)
(g) A concern with oral vaccines is the degradation of protein components in the stomach (due to low pH and gastric enzymes) and gut before they can elicit immune responses (Daniell et al. 2001b) but in plant edible vaccines bioencapsulation of antigen in the rigid plant cell walls could provide protection from intestinal degradation (Webster et al. 2002).
(h) For production of edible vaccines, costly equipments and machines are not necessary as they could be easily grown in farms as compared to cell culture grown in fermenters.

(i) Plant derived vaccines can be designed to contain numerous antigens for various disease. These multicomponent vaccines are called second generation vaccines and provide immunization against many diseases in a single dose.
(j) An edible vaccine provides higher safety of individual as compared to traditional vaccine as edible vaccines are subunit preparation and do not involve attenuated pathogens which sometimes causes disease when administered as vaccine.
(k) They can be ingested by eating the plant/part of the plant. So, the need to process and purify does not arise.
(l) If the local/native crop of a particular area is engineered to produce the vaccine, then the need for transportation and distribution can be eliminated.
(m) Plant cells are suitable for vaccine production due to their capability to correctly fold and assemble, not only antibody fragments and single chain peptides, but also full-length multimeric proteins.
(n) Low downstream processing requirements for proteins administered orally.
(o) As the antigen is present in edible plant product and can be administered by directly eating the plant tissue, it surpasses the purification requirement which otherwise is a costly process.
(p) New or multiple transgenes can be introduced by sexual crossing of plants thus creating novel vaccines against multiple diseases.
(q) As plants are a safe biological system and harbor no pathogen of human infections, there are no ethical problems associated with plant edible vaccines unlike the vaccines produced from animal cell cultures and transgenic animals.
(r) Expression of antigen in plant seeds provides a convenient system of vaccine storage for long time duration thus reducing storage and shipping costs under ambient conditions.
(s) Plant cells are able to perform complex posttranslational modification of recombinant proteins, such as glycosylation and disulfide bridging that are often essential for biological activity of many mammalian proteins, allowing for the retention of native biological activity (Lienard et al. 2007; Rybicki 2010; Tremblay et al. 2010)

10.6 Applications of Plant Edible Vaccine

The first report of the concept of using a plant expression system for the production of an edible vaccine appeared in a patent application published under the International Patent Cooperation Treaty (Curtiss and Cardineau 1990). The study aimed to express a surface protein (spaA) from *Streptococcus mutans* in tobacco plants to a level of approximately 0.02 % of the total leaf protein. The gene for spa production was introduced in tobacco through *Agrobacterium*-mediated transformation. Mice when feeded on this transgenic tobacco tissue showed oral immunogenicity to spaA protein. However these mice were not further challenged directly with the pathogen but the antibodies recovered from the mice serum demonstrated biological activity

against intact *S. mutans*. However much needed impetus to plant derived vaccine research was given by Arntzen and co workers in 1992 by presenting the concept of plant edible vaccines. In same year an important research paper was published by Mason et al. on the expression of hepatitis B surface antigen (HBsAg) in tobacco plants (Mason et al. 1992). In parallel with evaluation of plant-derived Hepatitis B surface antigen, Mason and Arntzen explored plant expression of other vaccine candidates including the labile toxin B subunit (LT-B) of entertotoxigenic *Escherichia coli* (ETEC) and the capsid protein of Norwalk virus (NVCP). It was found that the plant derived proteins correctly assembled into functional oligomers that could elicit the expected immune responses when given orally to animals (Haq et al. 1995; Mason et al. 1996, 1998). Subsequently, a number of attempts were made to express various antigens in plants (Table 10.1).

Table 10.1 List of various antigens successfully expressed in plant system

Disease	Antigen	Plant system	Expression	Reference
Hepatitis B	HBsAg	Potato	HEV-E2 gene correctly expressed and antigen had normal immuno-activity in mice	Richter et al. (2000)
Human rotavirus	HRV-VP7	Potato	Elicited serum IgG and mucosal IgA by oral delivery to mice	Wu et al. (2003)
Measels	Loop forming B cell epitope (H386-400)	Carrot	Intraperitonal immuni-zation of mice with carrot extracts induced high titers of antibodies	Bouche et al. (2003)
Tetanus	Tet C	Tobbaco chloroplast	Intranasal immunization resulted in antibody induction	Tregoning et al. (2004)
Diarrhea	PEDV-COE	TMV via Tobacco	Induced both systemic and mucosal immune responses by feeding to mice	Kang et al. (2004)
Cholera	CTB-gp120	Potato	Induced significantly greater immune responses in mice	Kim et al. (2004)
Anthrax	PA (protective antigen)	Tobbaco	PA from transformed tobacco plants showed similar biological activity as native PA	Aziz et al. (2002)
Cholera	CTB	Tomato	CTB protein expression in ripening tomato was 0.081 % which induced both mucosal and serum antibodies in mice	Jiang et al. (2007)

(continued)

Table 10.1 (continued)

Disease	Antigen	Plant system	Expression	Reference
Cervical human papillomavirus-like particles (HPV) disease	HPV type 11 L1 major capsid protein	Potato	Oral immunization induced anti-VLP immune response in mice	Warzecha et al. (2003)
Respiratory syncytial virus (RSV)	RSV-F protein	Tomato	Oral immunization of mice induce both serum and mucosal RSV-F specific antibodies	Sandhu et al. (2000)
Gastroentritis	S (N-gS) from Transmissible gastroenteritis coronavirus (TGEV)	Potato	Intraperitonal immunization in mice resulted in serum IgG specific for TGEV, oral immunization through direct feeding developed serum antibodies specific for gS protein	Gómez et al. (2000)
Hepatitis B	HBsAg	Banana	HBsAg derived from transgenic banana leaves was 1.146 g/ml and is similar to human serum derived one in buoyant density properties	Kumar et al. (2005)
Hepatitis C	HVR1	Tobbaco	Intranasal immunization of mice elicited both anti-CTB serum antibody and anti-HVR1 serum antibody	Nemchinov et al. (2000)

10.6.1 Hepatitis B

Hepatitis B virus (HBV) is one of the major causes of chronic viremia in humans (Purcell 1994). The hepatitis B virus is estimated to have infected 400 million people throughout the globe, making it one of the most common human pathogens. Since immunization is the only known method to prevent the disease of Hepatitis B, any attempt to reduce its infection requires the availability of large quantities of vaccine, hepatitis B surface antigen (HBsAg) (Malik et al. 2011). Mason et al. 1992 demonstrated the expression of HBsAg at levels equal to 0.01 % of total soluble protein in tobacco. However, The low level of expression of the HBsAg in transgenic tobacco (0.01 % of soluble protein) and the alkaloids present in the crude plant extract prevent direct feeding studies. Thus, Mason et al conducted further studies with tobacco-derived recombinant HBsAg (rHBsAg) protein that was recovered from leaf extracts as a VLP with an average size of 22 nm, which are similar to those

found in the sera of infected humans and in the commercial vaccine (Cabral et al. 1978). These plant-derived VLPs mimic the appearance of recombinant yeast-derived HBsAg particles (Scolnick et al. 1984), which is the material that is used in commercially available recombinant vaccine for hepatitis B (Recombivaxm; distributed by Merck, Sharpe, and Dohme) and can be injected as vaccinogen directly. In addition, the plant-derived material had similar buoyant density and antigenicity to human and yeast-derived HBsAg, indicating faithful preservation of protein folding characteristics in the plant system'. Thus a crude extract of rHBsAg from plants was used in parenteral immunization studies with mice (Thanavala et al. 1995). The extract caused an immune response that was similar to the one achieved with Recombivaxm, So the studies concluded that the rHBsAg from plants demonstrate that B- and T-cell epitopes of HBsAg are preserved when the antigen is expressed in transgenic plants, and that the recombinant antigen is produced as a VLP that mimics the currently available commercial vaccine. Subsequently many papers were published characterizing the recombinant product which assembled into virus like particles (VLPs), and could invoke specific immune responses in mice upon parenteral delivery.

Further studies were made by Arntzen group to prove that plant-derived HBsAg can stimulate mucosal immune responses via the oral route, for which they employed potato tubers as an expression system and optimized it to increase accumulation of the protein in the plant tubers (Richter et al. 2000). The resulting plant material proved superior to the yeast-derived antigen in both priming and boosting of immune responses to oral immunogen in mice (Kong et al. 2001; Richter et al. 2000). The HBsAg has also been expressed in banana (May et al. 1995) and lettuce (Kapusta et al. 1999). Recently Kumar et al. (2005) transformed embryogenic cells of banana with the 's' gene of hepatitis B surface antigen (HBsAg) using *Agrobacterium* mediated transformation. The expression levels of the antigen in the plants grown under in vitro conditions as well as the green house hardened plants were estimated by ELISA. Maximum expression level of 38 ng/g F.W. of leaves was noted in plants transformed with pEFEHBS grown under in vitro conditions, whereas pHER transformed plants grown in the green house showed the maximum expression level of 19.92 ng/g F.W. of leaves. HBsAg obtained by them from transgenic banana plants was found to be similar to human serum derived one in buoyant density properties. Kumar et al. (2005) advocated that although expression levels of the antigen are low in banana fruits, the expression levels of the vaccine antigens can be increased by the use of promoter of abundant pulp protein (Clendennen et al. 1998), or promoters of the proteins found in abundance in the ripe banana fruits (Peumans et al. 2002).

10.6.2 Rabies

McGarvey et al. (1995) have reported the expression of rabies virus (RV) glycoprotein in transgenic tomatoes. Although McGarvey et al. did not reported on the immunogenicity of the tomato RV glycoprotein, Yusibov et al. (1997) have reported the

induction of neutralizing antibodies in mice that were parenterally vaccinated with a peptide of RV glycoprotein fused to a plant virus coat protein. antigen-capsid fusion is one of the alternative strategies of producing a plant-based vaccine where plants are infected with recombinant viruses carrying the desired antigen in viral coat protein. The infected plants have been reported to produce the desired fusion protein in large amounts in a short time. It should, however, be kept in view that recombinant viruses need to be highly purified for parenteral administration or partially purified for oral administration. Modelska et al. (1998) reported that immunization of mice intraperitoneally or orally by gastric incubation or by feeding of plants infected with the recombinant alfalfa mosaic virus (AIMV) carrying rabies peptide CPDrg showed local as well as systemic immune response. After immunization, 40 % of the mice were protected against the challenge with a lethal dose of the virus. This report also demonstrated stronger immune responses in mice that consumed the chimeric viruses *in planta* rather than after purification (Modelska et al. 1998). Recently, Loza-Rubio and coworkers developed transgenic maize expressing the rabies virus glycoprotein of the Vnukovo strain and they evaluated its immunogenicity in mice by the oral route. Animals were fed once with 50 μg of protein and challenged 90-days later with a rabies virus isolated from vampire bats. The edible vaccine induced viral neutralizing antibodies which protected mice 100 % against challenge (Loza-Rubio et al. 2008).

10.6.3 Foot and Mouth Disease

The VP1 capsid protein of the foot-and-mouth disease virus (FMDV) was also successfully expressed in transgenic *Arabidopsis thaliana* (Carrillo et al. 1998). Carrillo and co-workers have shown that mice injected intraperitoneally with the partially purified VP1 protein are totally resistant to a challenge with a virulent strain of the virus. Oral administration of the plant-derived VP1 subunit of FMDV has not been demonstrated. Because FMDV virions are complex structures containing several subunits therefore it is unlikely that the VP1 peptide alone will assemble into stable VLPs.

10.6.4 Cholera

Another important study was regarding Enterotoxigenic *Escherichia coli* (ETEC) and *Vibrio cholera* which are the primary pathogens responsible for acute watery diarrhea. Cholera is a devastating diarrheal disease that has caused recurrent pandemics throughout the world since 1871 (Yu and Langridge 2000). The heat-labile enterotoxin (LT) of ETEC is closely related to cholera toxin (CT). Haq et al. (1995) proved that a bacterial antigen (LT-B) could be expressed in edible tissues of transgenic plants and could assemble into pentameric ring structures (as shown by

its ability to bind gangliosides). In addition, and most importantly, LT-B expressed in plants was shown to induce the production of both serum and mucosal antibodies in mice fed with transgenic potato tubers. However, the low level of expression of LT-B in potato tubers, implied that people would have to eat an unreasonably large amount of tuber to receive the desired dose. Mason et al. (1998) improved expression levels of LT-B in transgenic plants by the construction of a 'plant-friendly' synthetic LT-B gene. The protein product of this synthetic gene accumulates to ~0.15 % of the soluble protein, which is .10-fold higher than the best levels obtained previously by Haq et al. (1995). These higher expression levels have allowed this 'edible vaccine' to be tested in humans, representing the first human clinical trial of a plant-derived vaccine where 100 g of raw potato tubers expressing LT-B of ETEC in three doses had to be consumed in order to overcome digestive losses of the antigen and to elicit a significant immune response (Tacket et al. 1998). These results showed, for the first time, that food plant–based vaccines are immunogenic in humans.

Cholera toxin, which is very similar to *E. coli* LT, has also been expressed in plants. Hein et al. (1996) generated tobacco plants expressing CT-A or CT-B subunits of the toxin. CT-A produced in plant was not cleaved into A1 and A2 subunits, which happens in epithelial cells. While CT-B undergone similar processing in plants as the CT-B derived from *V. cholerae*, and was thus recognized by mouse anti-CT-B antibody. Antigenically it was found to be similar to the bacterial protein. Even after boiling transgenic potato tubers till they became soft, approximately 50 % of the CT-B was present in the pentameric GM_1 ganglioside-binding form (Arakawa et al. 1997, 1998). Higher expression levels (up to 31.1 % TSP) were obtained when CTB-2L21 fusion protein was expressed in transgenic chloroplasts (Molina et al. 2004).

10.6.5 AIDS

According to the WHO, HIV-induced acquired immunodeficiency syndrome (AIDS) kills 1–3 million people annually (UNAIDS 2002). Stable chimeric CPMV particles that express epitopes derived from human rhinovirus 14 and HIV-1 were described by Porta et al. in 1994. The inserted epitopes were immunogenic in rabbits. Initial success was also achieved in splicing HIV protein into CPMV by Prakash (1996). Two HIV protein genes and CaMV as promoter were successfully injected into tomatoes with a needle and the expressed protein was demonstrable by polymerase chain reaction (PCR) in different parts of the plant, including the ripe fruit, as well as in the second generation plant.

10.6.6 Norwalk Virus

Norwalk virus is known to cause acute gastroenteritis in humans. Norwalk virus capsid protein (NVCP) from the diarrhea causing Norwalk virus, expressed in transgenic tobacco and potato with 0.23 % of total soluble protein, also assembled

VLPs and stimulated serum IgG and gut IgA specific for NVCP when fed to mice (Mason et al. 1996). The clinical trial was conducted at the Center for Vaccine Development with NVCP potatoes (Tacket et al. 2000). Twenty adults ingested either two or three doses each of 150 g raw potato containing 215–750 lg NVCP. Nineteen of twenty adults showed significant increases in the numbers of specific anti- NVCP antibody-secreting cells of the IgA subtype, and six developed increases in IgG antibody secreting cells. This study proved that orally delivered plant-expressed VLPs could stimulate immune responses and further that GM1 binding activities not required for oral immunization. These results suggest a new strategy for the development of plant vaccines. Individual soluble protein antigens are relatively ineffective for oral immunization because of intestinal digestion and lack of antigen tropism for GALT (gut associated lymphoid tissues). In contrast, VLPs are stable in the acidic environment of the stomach and resistant to enzyme digestion in the small intestine. Most important, VLPs preserve conformational epitopes located on the surface of viral particles, which are recognized by the host immune system. Compared with other viral antigens, the plant-produced VLP may be the more effective antigen for protection against infectious enteric viral diseases (Yu and Langridge 2000). Attempts are underway to engineer bananas and powdered tomatoes expressing norwalk virus.

10.6.7 Malaria

Over two billion individuals reside in the malaria endemic areas and the disease affects 300–500 million people annually. As a result of malarial-infection, an estimated three million lives are lost annually, among them are over one million children (majority under 5 years of age).The world malaria situation has become significantly worse in recent years as the main forms of malaria control, spraying programmes and chemotherapy, becoming less effective in the development of vector and parasite resistance. In a study by Beachy et al. (1996) a 13-amino-acid epitope of *zona pellucida*, ZP3, protein and another epitope from malarial sporozoites have been expressed as fusion proteins with TMV capsid protein with the idea of developing anti-fertility and anti-malarial vaccines. The antigenicity of the products has been found to be positive. Currently, three malarial antigens are under investigation for the development of plant-based malaria vaccine, merozoite surface protein (MSP) 4,MSP 5 from *Plasmodium falciparum,* and *MSP* 4/5 from *P. yoelli.* Wang et al. (2004) has demonstrated that oral immunization of mice with recombinant MSP 4, MSP 4/5 and MSP1, co-administered with CTB as a mucosal adjuvant, induced antibody responses effective against blood-stage parasite. The study however involved complexity as the protein was expressed in *E. coli* and protection was only evident when high dose antigen was administered. Thus it is still uncertain if the oral delivery of a plant-derived malaria vaccine would induce significant immune responses in humans. It has been suggested that antigen expression level in plants are so low that an unrealistic quantity of plant material would have to be consumed to achieve meaningful immunity. For this approach to become realistic improve

antigenic expression has to be achieved. Moreover, due to high levels of antigen anticipated to be necessary, it is likely that strong adjutants will also be required (Wang et al. 2004). Hence, appropriate adjuvants have to be identified and tested. Finally, in the face of reports showing induction of tolerance or immunity through comparable oral immunizations vaccination regimens must be rigorously tested in preclinical studies (Arakawa et al. 1998).

10.6.8 Autoimmune Diseases

Applications of edible vaccines are expanding to autoimmune diseases where the body's own proteins recognized as foreign by the immune system. Autoimmune diseases include arthritis, myasthenia gravis, multiple sclerosis and type I diabetes. It was established by Arakawa et al. (1998) that food plants are feasible production and delivery systems for immunotolerization against autoimmune diseases. They found cholera toxin B to be useful as a carrier molecule for specific targeting to the gut-associated lymphoid tissue (GALT). A cholera toxin B–insulin fusion protein produced in transgenic potato plants successfully protected non obese diabetic mice from development of autoimmune diabetes mellitus type I.

Ma and Jevnikar (1999) expressed glutamic acid dehydrogenase in potatoes and fed them to non-obese diabetic mice, in which the reduced pancreatic islet inflammation suggested immuno- tolerization of cytotoxic T-cell-mediated autoimmune disease. An optimal oral dose of a plant-derived auto antigen can potentially inhibit development of the autoimmune disease (Carter and Langridge 2002; Sala et al. 2003). Edible vaccine development for the prevention or treatment of cancer is difficult since tumor antigens are also auto-antigens (Zhang et al. 1998). Recently, a poly-epitope isolated from a human melanoma tumor was integrated into the nuclear and chloroplast DNA of tobacco in an attempt to develop a plant-derived melanoma vaccine (Sala et al. 2003). McCormick et al. (1999) expressed a scFv antibody fragment of the immunoglobulin from a mouse B-cell lymphoma in tobacco with a viral vector and showed that mice injected with this vaccine were protected from challenge by a lethal dose of tumor. Another scFv fused to the potato virus X coat protein generated protection against lymphoma and myeloma (Savelyeva et al. 2001).

10.7 Potential Issues to Be Addressed

There were many questions regarding plant edible vaccines when the concept was given by Arntzen and group in 1990s. Some of them were answered in the course of successive development in this field while some still remain unanswered. Even after all the research and developments made in plant derived edible vaccines, there are some limitations and issues which are still to be addressed. A major impediment in the successful application of these vaccines is the low expression level of antigen

and uncertain dosage. In all the studies mentioned above the expression level of antigen in plant tissue was not of practical utilization as to achieve the required immunity very large amount of plant product is need to be consumed. In addition, not all vaccine candidate proteins are highly immunogenic in plant tissues and secondary metabolites found in plants may compromise the ability of the vaccine candidate protein to induce immunity (Teli and Timko 2004). Two solutions to overcome this limitation are being explored. First, techniques to enhance antigen accumulation in plant tissues are being explored. A number of factors, including genes encoding vaccine antigens can be optimized for plant codon use; plant promoters can be engineered to increase transcription levels; RNA splice sites and intron sequences can be removed codon usage, the type of 5′-untranslated sequence incorporated, the presence of specific intra- and extracellular targeting or compartmentalization sequences present, the site of gene integration into the genome, etc. affect transgene expression and ultimately vaccine epitope accumulation in plants. Optimization of coding sequences of bacterial or viral genes for transient expression, as well as defining the best subcellular compartment for product accumulation to obtain optimal quantity and quality, is also being studied (Teli and Timko 2004). To enhance the immunogenicity of the orally delivered antigens, the use of carrier proteins may also be required, especially for small, non-particulate subunit vaccine antigens (Walmsley and Arntzen 2000). Another approach is to use bacterial enterotoxins such as CT or LT (Mason and Arntzen 1995), mammalian and viral immunomodulator, or plant-derived secondary metabolites (Yu and Langridge 2001). Although the constitutive expression of foreign genes may lead to the accumulation of foreign proteins to levels toxic in the plant, inducible promoters, which stimulate gene expression at specific points in plant development, may not only prevent accumulation of toxic levels of the foreign gene product but may conserve the plant's photosynthate for generation of maximum plant growth, resulting in higher yields of recombinant proteins (Arakawa et al. 1997, 1998). Thus, temporal and organ- and tissue-specific promoters activated in fruit, tubers, or seeds must be explored (Fiedler and Conrad 1995).

Another problem is that the glycosylation of trans proteins in plants differs slightly from those produced in transgenic animals or animal cells in vitro (Lerouge et al. 1998). The addition of xylose and change from a b1!6 to a b1!3 linkage of fucose are typical in plants. A significant difference with transprotein production in plants is their inability to add sialic acid to glycoproteins (Lerouge et al. 1998). This sugar has been implicated in longer clearance times for proteins in the blood and therefore is a major factor for a select group of pharmaceutical proteins. However, plant glycan patterns may not represent a problem in terms of human health; they may affect conformational epitopes, or clearance of plant derived antibodies (Bakker et al. 2001; Ma et al. 2005). This potential problem is likely to be overcome as we learn more about the requirements for these processes in both plant and animal cells (Bakker et al. 2001).

Another concern is about the potential contamination of plant-derived recombinant proteins with potentially toxic factors due to the fact that some plant species contain numerous toxic alkaloids and other secondary metabolites. Careful selection of

appropriate plant materials for heterologous expression can help alleviate this potential problem (Teli and Timko 2004).

Another major concern is the possibility of development of immunotolerance to the vaccine protein or peptide. Also there will be a challenge in controlling transgene escape as the identification of "vaccine" fruit vs a normal fruit would be difficult. Fruit vaccines should be easily identifiable to avoid the misadministration of the vaccine, which may lead to complications such as immunotolerance. Also the oral vaccines are complicated by the need to protect the antigen from the effects of the acidic and proteolytic environment of the gut. Hence, to be effective, oral subunit vaccines generally require higher doses than oral replicating vaccines (Walker 1994; Mestecky et al. 1997) Allergic reactions to plant protein glycans and other plant antigens is a challenging issue. It has been suggested that plant derived recombinant proteins or antibodies may have increased immunogenicity or allergenicity as compared to mammalian counterparts (Ma et al. 2005; Penney et al. 2011). This is well explained by the fact that a state of tolerance or energy has been gained by the daily consumption of plant glycolproteins in our food (Ma et al. 2005).

Some other potential problems related to plant edible vaccines include:

(a) Plant and product contamination by mycotoxins, pesticides, herbicides and endogenous metabolites.
(b) Regulatory uncertainty, particularly for proteins requiring approval for human drug use (Doran 2000).
(c) Consistency of dosage from fruit, plant to plant, generation to generation is not similar
(d) Stability of vaccine in fruit is not known and differs from plant to plant.
(e) Some food cannot be eaten raw (e.g. potato) and needs cooking which will denature or weaken the protein present in it (Moss et al. 1999)
(f) Variable conditions for edible vaccine are also a major problem. Potatoes containing vaccine to be stored at 4 °C and could be stored for longer time while a tomato does not last long. Thus these vaccines need to be properly stored to avoid infection through microbial spoilage.

Thus while the plant edible vaccines are a lucrative option in the field of vaccination, there is much remains to be done in this field. With many potential issues to be addressed this area of health care is open for exhaustive research and development.

10.8 Transgenic Chloroplast Systems

Transgenic chloroplasts have become attractive systems for heterologous gene expressions because of unique advantages. Chloroplast genetic engineering offers several advantages, including high levels of transgene expression, transgene containment via maternal inheritance, and multi-gene expression in a single transformation event (Chebolu and Daniell 2009). The highest level of protein expression through transgenic tobacco chloroplast was obtained for *Bacillus thuringiensis* (Bt)

cry2Aa2 protein (46.1 % TSP) (De Cosa et al. 2001). As chloroplasts are genetically semi autonomous having their independent genome, many self replicating copies of transformed plastids can be produced in a single plant cell thus enhancing the antigen/protein yield considerably.

There are several other advantages of chloroplast transformation system over nuclear transformation. Environmental concerns about mixing genetically modified pollen with other crops or weeds have been continually raised (Stokstad and Vogel 2003). This objection may be partially addressed by engineering the foreign gene into the chloroplast DNA (Ruf et al. 2001). As chloroplast genome is maternally inherited it does not follow Mendelian pattern of inheritance (Zhang et al. 2003) so gene pollution caused by transgene escape through pollen can be controlled. Even if the pollen from plants that exhibit maternal inheritance contains metabolically active plastids, the plastid DNA is lost during pollen maturation and is not transmitted to the next generation (Daniell et al. 2002). Therefore, the chloroplast expression system is an environmentally friendly approach. Chaperones present in chloroplasts facilitate correct folding and assembly of monoclonal antibody in transgenic chloroplasts (Daniell et al. 2004) and also result in fully functional human therapeutic proteins, as seen in interferon alpha and gamma (Falconer 2002; Leelavathi and Reddy 2003). Chloroplasts have the ability to process eukaryotic proteins, including correct folding of subunits and formation of disulfide bridges (Daniell et al. 2001b). Chloroplast-synthesized cholera toxin-B subunit binds to the intestinal membrane GM1-ganglioside receptor, thereby confirming the correct folding and disulfide bond formation through functional assay (Daniell et al. 2001a; Molina et al. 2004).

Also, chloroplasts have the ability to express multiple genes in a single transformation event. Expression of polycistrons in transgenic chloroplasts is a unique feature, which facilitates the expression of entire pathways in a single transformation event (De Cosa et al. 2001; Daniell and Dhingra 2002). De Cosa et al. (2001) for the first time expressed a complete bacterial operon in transgenic chloroplasts, resulting in the formation of stable cry2Aa2 crystals. This should facilitate expression of polyvalent vaccines or multisubunit proteins in transgenic chloroplasts (Chebolu and Daniell 2009). Problem of gene silencing is also eliminated in chloroplast expression systems. Inspite of higher expression level gene silencing was not observed in transgenic chloroplast derived plants (De Cosa et al. 2001). Chloroplasts can be a good place to store the biosynthetic products that could otherwise be harmful when accumulated in cytosol (Bogorad 2000). This was demonstrated when cholera toxin B subunit was accumulated in large quantities in transgenic chloroplasts and it had no toxic effect (Daniell et al. 2001a), whereas when accumulated in the cytosol in very small quantities, CTB was toxic (Mason et al. 1998). Similarly, trehalose, was toxic when accumulated in cytosol but was nontoxic when compartmentalized within chloroplasts (Lee et al. 2003).

However chloroplast transformation is also not untouched with certain limitations and issues. As with any fresh tissue molecular farming system, protein stability over time will change even with refrigeration. Extraction and purification must be performed at very specific times following harvest. Tobacco is currently a highly regulated crop and is not edible. Large volume products and edible vaccines would

not appear to be feasible using this system (Horn et al. 2004). Tobacco appears to be the only species in which plastid transformation has been established as routine (Svab and Maliga 1993; Daniell et al. 2002). However, recently Kanamoto et al. (2006) developed plastid transformation system for lettuce.

10.9 Recent Developments

Currently the researches in plant edible vaccine production are emphasizing on good manufacturing practices (GMP) with increased antigen expression being the everlasting objective of this technology. Recently a novel technique developed by Icon Genetics (Bayer Crop science, Germany), based on the Magnifection, has additional advantageous than the routine methods used for the production of subunit vaccines in plants. This system allows very fast production, high recombinant protein expression levels for example hepatitis B virus (HB core) with an accumulation level exceeding 7 % of total soluble protein in tobacco (Gomez et al. 2010; Yusibov and Rabindran 2008; Gleba et al. 2005; Huang et al. 2006).

Recently Alvarez et al. (2010) conducted an important study on enhancing recombinant protein expression in transgenic plants. They suggested that in maize, γ-Zein is the major storage protein synthesized by the rough endoplasmic reticulum (ER) and stored in specialized organelles called protein bodies (PB). Zera® (γ-Zein ER-accumulating domain) is the N-terminal proline-rich domain of c-zein that is sufficient to induce the assembly of PB formation. Fusion of the Zera® domain to proteins of interest results in assembly of dense PB-like, ER-derived organelles, containing high concentration of recombinant protein. To confirm this, they expressed F1-V antigen protein from *Yersinia pestis* (causal pathogen of plague) in three plant models (*Ncotiana benthamiana*, *Medicago sativa* (alfalfa) and *Nicotiana tabacum* NT1 cells) with and without a fused Zera® domain. They found that F1-V protein with fused Zera® domain showed three times more accumulation in plant cells than F1-V protein alone.

The list of diseases that could potentially be prevented with plant-based, edible vaccines is keeping on increasing. Clinical trials have already succeeded in increasing the production of antibodies against HIV in mice (Karasev et al. 2005) On the other hand, soybean has been genetically modified to produce monoclonal antibodies that act as carriers of cancer-attacking compounds. The possibility of developing HIV and cancer vaccines through transgenic plants could be a major step in the fight against these devastating diseases. Highly pathogenic H5N1 avian influenza responsible for global pandemics and with mortality rates exceeding 60 % encouraged global efforts to develop vaccines against this highly pathogenic avian influenza (HPAI). Shoji et al. (2009) recently described the production of HA from the A/Indonesia/05/05 strain of H5N1 influenza virus by transient expression in *Nicotiana benthamiana* plants. They demonstrated that immunization of mice and ferrets with this plant-derived HA protected ferrets against challenge infection with a homologous virus.

Sexually transmitted diseases are also now included in the domain of plant based vaccination. Maclean et al. (2007) have reported the expression of L1 capsid protein from HPV-16. By a transient expression assay, the authors determined not only that

L1 protein was capable of assemble into VLPs, maintaining its immunological properties, but that a human instead of a plant codon usage and the protein-direction to the chloroplast, were the best conditions to achieve high yields of recombinant L1 protein (11 % of TSP in transgenic tobacco). These results showed an efficacious way of producing VLPs onward HPV vaccine, maybe a cheaper one than the new HPV vaccine produced in insect cells. Immunizing experiments are needed to better prove this hypothesis. Obregon and coworkers showed a new strategy for increasing recombinant protein production in HIV by improved the stability of p24 core protein. This stable protein formed dimmers that were retained within the cell resulting in an enhanced expression, apparently related to protein folding processing and assembly, subcellular targeting and protein stability (Obregon et al. 2006). Recently, Saejung et al. (2007) reported, for the first time, the production of a dengue vaccine in plants. Takagi et al. (2005) reported GM rice expressing two T-cell epitope peptides of Cry j I and Cry j II allergens of Japanese cedar (*Cryptomeria japonica*) pollen.

Multicomponent edible plant vaccines providing immunologic protection simultaneously against several infectious diseases are under construction. Recently in this context (Shchelkunov et al. 2006) demonstrated a bivalent vaccine synthesis through a synthetic chimeric gene, TBI-HBS, encoding the immunogenic ENV and GAG epitopes of human immunodeficiency virus (HIV-1) and the surface protein antigen (HBsAg) of hepatitis B virus (HBV), was expressed in tomato plants. Tomato fruits containing the TBI-HBS antigen were fed to experimental mice and, on days 14 and 28 post-feeding, high levels of HIV and HBV-specific antibodies were present in the serum and feces of the test animals. Intraperitoneal injection of a DNA vaccine directing synthesis of the same TBI-HBsAg antigen boosted the antibody response to HIV in the blood serum; however, it had no effect on the high level of antibodies produced to HBV. Although no plant-based edible vaccines are currently commercially available, a secretory antibody vaccine was approved in the EU, a poultry vaccine against Newcastle disease was approved by the USDA, and a hepatitis B virus vaccine using a tobacco plant has been approved in Cuba (Kim and Yang 2010). Vaccines for diarrhea, hepatitis B and rabies, and antibodies for non-Hodgkin's lymphoma, colorectal cancer and dental caries, have been submitted for phase I or phase II clinical trials in humans (Ma et al. 2005). Korban and colleagues at the University of Illinois (Urbana-Champaign, Ill.) have reported a plant-based oral vaccine against respiratory syncytial virus (RSV) in tomato fruit (Korban et al. 2002) with the ultimate aim of moving the product into apple. HSV is a viral pathogen that causes respiratory diseases and is a leading cause of viral lower respiratory tract illness in infants and children worldwide. Aziz et al. (2002) paved way for developing edible vaccines against anthrax by successfully expressing the protective antigen against anthrax in potato tubers.

10.10 Conclusions

Plant edible vaccines have the potential to change the whole scenario of vaccination. Upto few years ago vaccination was only limited to six deadly diseases in children but now not only human but animal diseases of bacterial, viral even protozoan origin

are being successfully studied for vaccination. Not only this but the researches are ongoing to provide plant based vaccines for autoimmune diseases like diabetes and cancer which has risen the need of these vaccines for developed countries also which till now were emphasized to be important majorly for developing countries. Apart from many advantages of these vaccines there are some very relevant social, environmental and ethical issues concerning them which are need to be addressed. Besides future research is needed to overcome limitations like low expression, immunotolerance, glycosylation, immunogenicity, and stability of the transproteins if the practical application of these vaccines is to be realized. One very important point is the proper coordination between academia and industry to help these vaccines reach people. Both technical and regulatory hurdles have to be overcome. It will be a challenge to create a positive public perception regarding safety and efficacy of these vaccines after all the fuss created over the safety issues of transgenic crops during last few years. Lastly timely funding for this research and participation of corporate giant will certainly help in making this dream a reality soon.

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Chapter 11
Malaria and Phytomedicine

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11.1 Introduction

Malaria is one of the most important parasitic infections, accounting for an estimated 500 million clinical attacks worldwide and more than one million deaths per year, mostly in Sub-Saharan Africa (Greenwood et al. 2005). Evidences are there that approximately 1,000,000 children die every year from malaria related anemia and 600,000 children lend to cerebral malaria with a case fatality rate of 20 % (Carme et al. 1993; Guyatt et al. 2001; Murphy et al. 2001). Placental malaria also contributes 400,000 deaths annually to total malaria mortality (Steketee et al. 2001). Severe anemia, cerebral malaria, respiratory distress syndrome, and low birth weight are among the complications that contribute to 25–30 % malaria-attributable deaths for children under 5 years of age in Africa (Murphy et al. 2001).

In India there were 1.49 million cases and 767 deaths due to malaria in the year 2010 (Khan et al. 2012). Malaria is associated with seasonally warm semi-arid areas where nearly 124 million people are considered at risk of climate-related malaria (Worrall et al. 2004). Most of the cases of malaria in India occur in Orissa. Orissa has a population of 36.7 million (3.5 % of India), it contributes 25 % of a total of

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1.5–2.0 million reported malaria cases annually, 39.5 % of *Plasmodium falciparum* malaria, and 30 % of deaths caused by malaria in India. Uttar Pradesh (UP), India's largest state, contributes only 5 % of total cases (Raza et al. 2009).

Antimalarial treatment consists of few currently available anti-malarial drugs, such as artesunate/amodiaquine, artesunate/sulphadoxine pyrimethamine (SP), artemether/lumefantrine (Willcox et al. 2011).

Due to the development of resistance against chloroquine (CQ), the mainstay of malaria treatment worldwide has enhanced morbidity and mortality due to malaria especially in Sub-Saharan Africa (Trape 2001; Wongsrichanalai et al. 2002). Evidences are present showing diminished efficacies of artemisinin combination therapy (ACT) in South-East Asia (Noedl et al. 2008). Analyzing the current situation of development of anti-malarial drug resistance we have to consider all options for the development of new anti-malarials. Two major families of traditional medicinal plants are still providing the major source of anti-malarials, these are artemisinin and quinine. Drug resistance is a major problem in the treatment and prophylaxis of malaria. Previously, resistance was reported against all antimalarials except artemisinins and its derivatives, but recently resistance against artemisinin has also been reported (Noedl et al. 2008; Dondorp et al. 2009). Resistance against chloroquine has been reported in *P. falciparum* wherever falciparum-malaria is endemic (Hastings et al. 2000). In India resistance against chloroquine has been reported in Karbi-Anglong and Nocogang districts in Assam in 1973 and 1974 respectively (Sehgal et al. 1973). Now it has covered all over the India. Sulphadoxime-Pyrimethamne (SP), once used as a drug of choice for chloroquine resistant malaria, also acquired resistance. The resistance to SP was first reported from Thai-Cambodian border in 1960s (La Medicina Tropacalis 1987). Since then SP resistance has been reported from large parts of South-East Asia, Southern China and Amazon basin (Aramburu et al. 1999; Vasconcelos et al. 2000).

In India resistance to sulpha drugs have been documented from falciparum-predominant areas like North-Eastern states and Orissa. Resistance to SP was first documented in India by Chaudhry et al. 1987 at Delhi, India (Chaudhury et al. 1987). The first quinine resistance case was reported from South America nearly half a century ago from Thai-Cambodian border in mid 1960s. In India resistance has emerged against quinine in North-Eastern states and Kolar district of Karnataka (Pickard et al. 2002). Resistance to mefloquine in India was first documented in Surat district of Gujarat (Mishra 1996). Artemisinin and its derivatives are the newest and most effective antimalarial drugs. The reduced susceptibility to falciparum strains to artemisinin derivatives has been recently reported in Cambodia-Thailand border (Noedl et al. 2008; Dondorp et al. 2009).

Keeping all these facts in mind, there is an urgent need to develop new antimalarial. In India plants and plant products are being used as medicines since antiquity. More than 80 % of World's population, mostly in developing world, depend on plant based medicines for their primary healthcare needs (WHO 1993). Potency, efficacy and safety profile of phytomedicine has attracted the pharmaceutical population towards herbal plant research. There are reports of increasing demand from medicinal plant (Kotnis et al. 2004). In India, practice of plants for treating various

illnesses of both man and animals is prevalent. Also, India is blessed with a wide variety of plants having medicinal values and these plants are widely used by all sections of community either directly as folk remedies or indirectly as pharmaceutical preparation of modern medicine (Bhagwati Uniyal 2003).

11.2 Development of Phytomedicine

According to the World Health Organization (WHO), phytomedicine is defined as herbal preparations produced by subjecting plant materials to extraction, fractionation, purification, concentration or other physical or biological processes (WHO 2001). These preparations may be produced for immediate consumption or as the basis for other herbal products. Such plant products may also contain recipient or inert ingredients, in addition to the active ingredients.

The first generations of phytomedicine were simple botanical materials employed in more or less crude form. These medicines such as *Cinchona, Opium, Belladonna* and *Aloe* were selected based on empirical evidences as gathered by the then traditional medicine practitioners. The second-generation phytomedicinal agents were pure molecules whose compounds differ from the synthetic therapeutic agent only in their origin, for example taxol from *Taxus* spp., quinine from *Cinchona* and reserpine from *Rauvolfia* spp (Iwu et al. 1999). In the development of third generation of phytomedicine, the formulation is based on well-controlled double-blind clinical trial and toxicological studies to improve the quality, efficacy, potency, stability and the safety of the preparations (Akerele 1993; Petrovick et al. 1999). The development of resistance by *Plasmodium falciparum* to conventional drugs poses a threat to malaria control. There is therefore a need to find new, effective, and affordable remedies for malaria, including those derived from plants.

Antimalarials of plant origin can be divided into two broad categories; one is highly active compounds of complex structure, which are very difficult to synthesize and another is of moderate to low activity but of relatively simple structures and that is why they are easy to synthesize. The plants belonging to first category are potential candidates for the development of antimalarial phytomedicine, while the second category would represent the template to synthetic drugs.

11.3 Reverse Pharmacology

The reverse pharmacology approach was coined in India to develop phytomedicine from traditional medicine. This concept is championed by the Chinese in the 1950s (Lei et al. 2004). This concept is still going on to isolate compounds from traditional medicine (Patwardhan et al. 2009). Reverse pharmacology concept is tested to standardized phytomedicine in which clinical evaluation is prioritized from the beginning. Isolation of active principle is done at the end of pathway especially for

the purpose of quality control, agronomic selection and standardization, if it is justified by clinical results. This concept led to the new antimalarial phytomedicine from traditional herbal remedy, *Argemone maxicana* popularly known as PEELI KANTELI in India. This is in the process of being approved in Mali (Willcox et al. 2011).

11.4 Currently Available Phytomedicines Against Malaria

Quinine, the oldest (seventeenth century) herbal antimalarial, was extracted from the bark of cinchona tree. The medicinal properties of cinchona tree, indigenous to Peru and Bolivia, were originally discovered by Quechua.

The active principle extracted from Chinese herb *Artemisia annuna*, *A. gorgonum* shows antimalarial activity. This antiplasmodial activity is due to sesquiterpene lactone, arborescin (2 % in the volatile oil) (Ortet et al. 2008).

Atovaquone (Malarone®), which is a synthetic compound (2-alkyl-3-hydroxynaphthoquinone) is an analogue of lapachol from the *Tabebuia species* (Bignoniaceae) (Oliveira et al. 2009).

11.5 Potential Candidates

Fagara zanthoxyloides is an indigenous plant found in West Africa. It is widely used as chewing stick for tooth cleaning there. The roots of this plant contains fagaronine, an alkaloid, that showed in-vitro schizonticidal activity of *Plasmodium falciparum* (Olakunle et al. 2005). Fagaronine also showed activity against growth and cell differentiation of human erythroleukemia K562 cells and of L1210 murine leukemia cells (Prado et al. 2004).

Ethanol extract of the whole plant, leaves and roots at concentrations of 25–50 µg/mL of *Bidens pilosa* (Asteraceae), in Brazil showed in-vivo antimalarial activity (Brandão et al. 1997; Oliveira et al. 2004).

A member of flacourtiaceae, popularly known as guacatonga (*Casearia sylvestris*), has significant antiplasmodial activity of stemwood and rootwood (de Mesquita et al. 2007).

Leaves of *Virola surinamensis* contain an essential oil. Inhalation of vapour produced by *Virola surinamensis* is used as an antimalarial treatment as folk medicine in Amazonian riverbanks. The essential oil of the leaves (100 µg/mL) causes 100 % growth Inhibition from the young trophozoite to schizont and sesquiterpenoid nerolidol was identified as one of the active principle (Lopes et al. 1999).

Also, in-vitro synergistic effects were observed among different extracts from different plants, one such example is synergism between *Mitragyna inermis* (total alkaloids + ursolic acid), *Nauclea latifolia* (total alkaloids) and *Feretia apodanthera*

(methanol fraction) together with *Guiera senegalensis* (harman + tetrahydroharman) in different combinations (Azas et al. 2002). This research suggests that these associations can be considered as good candidates for antimalarial combination therapy.

The leaves and stem bark of *Harungana madagascariensis* (Hypericaceae) of Guinea-Bissau are used for the treatment of anaemia, nephrosis, malaria, gastrointestinal disorders, irregular menstruation, dysentery, female infertility pharyngitis and fever (Jiofack et al. 2009). The stem bark ethanol extract showed significant antimalarial activity, thus indicating their positive role and justifying the use of this species in traditional medicine (Iwalewa et al. 2008). According to Lenta et al. 2007, extracts from *Harungana madagascariensis* shows antimalarial activity against the W2 strain of *P. falciparum*, of which bazouanthrone (IC50 of 1.80 mM) was the most active (Lenta et al. 2007).

In North Angola, a popular shrub (*Bridelia ferruginea*) is used in the treatment of malaria. Kolawole & Adesoye carried out a study to evaluate the antimalarial activity of the species with a sensitive strain of *P. berghei* (Kolawole and Adesoye 2010). Another popular plant root (*Cochlospermum angolense*) in Angola used in the prophylaxis of malaria, also exhibited in vitro activity against *P. falciparum* (Presber et al. 1992).

Piper umbellatum of family Piperaceae, common species in Angola, Mozambique and Brazil, especially in the Amazon region has been used to treat malaria and there has been a study describing the active compound of this species (Roersch 2010; de Andrade-Neto et al. 2008). The leaves, top and roots of *P. umbellatum* are also used in the treatment of poisoning, foetal malpresentation, filariasis, rheumatism, haemorrhoids, metaphysical power and dysmenorrhoea (Jiofack et al. 2009).

Becker et al. (2000) observed in vitro antiplasmodiaal activity of *Dicoma anomala* subsp. gerradii (Asteraceae). The active principle was dehyrobrachylaenolide. This compound was found active against the D 10 (Chloroquine sensitive) and K1 (Chloroquine resistant) strain of *P. falciparum* (Becker et al. 2000).

Recently Habila et al. (2011) observed and isolated active principle from *Szygium aromaticum* (clove), the Olenolic acid (JH 16) and attached this olenolic acid to cinnamic acid resulting cinnamic 3 β- hydroxyolean-12-en-28 carboxylic anhydride (JH 26). JH 26 has significant antimalarial activity (in vivo) against standard antimalarials (Habila et al. 2011).

Distephanus angulifolius (Asteraceae), commonly known as "Trailing Vernonia" a scrambling shrub, in South Africa is found from the Eastern Cape to Mozambique. The isolated compounds showed IC50 values in the range 1.6–3.8 μM and 2.1–4.9 μM against chloroquine sensitive D10 and chloroquine resistant W2 *P. falciparum* strains, respectively (Pedersen et al. 2009).

Leaves extracts of *Scleria striatinux* (Cyperaceae), a local spice in parts of Cameroon, was shown to exhibit low activity against both D6 chloroquine-sensitive (IC50 = 80.4 μg/mL) and W2 resistant (IC50 = 89.4 μg/mL) strains of *P. falciparum* (Efange et al. 2009).

A member of family lamiaceae (South Africa), the Salvia radula, exhibited the best antiplasmodial activity among various species of Salvia (Kamatou et al. 2008).

The active principle from *Carpesium rosulatum* (Asteraceae), the Ineupatorolide, which is chloroform soluble, was found to have high in vitro antimalarial activity (0.019 μM) against the chloroquine resistant D10 strain of *P. falciparum* (Moon 2007). It also showed schizonticidal activity against *P. berghei* (Chung et al. 2008).

Mohamad et al. 2009, isolated limonoids named ceramicins from *Chisocheton ceramicus* (Family Meliaceae) A, B, C, and D in which ceamicin B showed most potent in vitro antiplasmodial activity against *P. falciparum* strain 3D7 (IC50 = 0.56 μM), whereas ceramicin C and D exhibited a relatively good activity (IC50 = 4.83 μM, IC50 = 5.06 μM respectively) and ceramicin A displayed a weak activity (IC50 = 100.37 μM) (Mohamad et al. 2009).

The ethanol extract of *Zhumeria majdae* (Labiateae) showed good antiplasmodial activity in vitro against chloroquine sensitive (D6, Sierra Leone) and chloroquine resistant (W2, Indo China) strains of *P. falciparum*, with IC50 values of 8.8 and 7.5 μg/mL, respectively. The active principle extracted was 12,16-dideoxy aegyptinone B, which exhibited a significant antiplasmodial activity with IC50 values of 4.4 and 4.7 μM against D6 and W2 strains, respectively (Moein et al. 2008).

Leaf extracts of *Piptadenia pervillei* Vatke (Fabaceae) demonstrated potent antimalarial activity. The active principle were four phenolic compounds, (+)-catechin, (+)-catechin 5-gallate, (+)-catechin 3-gallate and ethyl gallate. Compounds (+)-catechin 5-gallate and (+)-catechin 3-gallate displayed the highest in vitro activity against the chloroquine resistant strain FcB1 of *P. falciparum* with IC50 values of 1.2 μM and 1.0 μM, respectively, and no significant cytotoxicity against the human embryonic lung cells MRC-5 was measured (Ramanandraibe et al. 2008).

11.6 Concluding Remarks and Future Trends

Medicines from plant origin have been used since long for the treatment of malaria and other fevers. Two main groups of phytomedicines are used to treat malaria fevers. These groups are artemisinin and quinine derivatives. Antimalarial drug resistance is an alarming situation in the treatment of malaria. This makes the treatment of malaria very difficult with modern medicines (White 2004). In this situation phytomedicines can become an important source of treatment module of drug resistant malaria. Since antiquity, plants have been important weapons to offence malarial parasites. Therefore plants may provide safe, effective and alternate treatment protocol for malarial parasite (Varughese et al. 2010). Any medicine, whether phytomedicine or modern medicine, has its limitations for its use. Most of the modern medicine has its experimental animal and human trial data but traditional medicines do not have such type of data. Phytomedicine has few clinical data on safety and efficacy, optimum dosages, concentration of active principle in a plant species. In spite of all these, limitations can be subsidised through research. Thus, the Research Initiative on Traditional Antimalarial Methods (RITAM) was founded in 1999 with the aim of nurturing research on phytomedicine for malaria (Bodeker et al. 2000), RITAM has conducted systemic literature, reviews and also prepared guidelines to

standardise and improve the quality of phytomedicine against malaria and also plant based insect repellent and vector control. Two main challenges are encountered in the field of phytomedicine and malaria, first is search of new chemical entities (NCE) of natural or synthetic origin and second is development of phytomedicine (Oliveira et al. 2009). On the bases of facts and results of in vivo and in vitro activity against malarial parasite there is no doubt about the possibility of discovery of antimalarials of plant origin, as quinine, artemisinin and atovaquone and their derivatives are remarkable examples of the plant based antimalarials, which are particularly useful for chloroquine resistant strains of plasmodium.

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Chapter 12
In Vitro Conservation Protocols for Some Rare Medicinal Plant Species

Anwar Shahzad and Taiba Saeed

12.1 Introduction

Plants have evolved with the ability to synthesize chemical compounds that help them defend against attack from a wide variety of predators such as insects, fungi and herbivorous mammals. By chance, some of these compounds, whilst being toxic to plant predators, turn out to have beneficial effects when used to treat human diseases. Such secondary metabolites are highly varied in structure, many are aromatic substances, most of which are phenols or their oxygen-substituted derivatives. At least 12,000 of such compounds have been isolated so far; a number estimated to be less than 10 % of the total. Chemical compounds in plants mediate their effects on the human body by binding to receptor molecules present in the body; such processes are identical to those already well understood for conventional drugs and as such herbal medicines do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicines to be in principle just as effective as conventional medicines but also gives them the same potential to cause harmful side effects. Many of the herbs and spices used by humans to season food, yield useful medicinal compounds (Lai and Roy 2004; Tapsell et al. 2006).

Population growth, urbanization and the unrestricted collection of medicinal plants from the wild is resulting in an over-exploitation of natural resources. Therefore, the management of traditional medicinal plant resources has become a matter of urgency. The most frequently used medicinal plants are slow-growing forest trees, bulbous and tuberous plants, with bark and underground parts being the parts mainly utilized. A strategy which would satisfy the requirements of sustainable harvesting, yet simultaneously provide for primary health care needs, would be the

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substitution of bark or underground parts with leaves of the same plant. However, with an ever-increasing global inclination towards herbal medicine, there is not only an obligatory demand for a huge raw material of medicinal plants but also of right stage when the active principles are available in optimum quantities at the requisite time for standardisation of herbal preparations (see Chaturvedi et al. 2007).

The World Health Organisation estimates that some 80 % of the developing world relies on traditional medicines and that, of these, 85 % use plants or their extracts as the active substance (Sheldon et al. 1998). This means that close to three billion people rely on plants for medicine. Ours is an era of profound changes on the surface of earth, changes driven by an unprecedented level of human demands on the resources of our planet (Gadgil and Meher-Homji 1986). Most plausible scenarios today suggest that we are likely to lose as much as half of an estimated total of ten million species of living organisms by the end of this century (Ehrlich and Ehrlich 1981; Gadgil and Meher-Homji 1986). Nearly 25 % of the estimated 250,000 species of vascular plants in the world may become extinct within the next 50 years (Raven 1987; Schemske et al. 1994). Since such a loss will be irreversible it is obviously a matter of prime human concern to avert it.

Species with small populations that are not at present endangered or vulnerable but are at risk are called rare. However rare species are those species which are usually localised within restricted geographical areas or habitats or are thinly scattered over a more extensive range (Singh et al. 2006). Conservation of such rare medicinal plants is therefore important to ensure sustainable human development. There are four complementary strategies for biodiversity conservation: *in situ* strategy, *ex situ* strategy, reduction of anthropogenic pressures and rehabilitation of endangered species (Singh et al. 2006). The rapid decline of plant resources due to their conventional use needs *ex-situ* and *in-situ* conservation strategies.

In situ (in site) methods allow conservation to occur with ongoing natural evolutionary processes while *ex situ* conservation includes germplasm banks, common garden archives, seed banks, DNA banks and techniques involving tissue culture, cryopreservation; incorporation of disease, pest and stress resistant traits through genetic transformation and ecological restoration of rare plant species and their populations. *Ex situ* conservation provides a better degree of protection to germplasm compared to *in situ* conservation. However both *in situ* and *ex situ* conservation are complementary and should not be viewed as alternatives (Wang et al. 1993). *Ex situ* conservation has gained international recognition with its inclusion in Article 9 of the Convention on Biological Diversity (Glowka et al. 1994). The intervention of Biotechnology or to be precise, plant tissue culture for accelerating clonal multiplication of desired rare medicinal plants through micropropagation and their conservation through establishing Tissue Banks are warranted in the right earnest. *In vitro* propagation of rare and threatened plants is generally undertaken to enhance the biomass and conserve the germplasm especially when populations number are low in the wild. *In vitro* culture techniques have been used in many germplasm repositories all over the world to supplement other *ex situ* methods for conservation of plant species particularly those which are either vegetatively propagated, produce recalcitrant seeds or are rare/endangered (Bapat et al. 2008).

Tissue culture is the culture and maintenance of plant cells or organs in sterile, nutritionally and environmentally supportive conditions. In commercial settings, tissue culture is primarily used for plant propagation and is often referred to as Micropropagation (George et al. 2008). The controlled conditions give the culture a sustainable microenvironment for the successful growth (Yadav et al. 2009). Plant tissue culture now has direct commercial applications as well as value in basic research into cell biology, genetics and biochemistry. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation which include

1. The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits, to produce mature plants and multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
2. Regeneration of whole plants from plant cells that have been genetically modified.
3. Production of plants from seeds that have very low chances of germinating and growing, i.e.: orchids and nepenthes. Other applications like germplasm maintenance, hybrid productions for incompatible species, production of haploid plants etc. (Sunderland and Cocking 1978; Georgiev et al. 2009).

The main areas of research in plant tissue culture viz. micropropagation, anther and microspore culture, somaclonal variations and mutagenesis, protoplast culture and somatic hybridisation are some of the effective tools for regeneration and conservation of endangered plants (Bapat et al. 2008). Production of phytochemicals from cell cultures is advantageous and *in vitro* studies on secondary metabolites, biotransformation, cryopreservation of valuable cell lines, immobilisation and understanding enzymatic pathways will generate new data as well as counter the reduction of production on medicinal plants (Bapat et al. 2008).

12.2 Clonal Multiplication of Some Rare Medicinal Plants

The development of reliable *in vitro* protocols are of great importance for conservation of rare and threatened plant species by virtue of producing uniform planting material for offsetting the pressure on the natural populations especially for medicinal and ornamental plants. Concerted international and national efforts have been initiated to conserve and to sustainably use the biodiversity. Significant *in vitro* protocols developed for some selected rare medicinal plants for the feasibility of their large scale propagation have been summarised below:

12.2.1 Rotula aquatica Lour.

R. aquatica (Boraginaceae) is a rare rhoeophytic woody aromatic medicinal shrub with virgate branches. It is distributed in India, Srilanka, Tropical South East Asia and Latin America. The plant inhabits sandy and rocky beds of streams. The plant

is a mandatory component of many ayurvedic drug preparations. For example, the root tuber which is the officially recognised component of these natural medicines, is astringent, bitter, diuretic, laxative for piles and is also useful in treating coughs, cardiac disorders, dysuria, blood disorders, fever, ulcers and uterine diseases (Sivarajan and Balachandran 1994). The decoction of root is used for treating stones in the bladder and veneral diseases. The root tubers contain the sterol, rhabdiol and the ureide allantoin (Anonymous 1972). In the Philippines, a decoction of the stem is used as a diuretic and sudorific. The tough split stems of the plant can be made into ropes (Anonymous 1972).

Sebastian et al. (2002) developed a propagation protocol using nodal explants from mature plants. Maximum number of shoots per node was achieved on Woody Plant Medium (Lloyd and McCown 1981) supplemented with 6.0 mg/l BAP. Rooting was obtained on ½ strength WPM supplemented with 0.5 mg/l IAA, which showed 5.7 ± 0.14 roots. Seventy per cent survival of the plantlets was recorded. Martin (2003a) reported axillary bud multiplication and indirect organogenesis. Maximum number of shoots (15) were obtained in combination of BA (1.0 mg/l) with IBA (0.5 mg/l) in MS medium through axillary bud multiplication. Chithra et al. (2005) described indirect somatic embryogenesis and encapsulation of somatic embryos from internode and leaf explants. Among the above stated protocols, the best protocol given by Martin (2003a) and Chithra et al. (2005) is as follows:

Martin (2003a)

1. Young shoots from mature plant of *Rotula aquatica* were collected and washed under running tap water followed by washing with detergent Extran (5 % v/v), for 5–10 min.
2. Surface sterilisation of the source tissues was done with 0.5 % (w/v) mercuric chloride solution for 7–10 min followed by several washing with sterile distilled water.
3. After that, nodal segments were cut into appropriate sizes (1–1.5 cm) and inoculated on sterile MS medium supplemented with various growth regulators NAA, IBA, IAA, BA, KN at varying concentrations.
4. All concentrations of BA and KN either alone or in combination facilitated axillary bud initiation, with BA being more efficient than KN with respect to initiation and subsequent proliferation of axillary buds.
5. BA (1.0 mg/l) + IBA (0.5 mg/l) was the most effective concentration for axillary bud proliferation, resulting in 15 shoots per node explant.
6. Subculturing was done on the same medium which enhance the rate of shoot multiplication, with the result that more than 20 shoots was developed. No decline in shoot number was observed, even upto the seventh culture however repeated subculturing results in stunted growth and fasciation of shoots. Further transferring the shoots on MS medium supplemented with a lower concentration of BA (0.5 mg/l) facilitates elongation of shoots.
7. For indirect organogenesis, transfer the callus that develop from the lower cut end of nodal explants on MS medium at all concentrations of BA either alone or in combination with an auxin, onto the half strength MS medium with

BA (1.0 mg/l)+ KN (0.5 mg/l) which resulted in maximum shoot regeneration (11.3 shoots) within 40 days.

8. *In vitro* rooting: Transfer the healthy shoots to full or half strength MS medium supplemented with growth regulators for rhizogenesis. NAA was found to be superior over IAA and IBA with respect to the induction of roots. Half strength MS medium supplemented with 0.5 mg/l NAA induce maximum number of roots i.e. 6.2 roots per shoot with well branched roots having hairs.
9. *Ex vitro* rooting can also be carried out in the following two ways:
   (a) By dipping the basal end of *in vitro* grown shoots in NAA, IBA, IAA (0.1–1.0 mg/l) solution for 25 days. NAA (0.5 mg/l) was found to be superior than other auxins, inducing a mean of 5.6 roots per shoot within 25 days. Rooted shoots were than transplanted to small pots with 75 % survival rate in greenhouse.
   (b) By directly transferring *in vitro* grown shoots to small pots filled with soil and sand (1:1) and maintaining them in greenhouse with 65 % survival rate.

Somatic Embryogenesis by Chithra et al. (2005):

1. Leaf and internode segments of *Rotula aquatica* were excised from young shoots. Washing was carried out similarly as given in the above protocol.
2. Place the sterilised explants on MS medium supplemented with BA, KN, 2,4-D or NAA at different concentrations either alone or in combination. All concentrations of different growth regulators either singly or in combination induces callus mainly from the cut ends of internode and leaf explants.
3. Friable callus obtained on MS medium with lower concentrations of NAA (0.54 or 2.69 μM) or 2,4-D (0.45 or 2.26 μM) were found to be suitable for the induction of somatic embryogenesis. Transfer these calli to half strength MS solid medium supplemented with the same concentration of NAA or 2,4-D on which the callus turned embryogenic.
4. Subsequent culture of embryogenic callus to half strength MS suspension containing 0.23 μM 2,4-D induces the highest number of somatic embryos; a mean of 25.6 embryos per 100 mg of callus. The embryo on this medium showed development upto the early torpedo stage.
5. Now transfer the torpedo stage embryos from liquid medium to half strength MS basal solid medium which allows development of 50 % of the torpedo stage embryos to the cotyledonary stage. Cotyledons of the embryo were initially white and later turned green. 90 % cotyledonary stage embryos undergo conversion to plantlets on the same medium.
6. Cotyledonary embryos were also encapsulated using 3 % sodium alginate and 50 mM $CaCl_2$ solution. The encapsulated beads were transparent and moderately hard and when cultured on half strength MS medium underwent 100 % germination. The embryos encapsulated in alginate beads became green within 7 days and gets converted to plantlets within 30 days.
7. Somatic embryos derived healthy plantlets were transplanted into thermocol cups and kept at room temperature, where they resume growth within 12 days and grew well. These plantlets exhibited 95 % survival rate and field established plantlets exhibited morphological characters similar to that of the source plant.

12.2.2 Ceropegia candelabrum L.

Ceropegia candelabrum L., (Asclepediaceae) the 'glabrous goglet flower' is a rare twiny, perennial medicinal herb found at the edges of moist deciduous forests. The officinal part of the plant, the root tuber, is rich in the alkaloid ceropegine (Nadkarni 1976). The tubers are valuable constituents in many traditional Indian ayurvedic drug preparations against many diseases, such as diarrhea and dysentery (Kirtikar and Basu 1935). The tubers that contain starch, sugar, gum, albuminoids, fats, and crude fiber are also useful as a nutritive tonic (Kirtikar and Basu 1935). Propagation of *C. candelabrum* through seed is held back by a low span of viability and a low germination rate of seeds, and scanty and delayed rooting of seedlings. Seed-derived progenies are not true-to type due to cross-pollination. Vegetative propagation by root tubers is tedious and is too low to meet the commercial needs. The development of an efficient method for rapid clonal propagation is important to meet the pharmaceutical needs and for conservation of this valuable rare medicinal plant.

Beena et al. (2003) develop rapid *in vitro* propagation protocol of *C. candelabrum* through axillary bud multiplication while Beena and Martin (2003) reported somatic embryogenesis in this plant. Both protocols are given below:

Beena et al. (2003)

1. Nodal segments of *C. candelabrum* were collected from tender parts of mature plants and washed under running tap water followed by washing with detergent Extran for 5 min. Surface sterilisation was done with 0.5 % $HgCl_2$ solution for 10–14 min followed by washing thoroughly with sterile DDW.
2. Sterilised nodal segments were cultured on Murashige and Skoog (1962) medium supplemented with different growth regulators (BA, KN, NAA, IAA, IBA) at different concentrations either alone or in combination. Nodal explants cultured on MS basal medium induced a mean of two shoots. All concentrations of BA/KN either alone or in combination facilitated axillary bud initiation. BA was the most efficient cytokinin for the axillary bud initiation and subsequent proliferation of axillary buds. BA alone at 6.66 μM induced a mean of four shoots per node explant. KN supplemented medium, resulted in reduced number of shoots, but gave shoots with longer internodes.
3. BA at 8.87 μM in combination with 2.46 μM IBA was most effective for axillary bud multiplication, which developed a mean of eight shoots per node explant. Excision of the node segments from these *in vitro* shoots and its culture facilitated the development of more than 10 shoots/node.
4. Half strength MS growth regulator free medium induced more roots compared to full strength MS. Of the three auxins, IBA was best for inducing roots and was followed by IAA. NAA was poor and was characterized by callus formation. Half strength MS supplemented with 0.49 μM IBA developed a mean of seven roots/shoot.
5. Shoots with well-developed roots transferred directly to small pots containing sterile (soilrite + sand at1:1) revived growth within 15 days. The plantlets exhibited 90 % survival rate in field conditions. Plantlets transferred subsequently to

field conditions grew well and exhibited morphological characters similar to wild plants. They developed root tubers and flowered normally. Starting from a single node explant, 250 rooted *in vitro* shoots could be obtained within a period of 120 days.

Beena and Martin (2003):

1. Leaf and internodal segments were excised from young shoot tips of mature plants and washing procedure was carried out similarly as mentioned in the above protocol.
2. After sterilisation segments were cut into appropriate sizes and were cultured on MS medium supplemented without or with BA, KN, NAA or 2,4-D each at different concentration. The effect of combinations of 2,4-D and BA or KN was also studied. The use of various concentrations of NAA, 2,4-D, BA, and KN alone and combinations of 2,4-D and BA or KN was efficient in inducing callus from both internode and leaf explants, but the texture, amount, and color varied depending on the type and concentration of the growth regulators.
3. Callus developed on MS medium containing 2.26–9.05 μM 2,4-D alone or in combination with 2.22 μM BA or 2.32 μM KN was friable and creamy colored, while the callus grown on other media was hard or semihard and pale green to dark green. Callus grown on MS medium containing growth regulators other than 2,4-D alone did not form somatic embryos. Friable callus developed on MS medium supplemented with 4.52 μM 2,4-D turned embryogenic upon subculture onto the solid half-strength MS medium with 2.26 μM 2,4-D.
4. The embryogenic callus (80 d from establishment) transferred to solid or liquid medium developed somatic embryos at different frequencies. Of all the treatments, half- or quarter-strength MS medium containing 0.23 or 0.45 μM 2,4-D induced somatic embryo formation at the highest frequency. The concentrations of 2,4-D higher than 0.45 μM resulted in callus proliferation. It was found that the presence of low concentrations of 2,4-D (0.23 or 0.45 μM) was mandatory for embryo formation, whereas elimination of 2,4-D was not beneficial for the induction of embryos.
5. Suspension and solid cultures displayed significant difference in the induction of embryos. Suspension cultures induced the highest number of somatic embryos. In this study a 100 mg callus induced more than 500 somatic embryos (5,000 embryos per g callus). The cultures showed globular- to early cotyledonary-stage embryos, but the globular-stage embryos dominated in culture. Late globular embryos showed a small suspensor-like structure, which persisted up to an early heart stage.
6. Upon transfer to quarter-strength MS agar medium without growth regulators, 50 % of somatic embryos underwent maturation and developed into plantlets. It was observed that a few embryos underwent dedifferentiation. The embryo-derived callus later developed somatic embryos (secondary embryogenesis).
7. Somatic embryo-derived plantlets were transferred to small cups containing soil and sand (1:1) and get acclimatized within 10 days. Plantlets were transferred to the

field and 90 % (54 out of 60) survived. Established plantlets were morphologically similar to the source plant. The protocol described in this study facilitates development of 2,500 *C. candelabrum* plantlets per g callus via somatic embryogenesis within 6 months.

12.2.3 Kaempferia galanga L.

Kaempferia (Zingiberaceae) is a genus of rhizomatous herbs distributed in tropics and sub tropics of Asia and Africa. About 10 species occur in India of which *K. galanga* and *K rotunda* are important (Gamble 1956; CSIR 1959*). K. galanga*-commonly called as Black Thorn(Eng); Ekangi or Chandramul(Beng.) is native to India. The plant is an economically important medicinal species used in several ayurvedic preparations (Sadimann 1992). The rhizome extract contains n-pentadecane, ethyl p-methoxy cinnamate, ethyl cinnamate, L- Δ3- careen, camphene, berneol, cineol, P-methoxy styrene, kaempferol and kaempferide (Anonymous 1959a). The methonolic exract of rhizome contains ethyl p-methoxy trans-cinnamate, which is highly cytotoxic to Hela cells (Kosuge et al. 1985). Leaves and flowers contains flavanoides (Ghani 1998a). The rhizome and tubers are used for curing bronchitis, asthma, malaria, skin diseases, wounds and spleenic discords (Kirtikar and Basu 1997). Larvicidal and anticancer principles have also been reported from the rhizome extract (Kiuchi et al. 1987).

Geetha et al. (1997) develop protocol for micropropagation of *K. galanga* using young sprouting buds. Rhizome buds of both the species sprouted within 10 days in MS medium containing 0.5 mg/l KN and 1.5 % sucrose solidified with 0.7 % agar and required number of uniform size explants could be established to carry out the multiplication trials. To identify a suitable medium for multiplication and rooting, MS medium supplemented with NAA (0.5 mg/l and 1.0 mg/l), IBA (0.5 mg/1 and 1.0 mg/l), BA (0.5 mg/l and 1.0 mg/1) and KN (0.5 mg/l and 1.0 mg/l) were tried singly and in combination. Among all the combinations used, NAA at 0.5 mg/l and BA at 1 mg/l were found to be optimum for the two species. In this medium, *K. galanga* produced 8–10 harvestable shoots. Good rooting of 1:10 and 1: 6 was achieved in the same medium. Multiple shoot formation and prolific rooting was achieved in a single medium, so that an additional step was not required for rooting of shoots. Shirin et al. (2000) develop rapid clonal propagation system using rhizomes as explant. *In vitro* plantlet production has been achieved on 0.75 × MS medium supplemented with 12 μM BA + 3 μM NAA. The procedure ensures 13 fold rate of plantlet production in every 4 weeks. Rahman et al. (2005) established an efficient protocol for rapid production of plantlets using rhizome tip and lateral buds explant of the field grown plant. The explants were cultured on MS medium with auxins (NAA, IBA and IAA) and cytokinins (BA and KN). BA (1.0 mg/l) and NAA (0.1 mg/l) was found to be the best medium for multiple shoot induction resulting in 20.50 ± 1.80 shoots when the explant were cultured three times on the same

medium. Kochuthressia et al. (2012) develop multiple shoot induction using rhizome segment as explant. Rhizome explants were cultured on MS medium (Murashige and Skoog 1962) supplemented with various concentrations of different hormones either singly (BA or KN) or in combination (BA + KN). When BA alone was used, the maximum number of shoots (7.14 ± 1.34) was obtained from explants on MS medium with 2 mg/l BA with 88.5 % shoot regeneration response. Maximum multiple shoots (10.85 ± 1.34) with 88.5 % of regeneration response were observed on MS medium supplemented with BA (2.0 mg/l) and KN (1.0 mg/l) and maximum shoot height obtained was 6.14 ± 1.86 cm. No additional step was required for rooting of the shoot. Shoots and roots simultaneously originated in the same medium fortified with BA and KN within 15 days of the second subculture. The protocol developed by Rahman et al. (2005) is given below:

Rahman et al. (2005)

1. Rhizome from field grown plants were collected and washed under running tap water followed by treatment with a solution of antiseptic (Savlon 5 %) for 10 min.
2. Surface sterilisation of the rhizome explants was done with $HgCl_2$ for 12 min followed by 2–3 times rinsing with autoclaved distilled water. After surface sterilisation explant was trimmed to appropriate sizes and cultured on MS medium fortified with BA (0.2–3 mg/l) either individually or in different combinations with auxins, NAA and IBA Multiple shoots were found to develop from rhizome explant within 4 weeks of culture on MS medium supplemented with either BA alone or in combination with NAA or IBA. Among the different concentrations of BA used singly the best response was observed on 1.0 mg/l BA supplemented MS medium with maximum number of shoots (8.50 ± 0.40) and almost 67 % of the culture showed shoot initiation.
3. The best shoot multiplication from the explant was observed on MS medium supplemented with BA (1.0 mg/l) + NAA (0.1 mg/l). In this treatment 100 % of the cultured plants produce multiple shoots where the number of shoots per culture was 20.5 ± 1.80 and average length of the shoots was 6.31 ± 0.24 cm.
4. Microshoots were isolated from the *in vitro* proliferated cluster of shoots and transferred to modified MS medium supplemented with 0.1–1.0 mg/l either of NAA, IBA or IAA. Among the three types of auxin tested, IBA was found to be most effective at different concentrations tested for producing roots on the bases of microshoots. Among different concentrations, IBA 0.2 mg/l resulted in the production of maximum number of roots (12) in 100 % of *in vitro* grown shoots within 6 weeks of culture.
5. Plantlets were transferred gradually to different potting mixture for hardening. About 100 % of the plantlets established under *ex vitro* condition when they were initially transferred on coco-peat as potting mixture. On the other hand 90 % plantlet survival rate was observed when they were transferred on ice cream pots containing garden soil, compost and sand (2:2:1). It was also observed that 85 % of the plantlets could be established under ex vitro condition when

they were transferred to earthen pots containing soil and organic manure. The reason behind maximum survival rate in cocopeat mixture is that it is a soil free material with very porous but good water holding capacity and do not allow microbial growth easily.

12.2.4 Primula scotica Hook.

P. scotica is a rare Scottish plant and it is one of the very few endemic plants which can only be found in North of Scotland.

The only protocol develop for micropropagation of *Primula scotica* is as follows:

Benson et al. (2000):

1. Commercially available seeds of *P. scotica* were washed under tap water followed by surface sterilisation with 10 % (v/v) Domestos hypochlorite solution. After washing with sterilised distilled water, seeds were cultured on hormone free MS medium.
2. Transfer the germinated seedlings to micropropagation medium comprising modified MS medium (Smith 1992) with 0.2 mg/l each of BA and IAA, 20 g/l sucrose and 7 g/l agar.
3. Culture the plants as individual populations of clonal plantlets derived from single seeds.
4. Subculture the shoots at 4–6 weeks interval onto either fresh micropropagation medium which contained the plant growth regulators (BAP and IAA) or on hormone free MS medium for about 8–10 months.
5. Transfer the *in vitro* grown shoots on either hormone free MS medium or on medium containing BA and IAA for rooting.
6. Transfer the *in vitro* grown plantlets to sterilised substrates containing perlite and vermiculite, sorba rods, compost and peat pots.

12.2.5 Chlorophytum borivilianum Sant. et. Fernand

C. borivilianum Sant.et Fernand. (Liliaceae), a rare species (Nayar and Sastry 1988) commonly known as safed musli, a monsoon herb, is distributed in India mainly in Rajasthan, Madhya Pradesh (Bordia et al. 1995) and Gujarat (Shah and Suryanarayana 1967). It is a traditional medicinal plant valued for the dried fasciculated storage roots. Tuberous roots of this monocotyledonous plant possess immunomodulatory, adaptogenic, aphrodisiac and diuretic properties due to the presence of steroidal saponins (Kirtikar and Basu 1975a; Ramawat et al. 1988; Tandon and Shukla 1995). Saponins are used in the industrial production of sex hormones, corticosteroids and steroid derivatives. The roots are used in the Indian system of

medicine for the treatment of general debility, weakness and impotency. They are also used as cardiac and brain tonic; as a curative agent in various diseases like piles, diabetes and as anti-pyretic, diuretic and hemostatic. *C. borivilianum* has been used in many herbal and Unani formulations in India along with other plants such as *Asparagus adscendens, A. racemosum*, *Curculigo orchioides*and *Withania somnifera* (Kirtikar and Basu 1975a; Ramawat et al. 1988). These are reputed to have aphrodisiac properties and form an important ingredient of herbal tonics prescribed in the ayurvedic system of medicine in India (Kirtikar and Basu 1975a).

This species perpetuates both vegetatively by tubers and sexually through seeds. It has a sluggish perpetuation through seed on account of poor seed germination (25 %). Further, seed-raised progenies display an appreciable spectrum of variation due to its preferential out-crossing nature (Lattoo et al. 2006). As the tubers constitute the commercial product there is always scarcity of the propagating material. In this situation, plant tissue culture offers an effective means for rapid multiplication of this species. Micropropagation technology is advantageous due to production of high-quality disease-free, true-to type plants independent of seasonal and other environmental conditions in a comparatively smaller space (Debergh and Zimmerman 1991).

Purohit et al. (1994a) develop an *in vitro* clonal multiplication protocol using young shoot bases as explant on MS medium supplemented with different concentrations of cytokinins (BA and KN) individually and in combination with different auxins (NAA, IBA, and 2,4-D). MS medium supplemented with 22.2 μM BA produce maximum number of shoots (11) per explant. IBA(9.8 μM) was found to be best for rooting, since a maximum of 16 roots per shoot were obtained with an average length of 6.4 cm without a trace of callus. Purohit et al. (1994b) also induce somatic embryogenesis as well as plantlet regeneration in safed musli. Callus was induced from immature zygotic embryos inoculated on MS medium containing 1.0 mg/l 2,4-D. After 6 weeks of growth and subsequent subculture on MS medium containing 0.5 mg/l 2,4-D, somatic embryos were developed which on repeated subculturing on MS medium containing 2,4-D (0.1 mg/1) undergoes maturation. Plantlets could be recovered from 20 % of these somatic embryos when inoculated on auxin free MS medium. Arora et al. (1999) obtained somatic embryogenesis from the callus produced by the shoot cultures of stem disc explants. Germination of somatic embryos was highest (48 %) on MS medium supplemented with 17.6 μM BA.

Pudake and Dhumale (2003) developed an improved method for large scale multiplication through shoot base and stem disc culture. *In vitro* multiplication was achieved on MS medium supplemented with 2 mg/l BA. Up to 90 % of plantlets were established in pots by a hardening treatment, where the plants were first transferred to sterile sand and kept in a mist chamber under high humidity. Gaikwad et al. (2003) compared efficiency of seedling, root, stem disc and leaf (basal half) as explants, suggesting leaf base as the best explant followed by stem disc. Dave et al. (2003a) developed a highly reproducible field tested and cost-effective micropropagation scheme, having the potential to produce more than 15,000 plantlets under optimized conditions in 20 weeks. Dave et al. (2003b) developed an *in vitro*

propagation technique for *C. borivilianum* using encapsulated shoot buds (synthetic seeds). Four-millimetre long shoot buds encapsulated in 3.0 % sodium alginate matrix polymerized by a 100 mM solution of $CaCl_2.2H_2O$ yielded the best results. This method of micropropagation opens up possibilities for the storage of shoot buds during the off-season to facilitate transport of germplasm with ease. Sharma and Mohan (2006) develop a novel method of shoot regeneration from immature floral buds along with inflorescence axis in *C. borivilianum*. MS medium with 2 mg/l KN + 0.1 mg/l 2,4-D proved to be the best medium for multiple shoot induction. Subculturing on MS medium with 2 mg/l BA results in maximum number (35) of shoot production. Rooting of shoots (86.7 %) with maximum fasciculated roots (5) occurred on Knops medium containing iron and vitamins of MS medium with 2 mg/l IBA and 0.1 % activated charcoal. Rizvi et al. (2007) develop an efficient and cost effective protocol employing liquid medium using *in vitro* cultures of young shoot apices. Murashige and Skoog (MS) basal medium supplemented with 3 % w/v sucrose, 22.2 μM BA and with 0.8 % w/v agar or without agar (liquid medium) was found to be best for multiple shoot induction. An average of 15 shoots per flask (7.5-fold increase over two shoots/ flask inoculum density) could be obtained in liquid medium against nine shoots (4.5-fold increase) on agar solidified medium during the same culture duration. *In vitro*-regenerated shoots exhibited optimal root induction and growth in three fourth strength basal MS liquid medium supplemented with 9.8 μM IBA.

Haque et al. (2009) develop an *in vitro* clonal propagation protocol by enhanced shoot crown explant proliferation. Average 73 shoots were generated on modified MS medium containing 3 mg/l KN and 2 mg/l BA. Rooting was initiated on the half strength modified MS medium supplemented with 1 mg/l IBA. Kemat et al. (2010) used young shoot buds as explant for rapid multiplication of safed musli. The explants were cultured onto medium containing basal salts of Murashige and Skoog (MS) and various concentrations of BA and KN for shoot induction. Treatment containing 3.0 mg/l BA produced the highest mean number of shoots per explants (18.90) and a mean length of shoots (6.0 cm) after 28 days of culture. Regenerated shoots were successfully rooted on MS medium supplemented with 1.0 mg/l IBA and 30 g/l sucrose. Rizvi et al. (2012) develop an efficient *in vitro* propagation through somatic embryogenesis from hypocotylar region with an average of 66 somatic embryos in liquid MS medium compared to only 54 on solid medium. Among the above stated protocols, the best protocol given by Haque et al. (2009) and Rizvi et al. (2012) is as follows:

Haque et al. (2009):

1. Dormant roots of *C. borivilianum* were taken and sprouted roots crown shoot buds were selected. Spray the roots having sprouted crown shoot buds with gentamycin (500 mg/l), 0.5 % fluconazole, 0.1 % bavistin and 0.1 % endosulphan at interval of 24 hrs for 7 days before the explant were used for tissue culture.
2. Explant was cut into appropriate thin slices of shoot crown (5–6 mm) possessing one juvenile adventitious shoot buds and washed thoroughly under tap water.

3. After washing explants were submerged in distilled water containing 0.05 % fluconazole, 0.1 % bavistin and 0.1 % endosulphan for 4 h. This was followed by surface sterilisation with 1 % sodium hypochlorite solution and 1–2 drops tween 20 for 15 min.
4. After four washing in sterile distilled water explants were trimmed from the sides and place on semisolid modified MS medium containing caseine hydrolysate 10 mg/l, sodium dihydrogen orthophosphate 380 mg/l, sucrose 30 g/l supplemented with different concentrations KN and BA or KN and BA with IAA, IBA and NAA for shoot proliferation.
5. Modified MS medium supplemented with KN (3 mg/l) and BA (2 mg/l) was found to be the best concentration which induces maximum number of shoots (73 shoots/ explant).
6. Rooting was initiated in the half strength modified MS basal medium containing 30 g/l sucrose (w/v) supplemented with different concentrations of IAA or IBA and/or NAA.1 mg/l IBA formed vigorous fibrous and thick roots during the culture period of 4 weeks. IBA (1 mg/l) was the most effective concentration in inducing rooting in this plant.
7. Four week old rooted tissue culture plants were planted in earthen or plastic pots with 95 % survival rate.

Rizvi et al. (2012):

1. Hypocotyls from *in vitro* raised seedlings which germinated on MS medium supplemented with 57.74 µM GA were implanted on optimal callus induction medium i.e. MS medium supplemented with 1.16 µM KN and 1.13 µM 2,4-D, earlier standardized amongst different levels of KN (0.47–2.32 µM) and 2,4-D (0.45–4.52 µM) tested in *C. borivilianum* (Rizvi et al. 2010).
2. Transfer the callus from callus induction medium to embryogenic medium (modified MS medium containing 1.79 mM NH_4NO_3, 10.72 mM KNO_3, 1.13 µM 2,4-D, 7.38 µM 2-iP and0.76 mM proline) which showed optimal embryogenic response.
3. Transfer the somatic embryos on embryogenic medium containing PEG and sucrose (3 % each) which induces maturation of somatic embryos.
4. The embryogenic medium supplemented with 3 % each of PEG and sucrose, showed 1.88-fold increase in number of mature somatic embryos compared to control medium (embryogenic medium with 3 % sucrose). Higher embryo germination (50 %) compared to only 30 % on control medium was recorded, when mature somatic embryos from medium containing PEG and sucrose (3 % each) were transferred to embryo germination medium.
5. Now, transfer the mature somatic embryos to liquid embryo germination medium containing 15.5 µM BA which induces maximum number of mature (cotyledonary stage) somatic embryos (66) as well as highest germination (57.5 %) at inoculum density of 0.4 g/ 40 ml of liquid medium.
6. Plantlets obtained from *in vitro* germinated somatic embryos after 6 weeks of culture of mature somatic embryos on embryo germination medium were hardened

in same culture at room conditions as described above for 2 weeks in quarter strength MS nutrient solution. These plants were then transferred to earthen pots containing sand:soil:farmyard *manure* in 1: 1: 1 (v/v) ratio and covered with transparent polythene bags with small holes. The polythene bags were removed after 2 weeks and the surviving plants were maintained in the glass house.

12.2.6 Plumbago indica L.

Plumbago indica L.*, (Plumbaginaceae)* commonly known as 'Raktochita' is a rare medicinal herb found in Assam and other parts of north-east India (Kanjilal et al. 1939). Several naphthoquinones, binaphthoquinones, flavonoids and phytosterols have been isolated from this plant (Harborne 1967; Dinda and Chel 1992; Dinda et al. 1995). Its root, root bark and milky juice of whole plant are used for medicinal purposes by the village people particularly of tribal areas as it contains two important alkaloids namely, napthoquinone and plumbagin (Ghani 1998b). 'Plumbagin'(2-methyl-5-hydroxy-1,4-naphthoquinone)is an orange yellow pigment which is commercially important for its broad range of pharmacological activities anti-Helicobacter pylori (Wang and Huang 2005), anti-tumor (Lin et al. 2003), anti-hepatoma (Parimala and Sachdanandam 1993), anti-intestinal carcinogenesis (Sugie et al. 1998), antimicrobial (Durga et al. 1990; Didry et al. 1994) and antimalaria (Likhitwitayawuid et al. 1998). In small doses it has stimulant action on the central nervous system, on muscle pain and on the secretion of sweat, urine and bile (Kirtikar and Basu 1918). Roots are reported to be vesicant, sialogogue and abortifacient, and are used in leucoderma, syphilis and leprosy. Roots are also recommended as a substitute for cantharides and are used for procuring abortion. Tincture of roots is used in dyspeptic and other digestive disorders and in piles (Kirtikar and Basu 1918). In traditional medicinal practices of Assam, the aerial parts fried with fresh fish are eaten to cure piles. Tender twigs fried with eggs of domestic duck are given in asthma and epilepsy. Root paste is applied on wounds to get relief from pain (Handique 1994). The species is becoming rare due to the massive collection by medicinal plant traders and also destruction of the natural habitat.

Chetia and Handique (2000) develop a protocol for high frequency *in vitro* shoot multiplication using nodal explants. Nodal explants inoculated on MS medium containing BA (3 mg/l) and AS (25 mg/l) developed small buds within 25–30 days and when transferred to MS medium with low concentration of BA (0.1–1.5 mg/l) induced maximum number (35) of shoots. Rooting experiment was not conducted in this study. Bhadra et al. (2009) develop an *in vitro* micropropagation protocol through induction of direct and indirect organogenesis using leaf and nodal segment as explants from 2 months old field grown seedlings. Rooting was induced on MS medium supplemented with 0.5 mg/l IAA. Protocol given by Bhadra et al. (2009)

contains rooting experiment while rooting was not carried out by Chetia and Handique (2000). Therefore the complete protocol for *in vitro* shoot multiplication and rooting given by Bhadra et al. (2009) is as follows:

Bhadra et al. (2009):

1. Leaf and nodal segments of approximate 0.5–1 cm excised from 2 months old field grown seedlings were surface sterilised with 0.2 % $HgCl_2$ solution for 10 min with occasional agitation followed by a dip in 70 % ethanol for 30 sec.
2. Segments were washed thoroughly with sterile distilled water and then cultured on MS medium supplemented with different concentrations and combinations of NAA, IAA, 2,4-D and picloram, and BA and KN. The nodal segments underwent direct organogenesis producing of multiple shoot buds (MSBs) on (i) 2.0 mg/l BA + 0.1 mg/l IAA, (ii) 2.0 mg/l BA + 1.0 mg/l IAA, (iii) 2.5 mg/l BA + 1.0 mg/l NAA, (iv) 2.0 mg/l BA + 2.0 mg/l NAA, and (v) 1.0 mg/l BA + 0.1 mg/l NAA supplemented media. Maximum number of MSBs were recorded on 2.0 mg/l BAP +1.0 mg/l IAA supplemented medium which resulted in direct organogenesis producing multiple shoot buds (7 ± 0.18 per explant).
3. On the other hand, nodal segments produced callus of different nature in some of the media combinations. Light green nodular callus was produced on (i) 3.0 mg/l BA + 1.5 mg/l KN + 1.0 mg/l NAA, (ii) 2.0 mg/l BA + 0.5 mg/l picloram while white and friable callus was produced on (i) 2.0 mg/l BA (ii) 2.0 mg/l BA + 2.0 mg/l IAA, and (iii) 1.5 mg/l BA + 0.5 mg/l IAA + 1.5 mg/l 2, 4-D supplemented media. Leaf explants produced only white and friable callus on three PGR combinations viz. (i) 2.0 mg/l BAP, (ii) 2.5 mg/l BAP + 1.0 mg/l NAA, and (iii) 1.5 mg/l BA + 0.5 mg/l IAA + 1.5 mg/l 2, 4-D supplemented media.
4. After two subsequent subcultures in a broad spectrum of auxin and cytokinin supplemented media, the light green nodular callus underwent differentiation producing huge number of MSBs. Medium fortified with 3.0 mg/l BA + 1.5 mg/l KN + 1.0 mg/l NAA proved to be best for induction of MSBs in this species. Thus indirect organogenesis took place and a combination of higher concentration of cytokinin and lower concentration of auxin proved to be effective for such kind of differentiation of the callus tissue.
5. In order to induce rapid elongation, the MSBs originated either through direct- or indirect organogenesis were grown on different PGR supplemented media. Medium fortified with 0.5 mg/l BAP + 1.0 mg/l IAA proved to be efficient for rapid elongation.
6. For rooting individual healthy shoots were transferred to one fourth strength of MS medium supplemented with 0.75 % (w/v) sucrose + 0.5 mg/l IAA which proved to be efficient for induction of strong and stout root system. This finding revealed that nutritional stress along with IAA was propitious for sprouting of roots.
7. Well rooted plantlets were then transferred to outside natural environment through sequential phases of acclimatisation. Over 90 % of the plants survived after transplantation to the garden.

12.2.7 Holostemma annulare (Roxb.), K. Schum

H. annulare (syn. *H. adakodian*; Asclepiadaceae) is a rare twiny, lacticiferous perennial medicinal shrub. It provides the essential raw material for more than 34 ayurvedic preparation and is one of the major ingredients of the drug, Jivanti, which is listed in the indigenous system of medicine (Kolammal 1979). Jivanti is assumed to be rejuvenative and bestows health and liveliness. The tuberous root, the officinal part, is a rich source of vitamin A, and a mash of it in cold milk is a remedy for diabetes (Kirtikar and Basu 1975b). According to Nair et al. (1992) the major southern Indian pharmacies require at least 150 metric tons of root tubers per anum for ayurvedic preparations. In addition to its medicinal functions, the leaves, flowers and fruits are useful as vegetables. Owing to the indiscriminate collection of root tubers as raw material for the ayurvedic drug preparations and other anthropogenic reasons, the plant is rare (Dan and Shanavaskhan 1991). Conventional propagation through seeds and cuttings of stem and root is too slow to provide the answer to meet the demand for this valuable plant in time. Seed derived progenies of *H. annulare* are not true-to-type due to cross-pollination. Propagation through seeds is an inadequate solution due to low viability, a low germination rate and scanty and delayed rooting of the seedlings. Vegetative propagation by root tuber is a very onerous procedure.

Sudha et al. (1998) established a rapid micropropagation system using shoot tips, terminal and basal nodes as explant. Shoot tips (0.5–0.8 cm) and terminal and basal nodes (1.0–1.5 cm) harvested from actively growing shoots of conventionally raised plants were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of BA and NAA. Multiple shoot formation (3.8) was observed in 68 % of basal nodes cultured on medium with optimum concentration of 4.43 μM BA and 0.54 μM NAA after 8 week. Terminal nodes were not suitable for inducing multiple shoots. Effects of other cytokinins (KN and 2-iP) and auxins (IBA) to enhance the regeneration potential of basal nodes were analyzed. Shoots were multiplied by subculture of basal nodes and stumps (the original explant tissue free of shoots, but with remnant axillary meristem and two or three protruding buds) in a reduced concentration of BA (2.21 μM) and NAA (0.27 μM). Liquid medium for multiplication was found to be ineffective due to a high degree of hyperhydricity. To make the multiplication process cost effective, culture bottles with polypropylene caps were used for multiplication. The best root induction (75 %) and survival (80 %) was achieved on 0.5 strength MS medium supplemented with 1.48 μM IBA.

Sudha et al. (2000) achieved plant regeneration from chlorophyllous root segments derived from *in vitro* rooted plants grown for 8–9 weeks in half-strength Murashige and Skoog (MS) medium containing 0.3 mg/l IBA. Up to 82 % solitary shoot bud initiation with small laterals was obtained from the excised (3–4-cm) root segments implanted horizontally on MS agar medium supplemented with 0.2 mg/l benzyladenine (BA) within 2 weeks. Rapid elongation of both shoot and lateral root initials was observed upon transfer of the root segments to half-strength MS medium

without growth regulators (basal medium). The shoots attained a length of 13–14 cm with 8–9 nodes within a period of 3–4 weeks of culture in basal medium. After 5–6 weeks incubation, the plantlets were hardened for 4 weeks in a mist chamber, and showed 80 % survival when planted in the field. Martin (2002) develop an efficient protocol for axillary bud multiplication and indirect organogenesis using leaf, node and internode as explants. Murashige and Skoog (MS) medium supplemented with 2.0 mg/lBA and 0.5 mg/l IBA induced an average of eight shoots per node and was the best for axillary bud proliferation. Subsequent cultures enhanced the number of shoots. In indirect organogenesis, callus developed from node and internode on MS medium fortified with 1.0–2.5 mg/l BA underwent organogenesis. Node callus on transfer to MS medium with 1.5 mg/l BA developed maximum number of shoots (15). Martin (2003b) established plant regeneration through indirect somatic embryogenesis in *Holostemma annulare* Schult. using leaf, internode and root as explants. Friable callus, developed from leaf, internode and root explants on Murashige and Skoog (MS) medium supplemented with 2,4-D (1.0 mg/l), was most effective for the induction of somatic embryos. An average of 40 embryos were obtained from 10 mg callus. Fifty per cent embryos exhibited maturation and conversion upon transfer to 1/10 MS basal solid medium. Geetha et al. (2009) develop cost effective and efficient protocol for the micropropagation and large scale production of quality planting material in *H. annulare*.

Martin (2002):

1. Leaf, internode and nodal segments were collected from the tender parts of mature plant and washed them with detergent extran (5 % v/v)for 5–10 min followed by surface sterilisation in $HgCl_2$ solution (0.5 % w/v) for 12–14 min. Rinse three times with sterile distilled water.
2. Nodal explants were then cultured on MS medium supplemented with various growth regulators at different concentrations either alone or in combination alongwith, $AgNO_3$ (0.5–5.0 mg /l), $CoCl_2$(10.0–100 mg/l), and additives (CW, 5–15 %). Node explants induced an average of two shoots on MS basal medium. The addition of 10 % CW to the MS medium resulted in the induction of an average of three shoots. The cytokinin BA showed the strongest effect with respect to the multiplication of axillary buds. Node explants induced an average of four shoots in the presence of 2.0 mg/l BA alone.
3. Nevertheless, BA in combination with IBA was most effective for axillary bud multiplication, which developed shoots at a rate of eight per node explant. Excision and culture of the node segments from the *in vitro*-derived shoots facilitated the development of increased number of shoots.
4. Callus induction was observed on internode and leaf explants of Holostemma ada-kodien on MS hormone-free medium. On MS medium fortified with 0.5–2.0 mg/1 NAA/IAA, either alone or in combination with 0.5–2.0 mg/1 KN/BAP, all types of explants induced callus. The texture of the callus varied according to the nature of the cytokinin and also on the auxin: cytokinin ratio. Callus developed on NAA/ IAA medium with BA was hard and nodular in texture and pale-green in colour with the combination of NAA/ IAA and KN,

the callus was cream-coloured and semi-hard. Callus formation also occurred on medium fortified with cytokinin alone.

5. The explant source of callus and the growth regulator inducing the callus exhibited a significant influence on organogenesis. Internode and node callus that developed on MS medium fortified with 1.0–2.5 mg/1 BA only underwent organogenesis. The highest number of shoots developed following the transfer of the callus to MS medium with 1.5 mg/1 BA. In the present study node callus developed an average of 15 shoots while internode callus exhibited low frequency of regeneration developing an average of five shoots. Callus from leaf explants did not develop shoots. Subculture of the organogenic callus (<1 g) resulted in proliferation and an enhanced rate of caulogenesis, with more than 55 shoots and numerous meristemoids.
6. Shoots developed through axillary bud multiplication as well as via indirect organogenesis after 30 days of culture exhibited abscission of leaves and shoot tips. Necrosis and abscission of leaves and shoots were due to the accumulation of ethylene, and this was alleviated by the addition of 3.0 mg/1$AgNO_3$ or 20.0–50 mg/1 $CoCl_2$ asethylene inhibitors. The addition of $AgNO_3$ or $CoCl_2$ to the medium favoured shoots with larger leaves but resulted in a significant reduction in the number of shoots. For rooting, transfer the healthy shoots to half strength MS liquid medium fortified with 0.05 mg/l IBA.
7. However, considering the negation of leaf and shoot-tip abscission by $AgNO_3$/ $CoCl_2$ and the corresponding reduction in the number of shoots with respect to the revival of growth through the emergence of axillary buds below the point of abscission of the shoot tip, the addition of $AgNO_3$/ $CoCl_2$ was not advantageous.
8. IBA was more suitable for root induction than IAA and NAA. When cultured on half-strength MS solid or liquid medium fortified with 0.05 mg/l IBA, each shoot developed an average of eight roots. The roots were more elongated in the liquid medium than on the solid medium.
9. Rooted shoots were transferred directly to small pot filled with sterile soilrite + sand (1:1); growth revived after15 days of transplantation. Of the 120 plantlets transferred to soil, 90 % survived, and the plantlets exhibited morphological characters similar to those of the source plant.

Martin (2003b):

1. Leaf, internodal and root explant were collected from the tender parts of mature plants and washing procedure was done as given in above protocol. The materials were cut into appropriate sizes (leaf, 1 cm; internode, and root, 1 cm) and cultured on MS medium (Murashige and Skoog 1962).
2. Basal medium was supplemented with different growth regulator (NAA/IAA/2,4-D/BA/KN) at different concentrations either alone or in combination. Callus induction was observed on root, internode and leaf explants of *H. annulare* on MS basal medium. Growth regulators viz. NAA, IAA, 2,4-D, BA and KN either alone or in combination were efficient to induce callus. Nevertheless, amount, texture and colour of the calli varied depending on the type and concentration of the growth regulator.

3. Callus developed on MS medium enriched with 2,4-D (1.0–3.0 mg/l) alone or in combination with BA/KN (0.5 mg/l) was friable and cream coloured showing a high proliferation rate. In all other cases, the callus was hard or semihard and pale green to dark green. Callus was very hard and dark green on MS medium with BA (1.0–3.0 mg/l) alone. Friable callus developed on MS medium supplemented with 2,4-D (1.0 mg/l) was best for the induction of somatic embryos.
4. Subculture of the callus developed on 2,4-D (1.0 mg/l) onto solid or liquid half strength MS medium with 0.1 or 0.5 mg/l 2,4-D turned the callus embryogenic. Transfer of embryogenic callus onto solid or liquid media facilitated the development of somatic embryos at different frequencies. Of the different treatments, half or quarter MS medium containing 2,4-D (0.05 or 0.1 mg/ l) induced embryo differentiation at the highest rate.
5. Low concentration of 2,4-D was obligatory for the induction of somatic embryos. However, concentration of 2,4-D above 0.1 mg/l resulted in proliferation of callus. Suspension and solid medium cultures exhibited significant differences in formation of somatic embryos. Embryogenic callus transferred on solid medium gave rise to only few embryos. In contrast, suspension cultures induced the development of higher number of somatic embryos. An average of 40 somatic embryos were obtained from about 10 mg callus. The cultures showed globular to early cotyledonary stage embryos abreast to callus proliferation.
6. Fifty percent of the embryos underwent maturation and conversion upon transfer to 1/10 strength solid MS medium without growth regulators. The plantlets developed through somatic embryogenesis exhibited 90 % survival in the soil. Plantlets established in the field exhibited similar morphological characters to that of wild growing plants. The present protocol enables development of 2,000 plantlets /g callus within 6 months.

12.2.8 Wattaka volubilis (Linn.) Stapf

Wattakaka volubilis which is commonly known as 'Perukuruncha' in Tamil, is an important medicinal woody climber belonging to the family Asclepiadaceae. It is a fleshy climber with green flowers in drooping umbels and found throughout the plains. The whole plant is used for medicinal purposes and has been used for the treatment of various ailments since ancient times. Among various saponins obtained from the stem and flower of *W. volubilis*, two compounds are active against Ehrlich's ascites carcinoma (Yoshimura et al. 1983 and Pullaiah 2002). The roots contain glucoside, which lowers carotid blood pressure in mice and dogs when administered intravenously. The extract from the roots is applied to cure snake bites and given to women to cure headache after child birth. The leaves are common ingredients of many folk and herbal medicines (Ojewole 2002; Subapriya and Nagini 2005). Leaf extract has been reported to possess pharmacological activity, including anti-inflammatory activity (Owoyele et al. 2005). It is also used for treating rheumatic pain, cough, fever and severe cold (Saikia et al. 2006).

Chakradhar and Pullaiah (2006) develop a protocol on axillary shoot multiplication using nodal explants derived from *in vivo* plants and *in vitro* seedlings of *W. volubilis*. Nodal explants from both the sources were cultured on Murashige and Skoog medium fortified with various concentrations of cytokinins – BA, KN, TDZ either singly or in combination with NAA. KN proved best for inducing healthy shoots in both *in vitro* and *in vivo* derived explants. Maximum number of shoots (14.1 ± 0.84) with 80 % regeneration frequency was obtained from nodal explants of seedlings cultured on 5 mg/l KN + 0.1 mg/l NAA. The differentiated shoots from both could be rooted with 85 % frequency on half strength MS medium (1 % sucrose) with 0.6 % agar + 1 mg/l IBA + 0.2 mg/l KN.

Arulanandam et al. (2011) develop a micropropagation protocol using shoot tip and nodal segments as explant by culturing them on MS medium fortified with various concentrations of BA, KN and NAA. Maximum number of shoots (5.3 ± 1.4) was developed on MS media fortified with 1.5 mg/l BA in combination with 0.7 mg/l KN. Maximum shoot length (5.8 ± 1.1 cm) was also observed on the same medium. The *in vitro* raised shoots produced highest percentage of rooting (85 %) in MS media augmented with 1.0 mg/l IBA. Vinothkumar et al. (2011) also develop *in vitro* shoot multiplication protocol using nodal explants. The highest shoot multiplication rate (23.4 ± 0.48) was observed after 28 days of culture on MS basal medium supplemented with 0.6 mg/l BAP and 0.2 mg/l NAA. Elongated shoots produced roots within 6 weeks in half strength MS basal medium supplemented with 0.6 mg/l IBA and 3 % (w/v) sucrose.

The best protocol develop by Vinothkumar et al. (2011) which results in higher number of shoots as compared to two other protocols is as follows:

Vinothkumar et al. (2011):

1. Nodal segments of 2–3 cm were excised from well developed 3 year – old mature plants and used for induction of multiple shoots. Explants were washed thoroughly under running tap water for 25 min and treated with a surfactant, Tween 20 (15 drops per 100 ml of sterilized distilled water) for 10 min. Later these explants were surface sterilized with 70 % ethanol(w/v) for 5 min followed by washing thrice with sterilized distilled water.
2. The explants were inoculated on MS medium supplemented with different concentrations and combinations of BA (0.2–1.2 mg/l), KN (0.2–1.2 mg/l), NAA (0.2–1.2 mg/l) and GA_3 (0.2–1.2 mg/l). Fifteen days after inoculation, the nodal explants showed slight swelling prior to the emergence of shoot buds that develop from the pre-existing material.
3. Among the four different treatment combinations, after 4 weeks of incubation higher number of multiple shoots (23.4 ± 0.48) was observed in MS medium supplemented with 0.6 mg/l BA and 0.2 mg/l NAA 0.6 mg/l.
4. The healthy shoots (3–5 cm long) were transferred to MS medium supplemented with different concentrations and combinations of GA_3 (0.1–0.6 mg/l), BA (0.1–0.6 mg/l) and KN (0.1–0.6 mg/l). Among the three different concentrations, GA_3 along with BA showed excellent shoot elongation. In this combination, the highest success rate (94 %) with maximum shoot length (9.6 ± 0.04) was observed with 0.4 mg/l GA_3 and 0.2 mg/l BA after 4 weeks of inoculation.

5. Well developed elongated shoots were excised from culture tubes and cultured on half strength MS medium containing different concentrations of IBA (0.2–1.2 mg/l), IAA (0.2–1. 2 mg/l) and NAA (0.2–1.2 mg/l). The percentage of root frequency, number of roots per shoot and length of roots were recorded after 4–5 weeks of culture. Of the three types of auxins, IBA was found to be comparatively more effective than the other two auxins (IAA and NAA). IBA (0.6 mg/l) was found to be the best concentration of auxin for proper rooting where 95 % of the shoots rooted within 6 weeks of culture. The highest average number of roots was (12.2 ± 0.40) with root length (13.5 ± 0.13 cm). At higher concentrations of auxins profuse callus was produced at the basal end of microshoots which inhibited the growth and elongation of roots.
6. Rooted plantlets were thoroughly washed to remove the adhering gel and planted in specially made paper cups containing sand, garden soil and farmyard manure (1:2:1) and kept in the greenhouse for acclimatization.

12.2.9 Justicia gendarussa Burm. f.

Justicia gendarussa Burm F. is a rare medicinal plant belonging to the family Acanthaceae. It is a native plant of China but is found wild or cultivated in Sri Lanka, India, Malaya (Malaysia) and Philippines. The leaves are simple, opposite, lanceolate or linear-lanceolate, acute at base, tapering into rounded apex and glabrous and shining leaves (8–12.5 cm long, 1.2–2 cm broad) with prominent purple veins beneath. The plant is known for its medicinal properties in the Indian traditional system of medicine. In traditional medicine, the leaves of the plant are recommended to treat a number of ailments such as fever, hemiplegia, rheumatism, arthritis, muscle, pain, lumbago, headache and earache (Ahmad and Holdworth 2003; Anonymous 1959b). Leaves of J. gendarussa are also used to cure injuries (Zheng and Xing 2009). Water and 80 % ethanol extracts of the plant showed HIV-type 1 reverse transcriptase inhibition ratio higher than 90 % at a 200 μg/ ml concentration (Woradulayapinij et al. 2005). The antinociceptive potential and toxicity of the aqueous leaf extract of this plant have been studied by Ratnasooriya et al. (2007).

This plant is a rich source of potassium salts (Jayaweera 1980), phenolic dimmers called lignans (Mrunthunjaya and Hukkeri 2007) and triterpenoids (Chakravarty et al. 1982). Lignans have been used as lead compounds for the development of antirheumatic agents (Apers et al. 2003). A study conducted by Uawonggul et al. (2006) showed that the whole plant extract could be used as scorpion venom antidotes with low cytotoxicity. The antihepatotoxic properties of this plant have been evaluated by Yang et al. (1987). The antilarvicidal and adulticidal properties of this plant against the malarial vector, Anopheles stephensi have been reported (Senthilkumar et al. 2009). According to Periyanayagam et al. (2009), both aqueous and ethanolic extracts of the leaves of *J. gendarussa* inhibit angiogenesis in a dose-dependent fashion and provides a scientific basis for its traditional use in the treatment of arthritis, which is an angiogenesis dependent disease. Another study by

Krishna et al. (2009) revealed the antioxidant and hepatoprotective activity of the leaf extract of this plant. The conventional method of propagation in *J. gendarussa* is from cuttings and seeds. However, both these methods are affected by soil microbial contamination and delayed rooting of the cuttings and poor viability of the seeds. Therefore *in vitro* propagation of *J. gendarussa*, a valuable medicinal is carried out to meet the requirement of this plant for traditional medicine, which is considered as an important mode of health care in rural areas of several countries.

Agastian et al. (2006) develop an efficient *in vitro* propagation protocol by inducing prolific callus in leaf and nodal explants on MS medium supplemented with NAA. Organogenic and chlorophyllous calli were produced at lower concentrations of NAA(1.0 mg/l) and BA (0.1 mg/l). Thick and long (25 cm) roots were produced in large numbers on medium supplemented with same concentrations of NAA and BA as given above. Thomas and Yoichiro (2010) obtained *in vitro* regeneration of shoot buds from culture of nodal cuttings as well as shoot regeneration from callus. Optimum 87 % of cultures responded with an average shoot length of 4.4 cm on Murashige and Skoog (MS) medium supplemented with 17.7 μM BA. Callus was induced from the mature leaf segments on MS medium supplemented with KN alone or in combination with 2, 4-D. Optimum callus induction (78 %) was obtained on MS medium supplemented with 14 μM KN and 4.5 μM 2,4-D. When the callus was subcultured on MS medium fortified with BA or KN alone or in combination with NAA, shoot regeneration was obtained. The highest response (92 %) was observed on MS medium containing 17.7 μM BA and 5.4 μM NAA with an average number of 12.2 shoots per responding callus. The shoots obtained from callus and nodal cuttings were rooted with a frequency of 73 % on MS medium augmented with 9.8 μM IBA. Janarthanama et al. (2011) reported nodal segments cultured on Murashige and Skoog (MS) medium containing 1.0 mg/l BA with 10 % coconut milk showed better growth response and produced 10.5 ± 0.6 shoots per explant with an average length of 4.4 ± 0.3 cm after 35 days. Rooting of shoots was achieved on growth regulator free half strength MS medium which results in the induction of 5.3 ± 0.25 roots with an average height of 4.8 ± 0.2 cm after 30 days. Kumar et al. (2012) studied shoot initiation, callus development and phytochemical analysis in *Justicia gendarussa* using node, shoot tip and leaf as explant. Nodal segments grown on Murashige and Skoog (MS) medium containing BA (2 mg/l) showed better growth response and produced 3.2 ± 3 shoots per explant with an average length of 7.8 ± 1.2 cm after 35 days. Thick friable calli were produced at lower concentrations of NAA (0.5 mg/l) and BA (2 mg/l). Then the callus was transferred on media enriched in cytokinin for shoot production. Among the above given protocols the highest number of shoot regeneration were obtained by Thomas and Yoichiro (2010) which is mentioned below:

Thomas and Yoichiro (2010):

1. Nodal explants (about 3–5 cm long) were collected and before surface sterilization, shoot explants were defoliated and placed under flowing tap water for 30 min. Explants were sterilized by immersion in 50 % (v/v) ethanol solution for 30 s, followed by 8 min immersion with agitation in 0.1 % $HgCl_2$ (w/v).

2. Explants were rinsed three times in sterile distilled water and single node explants (about 2 cm long) were transferred into culture tubes containing MS medium supplemented with various concentrations (4.4, 8.9, 13.3, 17.7, 22.2 μM) of BA, KN and TDZ. Of the three different cytokinins (viz. BA, KN and TDZ) tested, BA was more effective than KN and TDZ in inducing shoot development for nodal explants. The percentage of shoot proliferation improved with increasing concentrations of BA up to the optimal level. BA at 17.7 μM was the most effective. This medium induced shoot proliferation in 87 % of explants with an average shoot length of 4.4 cm within 45 days.
3. Among the various KN and TDZ concentrations tested, shoot regeneration was optimal in presence of 13.9 μM KN and 18.2 μM of TDZ. In all the three cytokinins tested, some cultures (20 %) showed callusing at the basal cut end of the nodal cuttings. However, it did not affect the development of axillary shoot in any way. The presence of NAA reduced the frequency of bud break and percentage response whenever it was added in conjunction with BA, KN and TDZ.
4. Mature leaves were excised from 45-day-old fully grown *in vitro* shoots for callus induction. One leaf was cut into two or three pieces, depending on the length of the leaf so that each piece measured an average length of about 0.75 cm, and then inoculated in culture tubes containing MS medium supplemented with KN (4.7, 13.9, 23.2 μM) and/or 2,4-D; 2.2 and 4.5 μM for callus induction.
5. The frequency of callus occurrence on medium was low when 2,4-D and KN were used separately. No callus formation was observed on medium without plant growth regulators (the control) and with 2.3 μM 2, 4-D. There was an optimum response of 12 and 18 % explants when the MS medium was fortified with 4.5 μM 2, 4-D and 13.9 μM KN, respectively. However, this combination increased the callus induction frequency to 78 %.
6. Callus proliferation from the leaf segments occurred within 4 weeks and the callus remained whitish yellow and friable when KN and 2, 4-D were used individually in medium, while it was yellow and semifriable when a combination of KN and 2, 4-D was employed. The calli were separated from primary explants and transferred onto MS medium supplemented with KN (13.9 μM), and 2,4-D (4.5 μM), where the callus was maintained by regular subculturing every 4 weeks.
7. Calli developed in the above plant growth regulator combinations did not exhibit any shoot bud differentiation until transfer to a regeneration medium. For shoot regeneration, the calli were transferred to regeneration medium with different concentrations of BA (8.9, 17.7, 26.6 μM) or KN (9.3, 18.6, 27.9 μM) alone or in combination with NAA (2.7 and 5.4 μM). The maximum percentage of culture responses (92 %) and highest shoot number (12.3 shoots/callus) were observed on MS medium supplemented with 17.7 μM BA and 5.4 μM NAA.
8. Regenerated shoots measuring a size of about 2 cm were isolated and used for root induction. Half-strength MS medium supplemented with different concentrations (2.5, 4.9, 9.8, 14.7 μM) of IBA and NAA (2.7, 5.4, 10.7, 16.1 μM) were employed for root induction. Of the two auxins tested, IBA at 9.8 μM was the most effective, as it induced 73 % of the shoots to form roots. After 45 days of culture on this medium, each shoot formed an average of 3.8 roots, each with an average 1.9 cm

in length. There was no rooting for shoots grown in the presence of low concentration of NAA (0.5 μM); instead, callus was formed at the shoot base.

9. Plantlets with fully expanded leaves and well developed roots were successfully acclimatized and eventually established in soil. Out of the 50 plants transferred to soil, 45 survived. Thus, the *ex vitro* survival rate of the plants after transfer to fine garden soil: sand (1:1) was 90 %.

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Chapter 13
In Vitro Conservation Protocols for Some Threatened Medicinal-Plant

Anwar Shahzad and Arjumend Shaheen

13.1 Introduction

In the frame of global efforts to halt the loss of biodiversity by 2010, the Convention on Biological Diversity (CBD 1992; Glowka et al. 1994), and then the Global and European Strategies for Plant Conservation (GSPC, ESPC, available at www.plantaeuropa.org) have prioritized the *in situ* conservation of rare and threatened plant species and their back up by *ex situ* conservation in botanic gardens (Sharrock and Jones 2009). Recently the need to conserve plant diversity *ex situ* has been given added urgency as the impacts of climate change start to become obvious and concerns are raised about our ability to effectively conserve species *in situ* in the long run (Sharrock and Jones 2009). The 2008 IUCN Red List shows that the number of threatened plant species is increasing gradually. The number of threatened plants is 8,457, out of which 247 plants are found at different biodiversity hotspots in India. Many of them serve as sources of food, fuel, fibre, timber, medicine, etc. and function as integral parts of local agricultural production systems. The resurgence of public interest in plant-based medicine coupled with rapid expansion of pharmaceutical industries necessitate an increased demand of medicinal plants, leading to over-exploitation that threatened the survival of many medicinal plants.

Further, the degree of threat to natural populations of medicinal plants has increased because more than 90 % of the plant raw material for herbal industries in India is drawn from natural habitats. Not surprisingly, wild plant species used for medicinal purposes are receiving ever-increasing attention from the scientific community and commercial enterprises. At the same time, these species continue to support indigenous and local communities that have relied on them for centuries in their traditional medicines. But a number of factors now threaten wild medicinal

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plants – habitat destruction, over-harvesting and big business. In India, hundreds of medicinal plants like *Pterocarpus santalinus*, *Commiphora wightii*, *Taxus wallichiana*, *Picrorhiza kurrooa*, *Salvadora persica* and *Dioscorea deltoidea*, are at the risk of extinction due to over-collection to supply domestic and foreign medicinal markets, threatening the discovery of future cures for diseases. The special significance of medicinal plants in conservation stems from the major cultural, livelihood or economic roles that they play in many people's lives. Various sets of recommendations have been compiled relating to the conservation of medicinal plants, such as those associated with international conferences at Chiang Mai, Thailand, in 1988 and Bangalore, India, in 1998 (www.frlht-india.org) (Akerele et al. 1991; Bodeker 2002). Probably, the single most important 'role' for medicinal plants in biological and ecological conservation stems from the foundations that they can provide for the involvement of people in conservation of natural habitats (Schopp-Guth and Fremuth 2001). It is estimated that 70–80 % of people worldwide rely chiefly on traditional, largely herbal, medicine to meet their primary healthcare needs (Farnsworth and Soejarto 1991). The global demand for herbal medicine is not only large, but growing (Srivastava 2000). The market for Ayurvedic medicines is estimated to be expanding at 20 % annually in India (Subrat 2002), while the quantity of medicinal plants obtained from just one province of China (Yunnan) has grown by ten times in the last 10 years (Pei 2002). Factors contributing to the growth in demand for traditional medicine include the increasing human population and the frequently inadequate provision of Western (allopathic) medicine in developing countries. Natural and anthropogenic disturbances can have dramatic consequences for population growth, particularly for small populations of threatened plants.

Conservation of medicinal plants (MPs) is receiving increased attention in view of resurgence of interest in herbal medicines for health care all across the globe (Franz 1993; Gupta et al. 1998). The global market of herbal drugs has registered a steady increase in recent years (Martinez 1995, 1997; Olsen 1998), and now exceeds over US$ 20,000 million (Valiathan 1998). The world trade figures suggest that India ranks next to China in export (32,600 tonnes: US$ 46 million) of medicinal raw material annually (Lange 1997). All these figures indicate that MPs offer a great motivation for conservation (Marshall 1997) for all those concerned in human health care and economy. As a mega-diversity country, over 15,000 species of higher plants are so far recorded in India. Of these, nearly 50 % are reported to have medicinal value (All India Co-ordinated Research Project on Ethnobiology). In India, the rich plant diversity of the Himalaya is over 8,000 angiosperms, 44 gymnosperms, 600 pteridophytes, 1,737 bryophytes, 1,159 lichens etc. (Singh and Hajra 1996) and has been a source of medicine for millions of people in the country and elsewhere in the world. The Indian Himalaya region (IHR) supports over 1,748 (23.2 % of India) plant species angiosperms i.e., 1,685, gymnosperms – 12 and pteridophytes – 51 species of known medicinal value (Samant et al. 1998). The unique diversity of MPs in the region is manifested by the presence of a number of native (31 %), endemic (15.5 %) and threatened elements: 14 % of total Red Data plant species of IHR and 3.5 % of total MPs in different threat categories of CAMP (Samant et al. 1998). Medicinal plants of IHR are represented in varying life forms at various altitudes and habitats.

The economic potential of the MPs in some parts of IHR (Nautiyal et al. 1997) and their contribution in providing novel bio-molecules is recognised (Dhawan 1997). Further, the degree of threat to natural populations of MPs has increased because >90 % of medicinal plant raw material for herbal industries in India and for export is drawn from natural habitats (Tandon 1996; Gupta et al. 1998; Ved et al. 1998).

On the order of 40 % or more of the pharmaceuticals currently used in Western countries are already derived or at least partially derived from natural sources. Ayurveda, the indigenous system of Indian medicine, describes thousands of plant species in detail. With its varied climatic zones, India has a rich diversity of medicinal herbs. The forest harbour a large number of plant species, but deforestation has been responsible for the rapid loss of medicinal plant wealth, such that many valuable medicinal plants are under the threat of extinction. Pharmaceutical companies depend largely upon materials procured from naturally occurring stands that are being rapidly depleted. Application of traditional medicinal plants for human use has also been reported (Shimomura et al. 1997). Experimental approaches used for propagation of medicinal plants through tissue culture can be divided into three broad categories. The most common approach is to isolate organized meristems like shoot tips or axillary buds and induce them to grow into complete plants. This system of propagation is commonly referred to as micropropagation. In the second approach, adventitious shoots are initiated on leaf, root and stem segments or on callus derived from those organs. The third system of propagation involves induction of somatic embryogenesis in cell and callus cultures. This system is theoretically most efficient as large numbers of somatic embryos can be obtained once the whole process is standardised. Biotechnology involving modern tissue culture, cell biology and molecular biology offers the opportunity to develop new germplasms that are well adapted to changing demands.

13.2 Protocols of Some Threatened Plants

The following are some threatened plants, their importance, medicinal properties and their protocols of conservation through *in vitro* strategies:

13.2.1 Podophyllum hexandrum Royle

Podophyllum hexandrum Royle (May apple) belongs to the family Berberridaceae is a threatened perrenial herb and a source of highly valued podophyllotoxin. It is the rhizome of several *Podophyllum spp*. that has been found to be the source of podophyllotoxin, the active ingredient used as a starting compound for the chemical synthesis of etoposide (VP-16- 213) and teniposide (VM-26), effective agents in the treatment of lung cancer, a variety of leukemias and other solid tumours (Van Uden et al. 1989). The Indian species, *P. hexandrum* Royle contains three times more phyllotoxin than the American species *P. peltatum* (Fay and Ziegler 1985).

The rhizomes are being indiscriminately collected in large quantities from the wild to meet the ever increasing demand for the crude drug. As a result of this and a lack of organized cultivation, *P. hexandrum* has been reported as a threatened species from the Himalayan region (Gupta and Sethi 1983). The major problem for the cultivation of this plant is its long juvenile phase and poor fruit setting ability. Moreover, its seeds take long period to germinate (Handa et al. 1989). One of the most appropriate actions for safeguarding over exploited species is to improve propagation techniques and to encourage cultivation. Several studies have been conducted to propagate and conserve *P. hexandrum* by using tissue culture techniques like Arumugam and Bhojwani (1990). The reliable protocol for micropropagation of *P. hexandrum* is described below.

13.2.1.1 Protocol by Nadeem et al. (2000)

This protocol deals with the successful propagation of *P. hexandrum* using both conventional and *in vitro* techniques.

1. Vegetative multiplication was improved when rhizome segments were treated with α-napthaleneacetic acid (NAA) or indole-3-butyric acid (IBA) before planting where more than doubling in rooting percentage was observed with 100 μM IBA.
2. Another experiment was carried out to improve the rate of seed germination where pre treatment of seeds with sodium hypochlorite resulted in five-fold increase, while 250 μM gibberellic acid (GA_3) and a combination of GA_3 and 6-benzyladenine (BA 250 μM each) enhanced germination by nearly two-fold increase.
3. The most reliable experiment of this protocol is the *in vitro* study of *P. hexandrum* and is as follows.
4. The embryos were excised from disinfected sterilized seeds of *P. hexandrum* and transferred onto MS (Murashige and Skoog 1962) medium containing 3.0 % sucrose (w/v) and 0.8 % agar (w/v) fortified with different plant growth regulators (PGR's) of varying concentrations.
5. Excised embryos germinated within 7 days of inoculation on basal medium or on medium supplemented with BA (0.5–4.0 μM).
6. Embryo expansion occurred rapidly when the medium was supplemented with 0.5 or 1.0 μM BA and the lowest concentration of BA resulted in maximum expansion.
7. Multiple shoot formation took place when the excised embryos were placed on MS medium supplemented with 1.0–4.0 μM IAA and 1.0 μM BA.
8. Highest shoot multiplication was observed on MS medium containing both IAA and BA (1.0 μM each) with a maximum of 5.0 shoots/embryo.
9. After 4–5 weeks, the base of the cotyledonary leaf of embryos swelled to give rise to multiple shoot formation.

10. Rooting of excised microshoots was carried out when the medium was supplemented with IAA.
11. Rooted plantlets were then hardened and transferred to polybags after 45 days and kept under a polyhouse.
12. Callus induced from the basal end of the embryo in most combinations gave rise to somatic embryogenesis when subcultured on a medium supplemented with 5.0 μM NAA and 0.5 μM BA.
13. For synthetic seed production the somatic embryos teased from embrygenic callus were embedded in 3.0 % Na-alginate solution and dropped into 5.0 μM $CaCl_2$ solution for hardening. The alginate beads were washed with sterilized distilled water.

13.2.2 Nothapodytes foetida (Wight) Sleumer

Nothapodytes foetida (Wight) an indigeneous small evergreen tree, belongs to the family Icacinaceae and the extract from this tree is used in the making of antileukaemia and antitumoural compound camptothecin. Clonal propagation of plant germplasm through tissue culture for rapid production of plants is an important prerequisite for in vitro conservation (Lynch 1999). Many of the indigenous species of India have not been investigated regarding their amenability to micropropagation (Mandal 1999). The most recent medicinal plant to come under serious threat from international traders is *Nothapodytes foetida*, a small tree found in rain forests of South India and Sri Lanka (Hoareau and DaSilva 1999). Govindachari and Vishwanathan (1972) reported the isolation of quinoline alkaloids camptothecin and 9-methoxy camptothecin from bark, stem, root, and leaf of the plant. A novel camptothecin derivative was isolated from the wood of this tree (Aiyama et al. 1998). A new naturally occurring alkaloid camptothecin and nothapodytines together with 17 known compounds were isolated and characterized from the stem of *N. foetida*. Several *in vitro* studies have been conducted to propagate and conserve *Nothapodytes foetida* like Thengane et al. (2001), Tejavathi et al. (2012). The most reliable protocol for this plant species is as follows.

13.2.2.1 Protocol by Ravishankar Rai (2002)

1. Sterilized seeds of *N. foetida* were inoculated on MS basal medium devoid of any plant growth regulators (PGR's).
2. After 4 weeks of germination hypocotyl segments were aseptically divided and cultured on MS shoot proliferation medium supplemented with different PGR's.
3. Among the different cytokinins tested viz. BA (Benzylaminopurine), KN (Kinetin), TDZ (Thidiazuron), 2.2 μM TDZ proved optimum with a maximum production of 18.3 shoots/explant.

4. Rooting was found to be optimum on 1/4 MS + IAA 5.7 μM + IBA 2.4 μM with a rooting percentage of 87.5 % and a mean root length of 3.2 cm.
5. After 4 weeks of successful acclimatization plantlets were transferred to large pots containing soil:compost (1:1) and kept in the greenhouse.

13.2.3 Allium wallichii Kunth

Allium wallichii Kunth (Alliaceae) is an erect herb of 30–40 cm height with slightly thickened and clustered bulbs Wawrosch et al. (2001). It is a perennial plant with a restricted distribution in higher altitudes ranging from 2,000–4,000 m above the sea level in Nepal (Malla 1976). The bulbous rhizomes are used for the treatment of coughs and colds and against altitude sickness. The young leaves are cooked as a vegetable and dried ones are used as a spice (Manandhar 1980; Dobremez 1982). *A. wallichii* is a common ingredient in stomach tonics. The bulb is also boiled, fried with ghee (clarified butter) and used for the treatment of cholera and diarrhea (Coburn 1984). In the Ayurvedic medicinal system, the indications are tuberculosis, nerve defects, blood circulatory defects, and long life and rejuvenation (Bajracharya 1979). Due to over exploitation of this plant species from its wild habitats it has achieved the status of threatened and is likely to be endangered in near future if the scenario remains the same. It has been noted that micropropagation would be of advantage in the process of the domestication of *A. wallichii* (Malla 1994). The application of tissue culture techniques has been described for various species of the genus Allium, for example garlic (Novak et al. 1986), onion (Dunstan and Short 1977; Rauber and Grunewald 1988), chive (Rauber and Grunewald 1988) or *A. carinatum* (Havel and Novak 1985). So far very reports on *in vitro* propagation and conservation of this plant species are available. One of the most acceptable protocol of *A. wallichii* is as follows.

13.2.3.1 Protocol by Wawrosch et al. (2001)

1. After proper disinfection and sterilization seeds of *A. wallichii* were aseptically germinated on half strength MS medium and the seedlings raised were multiplied on full strength MS medium supplemented with 10 μM BA to produce secondary explants which in turn were used for the main experiment.
2. Either whole shoots or longitudinally split halves (LH) were inoculated on the full strength MS medium supplemented with 108 factorial combinations of the cytokinins BA (6-benzyladenine), KN (kinetin) and Z (zeatin) and the auxins IAA (indoleacetic acid), IBA (indolebutyric acid) and NAA (napthaleneacetic acid).
3. An average multiplication factor of 4.6 was obtained when LH explants were inoculated on MS medium with 20 μM BA and 5 μM IAA and the same rate of shoot formation was also found on media containing Z.

4. Rooting was found optimum on 10 μM IBA with a rooting percentage of 100 % and a mean number of 2.22 roots with a root length of 12.2 mm per microshoot on full strength MS medium.
5. Acclimatization of *A. wallichii* plantlets was successfully done and subsequently the plants were moved to the greenhouse where they exhibited vigorous growth.
6. In addition to test the performance of *in vitro* derived plants under the specific conditions in Nepal a set of 300 shoots were rooted on MS medium with 10 μM IBA using disposable plastic containers.

13.2.4 Limonium cavanillesii Erben

The genus *Limonium* Miller (*Plumbaginaceae*), formerly called *Statice,* includes mainly rosulate plants with showy inflorescences (Amo-Marco and Ibanez 1998). This genus is characterized by its high ornamental value as a cut flower for both fresh and dry-flower arrangements (Harazy et al. 1985; Martin and Perez 1995). *Limonium cavanillesii* is an endemic and threatened statice with only a few populations known (Aguilella et al. 1994), and restricted to a small area in the coastal zone of north-eastern Castellon Province (Valencia Community, Eastern Spain).

Tissue culture techniques have proved to be good and efficient methods for the conservation of threatened plant species, because many plantlets can be obtained from a minimum of original plant material and with low impact on wild populations (Harazy et al. 1985; Lledo et al. 1993, 1994, 1996; Lledo and Amo- marco 1993; Martin and Perez 1992, 1995). One of the most reliable protocol for this threatened plant species is as follows.

13.2.4.1 Protocol by Amo-Marco and Ibanez (1998)

1. Inflorescence segments, 40–50 mm long, were excised and after proper sterilization 20 mm long explants with one or two buds were cut from the inflorescence segments and placed in MS medium.
2. When the shoots developed from buds of immature inflorescence stems were 1 cm in height and had 4–6 leaves, they were excised and subcultured on establishment medium with 2 mg/l kinetin.
3. For further multiplication, rosettes with 4–6 leaves were used as single explant.
4. Six week old shoots formed in clusters on establishment medium supplemented with 2 mg/l kinetin were selected and cultured on different multiplication media in combination with BAP, kinetin or 2iP at different concentrations.
5. Among the different cytokinins tested kinetin 5 mg/l, 2iP 5 mg/l and BAP 0.1 mg/l were found to be optimum where a maximum of 10.2, 9.4 and 9.3 shoots per cluster were obtained respectively.

6. Rooting was found to be optimum on 0.1 mg/l IAA where a maximum of 5.7 roots were produced with a rooting percentage of 80 % after 28 days.
7. Rooted plants were successfully acclimatized and transferred to plastic pots and the plantlets grew vigorously after the transfer with a survival rate of 90 %.

13.2.5 Decalepis hamiltonii Wight & Arn.

Decalepis hamiltonii Wight & Arn., commonly called 'swallow root', belongs to Asclepiadaceae and is a monogeneric climbing shrub native of the Deccan peninsula and forest areas of Western Ghats of India (Giridhar et al. 2005). The roots of *Decalepis hamiltonii* are used as a flavoring principle, appetizer, blood purifier (Wealth of India 1990), and preservative (Phadke et al. 1994). Of late, the highly aromatic roots have been subjected to over-exploitation by destructive harvesting that has endangered the survival of this plant in its wild habitat. Moreover, the absence of any organized cultivation of this plant (M. Sanjappa, Botanical Survey of India, personal communication) calls for immediate conservation measures. George et al. (1998) described a method for the preparation of the active fraction from the root of *Decalepis hamiltonii* and its use as a bioinsecticide. Also, it was observed that the aromatic roots of *Decalepis hamiltonii* proved to be potent antimicrobial agents (George et al. 1999). Though Bais et al. (2000) reported micropropagation of Decalepis hamiltonii by using growth hormones such as 6-benzyladenine (BA), a-naphthaleneacetic acid (NAA),n and indole-3-acetic acid (IAA), there were certain drawbacks such as profuse callusing from the base of the explants, and fewer shoots formed from nodal explants with moderate response (i.e., 30–40 %). A rapid and efficient protocol for micropropagation of *Decalepis hamiltonii* by shoot multiplication and effective *in vitro* rooting is reported by Giridhar et al. (2005) and is as follows.

13.2.5.1 Protocol by Giridhar et al. (2005)

1. Shoot tips of *D. hamiltonii* were used as explants source collected from Gumbali forest ranges of Mysore district.
2. The shoot tip explants were washed with Tween-20 (50 %, v/v) for 5 min followed by thorough washing under running tap water for 15 min. Later, explants were surface-sterilized with 0.15 % (w/v) mercuric chloride for 3–5 min and later rinsed four or five times with sterile distilled water.
3. For all experiments, MS (Murashige and Skoog 1962) with 100 mg/l myoinositol was used. All culture media contained 2 % sucrose (w/v). The pH was adjusted to 5.8 ± 0.2 using 1 N HCl or 1 N NaOH before adding 0.8 % (w/v) of agar (Himedia, Mumbai) and was subsequently autoclaved under 105 kPa at a temperature at 1,218 ° C for 15 min.
4. The MS medium with various concentrations of cytokinins such as isopentenyladenine (2iP; 2.5–7.5 μM), kinetin (2.3–7.0 μM), thidiazuron (2.3–6.8 μM) and

zeatin (2.3–11.4 μM) individually, or with IAA (0–0.85 μM), were investigated to optimize hormonal requirement for multiple shoot induction from shoot tip explants.

5. Of the various treatments with MS medium, 2iP (4.92 μM) alone produced the maximum number of shoots per culture (6.5 ± 0.4) that were supported by shoot length of 7.5 ± 0.5 cm after 8 week of culturing. Kinetin (4.7 μM) or zeatin (9.1 μM) in combination with IAA (0.6 μM) produced a maximum of 5.4 ± 0.4 or 5 ± 0.4 shoots, respectively.
6. Elongated microshoots were excised and transferred to MS medium fortified with phenolic compounds and indole-3-butyric acid (IBA; 9.8 μM) for root induction.
7. A greater number of roots (5.6 ± 0.8) were produced along with maximum root length (4.5 ± 0.3 cm) on medium containing 1,024 M PG (phloroglucinol).
8. At both concentrations of PG, 100 % of the explants responded for rooting.
9. After successful acclimatization these micropropagated plants were hardened under greenhouse conditions with 80–90 % survival rate.

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Chapter 14
In Vitro Conservation Protocols for Some Endangered Medicinal-Plant

Anwar Shahzad and Aastha Sahai

14.1 Introduction

In the world today, there are lot of people who do not have adequate access to basic needs such as food, water, education, health services and clean environment among others. This is a major concern being addressed by many governments at all levels amidst the rapidly growing population on one hand and a deteriorating environment on the other hand. Medicinal plants address not only the need for access to medicine as a component of health services but also to the need for increased income for farmers and as a significant contribution to the national economy. Medicines are the core of health care, they cure diseases (such as antibiotics), relieve symptoms (such as analgesics), are preventive (such as anti-hypertension drugs) or substitute for endogenous compounds (such as insulin). Since prehistoric times plants has served as a natural source of medicinal compounds which provided remedies for possible ailments. Even today medicinal plants offer alternative remedies with tremendous opportunities. They not only provide access and affordable medicine to poor people; they can also generate income, employment and foreign exchange for developing countries. According to World Health Organization (WHO) estimate upto 80 % of people still rely mainly on traditional remedies. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modeled on plant substances. Plants have contributed hugely to Western medicine, through providing ingredients for drugs or having played central roles in drug discovery. Some drugs, having botanical origins, are still extracted directly from plants, others are made through transformation of chemicals found within them, while yet others are today synthesized from inorganic materials, but have their historical origins into the active compounds found in plants. There are undoubtedly

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many more secrets still hidden in the world of plants (Mendelsohn and Balick 1995). Today scientists are using these renewable resources to produce a new generation of therapeutic solutions for new age diseases like AIDS, swine flu, Alzheimer, Parkinson's and many more.

Interest and support for the conservation and development of medicinal plants is increasing in all parts of the world. This is due, in part, to a growing recognition given to the role of medicinal plants in the provision of culturally relevant and affordable health care in creating sustainable livelihoods and in the vital conservation of biodiversity. Moreover demand for medicinal plants is increasing in both developing and developed countries due to growing recognition of natural products being non-narcotic, having no side-effects, easily available at affordable prices and sometime the only source of health care available to the poor. Herbal remedies have stood the test of time for their safety, efficacy, cultural acceptability. Continuous exploitation of several medicinal plant species from the wild and substantial loss of their habitats during the past 15 years have resulted in the population decline of many high value medicinal plant species over the years (Planning Commission 2000; Kala 2003). There are many other potential causes of rarity in medicinal plant species, such as habitat specificity, narrow range of distribution, land-use disturbances, introduction of non-natives, habitat alteration, climatic changes, heavy livestock grazing, explosion of human population, fragmentation and degradation of population, population bottleneck and genetic drift (Kala 2000, 2005; Weekley and Race 2001; Oostermeijer et al. 2003). Over 70 % of the plant collections from wild involve destructive harvesting because of the use of parts like roots, barks, wood, stem and the whole plant in case of herbs. At present 95 % collection of medicinal plants is from wild. This has led to many medicinal species becoming endangered in last decade. To cope up with alarming situation the recent developments in biotechnology have come as a boon. Thus, utilizing the biotechnological approaches towards the improvement of *in situ* and *ex situ* conservation programmes are becoming vital. Micropropagation has been advocated as one of the most viable biotechnological tools for *ex situ* conservation of germplasm (Thakur and Karnosky 2007; Murch et al. 2008). Plant tissue culture techniques offer an integrated approach for the production of standardized quality phytopharmaceuticals through mass production of consistent plant material for physiological characterization and analysis of active ingredients (Debnath et al. 2006). Here in this chapter we will provide some important in vitro conservation protocols for some valuable endangered medicinal plant species.

14.2 General Methodology

Mature and juvenile explants were collected and washed thoroughly under running tap water for 20 min and treated with 1 % (w/v) Bavistin solution (fungicide) for 30 min. Thereafter, these were rinsed in 5 % (v/v) Teepol solution (mild detergent) for 15 min and transferred to laminar hood for further sterilization. Under laminar

hood explants/seeds were rinsed in 0.1 % $HgCl_2$ solution for 3 min and then rinsed thoroughly in sterilized DDW 3–4 times to completely remove toxic traces.

After proper sterilization explants/seeds were inoculated on basal nutrient medium. Basal medium consisted of MS salts and vitamins with 3 % sucrose and 1 % agar. For seed germination and *in vitro* rooting strength of MS was reduced to half. The pH of the medium was adjusted to 5.8 ± 0.05 using 1 N NaOH and HCl before autoclaving at 15 lb/in. for 15 min. Sterilized medium was dispensed in culture tubes (Borosil) and Erlenmeyer flasks of 100 ml (Borosil). Inoculated seeds were incubated in controlled culture room conditions having temperature of 25 ± 2 °C and 55 ± 5 % RH with an irradiance of 50 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (40 W, Philips) for 16 h photoperiod.

14.3 *Tylophora indica* (Burm. f.) Merrill (Asclepiadaceae)

Tylophora indica (Burm. f.) Merrill, commonly called Antamul or Indian ipecac, is an important endangered medicinal plant belonging to the family Asclepiadaceae. It is a perennial woody liana indigenous to India, which is also found in forests of Malaya and Ceylon, and exists up to 900 m altitude in the sub Himalayan tract. Propagation in nature is only through seeds which are largely limited by low percentage of seed set and seasonal dormancy. The leaves and roots are medicinally important parts of this species (Bhavan 1992) and are known to possess emetic, cathartic, laxative, expectorant, diaphoretic and purgative properties. It is traditionally used as a folk remedy in certain regions of India for the treatment of bronchial asthma, inflammation, bronchitis, allergies, rheumatism and dermatitis. It also seems to be a good remedy in traditional medicine as anti-psoriasis, seborrheic, anaphylactic, leucopenia and as an inhibitor of the Schultz-Dale reaction. The roots are suggested to be a good natural preservative of food. The major constituents in this plant are the alkaloids namely tylophorine, which is responsible for strong anti-inflammatory action (Gopalakrishnan et al. 1979), tylophorinine which is pharmacologically active, and tylophorinidine having anti-cancer properties (Mulchandani et al. 1971). The other alkaloids include septicine and isotylocrebrine. The leaves and roots are used medicinally against bronchial asthma, dysentery, whooping cough, bronchitis, and diarrhoea (Kirtikar and Basu 1975; Bhavan 1992; Varrier et al. 1994). The leaves are emetic, diaphoretic and expectorant (Chopra et al. 1958), and used to treat bronchial asthma (Nandkarni 1954), bronchitis and rheumatism (Chopra et al. 1958). They are also used to destroy vermin (Nandkarni 1954). The roots have a sweetish taste and aromatic odour. They possess stimulant, emetic, cathartic, expectorant, stomachic and diaphoretic properties and are used for the treatment of asthma, bronchitis, whooping cough, dysentery, diarrhoea, rheumatism and gout (Anonymous 1976). The roots have bacteriostatic properties (Bhutani et al. 1985). Several pharmaceutical companies (Acron Chemicals, Mumbai, India; Sabinsa Corporation, Piscataway, NJ, USA) are marketing *T. indica* extracts as anti-asthmatic herbal drugs.

It has also been used in the treatment of allergies, colds, dysentery, hayfever and arthritis, and is administered in respiratory infections, bronchitis and whooping cough (Nandkarni 1976). It has reputation as an alterative and as a blood purifier, often used in rheumatism and syphilitic rheumatism. Root or leaf powder is used in diarrhoea, dysentery and intermittent fever. Dried leaves are emetic diaphoretic and expectorant. It is regarded as one of the best indigenous substitute for ipecacuanha (Kirtikar and Basu 1975), so it was considered as Indian ipecacuahna in the latter half of the nineteenth century.

14.3.1 Studies Conducted

Many in vitro regeneration studies have been conducted for conservation of *T. indica*, However most of them reported callus mediated regeneration. Shoot regeneration from leaf derived callus was reported by Faisal and Anis (2003), Thomas and Philip (2005), Chandrashekhar et al. (2006) and Neema et al. (2007). However, Sahai et al. (2010a) reported both organogenesis and embryogenesis from the leaf callus of *Tylophora indica* on single concentrations of BA and TDZ. Faisal and Anis (2005) and Faisal et al. (2005) observed efficient shoot regeneration from stem and petiole callus of this species with 38.0 ± 1.92 shoots/culture and 56 ± 3.6 shoots/culture on 5.0 μM BA and 2.5 μM TDZ respectively. An important study was conducted by Sahai et al. (2010b) in which direct shoot regeneration as well as indirect embryogenesis was reported simultaneously through in vitro derived green root explants of *T. indica*. The study was significant due to the facts that direct organogenesis through nodule mediated shoot formation as well indirect embryogenesis with high efficiency was reported. As self multiplying meristematic nodules presents a good system for large scale plant production in bioreactors for commercial use, this study could help vastly both in conservation of this species and in fulfilling the commercial demand of tylophorine and tylophorinine in pharmaceutical industry.

Protocol (Sahai et al. 2010b)

- Green root explants (GRE) (1.5 cm long) were excised from in vitro maintained cultures of *Tylophora indica* and used as explants.
- GRE were inoculated on various concentrations of BA, 2iP and Kn individually as well as in combination with different concentrations of NAA, IAA and IBA.
- Best response was observed on BA (5.0 μM) + NAA (0.1 μM) containing MS medium where 46.8 ± 0.96 direct shoot buds/culture were obtained along with 18.07 ± 0.33 somatic embryoids/culture.
- Germinating somatic embryoids were transferred to half strength MS augmented with 0.1 μM GA for proper maturation into complete plantlets referred as emblings.
- Excised microshoots were transferred to auxin containing half strength MS medium for rooting. Best rooting was achieved on 0.5 μM IBA with 6.40 ± 0.40 roots/shoot having mean root length of 13.48 ± 0.72 cm.

- Rooted plantlets regenerated via organogenesis and embryogenesis were acclimatized successfully in field conditions with 90 % and 70 % survival rate respectively in vermiculite.

14.4 *Balanites aegyptiaca* (L.) Del. (Balanitaceae)

Balanites aegyptiaca (L.) Del. is a slow growing, drought hardy, fire resistant and stress tolerant evergreen multipurpose tree species belonging to the family 'Balanitaceae'. It is well adapted to different agro-climatic regions with arid and semi-arid climatic features (Von-Maydell 1984). Commonly it is known as 'Desert date' (vernacular: *hingota*). Fruit and roots yield 'Diosgenin' which is a steroidal sapogenin compound used in pharmaceutical industries as a natural source of steroid hormones. Generally diosgenin is used as starting material for partial synthesis of oral contraceptives, sex hormones and other steroids (Zenk 1978). Studies have found important role of diosgenin in control of cholesterol metabolism (Roman et al. 1995) and anti-tumor activity (Moalic et al. 2001; Corbiere et al. 2003). Traditionally various parts of this species have been reported to possess medicinal properties in different ethno-botanical surveys (Hamid et al. 2001; Neuwinger 2004; Ojo et al. 2006). It has been shown to possess anti-leishmanial (El-Tahir et al. 1998), anti-molluscidal (Jain 1987), larvicidal (Wiesman and Chapagain 2006), antibacterial (Doughari et al. 2007), antifungal (Maregesi et al. 2008), hepatoprotective (Ali et al. 2001), antidiabetic (Zaahkouk et al. 2003) and anti inflammatory (Speroni et al. 2005) activities. Besides, this species is known to provide food, fodder, timber, charcoal, and fuel source to local population thus, being of great ecological value. Although natural propagation of this species occurs through seeds, cuttings, and root suckers but none of them are efficient enough to conserve it in wild.

14.4.1 Studies Conducted

Several protocols are available for regeneration of *B. aegyptiaca* through various mature tree derived and seedling derived explants. However, only few of them reported noticeable regeneration. Ndoye et al. (2003) achieved production of 4.3 shoots/explants on BA (5.0 mg/l) through axillary bud proliferation from mature tree derived nodal explants. Gour et al. (2007) reported callus mediated regeneration system for *Balanites aegyptiaca* by taking different explants like apical buds, young thorns and cotyledon pieces from mature tree and root segments of *in vitro* raised seedlings. However, shoot morphogenesis (36.36 %) was achieved only of cotyledon-derived callus. Anis et al. (2010) developed in vitro regeneration protocol through mature tree derived nodal explants. Axillary buds proliferated to yield as many as 7.7 ± 0.40 shoots/explants on BA (12.5 μM) + NAA (1.0 μM)

containing MS medium. Previously Siddique and Anis (2009) reported in vitro regeneration through seedling derived nodal explants where 11.5 ± 0.7 shoots/explants were produced on the medium of same composition (BA (12.5 μM) + NAA (1.0 μM)).

Protocol (Anis et al. 2010)

- Nodal explants collected from mature tree of *B. aegyptiaca* were inoculated on MS medium containing different concentrations of BA, Kn and TDZ either singly or in combination with NAA.
- Axillary bud break resulted in shoot multiplication with maximum of 7.7 ± 0.40 shoots/explant on BA (12.5 μM) + NAA (1.0 μM) containing MS medium in 6 week old culture.
- Elongated microshoots were excised and cultured on full and half strength MS medium alone as well as in combination with various auxins for in vitro root induction.
- The maximum frequency of root formation (68 %), number of roots (5.3 ± 0.32) and root length (4.1 ± 0.38 cm) was obtained in half strength MS medium containing 1.0 μM IBA.
- Plantlets with fully expanded leaves and well developed roots were successfully transferred to soilrite and subsequently to field with 70 % survival rate.

14.5 *Eremostachys superba* Royle ex Benth. (Lamiaceae)

Eremostachys superba Royle ex Benth. is one such plant species which is currently on the verge of extinction. It is a highly ornamental herbaceous species of Lamiaceae which is endemic to Garwal Himalayas. It bears yellow nectariferous, entomophilous flowers on 1–20 long spikes per plant (Koul et al. 1997; Verma 2001) and perennates by means of a thick and deep rhizome. Despite its perennating habit and conspicuously well-adapted zygomorphic flower, it is a critically endangered plant species (*The Hindu* 1997; *The Daily Excelsior* 1997). *E. superba* is under severe threat due to habitat destruction and various anthropogenic stresses. In Jammu and Kashmir, its natural habitat is being converted to agriculture, and the plants are dug for the root-stocks that are fed to cattle to improve lactation and to cure mastitis (Verma 2001; Verma et al. 2003). On the hillside of Mohand it is prone to severe erosion. Browsing by herbivores, collection of flower twigs by travelers due to its highly ornamental value, poor fruit and seed set due to pollinator limitation and lack of seed germination are the major causes of its rapid diminishing population size in Mohand (Rao and Garg 1994). Therefore, there is an urgent need to design an effective strategy for drastic multiplication of *E. superba* to counteract genetic stochasticity from remaining population in a short span of time. In such a critical situation *in vitro* plant tissue culture found its prime importance. Present study is the first attempt of conserving this critically endangered plant species using *in vitro* tissue culture approaches.

14.5.1 Studies Conducted

Only single study is conducted regarding in vitro conservation of this critically endangered species by Sunnichan and Shivanna (1998).

Protocol (Sunnichen and Shivanna 1998)

- Embryos were excised from the mature seeds of E. *superba* and cultured on MS basal media where they grow into seedlings.
- After excising the tap roots of the seedlings they were again cultured on MS medium supplemented with cytokinins.
- Best response was obtained with nine shoots/culture on MS + 0.1 mg/l BAP in 4 weeks of culture.
- Single nodes were isolated from the shoot cluster and cultured on BA and Kn singly as well as with IAA.
- Best response with 19 shoots/culture was obtained on MS medium supplemented with BAP (0.3 mg/l) + IAA (0.1 mg/l) after 16 days of incubation.
- For elongation shoots were transferred to MS medium without any hormone.
- Elongated shoots were transferred to MS + IBA/IAA/GA for in vitro root induction. Best rooting (12.1 ± 0.3 roots/shoot of 2.7 ± 0.3 cm) was obtained on ¼ MS + IBA (2.0 mg/l) + GA_3 (3.0 mg/l) with 2 % sucrose.

14.6 *Tecomella undulata* (Sm.) Seem. (Bignoniaceae)

Tecomella undulata commonly known as 'Rohida' or 'Desert teak' is a deciduous or nearly evergreen tree of dry and desert regions of Arabia, southern Pakistan and northwest India, found up to an elevation of 1,200 mt. It is a multipurpose tree species of economic, medicinal and ecological importance. Its wood is strong, tough, durable and takes a fine finish for which it is used as a substitute of 'teak' for furniture making purposes. Moreover, the presence of lapachol in its heartwood imparts termite and fungal resistance properties making it more suitable for commercial use. Medicinal properties are attributed to the bark of young branches which is used as remedy for syphilis, urinary disorders, enlargement of spleen, gonorrhea, leucoderma and liver diseases while the seeds are used against abscess, jaundice, diabetes, cancer and obesity. Biochemical analysis revealed that leaves of *Tecomella undulata* contain oleanolic acid, ursolic acid and betulinic acid, compounds that are strong HIV prohibitors (Bhau et al. 2007). It has been identified as an important agroforestry tree species mainly due to its high survival rate even in extreme drought and dry arid conditions. However, high demand of this timber yielding species has led to it's over exploitation largely for fuel and timber which lead to its inclusion in endangered plant category. In nature its propagation is severally limited by poor regeneration, sluggish growth and short seed viability caused by denaturation of functional proteins after the extraction of seeds from fruits (Singh 2004).

14.6.1 Studies Conducted

Few reports are available regarding micropropagation studies in *Tecomella undulata*. Single report available through seed derived explants does not ensure clonal multiplication and could not overcome limitation of high heterozygosity with in the species (Aslam et al. 2006). Rathore et al. (1991) obtained multiple shoot formation (8–10 shoots/culture) from nodal segments on BA+IAA combination medium. Robinson et al. (2005) reported production of maximum of nine shoots/explants on MS+(0.75 mg/l) BAP+(0.01 mg/l) IAA from nodal segment explants. Mathur et al. (1995) achieved 26 shoots/explants on MS medium containing IAA (0.05 mg/l) + BAP (2.5 mg/l) from seedling derived cotyledonary node explants. In vitro regeneration from seedling derived callus was achieved with 20–22 shoots/culture on IAA (0.1 mg/l)+BA (2.5 mg/l) containing MS medium by Nandwani et al. (1996). Recently, Varshney and Anis (2011) reported efficient regeneration through seedling derived cotyledonary node explants on TDZ containing MS medium. However still there is need for reliable efficient protocol from mature tree derived explants of *T. undulata* in order to produce clonal plantlets which is the main necessity of a tissue culture protocol which could not be achieved using heterozygous seedling derived explants.

Protocol (Varshney and Anis 2011)

- Seeds of *T. undulata* were sterilized and inoculated on MS medium without any hormone for germination.
- Cotyledonary node (CN) explants were excised from 7 days old aseptic seedlings.
- CN explants were cultured on MS medium containing TDZ at various concentrations.
- Best multiple shoot induction was obtained on 0.7 μM TDZ with average of 25.00 ± 2.30 shoots of 4.06 ± 0.58 cm per explants. However the regenerated shoots were stunted and fascicated.
- Cultures were further transferred to MS medium devoid of TDZ to improve shoot quality and quantity.
- Two fold multiplication (43.00 ± 2.86 shoots/culture) was obtained with improved shoot quality after four subculture passages on MS medium.
- Ex vitro root induction was carried out in regenerated microshoots by giving various pulse treatments. Best rooting was obtained on 200 μM IBA with 4.33 ± 0.88 roots/shoot of mean root length of 5.06 ± 0.85 cm.
- During field acclimatization 80 % survival was recorded in soilrite.

14.7 *Gloriosa superba* Linn. (Liliaceae)

Gloriosa superba L. (Liliaceae) also known as Malabar glory lily or “kembang telang” (Java, Indonesia) is a perennial tuberous climbing herb, extensively scattered in the tropical and sub-tropical parts of the India, including the foothills of

Himalayas. *G. superba* is a native of tropical Africa and is now found growing naturally in many countries of tropical Asia including Bangladesh, India, Sri Lanka, Malaysia and Myanmar. *Gloriosa superba* is an imperative medicinal plant, all parts are used in the medicine, which contains two important alkaloid, colchicine and colchicoside. Different parts of the plant have a wide variety of uses especially within traditional medicine practiced in tropical Africa and Asia. The tuber is used traditionally for the treatment of bruises and sprains, colic, chronic ulcers, hemorrhoids, cancer, leprosy and also for inducing labour pains. Because of its similar pharmacological action, the plant is sometimes used as an adulterant of aconite (*Aconitum* sp.). The white flour prepared from the tubers is bitter and used as stimulant. It is given with honey in gonorrhea, leprosy, colic and intestinal worms and for promoting labor pains, a paste of tubers is applied over the supra-pubic region and vagina. Its warm poultice is locally applied in rheumatism and neuralgic pains (Samy et al. 2008). Seeds and root tubers contain a valuable alkaloid, colchicine, as the major constituent which is occasionally used in cytological and plant breeding research. Medicinally, the tuber is used as abortifacient, and in smaller doses it acts as a tonic, stomachic and anthelmintic. It is also used in the treatment of gout because it contains colchicine. Paste of the tuber is externally applied for parasitic skin diseases (Ghani 2000). Leaves are used to treat cancer related diseases, ulcer, piles, and scrofula (Evans et al. 1981). *Gloriosa superba* usually multiply by corm and seeds but due to low germination capability its regeneration is restricted. Therefore, in order to safeguard and preserve this important plant, biotechnological approaches would be very useful (Sivakumar and Krishnamurthy 2002).

14.7.1 Studies Conducted

In vitro regeneration studies resulted in two types of response in *G. superba* that is tuberization and shoot formation. Hassan and Roy (2005) reported 15 shoots per culture using apical and axillary bud explants in MS basal medium fortified with 1.5 mg/l BA + 0.5 mg/l NAA + 15 % (v/v) Coconut water and 2 g/l activated charcoal. Custers and Bergervoet (1994) reported micropropagation of *G. superba* by shoot cuttings and explants from node, internode, leaves, flowers, pedicels and tubers. *G. rothschidiana* (duphur) vs. *G. rothschidiana* (new accession) and *G. rothschidiana* vs. *G. superba* were cultured on MS basal medium with 3 % w/v sucrose, 0–10 mg/L Benzyl Adenine (BA) and 0.1 mg Indol Acetic Acid (IAA) and maintained for 24 days under 16 h photoperiod. Addition of low level of BA (1 mg/L) improved plant growth, whereas the high level of BA (10 mg/L) caused proliferation of multiple shoots. From rhizome meristem, by applying alternatively high and low BA level, a method of continued propagation was achieved which resulted in a four to seven fold multiplication of qualitatively good plantlets in every 18 week. The resulting shoots were incubated on MS medium, with 3 % sucrose and 0–1 mg/L IAA or NAA. Samarjeeva et al. (1993) studied clonal propagation of

G. superba from apical bud and node segment of shoot tip, cultured on solidified agar (0.8 % w/v) Gamborg's B5 medium containing BA, IAA, Kinetin, NAA, IBA or 2,4-D. Somani et al. (1989) reported *in vitro* propagation and corm formation in *G. superba.* The fresh sprouts were excised from corms of *G. superba* and dissected propagules with shoot and root primordia were placed on MS basal medium (Murashige and Skoog 1962) containing 3 % sucrose and 0.6 % agar. Explant germinated on the MS medium producing shoot and root, which formed new corm within 1 month. For shoot and cormlet regeneration, 1–4 mg/L kinetin was added to the medium. Mukhopadhyay et al. (2008) and Ghosh et al. (2007) reported in vitro tuberization in *G. superba.* Sivakumar and Krishnamurthy (2002) reported *in vitro* organogeneic responses of *G. superba.* They used MS medium supplemented with ADS and BA, 98 %. The callus induction occurred in non-dormant corm bud explants. The maximum number of multiple shoot (57 %) was observed in corm-derived calluses. Sivakumar and Krishnamurthy (2002, 2004) studied induction of embryoids from leaf tissue of *G. superba*.

Protocol (Sivakumar and Krishnamurthy 2002)

- Explants (roots, corm buds (dormant and non-dormant), young leaves, stems, pedicels, and shoot tips from aerial shoots) were collected from both in vitro and in vivo sources.
- Explants were placed on the surface of basal MS medium supplemented with 2,4-D, Kin, BA, and NAA with 2.5 % sucrose.
- Best callus formation was recorded on BA (4.44 μM) and NAA (2.69 μM) from dormant and non dormant corms.
- Proliferated calluses derived from both non-dormant and dormant corm bud explants were transferred to shoot induction medium supplemented with 2iP and Kin within the range of 2.32–9.84 μM.
- After shoot induction cultures were transferred to multiple shoot induction medium where 57 shoots were obtained on BA (11.1 μM)+ADS (6.8 μM) (Adenine sulphate).
- Shoots up to 2 cm long, irrespective of the source of explants, were transferred to MS medium supplemented with IBA. The transfer of shoots to the medium containing 4.92 μM IBA promoted rooting.

14.8 *Commiphora wightii* (Arnott.) Bhandari (Burseraceae)

Commiphora wightii (Arnott.) Bhandari (synonym *C. mukul*, Burseraceae; English name 'Indian bdellium') is a slow growing, endangered medicinal tree, indigenous to Indian subcontinent (Kumar et al. 2004). It is found in Bihar, Mysore, Deccan, Khandesh, Rajputana desert, Sindh, Baluchistan, Arabia and Gujarat. *Commiphora wightii* is now on the verge of extinction over much of its Indian range and is listed as endangered (IUCN 2010). The golden yellow oleo-gum-resin is a complex mixture of over two dozen ketones, several phenolics, diterpenoids and sterols (Dev 1987). The hypolipidemic and hypocholesterolemic activity of oleogum resin of *Commiphora*

wightii (Arnott.) Bhandari is due to the presence of two closely related steroidal ketones i.e. guggulsterone-E and -Z (GsE, GsZ). They are effective antagonists of the bile acid receptor (Wu et al. 2002), In addition to this hypolipidemic effect, gum guggul has been reported to have beneficial effects on inflammation (Kimura et al. 2001), atherosclerosis (Lata et al. 1991), ischemic heart disease (Chander et al. 2003), thrombosis (Olajide 1999), nodulocystic acne (Thappa and Dogra 1994) and thyroid disorders (Panda and Kar 1999). The recent reports explain the mechanism of its lipid lowering properties (Urizar et al. 2002) and its potential role in cancer therapy (Samudio et al. 2005), making them very attractive molecules for production through biotechnological methods using this species. Currently several formulations of guggul gum are available in the markets. Overexploitation and slow-growth associated with poor seed set make this plant an endangered species (Kumar and Shankar 1982; Kumar et al. 2003).

14.8.1 Studies Conducted

Only few noticeable studies have been conducted for micropropagation of *C. wightii*. Barve and Mehta (1993) reported clonal propagation of this species using nodal explants. Axillary branch formation took place with the highest frequency of (4.1 ± 0.5 shoots/explants) on MS medium supplemented with BA (17.8 μM), Kn (18.6 μM), glutamine 100 mg/l, thiamine HCl 10 mg/l and 0.3 % activated charcoal. Recently, Kant et al. (2010) reported shoot multiplication from cotyledonary node explants of *C. wightii* with 2.04 ± 1.22 shoots/axillary bud on BAP (4.44 μM) + NAA (2.68 μM). An efficient protocol of somatic embryogenesis was reported by Kumar et al. (2006) where 25.7 somatic embryos were recovered on modified MS medium containing 30 g/l sucrose and 10 μg/l ABA.

Protocol (Kumar et al. 2006)

- Zygotic embryos were dissected from seeds and cultured on B5 medium containing 2,4,5 T and Kn to obtain callus.
- These callus cultures induced somatic embryogenesis. Cotyledonary stage somatic embryos were isolated and cultured on MS medium with various concentrations of activated charcoal, abssisic acid and mannitol.
- These embryos started producing more embryos with maximum of 138 embryos on 10 μg/l of ABA.
- For optimal maturation, medium containing activated charcoal (0.5 g/l) + sucrose (10 g/l) proved best.

14.9 *Gentiana kurroo* Royle (Gentianaceae)

Gentiana kurroo Royle (Gentianaceae) is an critically endangered important, native Indian species used for medicinal purposes. Because of possible extinction, it is legally protected by law and cannot be exported (Ministry of Commerce, Government of India; Raina et al. 2003). It grows on grass slopes in north-western Himalayan

region at an altitude of 1,800–2,700 m. Roots and rhizomes of this species are of immense medicinal importance. The bitter glycosides (gentiopicrine and gentianin) and alkaloid (gentiamarin) present in roots are of medicinal importance. The root stock is valued as bitter tonic, antiperiodic, expectorant, antibilious, astringent, stomachic, antihelmintic, blood purifier and carminative (Kirtikar and Basu 1935). It is also used for curing skin diseases, bronchial asthma and urinary infections (Chopra et al. 1956; Anonymous 1956). Their biological function is the control of the human digestive system. Unfortunately, the pharmaceutical industry is largely dependent on natural population of *G. kurroo*, which is depleting their wild stands. Thus, there is an urgent need to conserve this species, for which plant tissue culture offers a viable tool.

14.9.1 Studies Conducted

Several protocols have been reported for micropropagation of *G. kurroo* using various explants. Sharma et al. (1993) achieved shoot multiplication in this species using shoot tip and nodal segments. They obtained 3.5 ± 0.7 shoots/explant on BA (8.9 μM) + NAA (2.7 μM) supplemented MS medium. Fiuk and Rybczyński (2008a) investigated morphogenic potential of cell suspensions derived from seedling explants. The highest number of somatic embryos were obtained for cotyledon-derived cell suspension on GA3-free medium, but the best morphological quality of embryos was observed in the presence of 0.5–1.0 mg/l Kin, 0.5 mg/l GA3 and 80.0 mg/l AS. Experiments have been carried out on seedling and primary leaf explants of *Gentiana kurroo* by Fiuk and Rybczyński (2008b). Somatic embryogenesis was recorded from callus cultures of all the explants. Cotyledons showed the highest morphogenic capabilities with 89.97 ± 75.28 embryoids/explants on 2,4-D (2.32 μM) + Kin (4.64 μM). In leaf explants dicamba (9.04 μM) with zeatin (1.14 μM) produced the greatest number of 19.0 ± 4.0 differentiated somatic embryos. The best embryo conversion into germlings was obtained on MS medium containing 4.46 μM kinetin, 1.44 μM GA3 and 2.68 μM NAA on ½ MS. Both media were supplemented with 4.0 % sucrose and 8.0 % agar. Depending on explant origin and conversion medium, 55.8–71.0 % of somatic embryos developed into germlings and plants.

Protocol (Fiuk and Rybczynski 2008b)

- Seeds of *G. kurroo* were inoculated on (MS) medium supplemented with 3.0 % sucrose, 1.44 μM GA3 and 0.8 % agar for germination.
- Seedlings were used as the source of explants (cotyledons, hypocotyl and roots) or were transferred to jars containing MS medium supplemented with 8.86 μM BAP, 1.07 μM NAA, 3.0 % sucrose and 0.8 % agar at pH 5.8 to develop long term axenic culture.
- Young leaves excised from these axenic cultures constituted source of explants.
- Cotyledons, hypocotyl and root explants were cultured on MS medium containing Kn and 2,4-D.

- For leaf explants combinations of auxins: 2,4-D, NAA and Dic alone or with cytokinins: Kin, Zeat, CPPU, BAP and TDZ was tested.
- Callus induced somatic embryogenesis initiated in all the seedling explants. However, the highest morphogenic capacities were possessed by cotyledons that were cultured on 2.26 μM 2,4-D and 4.64 μM Kin.
- The highest number of somatic embryos per explant was 40.0, 57.5 and 89.9 for root, hypocotyl and cotyledon derived explants, respectively.
- In leaf explants dicamba (9.04 μM) with zeatin (1.14 μM) produced the greatest number of 19.0 ± 4.0 differentiated somatic embryos.
- The best embryo conversion into germlings was obtained on MS medium containing 4.46 μM kinetin, 1.44 μM GA3 and 2.68 μM NAA on ½ MS.

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Chapter 15
In Vitro Conservation Protocols for Some Commercially Important Medicinal Plants

Anwar Shahzad and Shahina Parveen

Abbreviations

BA	6-benzylaminopurine
Kn	Kinetin
2iP	2-isopentanyl adenine
TDZ	Thidiazuron
GA_3	Gibberrelic acid
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
NAA	α-naphthalene acetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
PG	Phloroglucinol
AdS	Adenine Sulphate
CW	Coconut Water
CH	Casein Hydrolysate
PGR	Plant Growth Regulator
MS	Murashige and Skoog's medium

15.1 Introduction

Plants have been an important source of medicine for thousands of years. The medicinal value of these plants lies in some chemical substances that produce definite physiological action on the human body. The most important of these plants

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chemical constituents are alkaloids, flavanoids, tannins and phenolic compounds (Hill 1952). The natural medicines from plants have enormous possibilities for new and more effective means for curing the modern day ailments. Some of the oldest traditional systems of medicine include Chinese, Ayurvedic, Unani, Japanese and recently added Homeopathy and Chiropathy that is also around 200 years old. All these systems utilize plants as a whole or some plant parts for the treatment of various diseases. The World Health Organization (WHO) estimates that up to 80 % of people still rely on herbal medicines for their health care (Vines 2004). Traditional medical knowledge of medicinal plants and their use by indigenous cultures are not only useful for conservation of cultural traditions and biodiversity but also for community healthcare and drug development in the present and future (Pei 2001). In the developed countries, 25 % of the medical drugs are based on plants and their derivatives (Principe 1991).

India with its varied climate, soils and agroecology possess an immense plant diversity with over 15, 000 species of higher plants. Approximately 50 % (about 8, 000 species) of these plants are grouped as medicinal plants. Though India has rich diversity and one among the twelve mega diversity centers, the rich resource is decreasing at an alarming rate due to overexploitation. In India, it is reported that traditional healers use 2,500 plant species and 100 species of plants serve as regular sources of medicine (Pei 2001). During the last few decades there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of the world (Hanazaki et al. 2000; Al-Qura'n 2005; Gazzaneo et al. 2005). The increasing realization of the health hazards and toxicity associated with the indiscriminate use of synthetic drugs and antibiotics has increased the use of plants and plant-based medicines. Only a small percentage of medicinal plants traded in India are solely cultivated and about 90 % of medicinal plants utilized by the industries are collected from the wild population. Over-exploitation and destructive harvesting to meet such demand in fact threaten the survival of many rare species. Confronted by such unprecedented genetic erosion and disappearance of species and ecosystems, conservation of natural resources assumes paramount urgency. The growing demand is putting a heavy strain on the existing resources causing a number of species to be either threatened or endangered and many of them are facing extinction (Natesh 1999). Conventional methods of propagation are inadequate to meet the demands of the industries.

Micropropagation or *in vitro* regeneration is a potential biotechnological tool that provides a solution to the problem of medicinal plants decimation. Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture (PTC) methods. Tissue culture has now become a well established technique for culturing and studying the physiological behavior of isolated plant organs, tissues, cells, protoplasts and even cell organelles under precisely controlled physical and chemical conditions. The importance and application of plant cell and tissue culture in plant science are vast and varied. The last few years of research into plant cell, tissue and organ culture had shown the emergence of technology from technique. The establishment of micropropagation for rapid propagation, the use of shoot tip culture to produce

nuclear stock free from parasites, especially viruses, and the application of a variety of procedures including anther and pollen culture to speed up the process of producing better varieties, protoplast culture for hybrid plant production, and genetic manipulation all have contributed to the acceptance of plant tissue culture not only as a valuable tool for plant propagation and multiplication but also for the plant improvement and conservation.

Within a very short time and space, a large number of plantlets can be produced and the regenerants obtained through PTC are genetically identical (clones) or elite in nature. Most of the medicinal plants either do not produce seeds or seeds are too small and do not germinate in soils. Furthermore, the plants raised through seeds are highly heterozygous and show great variations in growth, habit and yield and may have to be discarded because of poor quality of products for their commercial release. Thus mass multiplication of important medicinal plants is a great problem. In this regard micropropagation is the best alternative way to provide true to type, rapid and mass multiplication under disease free or aseptic conditions. Callus mediated organogenesis exhibit great chances of somaclonal variations that could be exploited for developing superior verities particularly in vegetatively propagated plant species. Tissue culture protocols have been developed for a wide range of medicinal plants through different developmental pathways using various explants, a summary of such protocols is provided in Table 15.1.

Different regeneration pathways such as direct regeneration, indirect organogenesis via callus formation and somatic embryogenesis were explored in different species to get optimum multiplication rate utilizing different explants. Rooting and field establishment as a part of micropropagation protocol development has been applied successfully without much difficulty. The purpose of the present review is to focus the application of tissue culture technology for *in vitro* propagation via direct regeneration, organogenesis and somatic embryogenesis with suitable examples reported earlier. An overview of tissue culture studies on important medicinal plants and related species is presented.

15.2 Materials and Methods

15.2.1 Plant Material and Explant Preparation

Explants collected from naturally grown healthy and mature plants as well as from aseptic seedlings (raised through seeds under controlled conditions) were used as the source of plant material. Mature explants/seeds were first washed under running tap water for half an hour and then disinfected with 1 % (w/v) Bavistin (Carnbandazim Powder), a broad spectrum fungicide, for 15–20 min, followed by washing with 5 % (v/v) Teepol (a mild detergent) for 10–15 min by continuous shaking. Further washing was done under laminar air flow, where explants/seeds were treated with 70 % (v/v) ethanol for 30–60 s followed by rapid washing with sterilized double distilled water (DDW) and then surface disinfected with 0.1 % (w/v) freshly prepared mercuric

Table 15.1 List of important medicinal plants which have been propagated through tissue culture

Plant species	Explants	References
Acacia sinuata	CN, ST, C	Vengadesan et al. (2002, 2003a, b) and Shahzad et al. (2006)
Aloe vera	ST, NS	Singh and Sood (2009)
Artemisia annua	NS	Gulati et al. (1996)
Aegle marmelos	AB, NS	Ajithkumar and Seeni (1998) and Pati et al. (2008)
Atropa belladona		
Azadirachta indica	NS	Sharma et al. (2002) and Chaturvedi et al. (2004)
Bacopa monnieri	L, NS, IN	Tiwari et al. (2001) and Mohapatra and Rath (2005)
Cassia angustifolia	CN, C, CN, R	Agrawal and Sardar (2003, 2007), Siddique and Anis (2007), and Parveen and Shahzad (2011)
Cassia siamea	CN	Parveen et al. (2010)
Cassia sophera	CN	Parveen and Shahzad (2010)
Celastrus paniculatus	NS	Martin et al. (2006)
Catharanthus roseus	ST	Bajaj et al. (1988)
Clitoria ternatea	NS, R	Rout (2005) and Shahzad et al. (2007)
Cardiospermum halicacabum	NS, L	Thomas and Maseena (2006)
Commiphora wightii	NS	Barve and Mehta (1993)
Curcuma domestica	Rh	Salvi et al. (2002)
Curcuma longa	Rh	Prathanturarug et al. (2005)
Datura metel	NS	Muthukumar et al. (2004)
Digitalis lanata	ST, NS	Schöner and Reinhard (1986)
Dioscorea composita	NS	Alizadeh et al. (1998)
Gingko biloba	E, L, P	Camper et al. (1997) and Tolyat et al. (2009)
Glycyrrhiza glabra	SB, ST, NS	Thengane et al. (1998) and Vadodaria et al. (2007)
Gymnema sylvestre	S	Komalavalli and Rao (2000)
Holostema ada-kodien		Geetha et al. (2009)
Hyocyamus niger	H, C, S	Uranbey (2005)
Melia azedarach	R	Vila et al. (2005)
Mentha arvensis	NS	Dhawan et al. (2003)
Ocimum basilicum	NS, L	Sahoo et al. (1997) and Gopi and Ponmurugan (2006)
Ocimum sanctum	NS	Shahzad and Siddiqui (2000)
Oroxylum indicum	NS	Dalal and Rai (2004)
Picrorhiza kurroa	ST	Sood and Chauhan (2009)
Papaver somniferum	SE	Ovecka et al. (2000)
Piper longum	ST	Soniya and Das (2002)
Solanum nigrum	NS	Pandhure et al. (2010)
Saussurea lappa	ST	Arora and Bhojwani (1989) and Johnson et al. (1997)
Stevia rebaundiana	NS	Debnath (2008)
Tinospora cordifolia	NS	Raghu et al. (2006)
Veronica anagallis-aquatica	NS	Shahzad et al. (2011)
Vitex negundo	NS	Noman et al. (2008)
Withania somnifera	ST, L	Kulkarni et al. (2000)
Zingiber officinalis	S	Lincy et al. (2009)

AB Axilary bud, *E* Embryo, *H* Hypocotyl, *IN* Internode *L* Leaf *NS* Nodal segment, *P* Petiole, *R* Root, *Rh* Rhizome, *SB* Shoot bud, *SCE* Shoot crown explant, *SE* Somatic embryo, *ST* Shoot tip

chloride ($HgCl_2$) for (3–6) min and then finally rinsed with sterilized DDW (4–5 times). After sterilization, seeds or explants [trimmed at both the ends to appropriate size (1–1.5 cm)] were cultured/inoculated on sterile media.

15.2.2 Culture Media and Conditions

MS medium (Murashige and Skoog 1962) containing 3 % (w/v) sucrose and 0.8 % (w/v) agar were used in most of the experiments (except liquid culture in which medium was prepared without agar). The pH of the medium was adjusted to 5.8 using 1 N NaOH or 1 N HCl. The media were sterilized by autoclaving at 1.06 Kg cm^{-2} pressure and 121 ºC temperature for 20 min. All the cultures were maintained at 24 ± 2 ºC under a 16 h photoperiod with a photosynthetic photon flux (PPF) density of 50 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Philips, India).

15.2.2.1 Establishment of *In Vitro* Cultures

MS basal medium supplemented with various concentrations of different cytokinins (BA, Kn, 2iP and TDZ) either singly or in combination with auxins (2,4-D, 2,4,5-T, IAA, IBA and NAA) was used to evaluate the morphogenic potential of various explants for direct regeneration or organogenesis via callus formation or somatic embryogenesis.

15.2.3 Rooting in Microshoots

15.2.3.1 *In Vitro* Rooting

For the development of complete plantlet, induction of roots in the micropropagated shoots is very important. Microshoots of appropriate size were isolated from the cultures and transferred to rooting media comprising of MS basal and half strength MS augmented with various auxins or devoid of auxins.

15.2.3.2 *Ex Vitro* Root Induction

Besides *in vitro* root induction, some of the studies reported rooting under *ex vitro* conditions. For *ex vitro* rooting microshoots (3–4 cm) were excised from the cultures and their basal cut ends were dipped in IBA or any other auxin for half an hour. After that subsequently planted in thermocups containing sterilized soilrite and covered with polythene bags with a few perforations. The shoots were irrigated with one-fourth strength of MS salt solution for 2 weeks followed by tap water. Hardening

and acclimatization of *ex vitro* rooted shoots were done under controlled conditions. Polythene bags were removed gradually upon emergence of new leaves in order to acclimatize the plants and shoots were taken out from the soilrite to observe the rooting percentage, number of roots per shoot and root length. The rooted plantlets were then transferred to earthen pots containing sterilized soil and manure (1:1) and maintained in greenhouse under natural environment.

15.2.4 Hardening and Acclimatization

Hardening and acclimatization is a very essential and crucial step for micropropagation and the success of tissue culture techniques depends on field establishment of regenerated plants. Well developed plantlets were removed from the rooting medium, washed gently under running tape water to remove any adherent gel and transplanted into thermocol cups containing sterile soilrite. Thermocol cups were covered with transparent polythene bags to ensure high humidity and irrigated after every 3 days with ¼ strength MS salt solutions (without vitamins) for 2 weeks. Polythene bags were removed gradually after 2 weeks to acclimatize plantlets to field conditions. After 4 weeks plantlets were successfully transferred to field conditions/natural habitat.

15.3 *In Vitro* Regeneration in Some Selected Medicinally Important Plant Species

Medicinal plants are now under great pressure due to their excessive collection or exploitation. The degree of threat to natural populations of medicinal plants has increased because more than 90 % of medicinal plant raw material for herbal industries in India and also for export is drawn from natural habitat. Plant tissue culture is an alternative method of commercial propagation and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants. The following account provides a brief survey of tissue culture studies in some of the most important plant species.

The genus *Cassia* belongs to family Fabaceae and subfamily Caesalpinaceae. It comprises of a large number of a species and most of them are of medicinal value. Some of the important species are *C. alata*, *C. angustifolia*, *C. fistula*, *C. occidentalis*, *C. siamea*, *C. sophera* and *C. tora* etc.

15.3.1 Cassia angustifolia Vahl.

Commonly known as Alexandrian or Tinnevely senna is a variable, branching, erect shrub well known for its laxative and purgative properties and is used as a drug in the Unani system of medicine. Presently it is also used in the Ayurvedic and Allopathic systems of medicine. It is mainly cultivated for the leaves referred as

'senna leaves' and being exported on a large scale. Senna leaves and pods contain an important alkaloid known as "sennoside" and are used as an infusion, decoction, powder, syrup and confection. The plant is known to cure a large number of diseases like splenic enlargement, anemia, jaundice, typhoid, cholera, bronchitis, gout, rheumatism, biliousness and leprosy. It is an excellent laxative, employed in the treatment of amoebic dysentery, as an anthelmentic and as a mild lever stimulant. India is the major supplier of the leaves and pods as well as the glycosides to the World market. For large scale cultivation and conservation of this valuable medicinal plant, *in vitro* techniques played an important role. Recently *in vitro* protocols for the regeneration of *C. angustifolia* have been developed using various explants derived from aseptic seedlings. Agrawal and Sardar (2003) used cotyledonary node (CN) explant for regeneration of senna on MS medium supplemented with 1.0 μM BA. Only 2.4 shoots per explant were induced however, the number of shoots increased to a maximum of 17.6 shoots per CN explants when cultured on MS basal medium after giving a treatment of 1.0 μM TDZ for 4 weeks (Siddique and Anis 2007). The regenerated shoots were rooted under aseptic conditions by giving a pulse treatment of IBA (200 μM) for about half an hour. In another report by Siddique and Anis (2007) maximum of 21.7 shoots were produced from nodal segment (NS) explant on MS basal medium. Initially the explants were cultured on MS + TDZ (5.0 μM) + NAA (1.0 μM) for 4 weeks for the induction of an average of 12.9 shoots from single NS and when these cultures were transferred to MS basal medium without any PGR, the number of shoots increased to 21.7 per explant. Leaflets and cotyledonary leaf explants were reported to induce regenerative calli on various hormonal supplements. Agrawal and Sardar (2006) reported regeneration in senna through indirect organogenesis (via callus formation) by culturing leaflets and cotyledonary leaves on MS + BA (1.0 μM) + 2,4 D (1.0 μM). For somatic embryogenesis immature cotyledons were dissected from semi mature seeds and inoculated on MS medium containing different cytokinins and auxins in various combinations (Agrawal and Sardar 2007). 70 % cultures on MS + BA (2.5 μM) + 2,4 D (1.0 μM) produced 14.3 somatic embryos. Besides leaflet and cotyledonary leaves a different explant that is excised root segments has been tried by Parveen and Shahzad (2011) for the induction of regenerative calli and it was observed that root explant proved to be better than both leaflet and cotyledonary leaf explants as it provided a very large number of shoots (42.7) per culture. Root segments excised from aseptically grown seedlings were cut and cultured on MS medium supplemented with different cytokinins and auxins, the best organogenic callus was induced on MS + TDZ (1.0 μM). For shoot regeneration this callus was transferred to various combinations of cytokinins and auxins and among those MS + BA (2.5 μM) + NAA (0.6 μM) proved to be optimal for inducing highest number of shoots.

15.3.2 Cassia sophera L.

The plant is shrubby herb or under shrub in habit about 0.5–3.0 m in height, found throughout India, highly medicinal and extensively used in Homeopathy. It exhibited anticancer, antidiuretic and purgative properties. An infusion or decoction of

leaves is used to cure cough, cold, bronchitis, asthma, hiccups, and jaundice. Also reported to cure rheumatic and inflammatory fevers, skin diseases, piles, ringworms, sores, scabies and psoriasis. The powdered seeds mixed with honey are used in diabetes.

Because of its high medicinal value the plant is in great demand by various pharmaceutical companies and is exploited from the wild. To prevent its mass depletion from the nature and production of large number of planting propagules in a very short span of time, *in vitro* regeneration is the only option. Parveen and Shahzad (2010) for the first time tried to develop a regeneration system for this valuable plant by culturing cotyledonary node (CN) explant on TDZ supplemented MS medium. Explants were collected from the *in vitro* raised seedlings and incubated under controlled condition on different concentrations of TDZ. After 6 weeks of culture 6.7 shoots were induced from single explant on 2.5 μM of TDZ. For further multiplication and development of shoots MS basal medium alone or containing different concentrations of BA were tested. Through this protocol a maximum of 14.9 shoots/explant could be produced, the shoots could be rooted successfully on rooting media and eventually developed into a complete healthy plantlet leading to a 100 % survival rate in natural conditions

15.3.3 Cassia siamea Lam.

It is a tree species of the genus *Cassia* and well known as fast growing, multipurpose, medicinal as well as an economically important agroforestry species. Cultivated as an avenue and shade tree, highly suitable for afforestation of degraded and wastelands, increases soil fertility, check soil erosion and provides best quality timber wood. It has been considered as a tool in various agroforestry programme to replenish wasteland. Besides being an economic tree it also posses medicinal value and contained an important alkaloid "sennoside". Till date not much attention had been given for its conservation and *in vitro* propagation. Conventionally it is propagated through seeds but due to poor seed germination, low viability of seeds and high mortality of seedlings by various disease caused by different pathogens, its cultivation become a difficult task. Thus to ensure large number of quality propagules for various afforestation and agroforestry programmes, we have to rely on tissue culture techniques. In this regard for the first time an attempt has been made by Parveen et al. (2010) to develop an *in vitro* regeneration system for *C. siamea*. CN explants were taken from aseptically grown seedlings and cultured on MS medium supplemented with various plant growth regulators (PGR). This protocol provides a maximum of 12.2 shoots/explant on MS + BA (1.0 μM) + NAA (0.5 μM). The regenerated shoots were isolated, rooted and established as complete plantlets under controlled conditions. The micropropagated plants showed 85 % survival under field conditions.

15.3.4 Acacia sinuata (Lour.) Merr.

Acacia sinuata is a multipurpose leguminous tree with considerable traditional socio-cultural values. It occurs in Tropical forests throughout India and South Asian Countries. It is important for fuel, timber, shelterbelts and soil improvement. The dry pods of the tree, called '*shikakai*' are extensively used as a detergent, in beauty cosmetics, for personal cleanliness, as a hair conditioner and in pharmaceuticals. The powdered pods are superior alternatives to soaps, for treatment of skin diseases and for removal of dandruff. Pods are also used medicinally as an astringent for fever, and as an antidiarrheal and anthelmintic agent (Kirticar and Basu 1989; Vaidyaratnam 1994). *A. sinuata* is commonly propagated through seeds however, seed loss in the natural habitat, poor seed viability and low germination all hinder successful establishment of this species (Dewan et al. 1992). In addition, increasing use of pods for commercial purposes deprives its wider distribution in forested areas. There are difficulties in propagation through conventional methods such as cutting and layering and poor rooting. Micropropagation, which is often used successfully for the multiplication of several woody plants, represents an interesting alternative for this species. *Acacia* species, in general, are recalcitrant to regenerate and they pose various problems during *in vitro* conditions (Nangia and Singh 1996).

Although *in vitro* regeneration via organogenesis has been achieved by Vengadesan et al. (2000) from hypocotyl derived callus of *A. sinuata* on MS medium. Calli were induced from hypocotyl explants excised from 7-day-old seedlings on MS medium containing 6.78 µM 2,4-D and 2.22 µM BA. Regeneration of adventitious buds from callus was obtained when cultured on MS medium supplemented with 10 % coconut water, 13.2 µM BA and 3.42 µM IAA. Regenerated shoots produced prominent roots when transferred to half strength MS medium supplemented with 7.36 µM IBA. Rooted plantlets, thus developed were hardened and successfully established in the soil. This protocol yielded an average of 20 plants per hypocotyl explant over a period of 4 months.

In another study conducted by Vengadesan et al. in 2002, cotyledonary node (CN) and shoot-tip explants excised from 15-day-old *in vitro* grown seedlings were used to initiate cultures in *A. sinuata*. Maximum number of shoots was induced from CN explants on MS medium containing 6.66 µM BA and 4.65 µM Kn. *In vitro* regenerated shoots produced roots when transferred to half strength MS medium supplemented with 7.36 µM IBA. From each CN explants 30 shoots were obtained within 90 days after two subcultures. The regenerated plantlets were transferred to field with 55 % survival rate. Nodal explants (NS) from a mature elite tree of *Acacia sinuata* has also been used by Vengadesan et al. in 2003a for *in vitro* regeneration. Maximum shoot proliferation (75.2 %) was achieved from explants collected during the December to March season in MS medium supplemented with 8.9 µM BA, 2.5 µM TDZ and 135.7 µM Adenine Sulfate (AS) at the end of the first transfer following initial culture (60 days after inoculation). Gibberellic acid (GA_3) at 1.8 µM

promoted shoot elongation. When transferred to half-strength MS medium augmented with 7.4 μM IBA, *in vitro*-regenerated shoots produced prominent roots. Rooted plants were hardened and successfully established in soil. This protocol yielded an average of 100 plants per nodal explant over a period of 3 months. For indirect organogenesis or induction of adventitious shoots cotyledon-derived callus of *A. sinuata* has been used (Vengadesan et al. 2003b). Calluses were induced from explants on MS medium containing 8.1 μM NAA and 2.2 μM BA. High-frequency regeneration of adventitious buds from callus was achieved when cultured on half-strength MS medium supplemented with 10 % coconut water, 13.3 μM BA and 2.5 μM zeatin. Addition of GA_3 (1.7 μM) along with BA (4.4 μM) favored shoots elongation. *In vitro* raised shoots produced prominent roots when transferred to half-strength MS medium supplemented with 7.4 μM IBA. Histological studies revealed that the regenerated shoots originated from the callus. This protocol yielded an average of 40 plantlets per cotyledon explant over a period of 3 months. Shahzad et al. 2006 also used mature green cotyledon explant for the induction of organogenic calli and plant regeneration. Calli were induced at different concentrations of TDZ and then subcultured on BA supplemented MS medium. Through this protocol a maximum of 16.6 shoots were produced per culture. Regenerated shoots were rooted under *in vitro* condition and well developed plantlets were hardened and transferred to net house with 90 % survival rate.

15.3.5 Clitoria ternatea L.

Clitoria ternatea belongs to the family Fabaceae and is distributed in Tropical Asia, Philippine Islands and Madagascar. It is an ornamental perennial climber with conspicuous blue or white flowers and in India it is commonly called 'butterfly pea'. The root is used in the treatment of various diseases like indigestion, constipation, arthritis and eye ailments. It is also employed in cases of ascetics, enlargement of the abdominal viscera, sore throat, skin diseases, etc. (Anonymous 1988; Morris 1999). The root, stem and flower are recommended for the treatment of snake-bite and scorpion-sting. The extract of *C. ternatea* was found to have anxiolytic, antidepressant, anticonvulsant and antistress properties (Jain et al. 2003). Due to unrestricted large-scale exploitation of this natural resource, coupled with limited cultivation and insufficient attempts for its replenishment, the wild stock of this species has been markedly depleted. Propagation from seed is unreliable due to poor germination and death of many young seedlings under natural conditions. *In vitro* culture is an alternative method for conservation and propagation of this species.

In vitro regeneration from nodal explants of *C. ternatea* has been standardized by Rout (2005) on MS medium supplemented with BA or Kn. Inclusion of NAA in the culture along with BA promoted higher number of shoots and after 4 weeks of culture 5.2 shoots per explants were produced. The regenerated shoots were rooted on half-strength MS basal supplemented with 1.34 μM NAA and 2 % sucrose.

About 85 % of the rooted plantlets were acclimatized and transferred to the greenhouse. An efficient protocol has also been developed by Shahzad et al. (2007) for high-frequency shoot regeneration of this valuable medicinal plant. Adventitious shoots were regenerated from young excised root segments of aseptic seedlings on MS medium supplemented with various concentrations of BA, Kn, NAA or 2,4-D either singly or in various combinations. The highest frequency (100 %) of shoot regeneration and maximum number (16.4) of shoots per explant was obtained on MS medium supplemented with 20.0 μM BA and 2.0 μM NAA. Organogenic calli were also induced from the root segments on a medium containing 2,4-D (10.0 or 20.0 μM) and BA (5.0 μM). The calli were differentiated into multiple shoots on MS medium containing 2.5–10.0 μM BA and 2.0 μM NAA. The microshoots were rooted on half strength MS medium supplemented with 5.0 μM IBA and transplanted successfully in field conditions. Further study showed that *in vitro* raised plantlets and *in vivo* grown plants were almost similar and it was confirmed by different studies like SDS PAGE electrophoresis, total soluble protein content and chromosomal study at mitosis. Barik et al. 2007 used CN explants excised from aseptic seedlings for the development of a rapid regeneration system for this valuable legume. Multiple shoots were induced on MS medium supplemented with BA, zeatin riboside, or TDZ. BA at 4.44 μM was most effective for shoot proliferation. Multiple shoots were also induced in nodal segments of *in vitro*-raised shoots grown on MS medium containing 4.44 μM of BA. Rooting was best induced in shoots grown on half-strength MS medium1.42 μM of IBA. Plants were acclimatized in vermicompost and established in soil where they flowered and formed mature seeds.

15.3.6 Azadirachta indica A. Juss

Neem (*Azadirachta indica*, Meliaceae) is highly valued on the Indian subcontinent owing to its medicinal and pesticidal properties. These properties are attributed to the presence of azadirachtin, a ring C-seco tetranortriterpenoid. Seeds are a rich source of azadirachtin (Isman et al. 1990). Azadirachtin either repels insects or prevents their larvae from entering the next instar for pupation. Conventionally, neem is propagated by seeds. Seeds require immediate planting after harvesting as they are recalcitrant. This limits distant distribution of seeds and storage for year-round availability of propagules. Seed propagation also has the disadvantage of heterogeneity as a result of cross-pollination. Application of a reliable clonal propagation system would unquestionably aid in multiplication of such elite types. A range of tissue culture techniques has been developed for crop plants that will effectively achieve rapid clonal propagation.

A tissue culture protocol for shoot formation from nodal and stem explants have been described by Yasseen (1994) obtained from mature tree and cultured on MS medium supplemented with 0.5 μM TDZ and 0.5 μM NAA. Stem node explants produced multiple shoots which were separated and cultured on MS with 0.01, 0.03, 0.5, or 0.9 μM TDZ with 0.5 μM NAA. Stem explants produced callus which

regenerated shoots upon transfer to a fresh medium. Regenerated shoots rooted in MS supplemented with 3.3 μM IBA, and were transferred to soil. Number of shoots increased with increasing TDZ concentration but shoots and root length decreased. The study conducted by Su et al. (1997) and Murthy and Saxena (1998) provided reproducible techniques for clonal multiplication of neem via somatic embryogenesis. Embryogenic calli were initiated from cotyledons or hypocotyls on MS medium supplemented with 0.5 mg/l NAA, 1.0 mg/l BA, 1.0 g/l hydrolysate and 50.0 g/l sucrose (Su et al. 1997). The calli when transferred to a liquid medium similar to the agar medium but with NAA replaced by 0.5 mg/l IAA, formed globular structures which further developed a rudimentary root, after 4–5 weeks incubation. Subsequently, these highly differentiated tissues when transferred into a hormone-free MS medium containing 1.0 g/l casein hydrolysate (CH) and 50.0 g/l sucrose, active embryo masses started to appear after 1–2 weeks. The embryo production was found to improve more than two fold by adding 0.2 mg/l zeatin to the medium. In another study conducted by Murthy and Saxena (1998) somatic embryos were initiated with mature seeds of neem when cultured on MS medium supplemented with TDZ. Regeneration occurred via direct embryo formation and through an intermediary callus phase. TDZ was very effective and induced somatic embryogenesis across a wide range of concentrations (1.0–50.0 μM). However, somatic embryogenesis was accompanied by callus formation at concentrations of 20.0 μM and above. Cell suspension cultures were established with the TDZ-induced callus and groups of large cell clumps were formed within 2–3 weeks. Plants were regenerated from both directly formed somatic embryos and somatic embryos derived from cell suspensions planted on semisolid medium devoid of growth regulators. Regenerated plantlets continued to grow after transfer to a greenhouse environment and were similar phenotypically to zygotic seedlings. *In vitro* clonal propagation of a 50-year-old neem tree through axillary shoot proliferation was achieved by Chaturvedi et al. (2004). Nodal explants collected between March and May gave the best shoot proliferation response (80 % aseptic cultures). Initially ½ MS medium was required for the establishment of nodal segment cultures. Multiple shoot formation occurred in the cultures of ½ MS + BA (1.0 μM) + GA_3 (0.5 μM). Number of shoots enhanced further on transfer of cultures to ½ MS + BA (1.0 μM) + CH (500 mg/l). However, both the media did not support shoot growth and the shoots remained compact and stunted. Therefore, for elongation and recurrent shoot multiplication, full MS + BA (1.0 μM) + CH (250 mg/l) medium was used where the shoots elongated well, and could be multiplied through single node segment cultures at a rate of 7–8-fold every 5 weeks on the fresh medium of the same composition and this rate of shoot multiplication was maintained for almost 5 years. Shoots were rooted on ¼ MS + IBA (0.5 μM), with a frequency as high as 82 %. Micropropagated plants were established in soil with 87 % survival rate. In 2010 Arora et al. developed *in vitro* propogation protocol for the cloning of a 40 year old tree of *A. indica* using nodal segments. Season of collection and maturity of explants showed direct influence on bud-break. Nodal segments collected during the month of April gave best response. Maximum bud-break (78.6–81 %) was obtained when middle order nodes (3rd or 4th node from apex) were taken. Amongst different cytokinins used, BA at the

concentration of 1.11 μM), was found most effective in inducing multiple shoots, whereas inorganic and organic constituents of the medium influenced growth and general condition of proliferating shoots. On an average 3.1 shoots per explant were regenerated in modified MS medium supplemented with 1.11 μM BA, 1.43 μM IAA and 81.43 μM adenine hemisulphate. Isolated shoots were rooted in the presence of 2.46 μM IBA. The *in vitro* raised plantlets were successfully transplanted in potted soil and finally grown under field conditions with 100 % survival. The genetic fidelity of *in vitro*-raised field-grown plants was ascertained by random amplified polymorphic DNA (RAPD) markers. Furthermore, the azadirachtin content of *in vitro* cloned plants was found comparable to the mother tree proving their chemical stability also. The protocol developed holds good for *in vitro* cloning of mature elite neem trees.

15.3.7 Melia azederach L.

Chinaberry or Persian lilac (*Melia azedarach*, Meliaceae) is an Asiatic multipurpose tree of world wide cultivation with economic and medicinal importance. It is a native of North-Western India, and naturalized in many subtropical countries. It withstands cooler climates better than its related tree "neem" (*Azadirachta indica* A. Juss) and is a fast-growing species with long lasting wood, used as a component of agroforestry systems with inter cropping annual species (Saymaiya and Shukla 1998). Moreover, Chinaberry is resistant to locust and ants and is a good source of natural compounds with potent insecticide and anitmicrobial action (Wan et al. 1994; Schmidt et al. 1997; Carpinela et al. 1999). The tree yields valuable timber resistant to termites, fodder, green manure, as well as oil from seeds. The tree bears seed only during the summer and its natural rate of multiplication is limited. Although the fruits are the poisonous part of the tree, they have been used traditionally for the treatment of a variety of diseases, especially dermatitis and rubella (del Mendez et al. 2002; Kim et al. 1999). In addition, a number of potent pharmaceutical limonoids and triterpenoids have been isolated from fruits and bark (Lee et al. 1991). In view of its economic and medicinal uses, attempts have been made to micropropagate this plant through tissue culture.

Micropropagation of *Melia azederach* has been established by Ahmad et al. (1990), Thakur et al. (1998) and Sharry and Abedini (2001) from an organogenic pathway. Thakur et al. (1998) used nodal explants of 3–6-week-old seedlings of *M. azederach* and cultured on MS medium supplemented with BA (17.75 μM). Multiple shoots were produced, shoots were isolated and induced to root on ½ MS medium supplemented with 4.92 μM. *In vitro* rooted shoots resumed growth after a short period of acclimatization and resulted in plantlets which were successfully established in soil.

A procedure has been developed by Vila et al. (2003a) for the regeneration of 'paradise tree' (*M. azederach*) from immature zygotic embryos via somatic embryogenesis. Somatic embryos were induced from explants cultured on MS medium

supplemented with 0.45, 4.54, or 13.62 μM TDZ. Development of somatic embryos was accomplished with MS at quarter-strength with 3 % sucrose. A large number of plants were regenerated from somatic embryos and successfully established in soil in a greenhouse. This system may be beneficial for mass propagation as well as for genetic manipulation of the 'paradise tree'. In another report by Vila et al. (2003b/2004) shoots were regenerated from the calli obtained from leaflets of *in vitro* grown seedlings. The best medium for the establishment of cultures was MS medium supplemented with 4.44 μM BA + 0.46 μM Kn + 16.29 μM AdS (Adenine Sulphate). Regenerated shoots were multiplied on MS + BA (0.44 μM) + Kn (0.37 μM) + 3.26 μM AdS. Shoots were rooted by giving a pulse treatment in MS + 12.26 μM IBA for initial 3 days and then subsequently transferring the shoots to hormone free MS basal medium. Plantlets were successfully acclimatized and transferred to field conditions. An efficient regeneration protocol for rapid multiplication of *M. azedarach*, has also been developed by Husain and Anis (2009). Nearly 90 % of the culture exhibited axillary bud sprouting and multiple shoot formation from nodal segments derived from 20-year-old candidate plus tree on MS medium supplemented with 5.0 μM BA. The highest shoot regeneration frequency (92 %) was obtained on MS + 5.0 μM BA + 0.5 μM IAA and 30.0 μM AdS. Addition of 250 mg/l ammonium sulphate, $(NH_4)_2SO_4$, and 100 mg/l K_2SO_4 prevented defoliation and tip burning without affecting the number of shoots. The percentage of shoot multiplication as well as the number of shoots per node remained the same during first three subculture passages, afterwards a decline was recorded. About 90 % of the *in vitro r*egenerated shoots were successfully rooted *ex vitro* by giving a pulse treatment of 250 μM IBA for 15 min, followed by their transfer to thermocol cups containing soilrite. The raised plantlets were successfully acclimatized first under culture room conditions, then to green house with 85 % survival rate.

15.3.8 Ocimum basilicum L.

Ocimum basilicum (sweet basil) belongs to the family Lamiaceae, is a herbaceous species rich in aromatic essential oils and is valuable for its pharmaceutical, aromatic and culinary properties. The plant is stomatic, antihelminthic, antipyretic, diaphoretic, expectorant, carminative, stimulant and pectoral (Sahoo et al. 1997; Phippen and Simon 1998). Roots bark, and leaves are cyanogenetic. The seeds have demulcent, stimulant, diuretic and diaphoretic properties (Sahoo et al. 1997). The conventional method of propagation of this species is only through seeds. However poor seed viability and low germination rate restricts its large scale propagation. *In vitro* micropropagation is an effective mean for rapid multiplication of the species using various strategies. An efficient protocol for *in vitro* propagation of this medicinal herb (sweet basil) through nodal explants, collected from field-grown plants has been developed by Sahoo et al. 1997. High frequency bud break and maximum number of axillary shoot formation was induced in the nodal explants on MS medium containing BA. The nodal explants required the presence of BA at a higher

concentration (4.4 μM) at the initial stage of bud break, however, further growth and proliferation required transfer to a medium containing BA at a relatively low concentration (1.1 μM). GA_3 at 1.2 μM added to the medium along with BA (4.4 μM) markedly enhanced the frequency of bud break. The shoot clumps that were maintained on the proliferating medium for longer durations, developed inflorescences and flowered *in vitro*. The shoots formed *in vitro* were rooted on half-strength MS supplemented with 5.0 μM IBA. Rooted plantlets were successfully acclimated in vermi-compost inside a growth chamber and eventually established in soil. All regenerated plants were identical to the donor plants with respect to vegetative and floral morphology.

The study conducted by Dode et al. 2003 described the procedure for micropropagation of *O. basilicum* using cotyledonary leaves from *in vitro* geminated seedlings. Explants were cultured in MS medium containing 0.2 mg l^{-1} NAA in combination with 0–5.0 mg/l BA. The highest efficiency of shoot formation after 45 days occurred in the medium containing 5.0 mg/l BA and 0.2 mg/l NAA. Higher concentrations of BA induced an increase in the regeneration percentage and a higher number of shoots/explant. An effective protocol for complete plant regeneration via somatic embryogenesis has been developed by Gopi and Ponmurugan (2006). Callus was initiated from leaf explant of young plant on MS medium supplemented with 1.0 mg l^{-1} 2,4-D. The calli showed differentiation of globular structure embryos when transferred to MS medium containing 0.5 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BA. The maximum globular structure embryos were further enlarged and produced somatic embryos in MS basal medium supplemented with BA (1.0 mg/l) + NAA (1.0 mg/l) + Kn (0.5 mg/l). Continued formation of globular embryo and germination of embryos occurred in this medium. Complete plantlets were transferred onto specially made plastic cup containing soilrite followed by their transfer to the garden soil. Survival rate of the plantlets under *ex vitro* condition was 80 %.

Daniel et al. (2010) reported micropropagation of *O. basilicum* using nodal explants on MS medium. Explants produced multiple shoots on the medium containing 0.5 mg/l BA with 0.5 mg/l IAA. The elongated shoots were separated and cultured for root induction. Rooting of *in vitro* raised shoots were best induced on ½ strength MS medium supplemented with 1.5 mg/l IBA with highest percentage of shoot regenerating roots (89 %). The well rooted plantlets were acclimatized and successfully established in the natural condition with 90 % survival.

15.3.9 Ocimum sanctum L.

Ocimum sanctum commonly known as 'holy basil' belongs to the family Lamiaceae. The plant is held sacred by Hindus all over the world and is of great medicinal value. The leaves on steam distillation yield a bright yellow volatile oil possessing a pleasant odour characteristic of the plant with an appreciable note of cloves. This oil possesses antibacterial and insecticidal properties and inhibits *in vitro* growth of *Mycobacterium tuberculosis* and *Micrococcus pyogenes* var. *aureus*. Besides oil,

leaves also contain ascorbic acid and carotene. The juice of leaves has diaphoretic, antiperiodic, stimulating and expectorant properties. It is also used in catarrh, bronchitis and is considered to have adaptogenic properties. It is anti-inflammatory, analgesic and a tonic for central nervous system (Anonymous 1966; Giri et al. 1987; Khosla 1995). The conventional method for propagation of *Ocimum sanctum* is via seed. However, poor germination potential restricts its multiplication. Unfortunately, this plant cannot be vegetatively propagated (Pattnaik and Chand 1996). *In vitro* micropropagation is an effective means for rapid multiplication of species in which conventional methods have limitations. In 1996 Pattnaik and Chand developed a procedure for *in vitro* propagation of *Ocimum americanum* and *Ocimum sanctum* using axillary shoot buds. Multiple shoots were induced on MS medium supplemented with BA. The optimum concentration for multiple shoot proliferation was 0.25 mg/l for *O. americanum* and 1.0 mg/l for *O. sanctum*. Addition of 0.5 mg/l GA_3 to the medium resulted in an increase in the frequency of axillary branching and multiple shoot formation. Shoot buds collected between September and December were more responsive in culture. Shoots of *O. sanctum* rooted on half strength MS medium supplemented with 1.0 mg/l NAA. The hardened plants were successfully transferred to soil in natural conditions. *In vitro* micropropagation of holy basil has also been achieved by Singh and Sehgal (1999) on MS medium utilizing young inflorescence explants. MS supplemented with 2,4-D or TDZ produced only non-morphogenetic callus. Direct multiple shoots differentiated within 2–3 weeks when explants were cultured on MS containing BA. Of the various levels of BA tested, MS + BA (1.0 mg/l) produced the maximum number of shoots. Addition of IAA (0.05 mg/l) with BA (1.0 mg/l) in the medium showed a marked increase in the number of shoots. About 92 % of the microshoots rooted on MS hormone-free medium within 2–3 weeks of culture and 85 % of the micropropagated plantlets were successfully established in soil.

In 2000, Shahzad and Siddiqui developed a protocol for *in vitro* organogenesis in *O. sanctum* from nodal explant callus. The explants were cultured on MS medium supplemented with different phytohormones. Phenolic exudates adversely affect the culture response, the explants turned brown and ultimately become necrotic. The addition of 50 mg/l Ascorbic acid (AA) checked the release of phenolic exudates. Treatment of explants with AA and omission of AA from the medium were quite beneficial for callus induction as well as caulogenesis and rhizogenesis from the callus and also directly from the explant. Organogenic calli were induced on MS medium supplemented with 2,4-D (2.0 mg/l), multiple shoots were differentiated on subculturing the callus on MS + BA (5.0 mg/l) + NAA (0.2 mg/l) + glutamic acid (50.0 mg/l). Early callus induction followed by profuse rhizogenesis was observed on MS + NAA (5.0 mg/l) + BA (0.5 mg/l) and lateral bud break has been obtained on MS + Ads (5.0 mg/l) + IAA (0.5 mg/l). The microshoots were rooted on MS + NAA (1.5 mg/l) and the plantlets developed were successfully transferred to soil.

Cotyledonary leaves of *Ocimum* species *viz*., *O. basilicum*, *O. sanctum* and *O. gratissimum* were used for comparative studies on somatic embryogenesis by Mathew and Sankar (2011). MS medium supplemented with 2,4-D and BA was used to initiate callus. MS with 2,4-D (1.0 mg/l) + BA (0.5 mg/l) was found suitable

for the development of callus with maximum weight and lesser days to induction for *O. basilicum* and *O. sanctum* whereas MS with 2,4-D (0.5 mg/l) + BA (0.5 mg/l) initiated callus of maximum weight with high % of response and lesser days to induction for *O. gratissimum*. High % of response to callus induction was found in MS with 2,4-D (1.5 mg/l) + BA (0.5 mg/l) for *O. basilicum* and *O. sanctum*. Differentiation into globular stage of somatic embryos was observed in all cultures but with variation in duration, % response and embryo colour, on transfer of sub-cultured callus to MS media containing different concentrations and combinations of BA, Kn and IAA. Maximum differentiation into globular shaped somatic embryos was observed in all concentration ranges of Kn with or without IAA and in BA (2.0, 3.0 mg/l) which had coconut water (CW) as an additional supplement.

15.3.10 Solanum nigrum L.

Solanum nigrum commonly known as black night shade is an important medicinal plant of family Solanacae, grown in dry parts of India up to an elevation of 2,100 m. It is an important herbaceous medicinal plant, generally used in traditional and folklore medicines. The whole plant is anti-periodic, anti-phlogiston, diaphoretic, diuretic, emollient, febrifuge, narcotic, purgative and sedative. The leaves, stems and roots are used externally as a poultice; wash etc. in the treatment of cancerous soles, boils, leucoderma and wounds (Moerman 1998). Extracts of the plant are analgesic, antispasmodic, anti-inflammatory and vasodilator. The plant has been used in the manufacture of locally analgesic ointments and the juice of the fruit has been used as an analgesic for toothaches. Most important aspect of this medicinal plant is that it contains two important alkaloids solamargin and solasonine which yield solasodine as glycone. Solasodine has embryogenic, teratonic as well as anti-fungal, antiviral and molluscidal effects (Kim et al. 1996). Solasodine has great demand in pharmaceutical industry owing to its demand in pharma industry the plant is extensively harvested from the wild. So it is necessary to develop efficient protocols for *in vitro* propagation of this important herbaceous medicinal plant.

Pandhure et al. 2010 developed *in vitro* regeneration system for this medicinally important plant by using nodal segment. During this investigation, direct multiple shoots were produced on MS medium supplemented with BA (6 mg/l) and IAA (0.5 mg/l). The well developed shoots were sub cultured on a rooting medium containing IBA. Plants were multiplied *in vitro* and hardened successfully. An efficient protocol for direct shoot regeneration from nodal segment and shoot tip explants of *S. nigrum* has been described by Sridhar and Naidu (2011). Explants were cultured on MS medium supplemented with different concentrations of BA (0.5–3.0 mg/l) or Kn (0.5–3.0 mg/l) alone or in combination with IAA or NAA (0.5–1.0 mg/l). All the explants were responded effectively for regeneration. High frequency and maximum number of multiple shoots was obtained in shoot tip culture, cultured on MS medium supplemented with 2.0 mg/l BA + 0.5 mg/l IAA. The present investigation also described the role of auxins on successful induction of *in vitro* flowering from

axillary bud, shoot tip and young leaf explants of *S. nigrum*. Maximum number of *in vitro* flowers (8.0) were obtained in shoot tip culture induced on MS + Kn (3.0 mg/l) + IAA (0.5 mg/l) followed by Kn (2.0 mg/l) + IAA (1.0 mg/l), where maximum of six (6.0) flowers per culture was obtained from axillary bud explant. All the *in vitro* raised shoots were transferred to MS rooting medium supplemented with IBA (0.25–1.0 mg/l). The best rooting response was observed in IBA (0.5 mg/l). The well rooted plantlets were transferred to polythene bags containing soil + vermiculite in 1:1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions for maximum survivability. In another study by Sridhar and Naidu (2011) shoot organogenesis has been achieved using leaf explants. The regeneration of shoots from leaf was found to vary with varying concentrations of BA. The combination of BA (2.0 mg/l) and IAA (0.5 mg/l) produced maximum number of shoots (32.8) from leaf explants of field grown *S. nigrum* plants. Where as in *in vitro* derived leaf explants maximum number of shoots (38.0) was obtained on BA (3.0 mg/l) and IAA (0.5 mg/l) combination. All the *in vitro* raised shoots with a length of 3.0–5.0 cm were transferred to rooting medium supplemented with different concentrations of auxins such as IBA, NAA and IAA (0.25–1.0 mg/l). The best rooting response was observed on 0.5 mg/l IBA. The well rooted plantlets were transferred to polythene bags containing soil + vermiculite in 1:1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions for maximum survivability. From the obtained results it is evident that leaf material is the best source for inducing maximum number of multiple shoots via direct shoot regeneration in *S. nigrum*.

Another protocol was developed for rapid multiplication of this medicinal plant, using mature nodal explants by Sundari et al. (2010). Multiple shoots were induced on MS medium supplemented with varying concentrations and combinations of auxins (NAA and IAA) and cytokinins (BA and Kn). Maximum number of shoots was developed with medium fortified with 13.5 μM BA. The excised shoots were rooted on half strength woody plant medium (WPM) supplemented with IAA (5.58 μM) and IBA (4.92 μM).

15.3.11 Stevia rebaudiana Bertoni

Stevia rebaudiana is one of 154 members of the genus *Stevia*, which produces sweet steviol glycosides (Robinson 1930; Soejarto et al. 1982). Belonging to the family Compositae, is one of the most valuable tropical medicinal plant. It is originally a South American wild plant (Katayma et al. 1976), but it could be found growing in semi-arid habitat ranging from grassland to scrub forest to mountain terrain. In ancient Indian traditional Ayurvedic system of medicine, *S. rebaudiana has* a long history of use by tribal people. The leaves of *Stevia* are the source of the diterpene glycosides, viz. stevioside and rebaudioside, which are estimated to be 100–300 times sweeter than sucrose (Ishima and Katayama 1976; Tanaka 1982). The leaf extracts have long been used in southern Africa to treat diabetes. The seeds of *Stevia*

show a very low germination percentage. Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition (Tamura et al. 1984).

Vegetative propagation too is limited by the lower number of individuals that can be obtained simultaneously from a single plant (Sakaguchi and Kan 1982). Thus, tissue culture is the only alternative for rapid mass propagation of *Stevia* plants.

Sivaram and Mukundan (2003) used shoot apex, nodal and leaf explants of *Stevia rebaudiana* to regenerate multiple shoots when cultured on MS medium supplemented with BA (8.87 μM) and IAA (5.71 μM). Rooting of the *in vitro*-derived shoots could be achieved following subculture onto auxin-containing medium. A survival rate of 70 % was recorded at the hardening phase on the substrate cocopeat. The presence of the sweet diterpene glycosides, viz. stevioside and rebaudioside, was confirmed in the *in vitro* derived tissues of *Stevia* using HPTLC techniques. Callus cultured on agar-solidified MS medium supplemented with BA (8.87 μM) and IBA (9.80 μM) showed the highest sweetener content.

Ahmed et al. (2007) developed an efficient regeneration system for clonal propagation of this sweetener plant. Multiple shoots were regenerated from nodal explants. The induction of multiple shoots from nodal segments was the highest in MS medium supplemented with BA (1.5 mg/l) + Kn (0.5 mg/l). For rooting different concentrations of IBA, NAA and IAA were used and highest rooting percentage (97.66 %) was recorded on MS medium with IAA (0.1 mg/l). The rooted plantlets were hardened and successfully established in soil. A protocol has also been developed by Debnath (2008) for plant regeneration and antimicrobial screening of *S. rebaudiana* through *in vitro* culture of nodal segments with MS medium supplemented with BA (2.0 mg/l) and IAA (1.13 mg/l) in combination were found to be most effective in inducing bud break and growth, and in initiating multiple shoot proliferation at the rate of 39 microshoots per nodal explant after 30 days of culture. By repeated subculturing a high-frequency multiplication rate was established for production of elite lines of this plant. Elongated shoots were transferred to rooting medium. MS medium supplemented with IBA (2.0 mg/l) was found to be best for rooting. *In vitro* and *in vivo* grown leaf extracts in different solvent system were screened for potential antimicrobial activity against medically important bacterial and fungal strains by agar well diffusion method. The chloroform and methanol extract exhibited a concentration dependent antibacterial and antifungal inhibition. Both *in vitro* and *in vivo* dried leaf extract showed similar antimicrobial activity. Therefore, commercial manufacture of active constituents from these improved elite lines would be useful and profitable. Ibrahim et al. (2008) studied the effect of different growth regulators on the cultivation of *S. rebaundiana*. Results indicated that BA increased multiplication rate, vitrification and somaclonal variation. However, the best results were recorded with MS nutrient medium without plant growth regulators during *in vitro* growth and development of *S. rebaudiana* MS basal medium supplemented with BA (2.0 mg/l) recorded the highest number of shoots, but these shoots were very thin and vitrified and not suitable for multiplication through several subcultures. The nutrient medium (MS basal medium) supplemented with 10 mg/l kn recorded 45 shoots/explant as compared to MS nutrient

medium which recorded 6.63 shoots/explant but growth parameters for *S. rebaudiana* plantlets grown in MS medium without kn is better than MS medium containing kinetin at high concentrations. Nutrient medium (MS basal medium) supplemented with IBA at low concentrations (0.01 mg/l) or without auxins achieved the best *in vitro* growth of *S. rebaudiana* plantlets (100 % root formation). IBA was better than NAA and IAA for shoot and root formation. Increasing NAA concentrations decreased gradually number of shoots. Regarding the effect of NAA on root formation, data indicated that the per cent of shoots formed roots was 87 % on MS basal medium without plant growth regulators as compared to MS basal medium supplemented with NAA at low concentrations 0.001, 0.01 and 0.1 mg/l NAA where root per cent was 80, 73 and 53 % respectively. On the other hand, NAA at 1.0 and 1.5 mg/l did not help shoots to form roots. Statistical analysis of variance showed no significant differences amongst IAA treatments for *in vitro* growth of plantlets. *S. rebaudiana* plants were adapted and grown well in planting media containing peat moss, sand and vermiculite at equal volume.

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