CHAPTER 9

ANTIMICROBIAL ACTIVITY

9.0 INTRODUCTION

9.1.1 Infection and diseases

Infectious diseases are the leading cause of death worldwide, especially in developing countries. In recent times, epidemics of infections due to drug resistant and unknown microbial organisms have posed enormous health concern. Also they are the underlying cause of death in approximately 80% of cases even in the United States of America (Pinner *et al.*, 1996). This situation has called for renewed strategies on treatment and prevention, of which the development of new antimicrobial agents is one of the strategies (Fauci, 1998).

In recent time, it is observed that there is rapid increase of pathogenic bacteria become multidrug resistant. This alarming situation has its origin in the excessive and often inappropriate use of antibiotics in human and animal health care for the treatment and prevention of bacterial infections. Since the development of the first commercially available antibiotic penicillin in the 1940s, the high expectations by man in the healing power of these 'wonder drugs' has not been fulfilled, as the drug resistance posed problems in it. To control this situation, newer drugs armed with competent mechanism should be developed. The plant derived antimicrobial agents may be the solution to these problems.

The continuous use of antibiotics has resulted in multi-resistant bacterial strains all over the world and as expected, hospitals have become breeding grounds for human associated microorganisms (Mainous *et al.*, 2001) and spreading the nosocomial infections. As an example, it is now estimated that about half of all *Staphylococcus aureus* strains found in many medical institutions are resistant to antibiotics such as methicillin (Roder *et al.*, 1999). The emergence among enterococci of resistance to another useful and widely effective antibiotic, vancomycin (Novak *et al.*, 1999), might accelerate the spread of vancomycin-resistant genes, via plasmids, throughout other species, eventually limiting the efficacy of this drug.

The discovery of two classes of antimicrobial peptides, non-ribosomally synthesized (Hancock *et al.*, 1999) present in bacteria, lower eukaryotes and plants; secondly ribosomally synthesized peptides, of wider distribution (Boman, 1995; Broekaert *et al.*, 1997; Hancock *et al.*, 1998; Hoffmann *et al.*, 1999; Thevissen *et al.*, 1999; Zasloff, 2002; Ezekowitz *et al.*, 2003), provided a new therapeutic strategy to fight microorganisms. The knowledge acquired in the past two decades and the discovery of new groups of antimicrobial peptides makes natural antibiotics the basic element of a novel generation of drugs for the treatment of bacterial and fungal infections (De Lucca, 2000; Hancock, 2000; Welling *et al.*, 2000; Selitrennikoff, 2001). In plants, a similar picture is slowly emerging *e.g.* A new family of antimicrobial peptides has been described from *Macadamia integrifolia* of which the first purified member has been termed MiAMP2c (Marcus *et al.*, 1997).

Plants have provided western medicine with an abundance of drugs and treatments for a variety of health problems (Lewis, 1977; Bruneton, 1999). While species used in traditional medicines continue to be the most reliable sources for the discovery of useful compounds, the screening of plants growing under various stresses (Ben *et al.*, 1992; Hanawa *et al.*, 1992; Kruger *et al.*, 1994; Broekaert *et al.*, 1997; Mohamed *et al.*, 1997; Dubery *et al.*, 1999; Pernas *et al.*, 2000) has provided yet another source for compounds with useful activities against microbes.

Recently, medicinal plants have become the focus of intense study regarding their conservation and potential pharmacological effects. Indeed, the search for new pharmacologically active agents, through the screening of natural sources such as microbial fermentations and plant extracts, has led to the discovery of many clinically useful drugs that now play major roles in the treatment of human diseases (Yue-Zhong, 1998; Leitao *et al.*, 2006; Funke, 2006).

Plants normally grow on different nature of soils which are extremely rich in microorganisms and infection remains a rare event. To keep out potential invaders, plants produce a wide range of selective antibacterial compounds either in a constitutive or an inducible manner (Cammue *et al.*, 1992). Among these compounds several low molecular weight proteins or peptides with antibacterial or antifungal activity have been isolated in recent years from various plants (Terras *et al.*, 1992; Hejgaard *et al.*, 1992; Roberts *et al.*, 1986) and are believed to be involved in a defence mechanism against phytopathogenic fungi by inhibiting microorganisms growth through diverse molecular modes, such as binding to chitin or increasing the permeability of the fungal membranes or cell wall. Also plants prevent the entry of invaders by localized production of antimicrobial low molecular weight secondary metabolites known as phytoalexins (Van *et al.*, 1989; Maher *et al.*, 1994). Moreover, the synthesis of many presumed defence related proteins are induced when plants are confronted with pathogens (Linthorst, 1991).

A large group of low molecular weight natural compounds that exhibit antimicrobial activity has been isolated from animals and plants during the past two decades. Among them, cationic peptides are the most widespread (Sergio *et al.*, 2003).

9.1.2 Historic use of plants as antimicrobials

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. Their role is two fold in the development of new drugs: (1) they may become the base for the development of a medicine, a natural blueprint for the development of new drugs, or; (2) a phytomedicine to be used for the treatment of diseases. There are numerous illustrations of plant derived

drugs. Some selected examples, including those classified as antiinfectives are presented below.

The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha* and related species, has been used for many years as amoebicidal drug as well as for the treatment of abscesses due to *Escherichia histolytica* infections (Cowan, 1999). Another important drug of plant origin with a long history of use is quinine. This alkaloid occurs naturally in the bark of *Cinchona* tree. Currently, the widely prescribed drugs are analogs of quinine such as chloroquine. Some strains of malarial parasites have become resistant to the quinines, therefore antimalarial drugs with novel mode of action are required (Cowan, 1999).

The higher plants have made important contributions in the areas beyond antiinfectives, such as in cancer therapies, the antileukaemic alkaloids, vinblastine and vincristine are used. They are obtained from the Madagascan periwinkle (*Catharanthus roseus* Syn. *Vinca roseus*) (Nelson, 1982). Other cancer therapeutic agents include taxol, homoharringtonine and several derivatives of camptothein are plant originated. A well-known benzylisoquinoline alkaliod, papaverine, has been shown to have a potent inhibitory effect on the replication of several viruses including cytomegalovirus, measles and HIV (Turano *et al.*, 1989). Most recently, three new atropisomeric naphthylisoquinoline alkaloid dimers, michellamines A, B, and C were isolated from a newly described species tropical liana *Ancistrocladus korupensis* from the rainforest of Cameroon. The three compounds showed potential anti-HIV with michellamine B being the most potent and abundant member of the series. These compounds were capable of complete inhibition of the cytopathic effects of HIV-1 and HIV-2 on human lymphoblastoid target cell *in vitro* (Boyd *et al.*, 1994).

9.1.3 Antimicrobial compounds from plants

It is estimated that today, plant materials are present in, or have provided the models for 50% Western drugs (Robbers, 1996). Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity. The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment.

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Most are secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total. In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odors; others (quinones and tannins) are responsible for plant pigment. Many compounds are

responsible for plant flavor (e.g., the terpenoid capsaicin from chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds. Useful antimicrobial phytochemicals can be divided into several Categories. Laboratories of the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro*. More of these compounds should be subjected to animal and human studies to determine their effectiveness in whole-organism systems, including in particular toxicity studies as well as an examination of their effects on beneficial normal micro biota. It would be advantageous to standardize methods of extraction and *in vitro* testing so that the search could be more systematic and interpretation of results would be facilitated. Also, alternative mechanisms of infection prevention and treatment should be included in initial activity screenings. Disruption of adhesion is one example of an anti-infection activity not commonly screened for currently. Attention to these issues could usher in a badly needed new era of chemotherapeutic treatment of infection by using plant-derived principles (Cowan, 1999).

9.1.4 Therapeutic benefit of natural antimicrobials

Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources. It was the discovery of penicillin that led to later discoveries of antibiotics such as streptomycin, aureomycin and chloromycetin (Trease, 1972). Though most of the clinically used antibiotics are produced by soil micro-organisms or fungi, higher plants have also been a source of antibiotics (Trease, 1972). Examples of these are the bacteriostatic and antifugicidal properties of Lichens, the antibiotic action of allinine in *Allium sativum* (Garlic), or the antimicrobial action berberines in goldenseal (*Hydrastis canadensis*) (Trease, 1972). Plant based antimicrobials represent a vast untapped source for medicines. There is a great need of continued and further exploration of plant antimicrobials. Plants based antimicrobials have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. Many plants have tropisms to specific organs or systems in the body. Phytomedicines usually have multiple effects on the body. Their actions often act beyond the symptomatic treatment of disease. An example of this is *Hydrastis canadensis*. *Hydrastis* not only has antimicrobial activity, but also increases blood supply to the spleen promoting optimal activity of the spleen to release mediating compounds (Murray, 1995).

9.1.5 Economic benefit

Worldwide, there has been a renewed interest in natural products due to the factors such as: consumer's belief that natural products are superior; their dissatisfaction with conventional medicines; changes in laws allowing structure-function claims which results in more liberal advertising and national concerns for health care cost (Cowan, 1999). Sales of products in this

market have increased dramatically in the last decade. Sales of botanical products in the United States have reached $3.1 billion of the $10.4 billion dollar dietary supplement industry 1996 (NBJ Sept, 1998). The industry anticipates growth in the order of 15–20% into the new millenium (Johnston, 1997). This growth rate will be maintained in an industry that is still considered to be in its infancy. Many plants which are previously collected from wild sources will need to be cultivated to meet the demands of the consumer. This represents many opportunities for the cultivation of medicinal crops for industries.

A market study show the exponential growth in the sale of plant based antimicrobials. In reviewing the top botanicals used as antiinfectives, the primary botanical used as an antimicrobial is *Hydrastis* with sales of 4.7% in 1995 (Gruenwald, 1997). While antiinfectives agents make up 24 % of the pharmaceutical market (1992 Census of Manufactures, 1994).

9.1.6 Plants with promising antiinfective activity

Many scientists emphasize on drug discovery from ethnomedicinal information using the 'Third Generation Approach'. This method differs from other methods as in this case clinical evaluation in humans takes place before the precise active constituents are known but the chemical composition and safety of the extracts are determined before formulation into dosage forms.

Plants containing protoberberines and related biflavones show antimicrobial activity. Some of these plants which are used in traditional African system of medicine and other plants are discussed below.

9.1.6.1 *Garcinia kola*, Bitter Kola (Guttiferae)

Garcinia kola is found in moist forest and grows as a medium size tree, up to 12 m high. It is cultivated and distributed throughout west and central Africa. Medicinal uses include purgative, antiparasitic and antimicrobial agents. The seeds are used in the treatment of bronchitis and throat infections. They are also used to prevent and relieve colic, cure head or chest colds and relieve cough. Also the plant is used for the treatment of liver disorders and as a chewing stick (Iwu, 1999).

The constituents include—biflavonoids, xanthones and benzophenones. The antimicrobial properties of this plant are attributed to the benzophenone, flavanones. This plant has shown anti-inflammatory, antimicrobial and antiviral properties. Studies show very good antimicrobial and antiviral properties. In addition, the plant possesses antidiabetic and antihepatotoxic activities (Iwu, 1999).

9.1.6.2 *Aframomum melegueta* (Zingiberaceae), Grains of Paradise

This is a spicy edible fruit that is cultivated and occurs throughout the tropics. It is a perennial herb. The medicinal uses of *Aframomum* include aphrodisiac, measles, and leprosy, taken for excessive lactation and post partum hemorrhage, purgative, galactogogue, anthelmintic and hemostatic agent. The constituents are essential oils—such as gingerol, shagaol and paradol. Studies show antimicrobial and antifungal activity and effective against schistosomes (Iwu, 1999).

9.1.6.3 *Xylopia aethiopica*, (Abbibacceae), Ethiopian Pepper

It is an evergreen, aromatic tree growing up to 20 m high with peppery fruit. It is native to the lowland rainforest and moist fringe forest in the savanna zones of in Africa. Medicinal uses of the plant are, as a carminative, as a cough remedy, and as a post partum tonic and lactation aid. Other uses are stomachache, bronchitis, biliousness and dysentery. It is also used externally as a poultice for headache and neuralgia. It is used with lemon grass for female hygiene. It is high in copper, manganese, and zinc (Smith, 1996).

Key constituents are diterpenic and xylopic acid. In studies, the fruit as an extracts has been shown to be active as an antimicrobial against gram positive and negative bacteria. Though, it has not been shown to be effective against *E. coli* (Iwu, 1999). Xylopic acid has also demonstrated activity against *Candida albicans* (Boakye-Yiadom, 1977).

9.1.6.4 *Cryptolepis sanguinolenta* Lindl. Schltr. (Periplocaceae)

A shrub that grows in the rainforest and the deciduous belt forest, found in the west coast of Africa. Related species appear in the east and southern regions of the continent. Its main medicinal use is for the treatment of fevers. It is used for urinary tract infections, especially *Candida*. Other uses are in inflammatory conditions, malaria, hypertension, microbial infections and inflammatory conditions of the stomach (Iwu, 1999).

9.1.6.5 *Amomum cannicarpum* (*Zingiberaceae*)

Zingiberaceae is one of the essential oil bearing plant families. These plants are mostly terrestrial, rhizomatous herbs. *Amomum* seeds are used as spices and their plant parts are used in traditional medicine for curing toothache, dysentery, diarrhoea, rheumatism, vomiting, dyspepsia, and lung diseases. *Amomum subulatum* or 'large cardamom' distributed in the eastern Himalayas is the most investigated *Amomum* species. Thirty-three constituents out of forty-one, containing 91.48% of the essential oil from the fruits of *A. cannicarpum*, are identified by GC-MS. The percentage of oxygenated sesquiterpenes in the oil is 47.97%, followed by monoterpene hydrocarbons (21.29%) and oxygenated monoterpenes (20.43%). The percentage of sesquiterpene hydrocarbons in the oil is relatively low (1.78%). The major

constituents of the oil are pinene (14.00%), elemol (10.45%) and cadinol (8.50%). The oil at a 33.3% (*V/V*) concentration in dimethyl sulfoxide showed good activity against the Gram-negative bacteria *Salmonella typhi*, *Pseudomonas aeruginosa* and *Proteus vulgaris* in comparison with streptomycin at 2 g per disc and against the fungi *Candida albicans* and *C. glabrata*, in comparison with the antifungal control, fluconazole at 2g per disc. They show no activity against *Bacillus subtilis*, *B. cereus*, *Klebsiella pneumoniae* and *Escherichia coli* (Sabulal *et al.*, 2006).

9.1.6.6 *Moringa oleifera*

Three fractions from the leaves of *Moringa oleifera* have antibacterial action against *Escherichia coli*, *Klebsiella aerogenes*, *Klebsiella pneumoniae*, *S. aureus*, and *B. subtilis*. They show strong inhibitory activity against *E. coli*, *S. aureus* and *B. subtilis* but clear zone of inhibition is also noted against *Klebsiella aerogenes* and inhibition against *Aspergillus niger*. Aqueous extract of *M. oleifera* leaves posseses significant antimicrobial activity against Gram positive and negative fungal species. The fractions are effective against the growth of pneumoniae, *A. fumigates*, *A. flavus* and *P. expansum*. The small proteins/peptides play an important role in plants of antimicrobial defense system and the plant resources can be utilized for isolation of antimicrobial peptides or small proteins (Dahot, 1998).

9.1.6.7 *Lantana camara* (Verbenaceae)

This plant has antimicrobial properties against three Gram-positive and two Gram negative bacteria, a non-acid fast bacterium, and the yeast, *Candida albicans* (Rajakaruna *et al.*, 2002).

9.1.6.8 Brazilian medicinal plants

The antibiotic activities of the ethanol extracts from 16 species of plants used in Brazilian folk medicine have been determined against *Staphylococcus aureus*, *Micrococcus flavus*, *Bacillus cereus*, *B. subtilis*, *Salmonella enteretidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens*, *Mycobacterium phlei*, *M. smegmatis* and *M. fortuitism*, and the yeasts *Candida albicans* and *C. krusei*. *Lafoensia pacari* and *Pterodon polygalaeflorus* show activity against the bacterial strains, and none amongst them are active against yeasts. The ethanolic extract from the leaves of *L. pacari* has minimum inhibitory concentration (MIC) values of 312.5 to 2500, 250, 625 and 1250 µg/mL, respectively, against eight different Gram-positive strains of *Staphylococcus aureus*, the Gram-negative *Proteus mirabilis* and the acid-fast bacilli *Mycobacterium phlei*, *M. fortuitum* and *M. smegmatis*. The ethanolic extract from the stem of *L. pacari* has the MIC value of 625 µg/mL against *S. aureus*. The crude extracts contain tannins, steroids, phenols, flavonoids, triterpenes and saponins. The activities are sufficiently high to present the possibility of future identification of the active components by bioassay-guided fractionation and purification (Maria *et al.*, 2006).

9.1.6.9 *Oxylipins* in plants have antimicrobial activity (Oxylipins)

Plant oxylipins are a large family of metabolites derived from polyunsaturated fatty acids and their biosynthesis depends upon the characterization of mutants or transgenic plants. Oxylipins have direct influence on the antimicrobial effect, stimulation of plant defense gene expression, and regulation of plant cell death. The precise contribution of individual oxylipins to plant defense remains essentially unknown. Most oxylipins are able to impair growth of some plant microbial pathogens and many oxylipins show inhibitory activity toward at least three different microbes. They strongly inhibit mycelial growth and spore germination of eukaryotic microbes. Oxylipins contribute to plant defense through their effects both on the plant and on pathogens, possibly through related mechanisms (Isabelle *et al.*, 2005).

9.1.6.10 *Aloe excelsa* (Aloe)

The fleshy leaves and roots of most species within the *Aloe* family are used in many traditional treatments (Mabberley, 1990). Traditional healers and indigenous people utilize mainly the leaf sap of this genus for the treatment of wounds, burns, rashes, itches, cracked lips and cracked skin (Cera *et al.*, 1980). *Aloe exclesa* leaf sap shows promising evidence of antibacterial and antifungal effect. A killing effect is seen even at the 10% dilution, indicating that the candidicidal compound is relatively potent. This evidence has shown that *A. excelsa* holds excellent potential as an antifungal agent against dermatophyte species *C. albicans, C. tropicalis* and *T. mentagrophytes* and other fungal species isolated from human superficial mycosis. As the number of organisms increases, the results become more credible. Further, these findings could be used to develop suitable dosage forms such as cream, ointment, and lotion as per the requirement of the treatment (Coopoosamy *et al.*, 2007).

9.1.6.11 *Sagittaria pygmaea* (Alismaceae)

Several plants of the genus *Sagittaria* has been used in traditional Chinese medicine for the treatment of various skin diseases. Phytochemical investigation reveals the presence of, ent-pimarane, ent-labdane and ent-kaurane direpinoides. *S.sagittifolia*, and several plant of this genus have series of new anti bacterial ent-rosane diterpinoides (Xue–Ting *et al.*, 2007).

9.1.6.12 Brazilian *Drosera*

The antimicrobial activity of three different extracts (hexane, ethyl acetate, methanol) obtained from Brazilian *Drosera* species (*D. communes, D. montana, D. brevifolia, D. villosa* var. graomogolensis, *D. villosa* var. villosa, Drosera sp. 1, and Drosera sp. 2) are tested against *Staphylococcus aureus, Enterococcus faecium, Pseudomonas aeruginosa, Escherichia coli, Salmonella choleraesuis, Klebsiella* pneumonia, and *Candida albicans* (a human isolate)

showed antimicrobial activity. Better activity can be observed with *D. communism* and *D. Montana* ethyl acetate extracts. Photochemical analyses from *D. communism, D. Montana* and *D. brevifolia* yields 5-hydroxy-2-methyl-1, 4- naphthoquinone (plumbagin); long chain aliphatic hydrocarbons are isolated from *D. communis* and from *D. villosa*, a mixture of long chain aliphatic alcohols and carboxylic acids, are isolated from *D. communism* and 3-O-acetylaleuritolic acid from *D. villosa* (Ferreira *et al.*, 2004).

9.1.6.13 Tannins in many perennial plants has antibacterial-promoting effects

Many tree leaves have antimicrobial factors, like tannins, essential oils, or other aromatic compounds (Kumar *et al.*, 1984). Nutritional and toxic effects of tannins present in various foodstuffs feed and fodder (Kumar *et al.*, 1984; Mehanso *et al.*, 1987). Antibacterial-promoting effects have been reported for plant tannins and flavornoids (Haslam, 1989; Scalbert, 1991; Chung *et al.*, 1998). Tannins and flavonoids present in plant leaves show antimicrobial activity against bacterial pathogens in ruminants, especially organisms such as *Staphylococcus aureus, Streptococcus* sp., coagulate-negative *Staphylococci,* Gram-negative rods, *Klebsiella* sp., *Escherichia coli* and *Enterobacter* sp. in *in vitro* disc diffusion method (Min *et al.*, 2008).

9.1.6.14 Antibacterial essential oils obtained from *Helichrysum* Species (Asteraceae)

The genus *Helichrysum* (Asteraceae) consists of about 500 species. There are 245 *Helichrysum taxa* found in Southern Africa which are divided in 30 groups (Hilliard, 1983). *Helichrysum cymosum* and *H. fulgidum* are aromatic perennial herbs with yellow flowers and characteristic odors, which are widespread in southern tropical Africa. Of all *Helichrysum* species occurring in Southern Africa, *H. cymosum, H. odoratissimum, H. petiolare* and *H. nudifolium* are among the best known and commonly used plants. The smoke of many *Helichrysum* species is used as ritual incense, called 'inphepho'. There are several different ways of administering these traditional medicines. To relieve cough and cold, a tea of leaves or the leaves boiled in milk are taken; for pain relief, leaves are burned and the smoke is inhaled. Leaves are widely used on wounds to prevent infection. Proven antimicrobial activity of these plants will provide scientific evidence for traditional use in wound dressing. Recent studies on essential oils from African *Helichrysum* species include those on oils of *H. bracteiferum, H. cordifolium, H. faradifani, H. gymnocephalum, H. hypnoides, H. kraussii, H. odoratissimum, H. rugulosum, H. rusillonii, H. se- laginifolium, and H. splendidum* (De Medici *et al.*, 1992; Lwande *et al.*, 1993; Theron *et al.*, 1994; Cavalli *et al.*, 2001; Bougatsos *et al.*, 2004). A systematic research on the chemical composition of *Helichrysum* species (Chinou *et al.*, 1996; Roussis *et al.*, 2002) has been reported, consisting of the study of the chemical constituents and antimicrobial activity of the essential oils obtained from the aerial parts of *Helichrysum cymosum* and *H. fulgidum* (Chinou *et al.*, 1996).

9.1.6.15 Antibacterial activity of plant essential oils

Majority of the essential oils show antibacterial activity. However Cinnamon, clove and lime oils are found to be inhibiting both Gram-positive and Gram-negative bacteria. Cinnamon oil can be a good source of antibacterial agents. Cinnamon oil has the most potential bactericidal properties. It can be used as an antibacterial supplement in the developing countries towards the development of new therapeutic agents. Additional *in vivo* studies and clinical trials are needed to test the potential of this oil as an antibacterial agent in topical or oral applications (Ignacimuthu *et al.*, 2006).

9.1.6.16 Antimicrobial activity of some ethno medicinal plants

Ethnomedicinal plants used in folkloric medicine such as *Acalypha fruticosa, Peltophorum pterocarpum, Toddalia asiatica, Cassia auriculata, Punica granatum* and *Syzygium lineare* exhibit antimicrobial activity against one or more microorganisms at three different concentrations of 1.25, 2.5 and 5 mg/disc. High antifungal activity is seen in the methanol extract of *Peltophorum pterocarpum* and *Punica granatum* against *Candida albicans*. Plants like *Toddalia asiatica, Syzygium lineare, Acalypha fruticosa* and *Peltophorum pterocarpum* could be potential sources of new antimicrobial agents.

9.2 Materials and Methods

9.2.1 Plant materials

Methanol extracts of *Urtica parviflora, Callicarpa arborea* leaves; *Morinda citrifolia* root and their respective isolated compounds (described in **Chapter 3**) were used as test drugs in this study.

9.2.2 Microorganisms

A total of 257 bacterial strains belonging to different genera were tested in this study. The test organisms were obtained from Department of Bacteriology, Calcutta School of Tropical Medicine, Kolkata, India and Institute of Microbial Technology (IMTECH), Chandigarh, India. All the strains are of human origin and were isolated in Himalayan Pharmacy Institute, Sikkim. Some *Staphylococcus* strains were kindly provided by Prof. (Mrs.) Sujata Ghosh Dastidar, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

9.2.3 Chemicals

Dimethyl sulphoxide (DMSO) and Mueller Hinton Agar were obtained from Ranbaxy Fine Chemicals.

9.2.4 Media

9.2.4.1 Liquid media

9.2.4.1.1 Peptone water

Peptone water having the following composition was used for the cultivation of bacterial strains as well as for spot inoculation.

Bacteriological peptone (Oxoid)	- 1.0%
Sodium chloride (Analar)	- 0.5%

The pH was adjusted to 7.2 to 7.4 and the volume was made up with distilled water.

9.2.4.1.2 Alkaline peptone water

The alkaline medium was prepared for the cultivation of *Vibrio cholerae*, as follows:

Bacteriological peptone (Oxoid)	- 1.0%
Sodium chloride (Analar)	- 0.5%
pH adjusted to	- 8.5 to 9.0

9.2.4.1.3 Nutrient broth

Bacteriological peptone (Oxoid)	- 1.0%
Beef extract (Oxoid)	- 0.5%
Sodium chloride (Analar)	- 0.5%
pH adjusted to	- 7.2 to 7.4

9.2.4.1.4 Solid media

9.2.4.1.4.1 Nutrient agar

This medium was used to isolate pure cultures of Gram-positive bacteria. It contained the following ingredients:

Agar (Oxoid)	- 3.0%
Beef extract (Oxoid)	- 0.5%
Bacteriological peptone (Oxoid)	- 1.0%
Sodium chloride (Analar)	- 0.5%
pH adjusted to	- 7.2 to 7.4

9.2.4.1.4.2 Bromothymol blue lactose agar

This medium consisted of the following ingredients:

Agar (Oxoid)	- 3.0%
Bacteriological peptone (Oxoid)	-1.0%
Beef extract (Oxoid)	- 0.5%
Sodium chloride (Analar)	- 0.5%

The pH was adjusted to 7.2 to 7.4 and 1.25 ml of bromothymol blue was added per 100 ml of the medium. After sterilization, 1.0% lactose was added, steamed for 30 minutes and poured in sterile petri dishes. This medium was used to isolate pure cultures of Gram- negative bacteria.

9.2.5 Preservation of bacterial cultures

All the strains of Staphylococci, Streptococci, Bacilli, *E. coli*, Klebsiellae, Salmonellae, Shigellae, Citrobacter, *Pseudomonas* spp. and Vibrios were preserved as stab slant cultures at a temperature of 4ºC and in freeze dried state after checking of purity and identification where necessary. Routine subculturing of the Gram-positive bacteria was carried out on nutrient agar and Gram-negative strains on bromoothymol blue lactose agar (Barrow *et al.,* 1993).

9.2.6 Standard antibiotics

The standard antibiotics used in the studies were amoxycillin (Lyka Labs, India) and gentamycin (Hindustan Antibiotics, Pimpri, Pune) obtained from the respective manufacturers.

9.2.7 Preparation of impregnated discs of extract and standard antibiotics

The discs of 7.25 mm diameter were prepared by punching of Whatman No.1 filter paper and were sterilized by dry heat at 160ºC for an hour in batches of 100 in screw capped Bijou bottles. The dried extract (semisolid) of *Urtica parviflora* leaf, *Callicarpa arborea* leaf and *Morinda citrifolia* root were weighed and dissolved in 0.5 ml of dimethyl sulphoxide, as the extracts are not fully soluble in water, and then diluted in sterile distilled water to make the required stock solutions. For each extract three stock solutions were prepared. Similarly the stock solutions of the control antibiotics were prepared by dissolving the required amount of amoxycillin or diluting required amount of gentamicin in 10 ml of sterile distilled water separately to prepare two fold serially dilutions of the antibiotics (0–1000 µg/ml concentrations). All the stock solutions were then kept at 4ºC and used for three months. For preparation of antibiotic impregnated discs 1.0 ml of the stock solutions of the antibiotic were added separately to each bottle of 100 discs. Each disc adsorbed 0.01 ml of the solution, so the entire 1.0 ml volume was adsorbed by 100 discs, each giving the required two fold concentrations of 0–1000 µg/ml. The procedure was repeated for preparation of impregnated

discs of the plant extracts and their isolated compounds. The discs were used in wet condition and for further use they were stored at 4°C, as the discs can retain their moisture and potency for at least 3 months in the screw capped bottles.

9.2.8 Antimicrobial activity of *Urtica parviflora* leaf

Microbial sensitivity tests were performed by disc diffusion method (Dash, 1977). The nutrient agar plates, containing an inoculum size of 10^5-10^6 cfu/ml of bacteria were used. Previously prepared crude methanol extract (Concentration 128-2000 µg/ml) and isolated compound (Concentration 0-1000 µg/ml) discs were placed aseptically on sensitivity plates. The discs containing no test compound and standard antibiotics (Amoxycillin and Gentamicin) served as negative and positive controls respectively. All the plates were then incubated at 37°C±2°C for 18 hr. The sensitivity was recorded by measuring the clear zone of inhibition on agar plate around the discs.

The MICs were determined by the standard agar dilution method (Dastidar, 1995). The crude methanol extract was dissolved in 0.5 ml of dimethyl sulphoxide, as they are not fully soluble in water, and then diluted by sterile distilled water to make solution. The drug solution was then added to the molten nutrient agar in different tubes to give final concentrations of 0–128 µg/ml and subsequently increasing it by two fold concentration up to 2000 µg/ml. The concentrations of the tubes were mixed thoroughly, pH adjusted to 7.2 to 7.4 and poured into sterile Petri dishes. Bacterial cell suspensions were spot inoculated on the plates using a bacterial planter (10 µl). The final number of cfu inoculated onto the agar plates was 10^5 for all strains. The inoculated plates were then incubated at 37°C±2°C for 18 h. The lowest concentration of the plate, which did not show any visible growth after incubation, was considered as MIC. The same method was followed for the isolated Compound I also. The agar plate containing only sterile distilled water and Amoxycillin was served as negative and positive control respectively.

9.2.9 Antimicrobial activity of *Callicarpa arborea* leaf

The antibacterial activity of ethanol extract of *Callicarpa arborea* leaf and its isolated compound II was determined as per the methods described above in **9.2.8** for *Urtica parviflora* leaf.

9.2.10 Antimicrobial activity of *Morinda citrifolia* root

The antibacterial activity of methanol extract of *Morinda citrifolia* root and its isolated compound III was determined as per the methods described above in **9.2.8** for *Urtica parviflora* leaf.

9.3 RESULTS

9.3.1 Antimicrobial activity of *Urtica parviflora* leaf

The methanol extract of *Urtica parviflora* leaf exhibited a significant *in vitro* antimicrobial activity against 257 stains of Gram-positive and Gram-negative bacteria including MRSC. All the three reference MRSC strains of bacteria were found to be sensitive between 256 and 1000 μg/ml concentration of the extract. The results of the antimicrobial spectrum of the leaf extract presented in **Table 9.1** showed that out of 257 bacteria, the growth of 168 isolates were inhibited at a concentration of 128 – 512 μg/ml. 76 isolates were resistant at <1000μg/ml, while remaining 13 isolates where resistant up to <2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 58 out of 63 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 92 were sensitive between 256-512 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the extracts was directed both against Gram-positive and Gram-negative bacteria. The isolated compound I was also tested for antimicrobial activity. The result is presented in **Table 9.2**, which revealed that all the isolates were sensitive at 128-256 μg/ml concentration of the compound I except the *Vibrio cholera* 14033. It was interesting to note that all the MRSC strains were susceptible to Compound I at concentration of 128-256 μg/ml, while they are resistant to both the standard antibiotics used.

9.3.2 Antimicrobial activity of *Callicarpa arborea* leaf

The methanol extract of *Callicarpa arborea* leaf exhibited a significant *in vitro* antimicrobial activity against 257 Gram-positive and Gram-negative bacteria. The results of the antimicrobial spectrum of the leaf extract presented in **Table 9.3** showed that out of 257 bacteria, the growth of 221 isolates were inhibited by the extract at a concentration of 128 – 512 μg/ml. 35 isolates were resistant at <1000μg/ml, while remaining 1 isolate was resistant up to <2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 51 out of 78 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 73 were sensitive between 128-256 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the extracts was directed both against Gram-positive and Gram-negative bacteria but more sensitive to Gram-negative strains. The isolated compound II was also tested for antimicrobial activity. The result is presented in **Table 9.4**, which revealed that all the isolates were sensitive at 128-256 μg/ml concentration of the Compound II. It was noted that all the MRSC strains were resistant to compound II at concentration of 128 μg/ml, while they are resistant to both the standard antibiotics used.

9.3.3 Antimicrobial activity of *Morinda citrifolia* root

The methanol extract of *Morinda citrifolia* root exhibited a significant *in vitro* antimicrobial activity against 257 Gram-positive and Gram-negative bacteria including multiresistant *Staphylococcus* (MRSC) strains. All the three reference MRSC strains of bacteria were found to be sensitive within 1000 μg/ml concentration of the extract. The results of the antimicrobial spectrum of the root extract presented in **Table 9.5** showed that out of 257 bacteria, the growth of 217 isolates were inhibited by the extract at a concentration of 128– 512 μg/ml, 37 isolates were inhibited at a concentration of 1000μg/ml, while the remaining 03 isolates where inhibited at concentration >2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 64 out of 78 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 73 were sensitive between 256-512 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the ethanol extract was directed both against Gram-positive and Gram-negative bacteria. The isolated compound III was also tested for antimicrobial activity. The result is presented in **Table 9.6**, which revealed that all the isolates were sensitive at 128-512 μg/ml concentration of the compound III except the *P. aeruginosa*. It was interesting to note that all the MRSC strains were susceptible to Compound III at a concentration of 128 μg/ml, while they were resistant to the two standard antibiotics used.

Table 9.1 *In vitro* antimicrobial spectrum of methanol extract of *Urtica parviflora*.

Bacterial Species	No. of strn.	MIC of leaf extract (µg/ml)					MIC of amoxycillin (µg/ml)						
		128	256	512	1000	> 2000	0.25	0.5	8	64	128	256	> 1000
E.Coli	70	03	21	21	24	01	-	07	03	03	05	15	37
Klebsiella spp.	12	-	-	03	09	-	-	-	-	-	-	02	10
Salmonella Spp.	18	-	07	06	05	-	-	-	-	-	01	04	13
Shigella spp.	34	02	02	10	14	06	-	-	-	-	02	10	22
Vibrio cholerae	15	-	03	04	06	02	-	-	-	-	-	03	12
Citrobacter spp.	15	-	02	03	08	02	-	-	-	-	-	01	12
Pseudomonas aeruginosa	15	-	01	09	03	02	-	-	-	-	-	02	14
Bacillus subtilis	06	1	03	02	-	-	-	-	04	01	-	01	13
Staphylococcus aureus	62	21	24	10	07	-	01	27	12	13	-	09	-
Streptococcus faecalis	10	05	04	01	-	-	01	05	04	-	-	-	-
Total	257	32	67	69	76	13	02	39	23	17	08	47	121

Inoculum size used 10^5 cfu per spot for all the organisms except *S.aureus*, where the inoculum size per spot was 10^6 cfu. The results are the mean value of triplicate tests.

Table 9.2 The MIC of 15 sensitive bacteria against methanol extract of *Urtica parviflora* and Compound I.

Name of the Organism	MIC (μg/ml)				Diameter of zone of inhibition (mm) in methanol extract
	MEUP	CM I	Amoxycillin	Gentamycine	
E.Coli 832	512	512	0.50	0.25	+
E.Coli TG_1	512	512	0.50	>256	+
E.Coli 871	256	512	0.50	0.50	+
E.Coli HD_{10}	512	512	0.25	0.50	+
S. aureus NCTC 6571	128	128	0.50	1.0	++
S. aureus NCTC 8530	128	128	0.50	0.50	++
S. aureus Bang 44	256	256	8.0	1.0	+
S. aureus ML 275	128	128	0.50	1.0	++
S. epidermidis 865	128	128	0.50	0.50	++
Bacillus lichenfermis 10341	512	256	0.125	0.50	+
Bacillus subtilis 8241	128	128	0.50	256	++
S.typhimurium NCTC 74	256	512	8.0	>256	++
V.Cholerae 14033	1000	1000	8.0	>256	+
Klebsiella pneumoniae.	256	256	256	0.50	++
Pseudomonas aeruginosa	256	256	2.0	0.50	++

MEUP: Methanol extract of *Urtica parviflora*; CM I= Compound I; '+' = ≤ 10mm; '++'= ≥12mm; inoculum size used 10^5 cfu per spot for all organisms except *S.aureus*, where 10^6 cfu where used. The results are expressed as Mean ± S.E.M (n=3).

Table 9.3 *In vitro* antimicrobial spectrum of *Callicarpa arborea* leaf extract.

Bacterial Species	No. of strn.	MIC of leaf extract (µg/ml)					MIC of amoxycillin (µg/ml)						
		128	256	512	1000	> 2000	0.25	0.5	8	64	128	256	> 1000
E.Coli	70	11	22	27	08	01	-	07	03	03	05	15	37
Klebsiella spp.	12	01	02	04	05	-	-	-	-	-	-	02	10
Salmonella Spp.	18	02	04	06	06	-	-	-	-	-	01	04	13
Shigella spp.	34	07	09	12	06	-	-	-	-	-	02	10	22
Vibro cholera	15	01	06	08	-	-	-	-	-	-	-	03	12
Citrobacter spp.	15	02	03	06	04	-	-	-	-	-	-	01	12
Pseudomonas aeruginosa	15	01	03	08	03	-	-	-	-	-	-	02	14
Bacillus subtilis	06	03	02	01	-	-	-	-	04	01	-	01	13
Staphylococcus aureus	62	13	22	24	03	-	01	27	12	13	-	09	-
Streptococcus faecalis	10	04	07	-	-	-	01	05	04	-	-	-	-
Total	257	45	80	96	35	01	02	39	23	17	08	47	121

Inoculum size used 10^5 cfu per spot for all the organisms except *S.aureus*, where the inoculum size per spot was 10^6 cfu. The results are the mean value of triplicate tests.

Table 9.4 The MIC of 15 sensitive bacteria against methanol extract of *Callicarpa arborea* leaf and Compound II.

Name of the Organisms	MIC (μg/ml)				Diameter of zone of inhibition (mm) in methanol extract
	MECA	CM II	Amoxycillin	Gentamicin	
E.Coli 832	256	128	0.50	0.25	++
E.Coli TG_1	256	128	0.50	>256	++
E.Coli 871	256	128	0.50	0.50	++
E.Coli HD_{10}	256	256	0.25	0.50	++
S. aureus NCTC 6571	128	128	0.50	1.0	++
S. aureus NCTC 8530	128	128	0.50	0.50	++
S. aureus Bang 44	128	128	8.0	1.0	+
S. aureus ML 275	128	128	0.50	1.0	++
S. epidermidis 865	128	128	0.50	0.50	++
Bacillus lichenfermis 10341	512	512	0.125	0.50	+
Bacillus subtilis 8241	128	128	0.50	256	+
S.typhimurium NCTC 74	256	256	8.0	>256	+
V.Cholerae 14033	128	128	8.0	>256	+
Klebsiella pneumoniae.	256	256	256	0.50	+
Pseudomonas aeruginosa	512	512	2.0	0.50	+

MECA: Methanol extract of *Callicarpa arborea* leaf; CM II: Isolated compound of *Callicarpa arborea*; '+'= ≤ 10mm; '++'= ≥12mm; innoculum size used 10^5 cfu per spot for all organisms except S.*aureus*, where 10^6 cfu where used. The results are means ± S.E.M (n=3).

Table 9.5 *In vitro* antimicrobial spectrum of *Morinda citrifolia* root extract.

Bacterial Species	No of strn	MIC of root extract (µg/ml)					MIC of amoxycillin (µg/ml)						
		128	256	512	1000	> 2000	0.25	0.5	8	64	128	256	> 1000
E.Coli	70	08	12	35	14	01	-	07	03	03	05	15	37
Klebsiella spp.	12	01	02	04	05	-	-	-	-	-	-	02	10
Salmonella Spp.	18	01	08	09	-	-	-	-	-	-	01	04	13
Shigella spp.	34	07	09	12	06	-	-	-	-	-	02	10	22
Vibro cholerae	15	02	02	08	02	01	-	-	-	-	-	03	12
Citrobacter Spp.	15	02	02	06	04	01	-	-	-	-	-	01	12
Pseudomonas aeruginosa	15	01	06	05	03	-	-	-	-	-	-	02	14
Bacillus subtilis	06	03	02	01	-	-	-	-	04	01	-	01	13
Staphylococcus aureus	62	29	20	10	03	-	01	27	12	13	-	09	-
Streptococcus faecalis	10	04	06	-	-	-	01	05	04	-	-	-	-
Total	257	58	69	90	37	03	02	39	23	17	08	47	121

Inoculum size used 10^5 cfu per spot for all the organisms except *S.aureus,* where the inoculum size per spot was 10^6 cfu. The results are the mean value of triplicate tests.

Table 9.6 The MIC of 15 sensitive bacteria against methanol extract of *Morinda citrifolia* root and Compound III.

Name of the Organism	MIC (μg/ml)				Diameter of zone of inhibition (mm) in methanol extract
	MEMC	CM III	Amoxycillin	Gentamicin	
E.Coli 832	256	256	0.50	0.25	++
E.Coli TG_1	512	512	0.50	>256	++
E.Coli 871	256	512	0.50	0.50	++
E.Coli HD_{10}	256	256	0.25	0.50	++
S. aureus NCTC 6571	128	128	0.50	1.0	++
S. aureus NCTC 8530	128	128	0.50	0.50	++
S. aureus Bang 44	128	128	8.0	1.0	++
S. aureus ML 275	128	128	0.50	1.0	++
S. epidermidis 865	128	128	0.50	0.50	+
Bacillus lichenfermis 10341	512	512	0.125	0.50	+
Bacillus subtilis 8241	128	128	0.50	256	++
S.typhimurium NCTC 74	128	128	8.0	>256	++
V.Cholerae 14033	512	512	8.0	>256	+
Klebsiella pneumoniae.	512	512	256	0.50	+
Pseudomonas aeruginosa	512	1000	2.0	0.50	+

MEMC: methanol extract of *Morinda citrifolia* root; CM III: Isolated compound of *Morinda citrifolia*; '+'= ≤ 10mm; '++'= ≥12mm; innoculum size used 10^5 cfu per spot for all organisms except S.*aureus*, where 10^6 cfu where used. The results are means ± S.E.M (n=3).

REFERENCES

Barrow, G. I. and Feltham, R. K. A. Cowan and steel's manual for the identification of medical bacteria, Cambridge University Press, Cambridge, 1993, 12.

Ben, Y. S., Rodov, V., Kim, J. J. and Carmeli, S. Preformed and induced antifungal materials of *Citrus* fruits in relation to the enhancement of decay resistance by heat and ultraviolet treatments, *J Agric Food Chem*, 1992, 40, 1217–1221.

Boakye-Yiadom, K., Fiagbe, N. and Ayim, S. Antimicrobial properties of some West African medicinal plants IV: Antimicrobial activity of xylopic acid and other constituents of the fruits of *Xylopia aethiopica* (Annonaceae), *Lloydia*, 1977, 40, 6, 543–545.

Boman, H. Peptide antibiotics ant their role in innate immunity. *Annual Review of Immunology*, 1995, vol. 13, p. 61-92.

Bougatsos, C., Ngassapab, O., Runyorob D. K. B. and Chinoua I. B. Chemical Composition and in vitro Antimicrobial Activity of the Essential Oils of Two Helichrysum Species from Tanzania, *Verlag der Zeitschrift für Naturforschung*, 2004, 368-372.

Boyd, M., Hallock, G., Cardellina, Y., Manfredi, J., Blunt, J., McMahon, J., Buckheit, R., Bringmann, G., Schaffer, M., Cragg, G., Thomas, D., and Jato, J. Anti-HIV michellamines from *Ancistrocladus korupensis*, *Med Chem*, 1994, 37, 1740–1745.

Broekaert1, W., Cammue, B., De Bolle, M., Thevissen, K., De Samblanx, G. and Osborn, R. Antimicrobial peptides from plants, *Critical Reviews in Plant Sciences*, 1997, 16, 3, 297-323.

Broekaert2, W. F., Cammue, B. P. A., Debolle, M. F. C., Thevissen, K., Desamblanx, G. W., Osborn, R. W., Antimicrobial peptides from plants, *Critical Reviews in Plant Sciences*, 1997, 16, 297–323.

Bruneton, J. Pharmacognosy-Phytochemistry, Medicinal Plants. 2ndEdition, Lavoisier Publishing, France, 1999, 1119.

Cammue, B. P. A., De-Bolle, M. F. C., Terras, F. R. G., Proost, P., Damme, J. V., Rees, S. B., Vanderleyden, J. and Broekaert. W. F. Isolation and characterization of a novel class of plant antimicrobial peptide from *Mirabilis jalapa* L. seeds, *J Biol Chem*, 1992, 267, 2228-2233.

Cavalli, J. F., Ranarivelo, L., Rtasimbason, M. and Bernardini, A. F. and Casanova J. Constituents of the essential oil of six Helichrysum species from Madagas- car. *Flavour Fragr, J,* 2001,16, 253-256.

Census of Manufactures-1992. U.S. Department of Commerce, Economics and Statistics Administration, Bureau of Census, 1994.

Cera, L.M., Heggers, J.P., Robson, M.C., Duraccio, M.R., The therapeutic effecacy of Aloe vera cream (Dermaide Aloe (TM)) in termal injuries. Two case reports, *J. Am. Anim. Hos. Ass,* 1980, 16, 768– 772.

Chinou, I. B., Roussis, V., Perdetzoglou, D., and Loukis, A. Chemical and biological studies on two Heli chrysum species of Greek origin, Planta Med, 1996, 62, 377-379.

Chung, K.T., Wong, T.Y., Wei, Y., Huang, Y.W., Lin, Y., Tannins and human health: A review, *Crit. Rev. Food Sci. Nutr*, 1998, 38, 421-464.

Coopoosamy, R. M. and Magwa, M. L. Traditional use, antibacterial activity and antifungal activity of crude extract of Aloe excels, *African Journal of Biotechnology*, 2007, 6, 18, 10, 20, 2406-2410.

Cowan, M. M. Plant products as antimicrobial agents, *Clin Microbio Rev,* 1999, 12, 564-582.

Dahot, U. M., Antimicrobial Activity Of Small Protein Of Moringa Oleifera Leaves, *Journal of Islamic Academy of Sciences*, 1998, 27, 11, 1, 27-32.

Dash, S. K., Dastidar, S. G. and Chakraborty, A. N. Antimicrobial property of Promazine hydrochloride, *Ind J Exp Biol,* 1977, 15, 197-199.

Dastidar, S. G., Chaudhary, A., Annadurai, S., Roy, S., Mukharjee, M. and Chakraborty, A. N. In vitro and in vivo antimicrobial action of Fluphenazine, *J Chemother,* 1995, 7, 201-207.

De Lucca, A. J. Antifungal peptides: potential candidates for the treatment of fungal infections, *Expert Opinion on Investigational Drugs*, 2000, 9, 2, 273-299.

De Medici D., Pieretti, S., Salvatore, G., Nicoletti, M., and Rasoanaivo, P. Chemical analysis of essential oils of Malagasy medicinal plants by gas chromatogra- phy and NMR spectroscopy, *Flavour Fragr*, 1992, 7, 275-276.

Dubery, I. A., Louw, A. E. and Van-Heerden, F. R. Synthesis and evaluation of 4-(3-methyl-2-butenoxy) isonitrosoacetophenone, a radiation-induced stress metabolite in *Citrus*, *Phytochemistry*, 1999, 50, 983–989.

Ezekowitz, A. and Hoffmann, J. Innate Immunity, *Humana Press*, 2003, 410.

Fauci, A. New and re-emerging diseases: The importance of biomedical research, *Emerg Infect Dis*, 1998, 4, 3.

Ferreira, D. T., Andrei, C. C., Saridakis, H. O., Faria, T. J., Vinhato E., Carvalho, K. E., Daniel, J. F. S., Machado, S. L., Saridakis, D. P. Antimicrobial Activity and Chemical Investigation of Brazilian Drosera, *Mem Inst Oswaldo Cruz*, 2004, 99, 7, 753-755.

Funke, I. and Melzig, M. F. Traditionally used plants in diabetes therapy - phototherapeutics as inhibitors of α-amylase activity, *Rev Bras Farmacogn*, 2006, 16, 1-5.

Grunewald, J. The herbal remedies market in the US, market development, consumer, legislation and organizations. *Phytopharm Consulting Communique*, 1997, 41.

Haggard, J., Jacobsen, S., Bjorn, S. E. and Kragh, K. M. Antifungal activity of chitin binding PR-4 type protein from barley grain and stressed leaf, *Federation of Eur Biochem Soc Letters*, 1992, 307, 389-392.

Hancock, R. E. W. Cationic antimicrobial peptides: towards clinical applications, *Expert Opinion on Investigational Drugs*, 2000, 9, 1723-1729.

Hanawa, F., Tahara, S. and Mizutani, J. Antifungal stress compounds from *Veratrum grandiflorum* leaves treated with cupric chloride, *Phytochemistry*, 1992, 31, 3005–3007.

Hancock, R. E. W. and Lehrer, R. Cationic peptides, a new source of antibiotics, *Trends in Biotechnology*, 1998, 16, 82-88.

Hancock, R. E. W. and Chapple, D. S. Peptide antibiotics, *Antimicrobial Agents Chemotherapy*, 1999, 43, 6, 1317-1323.

Haslam, E., Cai, Y. Plant polyphenols (vegetable tannins): gallic acid metabolism, *Nat Prod Rep,* 1994, 11, 41-66.

Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. and Ezekowitz, R. A. Phylogenetic perspectives in innate immunity, *Science*, 1999, 284, 5418, 1313-1318.

Ignacimuthu, S., Prabuseenivasan, S. and Jayakumar, M. In vitro antibacterial activity of some plant essential oils, *BMC Complementary and Alternative Medicine*, 2006, 30, 11, 6-39.

Isabelle, P., Sandrine, D., Grit, R., Jorge, V., Maria, J. R., Neil, K., Francis, C., Gareth, G., Marie, T. E. T., Sabine, R., Carmen, C., Mats, H. and Joelle, F. Evaluation of the Antimicrobial Activities of Plant Oxylipins Supports Their Involvement in Defense against Pathogens, *Plant Physiology*, 2005, 12, 139, 1902–1913.

Iwu, M. W., Duncan, A. R. and C. O. Okunji, New antimicrobials of plant origin. In: J. Janick (ed.), Perspectives on new crops and new uses, ASHS Press, Alexandria, VA. 1999, 457–462.

Johnston, B. One-third of nation's adults use herbal remedies, *HerbalGram*, 1997. 40:49.

Kruger, B. M. and Manion, P. D. Antifungal compounds in aspen: Effect of water stress, *Can J Bot*, 1994, 72, 454–460.

Kumar, R., Singh, M., Tannins, their adverse role in ruminant nutrition, *J. Agric. Food. Chem*, 1984, 32, 447-453.

Leitao, S. G., Castro, O., Fonseca, E. M., Juliao, L. S., Tavares, E. S., Leo, R. R. T., Vieira, R. C., Oliveira, D. R., Leitao, G. G., Martino, V., Sulsen, V., Barbosa, Y. A. G., Pinheiro, D. P. G., Silva, P. E. A., Teixeira, D. F. and Lourenço, M. C. S. Screening of Central and South . American plant extracts for antimycobacterial activity by the Alamar Blue test, *Rev Bras Farmacogn*, 2006, 16, 6-11.

Lewis, W. H. and Elvin-Lewis, M. P. F. Medical Botany, Plants Affecting Man's Health, John Wiley & Sons, New York, 1977, 515.

Linthorst, H. J. M. Pathogenesis related proteins of plants, *Critical Review Plant Science*, 1991, 10, 123-150.

Lwande, W., Hassanali, A., Wanyama, O. B., Ngola, S., and Mwangi, J. W. Constituents of the essential oil of Helichrysum odoratissimum (L.) Less, *J. Ess. Oil Res*, 1993, 5, 93-95.

Mabberley, D. J. The Plant-Book: A Portable Dictionary of the Higher Plants, Cambridge University Press, Cambridge, 1990, 25.

Maher, E. A., Bate, N. J., Elkin, W., Dixon, R. A. and Lamb, C. J. Increased disease susceptibility of transgenic tobacco plants with suppressed level of preformed phenyl propanoid products, Proceedings National Academy of Science, USA, 1994, 91, 7802-7806.

Mainous, I. A. and Pomeroy, C. Management of Antimicrobials in Infectious Diseases, *Humana Press*, 2001, 349.

Marcus, J. P., Goulter, K. C., Green, J. L., Harrison, S. J. And Manners, J. M. Purification, characterization and cDNA cloning of an antimicrobial peptide from *Macadamia integrifolia*, *European Journal of Biochemistry*, 1997, 244, 3, 743-749.

Maria, J. R., Neil, K., Isabelle, P., Sandrine, D., Grit, R., Jorge, V., Francis, C., Gareth, G., Marie, T. E. T., Sabine, R., Carmen, C., Mats, H. and Joelle, F. Evaluation of the Antimicrobial Activities of Plant Oxylipins Supports Their Involvement in Defense against Pathogens, *Plant Physiology*, 2006, 12, 139, 1902–1913.

Mehanso, H., Butler, L.G., Carlson, D.M. Dietary tannins and salivary praline rich proteins: Interactions, induction and defense mechanisms, *Annu. Rev. Nutr*, 1987, 7, 423-440.

Min, B. R., Pinchak, W. E., Merkel, R., Walker, S., Tomita, G. and Anderson, R. C. Comparative antimicrobial activity of tannin extracts from perennial plants on mastitis pathogens, *Scientific Research and Essay*, 2008, 2, 3, 2, 66-73.

Mohamed, F. and Sehgal, O. P. Characteristics of pathogenesis related proteins induced in *Phaseolus vulgaris* cv. Pinto following viral infection, *J Phytopathol*, 1997, 145, 49–58.

Murray, M. The healing power of herbs, Prima Publishing, Rocklin, CA, 1995, 162–171.

Nelson, R. The comparative clinical pharmacology and pharmacokinetics of vindesine, vincristine and vinblastine in human patients with cancer, *Med Pediatr Oncol*, 1982, 10, 115–127.

Novak, R., Henriques, B., Charpentier, E., Normark, S. and Tuomanen, E. Emergence of vancomycin tolerance in *Streptococcus pneumonia, Nature,* 1999, 399, 6736, 590-591

Nutrition Business Journal (NBJ). *Industry overview*, Sept. 1998.

Pernas, M., Sanchez-Monge, R., Salcedo, G. Biotic and abiotic stress can induce cystatin expression in chestnut, *FEBS Lett*, 2000, 467, 206–210.

Pinner, R. S., Teutsch, L. and Smonsen, L. Trends in infectious disease mortality in the United States, *J Am Med Assoc,* 1996, 275, 189-193.

Rajakaruna, N., Harris, C. S. and Towers, G.H.N. Antimicrobial Activity of Plants Collected from Serpentine Outcrops in Sri Lanka, Pharmaceutical *Biology*, 2002, 40, 3, 235–244.

Robbers, J. E., Speedie, M. K. and Tyler, V. E. Pharmacognosy and pharmacobiotechnology, Baltimore, MD; Williams & Wilkins, 1996, 337.

Roberts, W. K. and Selitrennikoff, C. P. Isolation and partial characterization of two antifungal proteins from barley, *Biochemica et Biophysica Acta*, 1986, 880, 161-170.

Roder, B. L., Wandall, D. A., Frimodt-Moller, N., Espersen, F., Skinhoj, P. and Rosdahl, V. T. Clinical features of *Staphylococcus aureus* endocarditis: a 10-year experience in Denmark, *Archives Internal Medicine,* 1999, 159, 5, 462-469.

Roussis, V., Tsoukatou, M., Chinou, I. B., and Harvala, C. Composition and antibacterial activity of the essential oils of two Helichrysum stoechas varieties growing in the island of Crete, *J. Ess. Oil Res,* 2002, 14, 459-461.

Sabulal, B., Dan, M., Pradeep, N.S., Valsamma, R. K. and George, V. Composition and antimicrobial activity of essential oil from the fruits of *Amomum cannicarpum* , *Acta Pharm*, 2006, 56, 473-480.

Scalbert A. Antimicrobial properties of tannin, *Phytochem*, 1991, 30, 3875-3883.

Selitrennikoff, C. P. Antifungal proteins, *Applied and Environmental Microbiology*, 2001, 67, 7, 2883-2894.

Sergio, H. M. and Gloria, A. Antimicrobial peptides, a natural alternative to chemical antibiotics and a potential for applied biotechnology, *Electronic Journal of Biotechnology,* 2003, 8, 15, 6, 2.

Smith, G. M., Clegg, C. K. and Grivetti, L. Mineral values of selected plant foods common to southern Burkina Faso and to Niamey, Niger, West Africa, *International J Food Sc Nutr,* 1996, 47, 41–53.

Terras, F. R. G., Schoofs, H. M. E., De Bolle, M. F. C., Van, L. F., Rees, S. B., Vanderieyden, J., Cammue, B. P. A. and Broekaert, W. F. Analysis of two novel classes of antifungal proteins from radish (*Raphanus sativaus* L) seeds, *J Biol Chem*, 1992, 267, 15301-15309.

Theron, E., Holeman, M., Pont-Guatier, M., and Pinel, R. Etudes du viellissement d'huiles essentielles Malgaches riches en 1,8 cineole, *Riv. Ital. EPPOS*, 1994, 13, 33-38.

Thevissen, K., Terras, F. R. G. and Broekaert, W. F. Permeabilization of fungal membranes by plant defensins inhibits fungal infection, *Applied and Environmental Microbiology*, 1999, 65, 12, 5451-5458.

Trease, G. and Evans, W. Pharmacognosy, Univ. Press, Aberdeen, Great Britain, 1972, 161-163.

Turano, A., Scura, G., Caruso, A., Bonfanti, C., Luzzati, R., Basetti, D. and Manca, N. Inhibitory effect of papaverine on HIV replication *in vitro*, *AIDS Res Hum Retrovir*, 1989, 5, 183–191.

Van, E. H. D., Matthews, D. E. and Matthews, P. S. Phytoalexin detoxification importance for pathogenicity and practical implications, *Annual review of Phytopathology*, 1989, 27,143-164.

Welling, M. M., Paulusma-Annema, A., Balter, H. S., Pauwels, E. K. and Nibbering, P. H., Tenehtium-99m labeled antimicrobial peptide discriminate between bacterial infection and sterile inflammations, *European Journal of Nuclear Medicine*, 2000, 27, 3, 292-301.

Xue, T. L., Yao, S., Biao, S., Williams, L. D., Hermar, H. Y. S., Qiong, Z. Antibacterial Diterpinoids from *Sagittaria pygmaoea*, *Planta Med*, 2007, 73, 84-90.

Yue-Zhong S, Recent natural products based drug development: A pharmaceutical industry perspective, *J Nat Prod*, 1998, 61, 1053-1071.

Zasloff, M. Antimicrobial peptides of multicellular organisms, Nature, 2002, 415, 6870, 389-395.