CHAPTER - 4

REVIEW OF LITERATURE
Since higher plants have proven to be an important source of antimicrobial compounds, discovery of new drugs through screening efforts is being taken up by many investigators. Many workers on the basis of their research studies have established that the extracts of plants inhibited the growth of many pathogenic bacteria and fungi at various concentrations and thereby acted as effective antimicrobial agents. Investigations have revealed that water and ethanolic extracts prepared from different plant species exhibited bacteriostatic or fungistatic activities at lower concentrations whereas at higher concentrations they were bactericidal or fungicidal. Phytochemical analysis of the plant extracts indicated the presence of biologically active compounds such as phenols, flavonoids, terpenoids, tannins, sesquiterpenes, glycosides and saponins, which may be responsible for the antimicrobial activity. The detailed review of antimicrobial studies with reference to medicinal plants has been summarized below.

4.1 Antimicrobial Studies on Plant Extracts

Venkataraman and Lalitha (1991) have reported that the Saponins isolated from the seeds of Indian Butter tree, *Madhuca butyracea* inhibited the growth of three fungi *viz.* *Penicillium expansum*, *Cephalosporium acrimonium* and *Helminthosporium oryzae* at the concentrations ranged
from 500 to 2000 ppm. It was found that the saponins caused leakage of cell components and lead to the movement of intracellular components into the extracellular medium thereby making the pathogens unable to grow.

Higher concentration of petroleum ether, chloroform and ethanol extracts of *Acalypha indica* at 750 μg, 500 μg and 250 μg/ml respectively inhibited the growth of fungi such as *Aspergillus niger* and *Candida albicans* and bacteria such as *Pseudomonas aeruginosa* and *Salmonella citrus* (Hiremath et al., 1992).

Chatterjee et al. (1993) reported that the crude leaf extracts of *Juniperus communis* at the concentration of 250 μg/ml found to be active against several gram positive and gram negative bacteria. However *Staphylococcus aureus* and *Streptococcus faecalis* showed resistance to the concentrations of the crude extract up to 20 μg/ml. They also suggested that some cheap, effective and economical herbal formulations might be prepared from the extract of this plant for certain bacterial infections and wound healing.

Water, ethanol, chloroform and hexane extracts of *Acalypha wilkesiana* were investigated for *in vitro* antimicrobial activities by agar diffusion and tube dilution techniques (Alade and Irobi, 1993). They observed that the water and ethanol extracts inhibited the growth of standard and local strains of bacteria and fungi including *Staphylococcus aureus*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Candida albicans* and *Aspergillus flavus*. The minimum inhibitory concentrations of the extracts
ranged between 0.25 and 32 mg/ml while the minimum cidal concentrations were between 1.0 and 64 mg/ml.

Screening of 132 plants from Argentine folk medicine for antimicrobial activity was conducted by Claudia Anesini and Christina Perez (1993). They used Penicillin G resistant strains of *Staphylococcus aureus*, *Escherichia coli* and *Aspergillus niger* as test organisms. Agar well diffusion method was followed using Ampicillin as the standard antibiotics. It was found that 12 plants were active against *Staphylococcus aureus*, 10 were against *Escherichia coli* and 4 against *Aspergillus niger*. Hot water extracts of the plants were used for the assay.

A total of 315 extracts from 63 traditionally used Ethiopian plants were subjected to antimicrobial screening using strains of *Staphylococcus aureus*, *Salmonella gallinarum*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida albicans*. The agar well diffusion method was used and a sample concentration of 1000 µg/ml was tested on the test microbes. It was found that all parts of the plants showed activity against one or more of the microorganisms. Direct aqueous extracts from six plants were found to be active against all of the test organisms (Desta, 1993).

Extracts of *Mitracarpus villosus* (Rubiaceae) leaves and inflorescences were investigated individually for in vitro antifungal activities by agar diffusion and tube dilution techniques. Ethanolic extracts produced definite antifungal activities against *Trichophyton rubrum*, *Microsporum gypseum*, *Candida albicans*, *Aspergillus niger* and *Fusarium*...
The aqueous extracts did not show any inhibition against any of the fungi tested. The zones of inhibition produced by the ethanol extracts ranged from 10 to 20.5 mm while ketoconazole control showed inhibition zone from 9 to 19 mm. The minimum inhibitory concentration of the extracts ranged from 0.5 to 4.0 mg/ml, while the minimum fungicidal concentration values ranged from 1 to 8 mg/ml (Irobi and Daramola, 1993).

Gundidza and Gaza (1993) tested the antimicrobial activity of *Dalbergia melanoxylon* using methanol, citric acid, water, dichloromethane and petroleum ether as solvents. Extracts were prepared from the bark of this plant. Agar well diffusion method was followed. After 24 hour, the diameter of the inhibition zone was measured, and after 7 days, the dry weight of the mycelia was calculated for the bacteria and fungi respectively. The percentage of inhibition was calculated using controls. The results showed that the citric acid extract exhibited strongest antimicrobial activity followed by ethanol fraction. The other solvent extracts showed no activity.

Irobi *et al.* (1994) examined the antimicrobial properties of water and ethanolic extracts of *Bridelia ferruginea* on several hospital strains which includes *Staphylococcus aureus, Streptococcus pyogenes, Proteus mirabilis, Proteus vulgaris, Streptococcus lactis* and *Klebsiella* sp. The zones of inhibition produced by the extracts in agar diffusion method ranged from 2 to 4 mm, while the chloramphenicol control produced zones that ranged from 15 to 36 mm. The extracts at the concentration of 5 mg/ml showed activity against the test organisms.

Hot water extracts of 132 plant samples from 54 families commonly used in Argentine folk medicine were screened for antibacterial activity
against *Salmonella typhi*. The agar well diffusion method was used with ampicillin as the standard. It was found that among the 132 plant samples, 24 species showed antibacterial activity against *Salmonella typhi* (Perez and Claudia, 1994).

Purohit *et al.* (1995) studied the antimicrobial activity of various extracts of *Evolvulus alsinoides* against selected bacteria and fungi. The *in vitro* evaluation of the solvent extracts of the plant showed moderate activity against bacteria and the water extract showed activity against fungi. Cup diffusion method was followed for the assay.

Sanaa *et al.* (1995) studied the antimicrobial activity of some Egyptian medicinal plants. Different solvents were used for the extraction and testing was made on 7 bacterial and 6 yeast species. Agar plate method was followed. The results showed that many plants showed antimicrobial activity against the tested organism and some solvent extracts such as benzene, ether, chloroform and ethyl alcohol, showed complete inhibition towards *Salmonella typhi*.

Vijaya *et al.* (1995) examined the antibacterial effect of compounds extracted from *Camellia sinensis* L. and the methanol extract of *Euphorbia hirta* against the dysentery causing *Shigella* spp. using the vero cell line technique. Cytotoxicity studies of the extracts were performed using the cell line and the non-cytotoxic concentration of the extract was tested for antibacterial activity against the pathogen. The extracts were found to be effective antibacterial agents.
Ethanolic extract of *Cassia alata* leaves was investigated for its antimicrobial activities on several microorganisms including bacteria, yeast, dermatophytic fungi and non-dermatophytic fungi. *In vitro* the extract exhibited high activity against various species of dermatophytic fungi but low activity against non-dermatophytic fungi. However, bacterial and yeast species showed resistance against *in vitro* treatment with the extract. The minimum inhibitory concentration of the extract over the desmatophytic fungi ranged from 62.5 to 125 mg/ml. The inhibition was observed on the macro conidia which resulted in structural degeneration beyond repair. The mechanism of inhibition was observed as cell leakage, by irregular wrinkle shaped macroconidia which lost its rigidity (Ibrahim and Osman, 1995).

The oil and curcumin isolated from *Curcuma longa* showed antifungal activity against 15 isolates of dermatophytes, 4 isolates of pathogenic molds and 6 isolates of yeasts. The dermatophytes were inhibited at the dilutions 1:40 to 1:320. The pathogenic fungi were inhibited at the dilutions 1:40 to 1:80. The 6 yeast isolates were however insensitive to the treatment (Apisariyakul et al., 1995).

Silva *et al.* (1996) studied the effect of ethanolic extracts of 12 plants selected through ethnomedical survey in Guinea-Bissau. *In vitro* antimicrobial activities on 10 bacteria and one fungi namely *candida albicans*, using agar diffusion and tube dilution method were performed. The results showed that all the tested extracts exhibited some activity against one bacteria, *Staphylococcus aureus* and the root bark extract of *Terminalia macropreva* showed some activity against *Candida albicans*. 
Taylor et al. (1996) studied the antimicrobial activities of Southern Napalese medicinal plants. Twenty plant species were analysed against 11 strains of bacteria and 4 strains of fungi. Methanolic extracts were assessed for antimicrobial activity. It was found that out of 20 samples of extracts, 15 extracts showed activity against bacteria and 14 showed activity against fungi. The antimicrobial activity was enhanced upon exposure to light.

Singh and geetha (1997) investigated the activity of plants viz. *Lawsonia inermis, Eclipta alba, Nyctanthes arbor-tristis, Vinca rosea* and *Datura stramonium*. Crude extracts of these plants were tested individually against several fungi. It was found that the extract of *Lawsonia inermis* was highly toxic to all the fungi tested.

Murugesan and Mahalakshmi (1998) reported that the aqueous extract of *Azadirachta indica* inhibited the growth and multiplication of *Xanthomonas oryzae*, the causal agent of leaf blight of paddy at the concentrations of 20-28 μg/l. The percentage of inhibition was 15-97. Increasing concentrations of leaf extract markedly inhibited the growth rate of that bacteria.

The antimicrobial activity of *Cardiospermum halicacaebum* on four species of *Xanthomonas* showed that the ethylacetate extract of fresh leaves of *Cardiospermum halicacaebum* inhibited the growth of all species of *Xanthomonas* and the percentage of inhibition was upto 80 at 200 μg/l concentration (Raman et al., 1998).
Antibacterial tests on the floral parts especially, the petals of 51 plant species belonging to 26 families were performed by Mathur et al. (1998). The screening test was made on the laboratory strains of *Escherichia coli*. Disc diffusion assay was used. The results showed that the petals of 20 plant species belonging to 12 families showed antibacterial activity against the bacterium.

Singh et al. (1999) studied the effect of *Cyperus rotundus* rhizome extract on *Fusarium udum*. They analysed the effect of aqueous, methanol, petroleum ether, chloroform and ethyl acetate extracts of the rhizome on the spore germination of *Fusarium udum*. It was found that the percentage of spore germination, germ tube length and mode of spore germination were affected considerably. It was also reported that at higher concentrations characteristic bulging of spores resulted. Ethyl acetate extract exhibited inhibitory effect on spore germination at 1000 µg/l.

Kudi et al. (1999) studied the antibacterial activity of some Nigerian medicinal plants on several bacteria using hole-plate diffusion method. Most of the extracts showed activity against gram-positive bacteria. However two plants *Anacardium occidentale* and *Anogeissus schimperi* however showed antibacterial activity against gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*.

Hernandez et al. (1999) studied the biological activity of *Vitex trifolia* on Fusarium species. The hexanic extract of leaves completely inhibited the growth of the test fungus within the first two days of the experiment. However it was found that the activity dropped gradually at the sixth day.
Bansal and Gupta (2000) evaluated the effect of leaf extracts of seven medicinal plants against the wilt pathogen of fenugreek, *Fusarium oxysporum*. Five different concentrations viz. 20, 40, 60, 80 and 100% were tested against the 7 day old *Fusarium oxysporum* on its spore germination and mycelial growth. The results stated that, among the seven plants tested, the leaf extracts of three plants namely *Azadirachta indica*, *Ocimum bacillium* and *Lantana camara* controlled the spore germination and mycelial growth of *Fusarium oxysporum* at 100% concentration.

Perumalsamy and Ignacimuthu (2000) studied the antibacterial properties of 30 Indian folklore medicinal plants used by the tribal healers. The bacteria used in the experimental study were *Bacillus subtilis*, *Escherichia coli*, *Klebsiella aerogens*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Among the 20 plant species, the extracts of *Cassia occidentalis* and *Cassia auriculata* exhibited significant broad spectrum of activity against *Bacillus subtilis* and *Staphylococcus aureus*.

Essawi and Srour (2000) screened some Palestinian medicinal plants for antibacterial activity. Fifteen plants were tested against eight different species of bacteria. Each plant species had unique character against different bacteria. The plant *Thymus vulgaris*, and *Thymus origanum* showed maximum activity against both gram-positive and gram-negative bacteria tested. The organic solvent extracts showed greater activity than the aqueous extracts.

John Britto and Senthilkumar (2001) reported the antimicrobial activity of leaf extracts of *Solanum incanum*. The aqueous and methanolic
leaf extracts of this plant was tested against six strains of *E. coli*, viz., C₂H₅ 7, KL96, KL16, HFrC, DH₅α, and Y₁₀ 90. The highest antibacterial activity was observed in KL16, HFrC and Y₁₀ 90, and moderate activity was observed against KL96, DH₅α, and C₂H₅ 7. The results indicated that the leaf extracts of this plant had bacteriostatic effect at higher concentrations.

John Britto (2001) analysed the comparative antibacterial activity of leaf and stem extracts of *Solanum incanum* against some mutant strains of *E. coli*. It was found that both the stem and leaf extracts of this plant were active against all strains of *E. coli*. However the extent of inhibition varied among the strains. In KL16, the leaf extracts showed relatively more activity than the stem extracts.

The anti-helicobacter pylori effect of the rhizome and leaves of *Aristolochia paucinervis* was studied by Gadhi *et al.* (2001). They tested the effect of methanol and hexane fractions of the leaves and rhizomes of this plant against a reference strain of helicobacter pylori by using agar dilution method. The results showed that both the methanol and hexane fractions of the leaves and rhizomes exhibited inhibitory activity at various concentrations. This inhibitory activity of the extracts were confirmed against the clinical isolates of helicobacter pylori.

Aburaji *et al.* (2001) studied the effect of methanolic extracts of 19 Jordanian plants combined with 7 different antibiotics and tested on the resistance of *Pseudomonas aeruginosa*. A resistant strain isolated from a patient and a standard strain of the same were used in the study. The result
showed that there were significant variations in the effects of some combinations used on the resistant and standard strains.

The antibacterial effect of *Cascabela thevetica* plant extract against the pathogenic microorganisms such as *E. coli* (MTCC 739), *Salmonella typhi* (MTCC 737), *Klebsiella pneumoniae* (MTCC 432) and *Staphylococcus aureus* (MTCC 737) were analysed by Upadhyay and Kaushik (2002). The result revealed that the antibacterial activity is attributed to the presence of alkaloid which was confirmed by GLC and Dragendorff's alkaloid test. The results were compared with reference antibiotic tetracycline (1 unit strength).

The antibacterial activity of 25 plants collected from arid zone of Rajasthan State were tested against human pathogenic bacteria, *Salmonella typhi*. Aqueous and methanol extracts of stem (1:10 w/v) were screened for their antimicrobial potential. A total of 50 extracts from different species were prepared during this investigation and about 12 plant extracts were found to have growth inhibitory effect against the test organism (Gehlot and Bohra, 2002).

The influence of leaf extracts of different plants on the toxic strains of *Aspergillus flavus* was studied by Bohra and Purohit (2002). It was found that maximum inhibition of *A. flavus* was observed in *Azadirachta indica* (73.18%) while *Argemone mexicana* stimulated the growth of the fungus.

The fungistatic activity of *Semecarpus anacardium* nut extract on the mycelial growth and sporulation of *Aspergillus fumigatus* was studied by Kanika Sharma *et al.* (2002). They observed that there was a remarkable
decrease in the growth and sporulation of *A. fumigatus* following the treatment with the plant extract at the concentration of 400 mg in ethanol. Reduction of sporulation up to 53.7% was also observed at this concentration.

### 4.2.1 Phytochemical analysis of plant extracts

A variety of phytochemicals have been studied for their antimicrobial activity against pathogenic microbes. Numerous reports on the use of crude plant extracts and their active principles against selective microorganisms *in vitro* are also available in the literature. Phytochemical analysis of the plant extracts have revealed the presence of biologically active compounds such as flavanoids, tannins, phenols, terpenoids, sesqui terpenes, glycosides, saponins, alkaloids etc. (Odebiyi and Sofowora, 1978; leven *et al*., 1979; Reinhold *et al*., 1981; Harborne, 1982; Bever, 1986; Barnabas and Nagarajan, 1988).

Toda *et al*. (1991) isolated the phytochemical flavonoids, catechins from tea leaves, which showed antibacterial activity against *Staphylococcus aureus* at the concentration of 50 μg/ml.

Inumma *et al*. (1992) isolated Isoflavanones and Chalcones from Erythrina plants which inhibited the growth of *Staphylococcus sp*. at the concentration of 50 μg/ml.

Chromatographic fractionation of root barks of *Uvaria narum* led to the isolation of various compounds such as terpenoids and acetogenins which exhibited antimicrobial and anthelmintic activities in their individual screening comparable with standard drugs (Padmaja *et al*., 1993).
Luisa et al. (1994) isolated 12 pure compounds from Mexican Asteraceae species and tested them for in vitro antimicrobial properties against gram-positive and gram-negative bacteria and also against Candida albicans. The twelve compounds were identified as terpenoids and except taraxasterol, no significant activity was observed.

Preliminary phytochemical analysis as well as antibacterial activities of crude extracts of Acalypha torta against some anaerobic bacteria was studied by Irobi and Banso (1994). The result of their study indicated that the aqueous, ethanol and methanol extracts of Acalypha torta possess in vitro antibacterial activity against all the anaerobes used in their study. The extracts exerted bactericidal action on the bacteria at the doses of 2-32 mg/l. Preliminary phytochemical analysis of the leaves indicated the presence of biologically active compounds such as phenols, tannins, sesquiterpenes and saponins.

The ethanolic extract, chloroform fraction, hexane fraction and n-butanol soluble fraction of Piper longum fruits exerted in vitro antiamoebic activity against the Amoebiasis causing Entamoeba histolytica. The amoebicidal action was at 1000 μg/ml (ethanolic extract) and 500 μg/ml (chloroform extract). The hexane fraction and n-butanol fraction did not show any activity. The pure compound piperine possessed the antiamoebic activity (Sheela et al., 1996).

A comparative study on the antibacterial activity of phytochemical flavanones against methicillin resistant Staphylococcus aureus (mRSA) was examined by Tsuchia et al. (1996). They isolated flavanones from Fabaceae
and tested its antibacterial study against mRSA. Among the 13 flavanones tested, tetra hydroxy flavanones showed intensive activity to growth inhibition of all mRSA strains at the concentration of 3.13 to 6.25 µg/ml.

Raman (1999) isolated some orthoquinonoid and anthraquinonoid natural products from *Thespesia populnea* which forms a source of a variety of natural products especially flavonoids.

Brindha and Saraswathy (1999) isolated the active principles from plants such as *Oldenlandia, Plumeria* and *Pentatropis* by phytochemical methods. the compounds noticed were steroids, triterpenoids and phenolics.

Two new flavones namely echiodinin and echioidin were isolated from *Andrographis echioides* (Jayaprakasam et al., 1999). They also reported that a number of unusual flavones and flavone glycosides were found to exist in this plant.

Srivastava and Srivastava (1999) isolated and characterized two new constituents from the roots of *Terminalia alata*. The constituents had been confirmed as glycosyl-glucosyl rhamnoside and glycosyl-hexagallate on the basis of chemical and various spectroscopic techniques.

A new and rare prenylated flavone-C-trioside was isolated from the fresh white flowers of *Polycarpaea corymbosa* (Jaswant and Raghunathan, 1999).

Manogaran and Sulochana (1999) fractionated a flavonol glucoside, quercetin-3-o-neohesperidoside from the fresh flowers of *Allamanda cathartica*. 
The phytochemical investigation of the roots of *Peltophorum pterocarpum* revealed the presence of polyphenolic glycosides (Shubashini *et al.*, 1999).

Thamarai Selvi and Mohan (1999) isolated a new flavonoid prenyl dihydroxy methoxy flavanone from the resinous exudates of *Azadirachta indica*. They also isolated two steroids namely fucosterol and isopropenyl cholesterol from the same part of the plant.

Quercetagetin and its glucoside was isolated from the yellow portion of the fresh petals of *Hibiscus vitifolius* by Padmavathy and Nagarajan (1999). The flavonol glycoside isolated found to be different from that o the flavonol nibifolin isolated by previous workers.

Preliminary phytochemical evaluation on the leaf extracts of *Carissa carandas* revealed the presence of different phytoconstituents *viz.* alkaloids, phenolic compounds, glycosides and saponins (Rajasekaran *et al.*, 1999).

Fresh flowers of *Jasminum grandiflorum* and *Jasminum officinale* had been analysed for their phytochemical constituents. It was found that polyphenols such as isoquercitrin and quercetin were present. The isolated compounds were active against Bacterial species such as *Bacillus subtilis* and *Escherichia coli* (Sukumar and Lakshmi Vasan, 1999).

Isolation and characterization of chemical constituents of root bark of *Ventilago bomaiensis* Dalz was done by Sivaram Babu and Sridharbabu (2002). The powder of the root bark of extraction with acetone and isolation by chromatographic methods found to contain eight anthraquinone group of compounds. They have been characterized by spectroscopic methods
including NMR and structures were established with authentic samples data. All these eight compounds were isolated for the first time.

Phytochemical screening of *Centella asiatica* L. showed the presence of alkaloids, steroids, anthracene glycosides, saponins, volatile oils, fatty acids, phenolics, tannins, carbohydrates and triterpenes in the dried callus extracts as well as dried leaf extracts of this plant. The various organic solvents used to extract the secondary metabolites were benzene, chloroform, ethyl acetate, acetone, butanol, ethanol and methanol (Alagumanian *et al.*, 2002).

The aerial parts of the plant *Alangium salviifolium* were extracted with ethanol and the ethanolic extract after acidification and concentration further extracted with chloroform. The *chloroform extract was chromatographed by preperative TLC technique. Four compounds were isolated. They were characterized to be 2-isopropyl-6-methoxy-7 hydroxy chromone and 2-isopropyl-6-methoxycromone (John Britto *et al.*, 2002).

Isolation and characterization of a flavanoid from *Vitex altissima* was done by Alex Ramani and Kalaiselvi (2002). The aerial parts of the shade dried plant of *Vitex altissima* were extracted with ethanol. The concentrated ethanolic extract was acidified and further extracted with ethyl acetates solvent. The ethyl acetate extract was chromatographed by preparative TLC using benzene : chloroform : ethylacetate in 8 : 7 : 5 as the eluant. The isolated compound VAEI was subjected to chemical and spectroscopic analyses. The compound was identified a 5,4-dihydroxy 8-methoxy flavonoid-7-o-glucoside.
4.2.2 Phytochemical studies on *Momordica dioica*

Some phytochemical studies were recently made on this plant which are summarized below:

4.2.2.1 Steroidal glycosides

Sadyojatha and Vaidya (1996) isolated two steroidal glycosides from chloroform extract of root powder of *M. dioica* and also an alkaloid. Glycosides were screened for antimicrobial activity. MIC upon comparison with standard drugs revealed a moderate antibacterial and a poor antifungal activity.

4.2.2.2 Triterpenes and Steroids

Luo Lei (1997) isolated seven constituents from roots of *M. dioica*. Their structures were elucidated by spectra analyses (MS, IR, UV, IH NMR, 13 C NMR). Two new compounds, 3 β-o-benzoyl-11-oxoursolic acid and 3 β-o-benzoyl-6-oxoursolic acid were established. Five known compounds were also established which are oleanolic acid, gypsogenin, hederagenin, α-spinasterol and stearic acid.

Luo Lei *et al.* (1998) established the presence of three triterpenes and two steroidal compounds from the roots. The compounds are α-spinasterol octadecanone; α-spinasterol-3-o-β-D-glucopyranoside; 3-o-β-D-glucopyranosyl gypsogenin (a new compound); 3-o-β-D-glucopyranosyl gypsogenin; and 3-o-β-D-glucopyranosyl hederagenin.

Ali Mohammed and Srivastava (1998) isolated for the first time from the fruit rind of *M. dioica* two new aliphatic constituents characterized as
6-methyl tritriacont-5 on-28-ol and 8-methyl hentriacont-3-ene, along with the known sterol pleuchiol. Momodicaursenol, an unknown pentacyclic triterpene was also isolated from seeds and identified as Urs-12, 18(19)-dien-3-β-ol.

Li Zuqiang et al. (1999) analysed the five anti-cancer compounds isolated from the dried root of *M. dioica* and identified them as bryonolic acid, 23, 24-dihydro cucurbitacin-F-25-acetate, a-spinasterol-3-o-6-D-glucopyrenosides, gypsumogenin and hederagenin.

### 4.2.2.3 Protein types

Sex linked polypeptides were investigated by Sinha et al. (2001). Soluble proteins from the tuberous roots were analysed by SDS-PAGE to compare the protein profiles of the sex forms. Twentyeight bands with molecular masses ranging from apprx 15 KD Protein to more than 94 KD proteins were found to be common in both staminate and pistillate plants. The pistillate plant only had 22 KD polypeptide which was not detected in staminate ones. Immunoblot assay demonstrated that antibody raised against P-22 not only cross-reacted at 22 KD antigen of the pistillate plant but also with 29 KD and 32 KD polypeptides of the staminate and pistillate plants. These indicate that the 3 polypeptide are electrophoretically distinct but antigenically similar. 22 KD protein found in pistillate is sex linked. Variation in the intensity of 29 KD and 32 KD Polypeptides of staminate and pistillate plants suggests that the interplay of the above 2 sex-linked polypeptides may contribute to the dioecism in *M. dioica*. 
4.3.1 MICROPROPAGATION

During the last few years tissue culture techniques have been extensively exploited for rapid and large scale propagation of a number of rare and endangered medicinal plants. The major advantage of this technique is the combination of high volume production with low cost propagation. Regeneration of complete plantlets in vitro from apical as well as axillary buds have been reported, for a number of medicinal species (Purohit et al., 1994; Pattnaik and Chand, 1996). This technique also lead to the production of useful medicinal compounds which are therapeutically important. A brief review on the micropropagation of some important medicinal plants are summarized below.

Sen and Sharma (1991) demonstrated that shoot multiplication can be achieved in vitro from shoot tips of aseptically germinated seedlings of Withania somnifera L. using low concentrations of 6-BA. viz. 2.2, 4.2 and 8.9 μm. Maximum number of shoots were obtained when 2.3 μm of 2,4-D or 2.5 μm IBA was added to the medium. Direct multiple shoot initiation was also obtained from germinating seeds in the presence of BA alone. Rooting was successful in excised shoots grown in MS medium.

Akram et al. (1993) raised in vitro rooting from the stem calli of Rauwolfia serpentina in MS medium supplemented with NAA, 0.1 mg/l and BAP 0.1 mg/l. The differentiated roots were excised and further subcultured.

Sudha and Seeni (1994) outlined a procedure for clonal propagation of Adhatoda beddomei Clarke (Acanthaceae) a rare medicinal shrub. Callus
free auxillary meristem was proliferated from the nodal explants and an optimum number of 5-10 shoots per explant were obtained in six weeks using 3.0 mg/l BAP and 0.5 mg/l IAA.

Shoot tips of 7 days old *Amaranthus hypochondriacus* seedlings were grown *in vitro* on Murashige and Skoog medium supplemented with four concentrations of IAA (0.5, 1.0, 2.0, 3.0 mg/l) plus fixed concentration of kinetin (0.5 mg/l). Maximum differentiation of shoot tips into shoots with slight callus at the base and bushy roots was obtained on MS medium supplemented with 2.0 mg/l IAA plus 0.5 mg/l kinetin followed by 1.0 mg/l IAA plus 0.5 mg/l kinetin. Further regenerated plants were transferred to soil and their survivality was also noted (Pramod Kumar and Dube, 1997).

Sahoo and Chand (1998) developed a protocol for rapid clonal propagation of a medicinally important plant species, *Tridax procumbens* L. through *in vitro* culture. High frequency bud break (83%) and multiple shoot formation was induced from nodal segments cultured on MS medium supplemented with 1.0 mg/l BAP. Rooting of the excised shoots from secondary or subsequent cultures was best induced on half strength MS containing 1.0 mg/l IBA. Vermi-compost was used as the planting substrate for hardening. Micropropagated plants established in pots containing garden soil flowered within four weeks following transfer to outdoors and they were uniform and identical to the donar plants with reference to vegetative and floral morphology.

*In vitro* micropropagation of *Holarrhena pubescens* Wallich ex G.Don, a medicinal plant has been successfully regenerated by culturing shoot tips on revised Murashighe and Skoog medium supplemented with
3.0% sucrose, 2.0 gm l\(^{-1}\) of glycine and 2.0 mg/l of BA. The shoot tips collected from nine-month old seedlings gave best results. The regenerated shoots were rooted on revised MS medium supplemented with 1.0 mg/l of IAA. The plantlets were acclimatized in pots containing peat-vermiculite mixture and successfully transferred to soil (Sumana et al., 1999).

Multiplication by adventitious shoot regeneration from root explants was successfully done for the propagation of a threatened Nepalese medicinal plant, *Swertia chirata* Buch. –Ham. ex Wall by Wawrosch et al. (1999). A two-step system consisting of an initial 3-week cultivation on modified MS medium supplemented with 3 μm 6-benzylaminopurine followed by another period of 3 weeks on hormone free medium was used. After rooting and acclimatization micropropagated plants could be successfully cultivated in Nepal.

Callus mediated shoot regeneration from stem explants of *Hybanthus enneaspermus* was described by Natarajan et al. (1999). They raised nodular calli on MS medium containing 2.0 mg/l, 2,4-D and 0.5 mg/l BAP. Shoot buds were induced and elongated on MS basal medium with 5.0 mg/l BAP. The shoots were rooted in MS medium with 2.0 mg/l IBA and then were successfully hardened and transferred to the field.

*Lagerstroemia reginae* Roxb., an ornamental and medicinal plant has been successfully micropropagated using seedling explants. Raising the level of NH\(_4\)NO\(_3\) from 1650 mg/l to 1900 mg/l in MS modified medium was found to be effective as nitrogen source. Sucrose at 3% level was very effective as a carbon source. BA was used with NAA at 0.5 mg/l and 0.1
mg/l respectively exhibited best results. The regenerated shoots were rooted in modified MS medium supplemented with 1.0 mg/l of IAA. The plantlets were maintained in the laboratory on vermiculite and peat mixture for one month and successfully transferred to soil (Sumana and Kaveriappa, 2000).

Tiwari et al. (2000) developed an efficient and rapid method for the in vitro propagation of Bacopa monnieri, a medicinally important herb, using liquid shake cultures. This was achieved by culturing nodal explants on liquid MS medium with or without 6-benzyladenine. Compared to single axillary shoot proliferation on a growth regulator-free agarified medium, the respective liquid medium induced 4 or 5 shoots per nodal explant 4 weeks after culture. All regenerated shoots were dark green, vigorous and rooted on the same medium. Addition of 6-benzyladenine (0.01-0.1 mg/l) resulted in the increase in morphogenetic response (number of shoots, mean shoot length, and number of roots per node explant) in both the type of culture media. The use of liquid shake cultures economize on the time and medium requirement for propagation.

John Britto et al. (2001) achieved a rapid and clonal propagation of Anisomeles indica L. through direct axillary proliferation from nodal explants of field grown plants, cultured on MS medium supplemented with BAP (2.0 mg/l) and NAA (0.05 mg/l). Regenerated shoots were transferred to rooting medium containing 2.0 mg/l IBA. The rooted plants were hardened and successfully established in soil.

High frequencies of multiple shoots were achieved with nodal explant culture of Hyptis suaveolens on MS medium supplemented with 5 mg/l BAP and 0.5 mg/IAA. The elongated shoots were cultured for rooting on MS
medium with 1 mg/l NAA. The rooted plants were successfully established in soil (John Britto et al., 2001).

Axillary buds of field plants of *Cunila galioides* Benth. were used to evaluate the effect of growth regulators and culture media on the *in vitro* shoot proliferation and growing. The highest multiplication rate was obtained using MS medium supplemented with 8.8 μm of benzyladenine. Repeated subcultures of shoot tips and single nodes at 4-week intervals for eight months on the above medium enabled mass multiplication of shoots without any evidence of decline. The best conditions for rooting were MS medium plus 0.5 to 2.5 μm of IBA. The rooted plants were successfully transferred to soil, exhibiting a normal development (Fracaro, 2001).

Ravishankar Raj and Thoyajaksha (2001) developed an efficient method of rapid multiplication of a rare and endemic plant *Paracautleya bhatii* Smith using rhizomes as explants. They cultured the rhizomes in MS medium supplemented with BA, Kn and NAA. The cultures were maintained at 25±2°C with a 16 hr photoperiod provided by cool white fluorescent tubes. Six weeks after culture 50% of the shoots that differentiated on MS were excised and cultured on the same medium. The remaining shoots bearing 4-8 roots/shoot, were hardened for 60 days, removed from the agarified culture medium, transferred to plastic pots containing 1:1 sand:soil maintained in the growth chamber for 3 weeks and under greenhouse conditions for 8 weeks and then transferred to soil. It was observed that about 25% of the cultured rhizomes produced both shoots and roots simultaneously.
4.3.2 Multiple Shooting

Shoot tip culture is an accepted technique in micropropagation. In this method the axillary or terminal buds are hormonally controlled to produce multiple shoots. It is based on the fact that shoot tips are capable of independent growth if proper conditions are provided. In this method the axillary or terminal buds are forced to produce multiple shoot with higher concentration of cytokinin or kinetin. Shoot tip culture is a favoured micropropagation technique because the shoot tips are less prone to pathogens and any number of plantlets can be produced through this mode of propagation. The shoot tip culture may be of two types: 1) shoot tip culture, and 2) single node culture. The various works on shoot tip culture and multiple shooting has been summarized.

Philip et al. (1992) achieved multiple shoots from the shoot tip explants of black pepper (*Piper nigrum*) when they were treated with 1.5 mg/l BAP on MS medium. This treatment activated the shoot tips to produce multiple shoots.

Umarani et al. (1993) produced multiple shoots from the shoot tips of *Sesbania* species when the shoot tips were treated with the MS medium supplemented with NAA (1 mg/l) and BAP (1 mg/l). Callus was found within 25 days which when subcultured on the same medium with increase in BAP concentrations (2 mg/l) and NAA (0.5 mg/l). The differentiated callus later transferred into multiple shoots.

Mirza (1996) initiated multiple shoots from aseptically grown seedlings of *Capsicum annum* in MS medium supplemented with BAP or
kinetin or in combination with IAA. BAP proved to be the superior hormone than the other with reference to multiple shooting. Sitakanta and Pradeep (1996) achieved multiple shoots from Ocimum species such as *O. canum*, *O. sanctum* and *O. americanum*. Axillary shoot buds were cultured on MS medium supplemented with BAP (0.25 mg/l). Incorporation of 0.5 mg/l GA$_3$ along with BA in the culture medium resulted in a marked increase in the frequency of axillary branching as well as multiple shoot formation.

Madhuri and Chandramati (1999) induced multiple shooting from the shoot tips of date palm (*Phoenix dactylifera*) in MS medium supplemented with BAP (5 mg/l) and NAA (0.1 mg/l).

Sivakumar and Krishnamoorthy (2000) achieved multiple shoots from the shoot tips of *Gloriosa superba* L. in MS medium supplemented with BAP with or without the addition of kinetin.

### 4.3.3 Nodal Culture

In many of the plant species the axillary bud remains inactive though they have the potential i.e. apical dominance. These dormant axillary buds can be made active either by causing an injury or by destroying the apical bud. This principle is used in single nodal culture where in the apical dominance is controlled by cytokinin. This causes the axillary bud to develop which when sub-cultured into similar medium proliferate into multiple shoots. Any number of plantlets can be produced through the process of subculturing.

Joshi *et al.* (1991) induced multiple shoots from the cotyledonary and epicotyledonary nodes of *Anogeissus pendula* when they were supplemented
with BAP (10 mg/l) and IAA (0.1 mg/l) on MS medium. This resulted in the formation of multiple shoots.

Gurdeep Kaur et al. (1992) observed multiple shoots from the cotyledonary node segments of *Anogeissus sericea* on MS medium containing 0.1 mg/l IAA, 4.0 mg/l BAP, 50 mg/l ascorbic acid, 25 mg/l citric acid and 25 mg/l adenine sulphate. This treatment activated the nodal segments to produce multiple shoots. Anita et al. (1992) established multiple shooting from the cotyledonary node explants of *Acacia nilotica* cultured on Gamborg et al.’s medium supplemented with cytokinins, kinetin or zeatin. Maximum multiple shoots were obtained in cytokinins (N6-benzyl) adenine at the concentration of 1.5 mg/l.

Adolfina et al. (1997) produced multiple shoots from the nodal segments of *Hedeoma multiflorum*, an important aromatic plant to the pharmaceutical, cosmetic and food industries, when cultured on half strength of MS medium supplemented with BA or NAA.

Ana Maria Jordan et al. (1998) observed multiple shooting on MS medium containing BA, KIN and NAA with reference to *Lavandula* species. Highest shoot multiplication rates were obtained when explants were grown in the presence of BA or KIN along with 15% coconut milk.

Smita Chetia and Handique (2000) observed multiple shooting from the nodal explants of *Plumbago indica*, a rare medicinal plant, which were cultured on MS medium with BA (3 mg/l) and IAA (0.1 mg/l). Shoot initiation were observed after 20-30 days of culture.