Chapter II

REVIEW OF LITERATURE
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2.1 Plants-a major source of drugs

Plants have been used by mankind as sources of therapeutic agents to combat diseases since time immemorial in the conventional systems of medicine such as ayurveda, siddha, unani and homeopathy. With the development of scientific methods and techniques, a number of medicinal plants from various countries were subjected to biochemical and pharmacological investigations and the active constituents were isolated and characterized. Subsequently, these compounds—either in pure state or in the extracts containing them, were included in the pharmacopoeias of several countries. According to one estimate about 35,000-70,000 species have been used in various cultures of the world for medicinal purpose (Farnsworth and Soejarto, 1991). These have been used in the form of crude drugs which were dried parts of the medicinal plants (root, stem, wood, bark, leaves, flowers, fruits, seeds and in some cases whole plants) or their extracts. The active principles from these plant parts or their extracts were introduced as drugs in modern medicines following pharmacological, toxicological investigations and clinical trials. Although the number of plant based drugs used in modern medicine is low compared to the synthetic products; the plant based drugs have been extremely valuable and helpful in the alleviation of human sufferings. Some of the claims made by traditional medicinal systems for their drugs have received clinical support from modern medicinal science (Sukhdev, 1997). As the synthetic drugs cause several side effects, the plant
based medicine has become popular throughout the world nowadays. The contribution of medicinal plants in discovery of new drugs has been enormous in terms of value and activity for treating diseases like cancer, hypertension and several other ailments.

The species used for isolation of active ingredients may be indigenous species growing wild or cultivated or hybrids or other cultivated varieties that have been developed through selection for particular characteristics (Anonymous, 1992). In India, about 90% collection of medicinal plants is from wild sources and since 70% of collections involve destructive harvesting, many plants have become endangered or vulnerable or threatened (Anonymous, 1992). The increasing demands of the pharmaceutical industry have created problems of supply. One of the major difficulties being experienced by the industry is that of obtaining sufficient quantities of medicinal plants for the manufacture of genuine medicines. In our country there are only a few herbal drugs which are cultivated on large scale. One of the major difficulties of large-scale medicinal plant cultivation is the lack of scientific and appropriate agrotechnology for different climatic zones of the country. However several organisations viz, Indian Council of Agricultural Research (ICAR), Council for Scientific and Industrial Research (CSIR) laboratories, various Indian Universities have taken up the work towards development of appropriate agrotechnologies and of high yielding varieties of medicinal plants. But much efforts are still needed keeping in view the demands of trade and industry. Enormous importance should be given in mounting research efforts to develop in vitro micropropagation protocols for rapid regeneration of these plants on a large scale. There is also the need for
generating somaclonal variants of these species through plant tissue culture approaches for selection of elite clones having features fulfilling specific industrial requirements.

In the present study the three chosen plant species viz. *Centella asiatica*, *Adhatoda vasica* and *Asparagus racemosus* have high demand in the indigenous drug industry (Singh *et al.*, 2001). The drugs from these plant species have also received pharmacological/clinical support from contemporary medical system (Sukhdev, 1997).

*Centella asiatica* (L.) Urban is a member of Apiaceae family, commonly known in India as 'Indian pennywort' or 'Mandookaparni'. In the traditional system of Indian medicine, *Centella asiatica* is a nerve tonic and is used in the treatment of leprosy, asthma, bronchitis, dropsy, leucorrhoea, skin disease and urethritis (Kakkar and Gomez, 1988). The wound healing activity of the species is ascribed to a triterpenoid saponin, asiaticoside (Fig 1) while antitumour properties are attributed to madecassic acid. *Centella* extracts have antitumour activity *in vitro and in vivo* (Babu *et al.*, 1995).
Adhatoda vasica Nees (Acanthaceae) is an important medicinal plant. The medicinal importance of Adhatoda vasica had been reported in the ancient Indian medicinal treaties of Ayurveda. The plant grows in the wild and is being exploited for extraction of its principal alkaloid vasicine (Fig 2) which is used in the preparation of ‘vasaka’, a well known drug in the Ayurvedic system of medicine. This drug is recommended by the Ayurvedic medicine for a range of ailments viz., bronchitis, asthma, jaundice, diseases of the respiratory system, diphtheria and gonorrhoea (Kapoor, 2001). The pharmaceutical investigations of vasicine and vasicinone- alkaloids derived from Adhatoda vasica had been reported (Gupta et al., 1977). The efficacy of vasicine as a uterotonic abortifacient had also been proved (Gupta et al., 1978). Several alkaloids
from *Adhatoda vasica* have been found to have pronounced protective activity against allergy induced bronchial obstructions (Dorsch and Wagner, 1991). Extract of the plant *Adhatoda vasica* has been used as an effective Ayurvedic medicine in the treatment of tuberculosis. Bromhexine and ambroxol which are the semisynthetic derivatives of vasicine are also found to have growth inhibitory effect against *Mycobacterium tuberculosis* (Grange and Snell, 1996). The roots and leaves of the plant *Adhatoda beddomei* C.B. Clarke, an allied species are also used in both traditional and modern systems of medicine for its alkaloid vasicine (Sudha and Seeni, 1994).

*Asparagus racemosus* Willd. (Liliaceae), commonly known as 'satawar'or 'shatavari' is a scandent climber. The root of this plant is known to possess anti-diarrhoeal, anti-ulcer, refrigerant, tonic, nutritive, demulcent, diuretic, galactagogue, aphrodisiac and antispasmodic actions and has gained importance by its use in Ayurveda, Siddha and Unani systems of medicine (Nadkarni, 1954). The alcohol, ethyl acetate and acetone extract of powdered dry roots of *Asparagus racemosus* yield pharmacologically active
(antioxytocic) saponins, one of which is L-asparagine (Fig 3)(CRC Handbook of Ayurvedic Medicinal plants).

The dry roots of the plants are used as drug (Krinck, 1978). The roots are

![Structure of the saponin, L-asparagine in Asparagus racemosus](image)

Fig 3 Structure of the saponin, L-asparagine in Asparagus racemosus

said to be tonic and diuretic (Tewari et al., 1993). The cultivation of Asparagus is not extended at the commercial scale and its demand is met through the wild resources from the forest. Looking at the high demand of shade dried roots of Asparagus a study was undertaken to determine the optimum plant density for obtaining high root yield of A. racemosus and A. adscendens in sandy soils of the north Indian plains. Significantly higher yield of roots were recorded with greater plant density of $1.11 \times 10^5$ plant/ha in A. racemosus by 1.6 fold as compared to A. adscendens (Ram et al., 2001). The aqueous extracts of both fresh and dried roots of Asparagus racemosus were found to have amylase and lipase activities (Dange et al., 1969).

2.2 Micropropagation

In recent years, there has been an increased interest in in vitro culture techniques which offer viable tools for mass multiplication and germplasm
conservation of rare, endangered and threatened medicinal plants (Ajitkumar and Seeni, 1998; Sahoo and Chand, 1998). Plant tissue culture can also be used to generate variants (mutants) having industrially relevant features. Another significant aspect with respect to these species is that in spite of their immense potentials as sources of important drugs, there are some limitations in propagation of some of these species through the conventional methods as they are cross pollinated with high degree of genetic variability. Besides, seed viability is also very low in many a cases. Tissue culture techniques play a crucial role in overcoming these problems.

*Centella asiatica* L (Apiaceae) is an important medicinal plant used in several ayurvedic preparations. The requirement of this plant is now met from the natural populations, leading to their gradual depletion. Micropropagation techniques and application of biotechnology have given a wide scope for improvement of this important medicinal plant. A simple and a rapid method for the *in vitro* multiplication of *C. asiatica* from leaf explants had been established (Banerjee *et al.*, 1999). Leaf explants without the petioles were found to be more responsive than those with petioles. BA (2mg/l) along with IBA (0.1 mg/l) caused maximum sprouting where 80% of the leaf segments without petioles showed initiation along the margins and cut ends within two weeks, and only 30% of leaves with petioles responded, showing initiation near the distal cut ends of petioles after 4 weeks of culture initiation. In another attempt nodal segment harbouring single axillary buds proliferated well in ½ strength MS medium supplemented with 2.0 mg/l BA, 0.5 mg/l KIN and 0.25 mg/l IBA (Josekutty, 1998). Prolonged culture on this medium or transfer to ½ strength MS medium without hormones resulted in profuse
rooting. A protocol was also described for rapid and large scale in vitro clonal propagation of *C. asiatica* L. through enhanced axillary bud proliferation in nodal segments isolated from mature plants (Tiwari *et al.*, 2000). MS medium supplemented with 6.7 μM BA and 2.88 μM IAA was found most suitable for shoot elongation. Rooting was highest (90%) on full strength MS medium containing 2.46 μM IBA.

However, initiation of *Centella* nodal culture proved rather difficult due to heavy fungal and bacterial contamination (Tiwari *et al.*, 2001). Treatment with solution of systemic fungicide (Bavistin) and antibiotic (Trimethoprim) were required which resulted in drastic reduction in contamination with nearly 80% cultures were contamination free. Shoot tips have been a source material for obtaining virus-free and genetically aberrant plants. Plantlets from shoot tip explants have been described by several workers in different species (Deshpande *et al.*, 1999; Philomina and Rao, 2000). The suitability of shoot tip explant for regeneration and its sensitivity to various hormones is due to the activity of meristematic cells, which are actively dividing and are known to have dense cytoplasm with much more uniform and homogeneous composition (Mathur *et al.*, 2002).

*Adhatoda vasica* Nees is an important medicinal plant for the presence of bitter crystalline alkaloid called vasicine and an organic acid – adhatodic acid, another alkaloid and an odorous volatile principle (Iyengar, 1984). It is an evergreen woody perennial bush. The influence of callusing at the excised ends and the effect of browning and phenolic exudation in woody perennial plant species preventing multiple shoot induction was reported earlier (Komalavalli and Rao, 2000; Reddy *et al.*, 1998; Patnaik and Debata, 1996).
These problems were overcome by modification of the media. Even in *Adhatoda beddomei* C.B. Clarke there was evidence of decline due to browning followed by necrosis of the cultures after 4 weeks irrespective of the concentrations and combinations of the cytokinins tried (Sudha and Seeni, 1994). However an optimum combination of 3.0 mg/l BA, 0.5 mg/l 2-ip and 1.0 mg/l NAA favoured the differentiation of 2-3 shoot buds in 3 weeks and development of 5-10 shoots (0.5-2.0 cm) in 85% of the explants in 6 weeks. Combinations of cytokinins thus found to activate the axillary meristems to form shoot buds but sustenance of the shoot growth depended on the synergistic influence of the auxin.

Efficient tissue culture method for rapid multiplication of *Adhatoda vasica* through nodal segment culture was established (Jaiswal et al., 1989) for the production of vasicine. The vasicine content also varied with varying concentrations of BA. Shoots with broad leaves and multiple shoot buds cultured on medium with 0.5 mg/l BA synthesized the highest amount of vasicine (Jaiswal et al., 1989). However there was persistent contamination problem when nodal explants were used for micropropagation of *Adhatoda vasica*. The use of nodal explants may also accentuate the problem of phenolic exudates. In the present study therefore, shoot tip were used as explants for micropropagation of *Adhatoda vasica*. *Adhatoda beddomei* is also as important source of drug *vasaka* in the Indian indigenous system of medicine (Ayurveda) where it is considered therapeutically superior to the allied species, *Adhatoda vasica*. It rarely set seeds and natural vegetation and conventional propagation through vegetative cuttings were also slow and insufficient for conservation. Therefore an attempt was made to establish a
rapid propagation protocol through axillary bud proliferation (Sudha and
Seeni, 1994).

Efforts towards development of in vitro micropropogation technique is rather meagre for the genus Asparagus, despite its horticultural and medicinal importance. Vegetatively propagated Asparagus plants are likely to accumulate pathogenic viruses and the latent viruses can be seed transmitted. Yang and Clore (1976) compared shoot tips (0.1-0.3 mm explants with 1-3 leaf primordia) with meristem tip (less than 0.1 mm with no leaf primordia) culture and found that 91% of the plants derived from meristem tip explants were virus free compared to only 43% of those which came from shoot tips. In another attempt, Dan and Stephens, 1991 developed a protocol for culture and plant regeneration of callus derived protoplasts of Asparagus officinalis cultivar Lucullus 234. Asparagus officinalis Cultivar Lucullus 234 is a highly disease resistant species against Fusarium moniliforme (sheld) and F. oxysporum. But due to incompatibility barriers sexual crosses were not possible between the resistant species A. officinalis and A. densiflorus 'sprengeri'. This problem was overcome by protoplast fusion (Davey and Kumar, 1983) for which the above attempt of protoplast regeneration of A. officinalis was made.

Murashige et al (1972 b) and Hasegawa et al (1973) developed a method for propagating Asparagus officinalis plants by shoot culture. Multiple shoots were obtained from shoot apex or lateral bud explants on Murashige et al., (1972 b) medium augmented with 0.3 mg/l NAA, 0.1 mg/l KIN and 40 mg/l adenine sulphate dehydrate. Even lateral buds from Asparagus officinalis spears were grown by Yang and Clore (1973) into single shoot on a semi-
solid basal medium (3% sucrose) containing 0.05- 0.1 mg/l NAA and 0.05- 0.1 mg/l KIN. Shoot arising from cultured nodes were cut into single node segments, which were recultured on the substrate or rooted. However, no report on efficient micropropagation protocol has been established in *A. racemosus* till date.

### 2.3 Callus culture and cell suspension culture

Callus is produced on explants *in vitro* in response to wound and growth substances either endogenous or supplied to the medium. Continuous subculture at 3-4 week intervals of small cell clusters taken from these calli can maintain the callus cultures for long periods. Initiation of cell division and subsequent callus production require a supply of cytokinin and auxin in the medium at correct proportion (Skoog and Miller, 1957). Auxin at a moderate to high concentration is the primary growth substance used to produce callus. Cytokinin is supplied in a lesser amount if not adequate within the explant. Ghosh and Sen, 1991 induced callusing in *Asparagus officinalis* using spear sections 3 mm below apex when cultured in MS medium supplemented with 1 mg/l NAA and 1 mg/l KIN. Even Levi and Sink, 1992 obtained callus in the medium supplemented with 0.1 mg/l NAA and 0.28 mg/l 2-ip.

Although callus tissue cultures may appear outwardly to be uniform masses of cells, in reality these structures are relatively complex with considerable morphological, physiological and genetic variation within the callus. Cell divisions do not take place throughout the culture mass but are localised primarily in a meristematic layer on the outer periphery of cells. Organogenesis begins with dedifferentiation of parenchyma cells to produce
centres of meristematic activity (meristemoids)(Sharp and Flick, 1981; Thorpe, 1979). Shoot and root initiation from the meristemoids follow when an appropriate ratio between the auxin and cytokinin is maintained. Reinert J, 1959 discovered the formation of miniature embryo like structures called embryoids in carrot grown on agar using high auxin concentration as the inducing agent. Since then, specific tissues in various species have been found to have either a capacity (competence) for somatic embryogenesis in culture systems or can be induced to develop competency for the same in culture by specific treatments to the medium. In *Asparagus cooperi* somatic embryogenesis was induced from callus following KNO₃ treatment (Ghosh and Sen, 1989). Such somatic embryos provide ideal materials for rapid propagation of stable regenerants. However complete plantlets could not be obtained in MS basal medium only. The optimum results were secured when embryos were cultured in the basal medium (MS) supplemented with Zeatin or GA₃ alone (Ghosh and Sen, 1991).

Reuther, 1977 induced callus by culturing shoot tip or shoot segment explants on LS medium supplemented with 1 mg/l NAA and 1 mg/l KIN. The calli when subcultured at stage II to LS medium with 1 mg/l IAA and 0.1 mg/l BA, the callus became organogenic and gave rise to shoot initials and somatic embryos. Even embryogenic calli were obtained in a variety of *Asparagus* explants when cultured on MS medium in which vitamin content has minor amendments. Ghosh and Sen, 1991 obtained somatic embryos in calli when induced from spear sections 3 mm below apex of *Asparagus officinalis* following transfer to medium with extra 1g/l KNO₃ or 1g/l casein hydrolysate.
Somatic embryos can be induced even by cell suspension culture in liquid culture media. A cell suspension culture is initiated by placing a piece of friable callus or homogenized tissue in liquid medium on a shaker so that the cells dissociate from each other and single cells can be obtained. Somatic embryos in cell suspensions can be obtained if the tissue is subjected to conditioning to induce embryogenic competence. Although embryogenic cell suspension cultures outwardly appear to be callus, on closer inspection these cell masses are well organised as proembryogenic masses (PEMs). PEMs continue to develop in suspension cultures until they are transferred to a stationary medium to develop somatic embryos. In *Acacia catechu* Willd. (Leguminaceae) callus proliferation occurred on cotyledon explants cultured on MS medium supplemented with 2, 4-D (3 mg/l and BA (0.5 mg/l) (Kaur and Kant, 1999). On transferring and agitating callus clumps in liquid MS medium containing 3 mg/l BA and 0.5 mg/l NAA, shoot bud differentiation was obtained.

The proembryogenic masses are often passed through sizing screens to get uniformity and synchrony of development. In *Asparagus officinalis* (Levi and Sink, 1992) the calli obtained in the medium supplemented with 0.1 mg/l NAA and 0.28 mg/l 2-ip were transferred to a suspension culture with continuous agitation and after 3 weeks the suspensions were sieved through 600 μm mesh and re suspended in medium with 10-20 mg/l NAA to get uniform embryogenic cell clusters and globular embryos. The growth of the embryos were promoted when transferred to medium with 0.1 mg/l NAA and 0.22 mg/l 2-ip and complete plantlets were obtained with 8.2 % sucrose initially, followed by transfer to medium with 8 % sucrose.
Plant tissue culture techniques like callus cultures were widely employed to obtain a stable supply of bioactive secondary products (Bajaj et al., 1988; Yeoman et al., 1996), including anti-cancer drugs like taxol (Edgington, 1991). Iyer et al., 1998 raised calli from cotyledons, hypocotyls, roots, leaves and internodes of *Nyctanthes arbor-tristis* in MS medium supplemented with 2,4-D, NAA and coconut milk. The alcohol extract of the callus showed the presence of iridoid glycosides by thin layer chromatography. The iridoid arbor-tristoside A had been reported to have pronounced anticancer activity (Mathuram et al., 1991; Stuppner et al., 1993; Mathuram et al., 1994). Castellar and Iborra, 1997 attempted to induce calli from stems of *Crocus sativus* L., the source of saffron which is a high value flavouring agent in MS medium supplemented with 5 mg/l BA and 10 mg/l NAA and the calli differentiated into stigmas when transferred to MS medium with 5 mg/l BA and 1 mg/l NAA. The saffron stigmas contain a secondary metabolite called crocin, a yellow pigment freely soluble in water. The yellow pigmentation due to presence of crocin progressively increased with calli growth, which was analysed by HPLC.

So far, there is no report on the use suspension culture for production of vasicine, asiaticoside and L-asparagine.

2.4 Antimicrobial activity of secondary metabolites of plant origin
The importance of medicinal plants in providing healthcare against various ailments including infectious diseases is well documented (Chopra et al., 1992; Iyengar, 1984). Moreover, the development of multi-drug resistance in the pathogenic bacteria and parasites has created major clinical problems in
the treatment of infectious diseases. This and other problems like toxicity of certain antimicrobial drugs on the host tissue (Idose et al., 1968; Maddux and Barrere, 1980) and non-availability of a suitable antifungal drug for systemic mycoses have triggered interest in search for new antimicrobial substances/drugs of plant origin. Considerable work on the antimicrobial activity of medicinal plants has been reported from different parts of the world (Ahmad et al., 1995; David, 1997; Desta, 1993).

The antimicrobial drugs injure microbes through several different mechanisms. Antimicrobial drugs either kill microorganisms directly (bactericidal) or simply prevent them from growing (bacteriostatic). They function specifically in one of the following ways-

1. inhibition of cell wall formation- The cell walls of most bacteria contain a rigid girdle of peptidoglycan. This structure, which is many layers thick in gram positive species and quite thin in gram negative ones, protects the cell against rupture from hypo tonic environments. Cells actively engaged in enlargement or binary fission must constantly synthesize new peptidoglycan and transport it to its proper place in the cell envelope. Drugs such as penicillin and cephalosporin react with one or more of the enzymes required to complete this process, causing the cell to develop weak points at growth sites and to become osmotically fragile (Fig 4). Antibiotics that produce this affect are considered bactericidal, because the weakened cell is subject to lyses.
Fig 4 Effect of antibiotics on cell wall synthesis of a growing cell (a) coccus is exposed to betalactam agent (cephalosporin) (b) weak points develop where peptidoglycan is incomplete (c) the weakened cell is exposed to a hypotonic environment (d) the cell lyses (e) scanning electron micrograph of bacterial cells in their normal state (× 10,000) (f) SEM of the same cells in the drug affected state showing surface bulges (× 10,000) (Talaro K and Talaro A, 1996)

(2) inhibition of nucleic acid synthesis- Antimicrobial drugs interfere with nucleic acid synthesis by blocking the synthesis of nucleotides, inhibiting replication or stopping transcription. Sulfonamides act as structural or metabolic analogs that mimic the natural substrate of an enzyme and vie for its active sites. In practice, sulpha drugs are similar to natural metabolic compound PABA (para amino benzoic acid) required by bacteria to synthesize folic acid. Folic acid in turn is a component of the co-enzyme tetrahydrofolic acid that participates in the synthesis of purines and certain amino acids. A sulfonamide molecule has extremely affinity for the PABA site
on the enzyme that synthesizes folic acid, thus it can successfully compete in a "chemical race" with PABA for the same site (Fig 5). Sulfonamides ultimately cause inadequate supply of folic acid for purine production, which invariably halts nucleic acid synthesis and prevents bacterial cells from multiplying.

![Diagram of competitive inhibition in nucleic acid synthesis](image)

Fig 5 Competetive inhibition of a pathway in nucleic acid synthesis. Sulfonamides are structural analogs of PABA, a chemical required to synthesise folic acid. The similar configuration of the two means that sulfonamides can bind to the active site on an enzyme involved in folic acid synthesis. Although it binds, sulfa still cannot complete the required synthesis (Talaro K and Talaro A, 1996)

(3) inhibition of protein synthesis- Antibiotics like streptomycin, gentamycin, tetracyclines inhibit protein synthesis by binding with the prokaryotic ribosome. Some drugs bind to the 30S (small) subunit, while others attach to the 50S (large) ribosomal subunit. Thus different steps in the protein synthesis mechanisms are affected: aminoacyl-tRNA binding, peptide bond formation, mRNA reading and translocation.
(4) Interference with the function of the cell membrane—isoniazid (INH) and ethambutol are examples of effective synthetic antimicrobial drugs against *Mycobacterium tuberculosis*, which inhibit synthesis of mycolic acid, which are components of cell walls restricted only to the mycobacteria. The cells of the genus *Mycobacterium* are long, slender, or curved rods with a slight tendency to be filamentous or branching. The high lipid content of the cell wall imparts the characteristic of acid-fastness and is responsible for the resistance of the group to drying, acids and various germicides. It produces no exotoxins or enzymes effecting infectivity. The majority (85 %) of tuberculosis (TB) cases are contained in the lungs, even though disseminated tubercle bacilli can give rise to tuberculosis in any organ of the body (extrapulmonary TB). Clinical tuberculosis is divided into primary, secondary (reactivation or reinfection) tuberculosis, and disseminated tuberculosis. Treatment of TB therefore involves administering drugs for a sufficient period of time to annihilate the bacilli in the lungs, organs and macrophages usually for 6-24 months. In the conventional treatment of patients with tuberculosis a combined therapy with at least two drugs including isoniazid, rifampin, ethambutol, streptomycin, pyrazinamide, thioacetazone or para aminosalicylic acid (PAS) are administered to avoid drug resistance. However other factors such as drug addiction, HIV pandemic and various socio-economic factors have contributed to the spread of the TB bacillus and led to the growing cases of MDR-TB. An estimate of 273,000 (95 % confidence limits, 185,000 and 414,000) new cases of MDR-TB occurred worldwide in 2000, 3.2 % of which all new TB cases were reported by a survey conducted over 64 countries (Dye et al., 2002). The treatment regime for HIV and MDR-TB is complicated by the fact
that most of the drugs used have not been studied for interactions with antiviral agents. Hence overlapping toxicities require intensive management and monitoring of these patients (Poznaik, 2001). Informations gained from sequencing the *Mycobacterium tuberculosis* genome will enable scientists to accelerate the development of reagents for improved tuberculosis control. Cloning and expressing the genes encoding the enzymes involved in cell wall biosynthesis will provide the tools for screening millions of novel compounds (Young, 2001) having inhibitory effects. Even an approach to target metabolic processes that are essential in nondonvding bacteria will be effective and the latent diseases through a drug that acts in synergy with the immune response could be targeted (Young D, 2001).

Considering the rich diversity of Indian medicinal plants, it is expected that screening and scientific evaluation of plant extracts for their antimicrobial activity may provide new antimicrobial substances. Ahmad I et al., 2000 has determined the MIC values against several bacteria and yeast, *Candida albicans* of the extracts (800-9000 mg/ml) of five plants *Emblica officinalis* L., *Plumbago zeylanica* L., *Holarrhena antidysenterica* Rox. bex Fleming, *Terminalia belerica* (Gaertner) Roxb. and *Terminalia chebula* Retz. Maximum potency (lowest MIC) was recorded in *Emblica officinalis* against *Staphylococcus aureus*, *Staphylococcus epidermis* and *Salmonella typhimurium*. Growth inhibition using agar diffusion assays against *E. coli*, *Psuedomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* were also studied using the crude extracts from 59 species representing mostly plant families of *Scrophulariaceae* and *Acanthaceae* (Grimes et al.,
1996). Growth inhibitory activity against one or more of the microbial species was detected in over 40% of the samples.

The herb, *Centella asiatica* L. has been used in the treatment of leprosy patients from very early times in India. The *in vitro* effect of an indigenously produced dry powder of *Centella asiatica* on the acid fastness and viability of *Mycobacterium tuberculosis* was investigated and found that it has no direct action on the acid fastness or viability of *M. tuberculosis* H37Rv *in vitro* (Herbert *et al.*, 1994). Even the antitumour effect of the crude extract of *Centella asiatica* as well as its partially purified fractions from chromatographic procedures through both *in vitro* short and long term chemosensitivity and *in vivo* tumour model test system were studied (Babu *et al.*, 1995). The partially purified fractions from the crude extracts of *Centella asiatica* has been reported to inhibit the proliferation of transformed cell lines (L-929) significantly. Hausen, 1993 also found the *C. asiatica* raw extract and its 3 acids namely triterpenic constituents asiaticoside, asiatic acid and madecassic acid to be weak sensitizers. However, the alcohol extract of *C. asiatica* increased cellular proliferation and collagen synthesis at the wound site of rats as evidenced by increase in DNA, protein and collagen contents of granulation tissues (Suguna *et al.*, 1996) which suggests that some kind of anti-tumour properties are present in this plant.

The presence of antiasthmatic activity of *Adhatoda vasica* plant extracts was also supported by Singh *et al.*, 2001. The leaves and roots are antispasmodic and efficacious in cough (CRC Handbook of Ayurvedic Medicinal Plants). The unknown alkaloids from *Adhatoda vasica* showed pronounced protection against allergen induced bronchial obstruction in
guinea pigs (Dorsch et al., 1991). The abortifacient effect of vasicine, the prime alkaloid of Adhatoda vasica like its uterotonic effect was more marked under the primary influence of oestrogens which are known to enhance the synthesis of prostaglandin indicating that the action of the vasicine was mediated through the release of prostaglandin (Gupta et al., 1978). The effects of even aqueous or 90 % ethanol extracts of Adhatoda vasica were studied in rats orally dosed for 10 days after insemination with special reference to foetal development. Leaf extracts of Adhatoda vasica were found to be 100 % abortive at doses equivalent to 175 mg/Kg of starting dry material (Nath et al., 1992). Grange et al., 1996 found that the benzylamines, bromhexins and ambroxol which were semi-synthetic derivatives of vasicine from the Indian shrub, Adhatoda vasica had a pH-dependent inhibitory effect on Mycobacterium tuberculosis. The plant extracts of Adhatoda vasica were found to be rich in enzymic and nonenzymic antioxidants such as catalase, peroxidase, total carotene, ascorbic acid, tocopherol and polyphenols. The plants extracts were found to possess antitubercular activity which were also good sources of antioxidants and therefore capable of preventing tissue damage by reactive oxygen species (Usha et al., 2001).

Alcoholic extract of the root of Asparagus racemosus was found to inhibit ochratoxin A (OTA)- induced suppression of production of cytokines. The extract also significantly inhibited the OTA-induced suppression of chemotactic activity (Dhuley, 1997). The claim of the use of Asparagus racemosus root in Ayurveda in the treatment of jaundice was scientifically validated by Muruganandan et al., 2000 by evaluating the immunomodulatory and antihepatoxic activities of Asparagus racemosus root extracts. He found
that the ethanolic root extract (100 mg/ Kg) enhanced the humoral and cell-mediated immunity in mice and exhibited antihepatotoxicity in rats. The crude saponins from the shoots of Asparagus were found to have antitumour activity (Shao et al., 1996). The saponins inhibited the growth of human leukaemia HL-60 cells in culture and macromolecular synthesis in a dose-dependent manner. The inhibitory effect of crude saponins on DNA synthesis was also found to be irreversible. In another attempt, Shao et al., 1996 isolated two oligofurostanosides from the seeds of Asparagus officinalis which also showed the same anti-tumour activity. However, so far no report has been found on anti Mycobacterium tuberculosis activity of the crude extract of Asparagus plant.

In another species of the genus Asparagus like in Asparagus adscendens, the aqueous and alcoholic extracts of the roots was studied on the muscle preparation of Setaria cervi, in vitro and on the survival of microfilariae in vitro. It was found that both alcoholic as well as aqueous extracts caused death of microfilariae in vitro, LC50 and LC90 being 8 and 16 ng/ml for aqueous, 3 and 12 ng/ml for alcoholic extracts respectively (Singh et al., 1997). Two anti protozoal compounds have been also isolated from the roots of Asparagus africanus Lam, which potently inhibit the growth of Leishmania major promastigotes, while moderately inhibits Plasmodium falciparum schizonts in 12 μM and 49 μM respectively (Oketch-Rabah et al., 1997). These concentrations only moderately affect the proliferation of human lymphocytes.
2.5 Approaches to the discovery of drugs from plant sources

Natural products have been the most successful source of drugs ever (Harvey, 2000; Newman et al., 2003). Historically the most important natural sources have been plants. Research progressed along two major lines: ethnopharmacology (medicinal herbs, substances of abuse, ordeal poisons) and toxicology (poisonous plants, venomous animals, arrow and fish poisons) (Heinrich and Gibbons, 2001). These strategies have produced many valuable drugs and are likely to continue to produce lead compounds (Tulp and Bohlin, 2002).

The interest in the use of ethnopharmacological information was for several reasons. At the beginning of the 1990s, there has been greater cross-cultural interest in the practices of native people than was formerly the case (de smet and Rivier, 1989). Farnsworth and colleagues showed that of the 119 important plants derived drugs used in one or more countries, 88 (77 %) were regarded as having been discovered as a result of being derived from a plant used in traditional medicine (Farnsworth and Soejarto, 1991). In addition, several additional contributions have appeared in the literature recently that report on the value of using an ethnopharmacological approach to the discovery of natural product drugs from plant sources. Another approach is chemotaxonomy which relies upon the fact that taxonomically related plants often biosynthesise chemically similar secondary metabolites. The discovery of digoxin from Digitalis lanata is a good example of how a chemotaxonomic approach can lead to a new drug, since this species was investigated phytochemically, based on the known existence of cardiac glycosides, such as digitoxin in another member of the same genus, D.
This approach is potentially useful in that, a higher yield of a given compound of interest can often be found for example, in other member of the same genus of a species under consideration (Anonymous, 1989).

Once significant activity is detected and confirmed for a given plant extract, the plant materials should be recollected under the same conditions, purification and structural characterization of the active constituents were done. Activity-guided fractionation is carried out using a combination of bulk solvent extraction and chromatographic procedures, which vary from lab to lab depending upon individual preference. Compound characterization and structure determination is performed routinely by the interpretation of physical and spectroscopic data, with compound degradations and total or partial synthesis carried out as deemed necessary.

High performance liquid chromatography (HPLC) has become one of the most widely used methods for the analysis of different compound mixtures. Simple, quick and accurate analytical procedures through HPLC with photodiode array detector was used for purity determination, wavelength optimization, similarity curves and component identification by spectral libraries in *Catharanthus roseus*, *Papaver somniferum*, *Adhatoda vasica* for their important alkaloids (Gupta *et al.*, 2000). Brain and Thapa, 1983 studied the degradation of vasicine in various solvents under normal laboratory lightning conditions and continuous UV irradiation at 365 nm. Quantification and detection of vasicine and vasicinone in *Adhatoda vasica* by HPLC method was also reported (Choudhury and Hirani, 1987) which involves the ion-pairing technique and uses an internal standard for quantification.
Fifteen collections of *Centella asiatica* L. were analysed for asiaticoside and madecassocide composition. The estimations were carried out by HPLC using C18 column, acetonitrile-water (3:7) as solvent and UV detector at 220 nm (Singh *et al.*, 1999). A pharmacologically important plumbagin in *Plumbago zeylanica* was estimated by rapid, accurate and simple HPLC method (Gupta *et al.*, 1999). The assay combines the isolation and separation of plumbagin on silica gel 60F<sub>254</sub> TLC plates followed by scanning of the spot at 265 nm detection modes using a CAMAG Scanner 3.

The alkaloids ajmaline, serpentine, reserpine and ajmaciline from *Rauwolfia serpentina* benthe ex kurz were identified by TLC and quantified by HPLC from tissue culture derived plants and wild plants. The identity of the alkaloids was confirmed by co-chromatography with authentic samples of ajmalicine, reserpine, ajmaline and serpentine (Roja and Heble, 1996). Similarly, quantitative determination of aristolochic acid in *Aristolochia indica* was also done by a rapid, sensitive and reproducible HPLC method based on photo-diode array detector (Singh *et al.*, 2001). The analysis of tropane alkaloids in solanaceous plants is of importance because of the extensive use of atropine and scopolamine in the pharmaceutical preparations. Various spectrophotometric (Worrel and Booth, 1953), HPLC (Verpoort and Svendsen, 1976) methods had been reported for quantitative determination of the tropane alkaloids.

### 2.6 Bioreactor raised secondary metabolites

Mass culture of plant cells *in vitro* has been proposed as a viable alternative for the production of vast arrays of high value, low volume phytochemicals. It
is greatly influenced by the culture conditions of which culture medium is the most important. The production of secondary metabolites generally occurs in the late stationary phase when the medium gets depleted of some of its important constituents. In such cases a ‘dual culture system’ is preferred. It involves biomass production in a medium for cell proliferation (growth medium) followed by transfer of healthy cells to a different medium (production medium) which does not support good growth of the cells but is favourable for product yield. The production of an anticancer drug paclitaxel from the plant Taxus baccata has been studied by Tabata, 2004 following this strategy. The study revealed that paclitaxel reaches a maximum level of 295 mg l⁻¹ in a large scale culture of T.x media cells.

In the scale up of a production process for secondary metabolites in plant cells by suspension culture, a number of basic laboratory-scale experiments have to be performed to yield essential data like growth rate, product formation rate, nutrient uptake, respiration rate and heat production. There are several examples showing changes in growth or productivity of plant cell clusters when scaled up (Scragg et al., 1987; Schiel et al., 1987). It is therefore essential to mimic in the laboratory the conditions occurring on a large scale as carefully as possible, or to study the differences on various scales to pinpoint the responsible factor.

For the last two decades considerable work has been done to design bioreactors for plant cell culture in large scale (Scragg, 1994). Conventional bioreactors like stirred tank reactor, bubble column or airlift bioreactors can be used for cell suspension with free plant cells or their small aggregates because it approximates microbial cultures. The effects that are known to
occur on a large scale can be studied in the same type of bioreactor at a smaller scale, mimicking the critical situations that occur in a large system. Noguchi et al., 1977 cultivated *Nicotiana tobacum* cell suspensions in a 20,000 l stirred tank reactor to produce while Schiel and Berlin, 1987 studied *Catharanthus roseus* in a 5000 l stirred tank reactor for the production of indole alkaloids.

Efficient mixing of plant cells cultured on large scale is extremely important to provide uniform physiological conditions within the culture vessels. Plant cells are sensitive to the shear stress due to rigid cellulosic wall and extensive vacuole, restricting the use of high agitation for efficient mixing. Plant cells are therefore often grown in modified stirred-tank bioreactors at a very agitation speeds. Air-lift bioreactors may provide even better and uniform environmental conditions at low shear. In suspension cultures of *Taxus chinensis* var mairei the shear rates over 719 s\(^{-1}\) damaged the cells by decreasing the mitochondrial activity, increasing the membrane permeability and causing cell hypersensitive responses. Consequently phenylalanine ammonia lyase (PAL) were activated and extracellular phenolics were accumulated in the cells leading to lower secondary metabolism (Shi et al., 2003). But Scragg et al., 1988 and Meijer et al., 1993 demonstrated that cells from various plant species are shear tolerant. He explained that recently initiated cell lines may be more susceptible to shear stress than cell lines cultivated for a long time in liquid medium. This conclusion was supported by the observation that the cultivation of freshly initiated (from calli) *Ginseng* cultures on a large scale was troublesome due to shear stress, while the
cultivation of *Echinacea* and *Rauwolfia* cell clusters in 75,000 l stirred tank reactors presented no shear problems (Westphal, 1990).

All plant cells are aerobic and require continuous supply of oxygen. However, plant cells require less oxygen than micro-organisms because of their slow metabolism. It has been observed that carbon dioxide level can drastically influence the length of the lag phase and is some cases, higher oxygen concentration even proved to be toxic to the metabolic activities of cells. The cell suspension culture of *Taxus wallichiana* in a 20 l airlift bioreactor accumulated higher amount of paclitaxel and baccatin III (factor of 2.0 and 1.2 respectively) than in the shake flasks, even the cell biomass was at maximum productivity in both the condition. This was mainly due to adequate aeration and mixing of the culture in the bioreactor (Navio osorio *et al.*, 2002).

During the late exponential phase of growth, cells become more sticky because of increased excretion of polysaccharides into the culture vessel, leading to the adhesion of plant cells to the reactor wall, probes and stirring device and formation of large aggregates. Proper mixing is therefore affected leading to lower secondary metabolite production. Even stresses, locations, climates, microenvironments and physical and chemical stimuli often called elicitors; qualitatively and quantitatively alter the content of bioactive secondary metabolites. Enzymatic pathways leading to the synthesis of these phytochemicals are highly inducible (Ebel and Cosio, 1994). This is true for alkaloids (Facchini, 2001), phenylpropanoids (Dixon and Paiva, 1995) and terpenoids (Trapp and Croteau, 2001; Turlings and Tumlinson, 1992) whose levels often increase by two to three orders of magnitude following stress or
elicitation (Darvill and Albersheim, 1984; Dixon, 1986). The enhanced catharanthine production in Catharanthus roseus cell cultures in a 20 l airlift bioreactor was due to the synergistic effect of the elicitors, malate and sodium alginate (Zhao et al., 2001). Even the growth rate and production of paclitaxel and baccatin III in cell suspension culture of Taxus increased by 8.3 and 4.0 factors respectively when the production medium was supplemented with an elicitor, methyl jasmonate (220 μg g⁻¹ FW) and two putative precursors, mevalonate (0.38 mM) and N-benzoylglycine (0.2 mM) (Cusido et al., 2002).

Another important characteristic of plant cell suspensions is the requirement for a high inoculum density in order to obtain growth. This is due to the requirement of plant cells in culture for a factor(s) which is released into the medium from the cells. There is a report on the success of large scale production of oil palm (Elaeis guineensis) suspension cells in a bioreactor where the biomass increased approximately by 3.5 fold per month. This was due to the synergistic effect of both inoculum density and conditioned media respectively (Gorret et al., 2004). Even for high salidroside production, which was identified as the most potent ingredient of the Chinese herb, Rhodiola sachalinensis, the optimum inoculum amount was 10% (Wu et al., 2003). With this inoculum amount the optimal concentration for 6-benzylaminopurine and IBA added in the liquid medium was 5 and 2.5 mg l⁻¹ respectively. The acidic culture medium and a faster shaking speed favoured the salidroside production. The addition of 2,4-D in the liquid MS medium and the utilization of L-tyrosal for chemical feeding enhanced salidroside production. Using a proper combination of culture condition and treatment, salidroside accumulation could reach 57.72 mg g⁻¹ DW that was 5-10 fold higher than that
detected in field grown plants. The corresponding salidroside yield was 555.13 mg l\(^{-1}\), a level suitable for cost effective commercial production to compensate the natural resource shortage of \(R.\ sachalinensis\).

2.6.1 Optimizing scale-up fermentation processes

Optimization of the fermentation process takes place once the feasibility of the production in the selected organism has been demonstrated. Before starting long and expensive optimisation work, it is important that the stability of the strain is established, at least for the number of generations necessary for cell banking and largest-scale fermentation, including the pre-cultures. The main aim of optimisation is to maximise the production, so this process can be initiated only once a laboratory-scale purification process and a minimum set of quality control tools are available to quantify and assess the quality of the product (Thiry and Cingolani, 2002).

The factors affected by scale are the number of generations, the mutation probability, medium sterilization, the quality of temperature and pH regulations, agitation, aeration and pressure. The best way to prepare the scaling-up of a process is to first scale-down to the pilot scale of the conditions of culture that will be used at the final scale of production (Kwanmin, 1989). Then, when the scale is increased, the broth will become more and more heterogeneous. In large fermentors, oxygen can be depleted in some area of the reactor (Enfors et al., 2001). Even though there is a trend to standardize as much as possible of the fermentation processes, it has been shown that even subtle changes would powerfully increase the productivity
like, adjunction of a cofactor or substrate can stabilize an enzyme and increase the final productivity (Thiry and Cingolani, 2002).