Chapter – I
GENERAL INTRODUCTION

Throughout the ages the humans relied on nature for their basic needs for the production of foodstuff, shelter, clothing, medicines, means of transportation, fertilizers, flavors and fragrances. Plants have formed the basis of sophisticated traditional medicine system that has been in existence for thousands of years. Herbal medicine system is still the mainstay of about three quarter of the world's population which relies upon traditional remedies for their health care (Collin, 1987).

Plant materials remain as an important resource to combat serious diseases in the world. The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in developing countries. The medicinal value of these plants lies in some chemically active substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds (Edeoga et al., 2005).

In recent years there has been renewed interest in natural medicines that are obtained from plant extracts. Nearly 40% or more of the pharmaceuticals currently used in western countries are derived or at least partially derived from natural sources (Rout et al., 2000).

Use of plant based drugs and chemicals for curing various ailments and personal adornment is as old as human civilization. Plants and plant based medicaments are the basis of many of the modern pharmaceuticals we use today for our various ailments (Abraham, 1981).

Many plants play a dominant role in the introduction of therapeutic agents. Even now, drugs from plants occupy an important niche in allopathic system of medicine. Medicinal plants have their values in the active principles present in various plant tissues, which produce specific physiological action on the human body.

The use of medicinal herbs in United States which was quite common in the 19th century and early 20th century, declined with increased knowledge of the germ theory of disease and the increased availability of synthetic drugs. The recent resurgence in the use of medicinal herbs is speculated to be due to some
disillusionment with conventional medicine and its increased costs as well as changes in the health care delivery system that make them more impersonal.

Due to unsustainable exploitation of eco-resources, several plant and tree species of great medicinal importance have become threatened and may extinct without proper planning for their conservation and multiplication.

There are approximately 1250 Indian medicinal plants that are used in formulating therapeutic preparations according to Ayurvedic or other traditions. Several of these plants came under the contemporary scientific scrutiny since the middle of 19th century.

The flora of India is very rich in plant diversity with an estimated 50,000 species, of which about 15,000 are flowering plants, of these 5,000 species are endemic to India, while several hundred species are threatened.

Due to increasing use of medicinal plants, their future is being threatened. Reserves of herbs and stocks of medicinal plants in developing countries are diminishing and in danger of extinction as a result of growing trade demands for cheaper health care products and new plant-based therapeutic markets in preference to more expensive target specific drugs and biopharmaceuticals.

To cope up with this alarming situation, advanced biotechnological methods of culturing plant cells and tissue provide alternative means for rapid propagation and conservation of rare and endangered and/or commercially important medicinal plants and provide source for extraction of secondary metabolites.

The technique of plant tissue culture has been widely applied in the rapid propagation of higher plants.

Tissue culture is a technique for in vitro regeneration of plants, especially the economically valuable plant or plants that are difficult to propagate in natural environment. This technique is gaining importance towards their utilization for practical application to agriculture, horticulture and other kinds of modern agro-industries. These include plant propagation via embryogenesis, micro propagation, seed germination, regeneration from callus, shoot bud initiation, root formation and embryo rescue (Evans et al., 1981a, 1981b).

Tissue culture that began as an intellectual curiosity has presently attained respectable status because of its significant contribution in the field of biotechnology.

There are also intricacies involved in micro propagating the medicinal plants and have been periodically elaborated by Murashige (1978), Evans et al., (1981a,
These reports and several others establish the efficiency of the *in vitro* techniques that can be utilized profitably for regenerating and also of temporal monitoring of metabolites in plants. Recently, there are various reports which indicate the use of different explants for callus initiation, maintenance and regeneration. Haberlandt, as early as in 1902, demonstrated the totipotency of plant cells using excised leaf segments. Later, in *Torenia fournieri* (Bajaj, 1972), *Lycopersicon esculentum* (Padmanabhan *et al*., 1974) regenerated shoot and root initials from leaf derived calli. The complete regeneration of plant from leaf derived calli was achieved by De Greef and Jacobs (1979) in *Beta vulgaris*. Successful *in vitro* regeneration of medicinal plants could be made possible through the use of varied explants such as leaf and stem segments, shoot buds, hypocotyls, cotyledons, roots, anthers and seedlings.

Plant tissue culture is being used globally for the ex situ conservation of plants. The endeavor is to adopt the method to multiply the medicinal herbs and monitor their secondary metabolites. The application of plant cell, tissue and organ culture has proved its potential for the improvement of threatened medicinal plants [Tiwari *et al*., 1998; Remashree *et al*., 1997; Satish *et al*., 2003 and Rajashekar *et al*., 2006]. The regeneration of whole plants through tissue culture is popularly called "micropropagation", by which a large number of plant species can be propagated all round the year and the plant breeder is no longer restricted by season in the production of large number of plants. The plants synthesize various medicinally important compounds such as alkaloids, glycosides, steroids, flavonoids etc. Similarly, *in vitro* derived calli can also synthesize these compounds. Hence *in vitro* culture is used as an alternative to whole plants for the production of useful secondary metabolites (Ammirato, 1987).

Somatic cells and tissues may lead to organogenesis depending upon the concentration of growth regulators. Several different organs, tissues and cells of plant form an integrated system in the development of a whole plant expressed by the subtle interactions among the different groups of hormones determining the morphogenetic pathways.

Skoog and Miller (1957) demonstrated that a low level of auxin and high level of cytokinin induces bud formation while the reverse proportion stimulates root initiation in tobacco pith callus. Observations on auxin-cytokinin mediated
organogenesis have been described in *Asparagus* (Harada, 1973) and *Petunia* (Rao and Harada, 1974).

Axillary shoots can grow in the absence of exogenous cytokinin as in potato (Hussey and Stracy, 1981). Formation of axillary shoots from nodal segments was reported in *Rauwolfia serpentina* (Tiwari *et al.*, 1998). Formation of adventitious shoots from internodal explants was reported in *Adenophora triphylla*, an important medicinal plant (Chen *et al.*, 2008). Direct multiple shoots were developed within six weeks from nodal explants in *Trifolium resupinatum* (Uranbey *et al.*, 2005). *In vitro* multiplication from nodal explants was achieved in *Paedaria foetida* (Srivastava and Srivastava, 2004) and *Psoralea corylifolia* (Faisal and Anis, 2006) Cytokinin was effective in inducing shoot buds and maintaining high rate of shoot multiplication in *Rauwolfia tetraphylla* (Faisal *et al.*, 2005a).

Regeneration of plantlets has been achieved from the stem culture either from direct organogenesis or through differentiation from the callus. Loo (1945) using meristem tips of *Asparagus racemosus*, could be able to induce callus proliferation. Later, Sevenster and Karstens (1955) in *Helianthus tuberosus*, Morel (1964) in orchids; Subramanya *et al.*, (1968) in *Trigonella foenum-graceum* and *Vigna unguiculata*; Rao and Narayanaswamy (1972) in *Tylophora indica*, Chaturvedi (1975) in *Dioscorea floribunda* cultured the shoot meristem to regenerate the whole plants successfully.

Direct shoot regeneration from stem explants has been developed for six cultivars of *Antirrhinum majus* by Cui *et al.*, (2004), high frequency plant regeneration from stem derived calli in *Astragalus metilotoides*. Komalavalli and Rao (1997) reported 30-day-old *in vitro* seedling axillary node inducing shoots in *Gymnema elegans* on MS medium. The highest rate of shoot regeneration was reported in *Paracautleya bhatti* (Rai and Thoyajaksha, 2001); formation of multiple shoots from axillary buds within five weeks of culture on MS medium in *Spilanthes acmella* (Haw and Keng, 2003) and in *Ranunculus asiaticus* (Baruto and Debergh, 2004).


Only within the past decade many number of studies dealing with the aseptic culture of flower buds has begun. Hicks and Sussex (1981) cultured the excised young flower primordial of tobacco (*Nicotiana tabacum*) on nutrient medium supplemented with kinetin and successful in obtaining organogenesis. Likewise, Ganapathi *et al.*, (1999) yielded somatic embryogenesis and plant regeneration from male flower buds in five cultivars of banana (*Musa* spp.). Altamura *et al.*, (1986) worked on *in vitro* floral morphogenesis in a double haploid tobacco. Sudarshana and Shanthamma (1988) regenerated plant from inflorescence culture of *Boerhavia diffusa*. Raman and Greyson (1978) reported the differential sensitivities to plant growth regulators by cultured “SINGLE” and “DOUBLE” flower buds of *Nigella damascena*. Young *et al.*, (1987) directly propagated clones from flower buds of tomato spp. (*Lycopersicon peruvianum* and *L.esculentum*).

Regeneration through somatic embryogenesis is reported in many species where in the somatic embryoids showed many structural similarities to those of zygotic embryos and under appropriate conditions on a suitable medium they grew into complete plantlets by Rangaswamy (1986) in angiosperms, George and Sherrington (1987) in *Ocotea cathariensis* Mez. and many others.

Somatic embryoids may induce in two different ways viz., direct and indirect somatic embryogenesis (Rangaswamy 1986). Bhojwani and Razdan (2004), Liu *et al.*,...
(1983) and Stamp and Henshaw (1987) have reported direct embryogenesis. Indirect somatic embryogenesis has also been reported by Bhojwani and Razdan (2004), Cheng and Raghavan (1985) and Pederson (1986).


Direct somatic embryogenesis has been reported at the cut edges of leaf, petiole and stem explants of *Epipremnum aureum* by Zhang et al., (2005). Somatic embryos were also developed from mature leaves of *Tylophora indica* reported by Chandra shekar et al., (2006). Embryogenic calli were induced within eight weeks from leaf explants of *Pennisetum glaucum* when cultured on MS medium supplemented with BAP and Kn as reported by Arockiasamy (2006), Anuradha et al., (2006) in *Nothapodytes foetida*, Kim et al., (2007) reported somatic embryos differentiated directly from cotyledon explants of *Podophyllum* MS medium with NAA after eight weeks of culture.

The concept of production and utilization of synthetic seeds was first suggested by Murashige & Skoog in 1977. Synthetic or artificial seeds have been defined as somatic embryos engineered for use in the commercial propagation of plants (Gray, 1987). Artificial seed production is a potential technique for plant multiplication and preservation, especially as it has been considered to be promising for propagation of no seed producing plants, transgenic plants and other plants that need to keep superior traits by means of asexual production. Encapsulation of propagules that were produced *in vitro* could reduce the cost of micropropagation in plantlets for commercialization and final delivery. This technology may be of value in breeding programs and allows the propagation of many elite genotype-derived plants in a short time. This technology has also been employed for germplasm storage and exchange purposes as reported by Danso & Ford-Lloyd (2003).

Currently, systems of artificial seed production have progressed substantially in this area, the most advanced being in seedling under *ex vitro* or field conditions, obtaining high percentages of conversion to plants (Fujii et al., 1987). Development
of efficient *in vitro* techniques to ensure its safe conversion is therefore of paramount importance.

Different coating materials were tested such as hydrogel sodium alginate, polyethylene imine and chitosan (Fujii *et al*., 1987). Although, a variety of natural and synthetic polymers are available for encapsulation, sodium alginate is the most commonly used gel-matrix because of its easy gelling properties, non-toxicity and low cost. Different concentrations of sodium alginate ranging from 1.5% to 6% have been used for different systems. It was found that among the different concentrations tested, 3% sodium alginate & exposure to 100mM CaCl$_2$.2H$_2$O solution for 30 min. produced uniform optimal beads which were suitable for handling.

Synthetic seed technology offers an excellent scope for conservation of rare hybrids, elite genotypes and genetically engineered Pitchouli plants. This technology has gained considerable importance during the last four decades as a potential, viable and valuable system for *ex situ* conservation of commercially important plants as reported by Kavyashree *et al*., (2004). Encapsulation and storage of the buds at freezing temperatures offers a long term storage capacity, maximum space and maintenance besides low production costs, ease of storage and transport as additional advantages as reported by Ghosh and Sen (1994).

Encapsulation of somatic embryos to conserve germplasm *in vitro* has been reported and studied in many plant species such as cereals, vegetables, fruits, ornamentals and medicinal plants (Onay *et al*., 1996 and Castillo *et al*., 1998). Recently, *in vitro* shoot tips or axillary buds have been used instead of somatic embryos for encapsulation (Bapat *et al*., 1987, Sharma *et al*., 1994 and Adriani *et al*., 2000).

Dawson (1942) for the first time showed the biosynthetic potentialities of plant cell cultures. Tulecke and Nickel (1960), Klein (1960), Staba (1963), Butcher (1977) others have provided the knowledge occurring from classical researchers on the same subject. Many investigators have succeeded in producing the enhanced concentration of secondary metabolites through *in vitro* cultures. To name some of them Furuya *et al*., (1972) in *Papaver somniferum*, Tabata *et al*., (1972) in *Scopalia parviflora*, Khanna *et al*., (1976) in *Ephedra foliata*; Hall and Yeoman (1982) in *Catharanthus roseus*; Rau and Forkman (1986) in *Callistephus chinensis*; Yamamoto and Yamada (1986) in *Rauwolfia serpentina*; Kamada *et al*., (1986) in *Atropa belladona*; Hashimoto *et al*., (1986) in *Hyocyamus niger*; Ishimaru *et al*., (1991) in

The phytochemical investigation of medicinal plants with the aim of isolating and identifying some active substances requires a correct choice of plant material. As a large number of plants has not yet been investigated from both phytochemical and pharmacological point of view. Different criteria can be considered such as chemotaxonomic criteria, information from traditional medicine, field observation, endemeity and degree of investigation of the plant (Hostettmann et al., 2001).

Antimicrobial activity of plants particularly of medicinal, finds application in identifying the phytoalexins. Owing to the omnipresence of pathogens, man has discovered and synthesized numerous compounds for disease management. In vitro evaluation of plants for antimicrobial property is the first step towards achieving the goal for developing eco-friendly plant protection.

The antimicrobial nature of plants has been attributed to the wide variety of compounds they synthesize such as alkaloids, essential oils, phenolics, flavonoids, terpenoids etc. The screening of bioactive compounds has always been of great interest to scientists looking for new sources of drugs useful in the treatment of infectious diseases. In recent years numerous reports of antimicrobial screening are available (Khanum et al., 2000).

The antimicrobial activities are well documented in the literature. Plants which have been used as medicine over hundreds of years constitute an obvious choice for study. It is interesting to determine whether their traditional uses are supported by actual pharmacological effects or merely based on folklore.

Calli are good source of chromosomal variation. Callus is obtained from root, hypocotyls, leaves and other parts of regenerated plants. These organs are made of numerous cells which remain in different states of differentiation. Normally in vivo meristematic diploid cells undergo selective division for the growth of an organ. Therefore, the genomic constituent is heterogeneous in original explants. Callus tissue may get such genomic heterogeneity possibly due to non-selective induction of asynchronous division of both diploid and endoreduplicated cells.
With some exceptions, chromosomal instability and variation in nuclear DNA content are usually observed in dedifferentiated callus cultures. Cytological abnormalities like endoreduplication, non-disjunction and fragmentation of chromosomes and others have been found to be responsible for such variation under the promotive influence of chemical (nutrient media, growth regulators etc.) as well as physical culture conditions \textit{in vitro}.

A number of studies have confirmed a close relationship between the ploidy instability and the loss of totipotency of cultured cells. Since most of the genetic manipulation techniques rely on efficient and reproducible regeneration procedures from \textit{in vitro} cultured cells and tissues, a precise understanding of the variations in chromosome number, nuclear DNA content and ploidy level during the neoplastic progression of undifferentiated callus cells is of greater importance for studying the morpho-organogenic competence.

During the medicinal plants survey, we came to know that \textit{Gynandropris pentaphylla} DC. (fig.1.1) and \textit{Enicostemma littorale} Bl. (fig.1.2) were used and being used even today by the traditional practitioners to cure several disorders and diseases. Keeping this point of view in mind, we selected these two plants for our present research work. Moreover, the \textit{in vitro} propagation and also certain bioactivity studies have not been much attempted in these plants as per the literature. So the present investigation was envisaged.

\textbf{A brief note on the selected plants:}

\textit{Enicostemma littorale} Blume Family: Gentianaceae

It is a glabrous perennial herb, leaves opposite, decussate, sessile, elliptic, lanceolate, flowers white or bluish in whorled or axillary clusters, capsule ellipsoid, seeds many, globose. The whole plant is used for medicinal applications.

It occurs in all plains, districts and to 1,500 ft. in the hills, chiefly however near the sea, often on black cotton soil (Gamble, 1935).

The plant is pungent and very bitter, anthelmintic, cures fevers and "vata" (ayurveda). It is much used as a stomachic and also a laxative (tonic). It is crushed and applied locally in snakebite (Blatter). It is reported to be effective against malaria (Kirtikar and Basu, 1975).
Gynandropsis pentaphylla DC. Family: Capparidaceae (Syn: Cleome gynandra DC.)

It is an erect rather showy glandular pubescent annual herb. Leaves long stalked digitately 3-5 foliate, flowers white or purple in corymbose racemes, capsules long striated with reniform, rugose brown or black seeds, leaves, seeds and roots are used for medicinal applications.

It is common in waste places in all districts, in the plains and at low elevations (Gamble, 1935).

It is tested among cattle feed in Indonesia. The leaves are eaten as a pot herb and as flavouring agent in sauces and they are also pickled. The leaves and seeds are used in indigenous medicine in the same way as mustard. Bruised leaves are rubifacient & vesicant and used as counter-irritant in head ache, neuralgia, rheumatism and other local pains. The leaves are also applied to boils to prevent pus formation. The juice of the leaves, alone or mixed with oil is used to cure ear ache. The leaves are taken internally in certain bilious disorders. A decoction of the roots is reported to possess mild febrifugal properties. The seeds are anthelmintic and rubefacient (Kirtikar and Basu, 1975).

The major objectives of the present investigation are:

- Establishment of protocols for the in vitro cultures of Enicostemma littorale and Gynandropsis pentaphylla and the histological studies of callus cultures.
- Suspension culture, embryogenesis and synthetic seed production.
- Cytological variations of cultured cells.
- Preliminary phytochemical analysis and antimicrobial activity of extracts.
Fig. 1.1: Habit of *Enicostemma littorale* Blume.

Fig 1.2: Habit of *Gynandropsis pentaphylla* DC.