CHAPTER-8
Summary and Conclusion
Cancer is an insidious disease for which definite treatment is rarely predictable. According to the definition of International Union against Cancer, "Cancer is a disturbance of growth characterized primarily by an extensive proliferation of cells with out apparent relation to the physical demands of the organ involved". Chemotherapy, radiotherapy and surgery are the three major modalities of treatment now available against cancer. Systemic chemotherapy remains the primary method to treat this disease and there is dire need to increase the production of available drug and discovery of new clinically efficacious agents.

Plants are proven source of variety of pharmaceutically valuable chemicals. These phytochemicals are collectively called as secondary metabolites. Several such secondary metabolites are being clinically used as anticancer drugs. Moreover, certain phytochemicals (secondary metabolites) act as chemopreventive substances. One of the major problem facing these plant derived drugs is its availability to conduct *in vitro, in vivo* and clinical trials, because the level of these active compounds are low and the synthesis of these compounds depends on the growth rate of the plants. In addition, the accumulation pattern, content of these compounds are vulnerable to geographical, environmental conditions and genetic make up of the plants. Further, the complex steriospecificity of these compounds is a major obstacle for the chemical synthesis of these drugs. In this context, plant tissue culture technique is a substitute to overcome these problems for the genuine supply of specific compounds.
The present work is an attempt to study the anticancer activity of secondary metabolites from selected medicinal plants employing cell and tissue culture techniques.

One part of the study is devoted to the production of a known anticancer quinoline alkaloid Camptothecin (CPT) from *Notapodytes foetida* (Icacinace) and *Ervatamia beyneana* (Apocynaceae). The other part deals with the study of anticancer activity of a folklore medicinal plant *Emilia sonchifolia* (Asteraceae) and comparative evaluation of callus and plantlet cultures of this plant.

Wall and Wani initially isolated CPT in 1962 from a Chinese plant *Camptotheca acuminate*. CPT and its semisynthetic forms are used for the treatment of colon, head and neck tumors and breast cancers. Along with anticancer properties CPT also posses activity against HIV 1 (151). Production of CPT in callus and cell suspension cultures of *C. acuminate* has been reported by several authors (27,169,170). Later this important drug has isolated from certain south Indian plant species (125,126,127).

In our study the plant *N. foetida* (Whight) Sleummer collected from Nilgiri Hills found rich amount of CPT. The percentage of CPT in bark was 0.086, in seeds 0.05, and the mature leaves 0.001 which were relatively high when compared with reported values of Roja and Heble 1994 collected from Mahabeleswar. The content of secondary metabolites varies with geographical and genetic constitution of the plant species. The difference in the amount of CPT in these plants may due to these factors.
Tissue cultures studies of *N. foetida* showed the following results. Different explants were cultured in different media containing auxins and cytokinins. Only the immature embryo showed good response in Murashige and Skoog (MS) medium and modified MS medium (mMS) [supplemented with B3 vitamins], where as the other explants such as leaf disc and inter node did not responded positively even after 3 weeks of culture. 4 weeks old calli from embryo were subcultured in different hormone concentrations of auxins and cytokinins in MS and mMS medium. Comparatively good growth rate was observed in mMS containing NAA 2 and KN 0.5 mg/L. Growth of the callus after 90 days the callus was declined and morphological differentiation such as nodular protuberance and leafy out growth was noticed in these cultures. Qualitative analysis of these callus cultures showed presence of CPT. HPLC analysis of revealed that increased amount of CPT in 90 day old cultures. This result indicates the co-relation between the growth, morphogenetic differentiation and the biosynthesis of CPT. Similar observations also reported in CPT production in *Ophiophriza pumilia* by Kijatima et al 1997 (205), vinblastine production in *Catharanthus roseus* by Paar et al. (226) and Endo et al. 1987 (22).

Cytotoxicity of CPT isolated from the callus and standard CPT were evaluated. At a concentration of 0.002 μg/ml of both standard CPT and callus derived CPT inhibited the 50% cell proliferation in L-929 cells.

*E. hynamana* (Apocynaceae) is a tree species found to contain several anticancer alkaloids (202). The major contributor of this property is CPT and its 9-methoxy CPT. Quantitative analysis of in different plant parts
showed that the amount of CPT was 0.006 % in young shoots and 0.0001 % in the bark.

Among the different medium tried for callogenic response Murashige and Skoog medium was found to be the best. The primary explants such as internode, leaf disc and seed embryo responded positively. Maximum callus formation was noticed in the medium containing 2,4-D 2mg/l and KN 0.5 mg/l.

A slight increase in 2,4-D (2.5 mg) in seed embryo culture induces somatic embryogenesis. In a combination of NAA 2 and KN 0.5 mg/l produce plant lets, where as NAA 4 mg/l alone induce rhizogenesis.

Biological assay such as tumor cell cytotoxicity of is a sensitive method to scrutinize the anticancer principle present in cultured plant tissues. For example, HeLa-S3 cell lines were used to detect vinblastine produced by Catharanthus roseus by Miura and Okazaki 1989 (162). In our study, the extracts of callus and in vitro derived root showed cytotoxicity towards L-929 cells. However, the toxicity was high in invitro derived root extract than the callus extracts. Hence, we conducted the in vivo anti-tumor of the in vitro derived root extract in mice. The root extract significantly inhibits the solid tumor development in mice in a dose dependent manner.

Phytochemical analysis of the extracts showed the presence of CPT in in vitro derived root extract. No CPT was detected in callus tissues. This result supports the hypothesis that morphogenetic differentiation can
enhance the production of secondary metabolites, as we observed in \textit{N. foetida}.

The general conclusions to the first part of the work are following.

1. An appreciable amount of CPT can be isolated from \textit{N. foetida} when compared with \textit{E. beyneana}.

2. Tissue culture studies of these CPT yielding plants shows that ability to synthesis CPT in intact plants retains in these cultures.

3. Production of CPT in cultures of these two plants comes to a common conclusion that the morphogenetic differentiation increases the production of CPT compared with the callus tissues.

4. In \textit{E. beyneana} callus and root extract showed cytotoxicity towards the tumor cells, but the phytochemical analysis of the callus tissues failed to detect the presence of CPT. Cytotoxicity of callus tissues may be contributed by the other anticancer alkaloids.

The above results confirm that the production of secondary metabolites increase during the morphological differentiation. Further the production of CPT can be enhanced by new biotechnological interventions such as cell suspension cultures and agrobacterium mediated hairy root cultures. The possible precursor, feeding experiments could be another promising attempt to scale up the production of these interesting secondary metabolites.

The results of the second part of the thesis shows the medicinal properties of \textit{Emilia sonchifolia}. 
The balance of oxygen stress and oxygen tolerance is considered important for the maintenance of health in animals and plants. Over production of free radicals, viz. superoxides, hydroxyl radicals have been implicated in several disease processes including carcinogenesis. Antioxidant compounds taken as food, cosmetics or drugs can support self-defense system against the toxic effects of free radicals.

Alcoholic extract of *E. sonchifolia* was found to be a potent inhibitor of superoxide and hydroxyl radical generation. At a concentrations 3μg and 28 μg/ml of the extract, inhibited 50 % superoxide and hydroxyl radical generation *in vitro*. Intra peritoneal administration of the extract (250 mg and 500 mg/kg. b.wt) in Swiss albino mice significantly inhibited carageenan induced paw edema. Nevertheless, the inhibition was not in a dose dependent manner.

Purified fraction (M-60) of the extract significantly inhibited superoxide and hydroxyl radical generation. The M-60 fraction also inhibited lipid peroxidation in rat liver homogenate. More over the same fraction (250 μg, 500 μg and100 μg/animal) inhibited skin tumorogenesis in mice induced by DMBA/croton oil. The inhibition was in dose dependent manner.

The alcoholic extract of *E. sonchifolia* found to be cytotoxic to DLA, EAC and L-929 tumor cells. At a concentration 1.5 mg and 1.2 mg caused 50 % cell death in DLA and EAC cell lines respectively. Whereas in long chemosensitivity assay on L-929 cells 15 μg/ml of the extract inhibited the
50 % cell proliferation. More over the extract dose dependently inhibited DNA synthesis as judged from the thymidine incorporation assay.

Oral administration of the extract (100 mg/kg.b.wt) significantly increased the life span of ascites tumor bearing animals. The increase in life span was 241 % in animals, which received the drug 24h after the tumor challenge. The same mode and same dose of the extract also significantly inhibited the solid tumor development in mice. The volume of tumor was 1.25 cm³ on 30th day.

In tissue culture of *E. sonchifolia* the primary explants such as leaf disc, petiole, internode and shoot tips responded positively in MS medium. Callogenic response was observed in all explants in the medium contains 2,4-D 2mg/ l and KN 0.5 mg/ l, whereas NAA/BA combination induced shoot induction was noticed in all explants.

Antioxidant and anticancer activity of the callus and plantlet cultures of the *E. sonchifolia* showed that plantlets possess high antioxidant and anticancer activity than the callus tissues.

The general conclusion of the second part of the work is following.

1. *Emilia sonchifolia* seasonal folklore medicinal plant found to possess antioxidant, anti-inflammatory, anti-carcinogenic, anti-tumor properties.
2. Preliminary phytochemical analysis indicated that the purified fraction is flavonoids.
3. *Emilia sonchifolia* is a seasonal plant. The plant tissue culture utilized its availability throughout the year. Moreover, the biological activity of the *in vitro* grown plantlets retains similar properties.

The overall results suggest that the medicinal plant tissue culture is a viable method for the production of pharmaceutically valuable compounds.