APPENDIX V: Synopsis of the thesis submitted 6 Months before final submission of the thesis
INTRODUCTION

Diabetes mellitus is one of the oldest diseases known to mankind. Clinically it is a condition characterized by increased blood glucose level (Hyperglycaemia) due to insufficient or inefficient insulin. Diabetes is the 3rd leading cause of death. Diabetes mellitus is broadly divided into two groups namely Insulin Dependent Diabetes Mellitus (IDDM) and Non-Insulin Dependent Diabetes mellitus (NIDDM). Medical science cannot claim that it knows all that needs to be known about this disease. This is the main reason for the persistent interest all over the world to explore alternative remedies from the so called alternative system of medicine.

Interest in traditional drugs is not new but has been spurred in recent years by methodological advances in phytochemistry, growing number of ethno-botanical studies, and an upsurge of interest in renewable resources and traditional medicine. Plants have always been an exemplary source of diabetes drugs (Grover et al.; 2002). The public interest and awareness of natural medicines have led the researchers to pay more attention to medicinal plants (Day, 1998).

Clerodendron phlomidis L., Bougainvillea spectabilis rosea L., Tabernaemontana coronaria L. and Enicostema littorale Blume were studied in the present study.

Clerodendron phlomidis L. (Arni) is used in diabetes, gonorrhea, measles etc. This plant has aromatic, astringent, demulcent, anti-convulsion, anti-diarrheal activities. In India parts of the plant are used in post-natal conditions in women and in gastrointestinal disorders. The roots are employed as an appetite stimulant (Kirtikar, K.R. and Basu, B.D. 1933; Sheba Rani et al., 1999).

Tabernaemontana coronaria L.(Chandani) is used as vermicide, in relief of toothache and removal of opacities of the cornea and other eye diseases. Milky juice of leaves is used for curing of ophthalmia, inflammation and wounds. Flower juice relieves the burning sensation of sore eyes and used in skin diseases. It is also used as antimicrobial, antiamoebic and antiviral drug (Beek et al., 1984).


Enicostema littorale Blume (Mamejavo) is used in the treatment of diabetes and malarial fever. It has anti-malarial, anti-bacterial, anti-pyretic and anti-oxidant activities and also has hepatotoxicity (Nadkarni, A. K.; 1954, Murali, et al.; 2002, Gopal et al, 2004; Upadhyay et al., 2004).

There is no doubt that plants play a dominant role in the introduction of new therapeutic agents, and also drugs from the higher plants continue to occupy an important position in modern medicine (Dev, 1997) because compounds used in today’s medicine have a complex structure, and synthesizing these bioactive compounds chemically at a low price is not easy (Shimomura et al., 1997). Callus cultures are large aggregates of
undifferentiated plant cells. Plant tissue culture technique approach has been found to be advantageous as it provides as continuous and reliable source of natural product year round without the destruction of the entire plant. High quality and desired compounds can be obtained through cell line selection and or addition of the precursor into the production medium. The state of undifferentiated growth is maintained by the precarious phytohormone balance, mainly the auxin and cytokinins added to the medium. Manipulation of the auxin to cytokinin ratio leads to the development of shoots, roots or somatic embryos from which whole plant can be produced. Callus culture can also be used to initiate cell suspension which is used in variety of ways in plant regeneration studies. Callus tissue also has proved suitable material for secondary metabolite extraction. Murashige and Skoog's (1962) media is widely accepted for growing different cultures.

Chemical reactions taking place in plant cells, notably reactions of degradation of food substances which provide the energy required by plant cells and biosynthesis reactions leading to the formation of compounds needed by the cells. As a result of these metabolic reactions various products are formed, out of which some products are further needed in growth (e.g. amino acids, proteins, carbohydrates, lipids, vitamins, nucleotides etc.). The products which are synthesized by metabolic reactions and are necessary for the growth and development of cell are called primary metabolites. Plants produce many other compounds, which are not required for normal growth and development by the metabolic pathways common to all plants are referred to as secondary metabolites, which are medicinally important (e.g. alkaloids, flavonoids, steroids, tannins lignin, antibiotics, coumarins, resins, cardiac glycosides etc). These active substances are present in storage organs of plants as roots, leaves, bark, seeds etc. Higher plants produce a great variety of secondary products which play a minor role in the basic life processes of the plant but often have and ecological role, such as attractant of pollinators and chemical defence against microorganisms, insects and higher predators. Some of the plants are rich in secondary metabolites which are potential source of drug and essential oils. Biosynthesis of metabolites although controlled genetically is affected strongly by environmental influence. Excellent reviews on these constituents have been published by Harbone J.B. (1980); Hardman, R.E. (1980); Khanna P. (1984) and Staba, E.J. (1985).

The production of useful compounds by plant cell cultures has become increasingly significant in the field of biotechnology. There are two important problems that have to be overcome for in vitro production of useful compounds. These are the selection of specific cells that produce high amounts of the desired compounds harvested at right time when the number of compound and amount is maximum. The successful selection of cells producing high amounts of secondary metabolites has been made possible because of the heterogeneity associated with cultured plant cells. It has therefore been possible to select cells and develop cell line with desirable characteristics. For example high vitamin producing cells (Matsumoto et al.1980; Yamada and Watanabe, 1980; Watanabe and Yamada, 1982) high alkaloid producing cells (Zenk et al., 1977; Ogino et al., 1978; Yamada and Hashimoto, 1982) have each been obtained in various plant species.
Alkaloids are compounds known for their potent pharmacological activities. They are important bioactive compounds and are freely present in plants. They are all nitrogen heterocyclic which occur mainly in plants as their salts of common carboxylic acids such as citric, lactic, oxalic, acetic, malic and tartaric acids as well as fumaric, benzoic, aconitic and veratric acids. Their amine character produce an alkaline solution in water and hence the origin of their name - alkaloids. There is a wide variety of structural types of alkaloid e.g. monocyclic, dicyclic, tricyclic, tetracyclic etc. as well as cage structures (Schultes et al. 1979; Gallagher et al., 1995)

Flavonoids constitute one of the most characteristic classes of compounds in higher plants. Many flavonoids are easily recognized as flower pigments in most plants. However, their occurrence is not restricted to flowers but include all parts of the plant. The chemical structure of flavonoids are based on a C15 skeleton with a chromane ring bearing a second aromatic ring B in position 2, 3 or 4 (Shimkim et all 1936; Corbett 1974). There are 14 major classes which are usually determined by the oxidation patterns of the C-ring only 8 flavonoids aglycones are widely distributed and common over 60% of all plants sampled had quercetin, kaempferol, or myricetin but they are often components of glycosides.

Steroids are pharmacologically important for human life. According to Staba (1980), steroids are divided into four types (1) sterols (2) sterolin (3) steroidal saponins (4) nitrogen containing steroidal saponins. Saponins may be either triterpenoid glycoside or glucoside of steroids with spiroketal side chain.

The comparative study of secondary metabolites content and their properties were investigated at different stages *in vivo* and *in vitro*. The secondary metabolites (Alkaloids, flavonoids and steroids were identified and compared at various stages of production and also grown on different media with combination of different PGRs to produce maximum number as well as maximum amount of secondary metabolites with the help of Spot Tests, Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) and other various analytical methods.

Hence with the help of Plant Tissue Culture technique the suitable media were identified which took short time for mass production of callus, secondary metabolites as well as regeneration of plantlets, organogenesis for their medicinal values. Thus the studies were undertaken with the following objectives:

**OBJECTIVES:**

1. To develop protocol for the establishment of explant for production of callus and plantlets.

2. To optimized growth for the production of secondary metabolites.

3. To evaluate the biochemical profile of *in vivo* and *in vitro* produced materials.
4. (a) To compare *in vivo* and *in vitro* produced bio-active substances through various analytical methods.

4. (b) To conclude whether *in vitro* produced material is equally potent or superior to *in vivo* material for production of phytoactive substances.

**EXPERIMENTAL MATERIALS, METHODS AND RESULTS**

**A. TISSUE CULTURE STUDIES**
Murashige and Skoog’s (MS)(1962) media was used as a basal nutritive medium. It was used as basal or hormonal medium supplemented with PGRs like 2, 4-D, IAA, IBA, NAA, BAP and kinetin. Various plant parts were taken as explant e.g. leaf, stem, node, internodes, buds, etc. Results were obtained according to different explant on different media supplemented with combination of various levels of auxins and cytokinins.

**B. BIOCHEMICAL STUDIES**
1) Reducing and non reducing sugars (Nelson N., 1944)
2) Total proteins (Lowry et al., 1951)
3) Starch (Chinoy J.J., 1939)
4) Total phenols (Bray H.G. and Thorpe W.V., 1954)
5) Free Amino acids (Lee Y.P. and Takahashi N., 1966)

**C. ENZYME ACTIVITIES**
1) Peroxidase (George, P., 1953)
2) IAA-Oxidase (Mahadevan, S., 1964)
3) Invertase (Hatch M.D. and Glasziou, K. T., 1963)
4) Protease (Cruz et al., 1970)
5) Amylase (Summer J.B. and Howell S.F., 1935)
6) Polyphenol Oxidase (Kar, M. nd Mishra, D., 1976)
7) Catalase (Chance B. and Maehly A.C., 1955)
8) Enzyme protein ((Lowry et al., 1951)

**D. PHYTOCHEMICAL STUDIES**
1) **SPOT TEST ANALYSIS**
   Spot test provides qualitative results in short time for the presence of various substances (Peach K. and Tracey M.V. 1956; Daniel M., 1991)

2) **TLC (Stahl E., 1969)**
   Chromatographic analysis was carried out with different plant material for comparison between *in vivo* and *in vitro* produces substances at different levels using spraying reagents as well as through iodine as detecting gas and under UV light.
   - Alkaloids (Constabel et al., 1981)
   - Steroids (Tomita et al., 1970)
   - Flavonoids (Subramanian S.S. and Nagarjan S., 1969)
3) HPTLC (Sethi P.D., 1969)
It was carried out from in vivo and in vitro plant materials at Anchrom laboratory, Bombay. Phytoactive substance present in the pure alkaloids, flavonoids and steroids extracts of in vivo and in vitro were separated and analysed at different wavelengths i.e. 200, 254, 260 nm as well as absorption spectra of individual substance was obtained from it for identification of substances.

OBSERVATIONS

Important observations from the above studies are as follows:

1. Leaves of C. phlomidis responded to range of different concentrations of Kin and 2, 4-D. Variation in the appearance of callus was negligible. The callus obtained was pale green and friable. Maximum G I value - 24.28 was obtained from the callus grown on medium containing 6mg/l kin and 6mg/l 2, 4-D. NAA at different concentrations was lethal.

2. Callus of T. coronaria was obtained on medium with lower concentrations of Kinetin and auxin i.e. 1- 2 mg/l Kin and 2, 4-D each. The callus obtained was green, compact, shiny and sticky. The callus at higher concentrations i.e. 3-6 mg/l Kin and 2, 4-D each, was white and friable. Growth of callus was observed within 2-3 days after inoculation. The callus with 1mg/l Kin and 1mg/l 2, 4-D gave the highest GI value -6.28.

3. Callus of B. spectabillis was reddish brown and friable. Lower concentrations of Kintin and auxin did not result in the callus formation. Callus was obtained on medium containing 4-6 mg/l Kin and 2, 4-D each. However best results were obtained with 4 mg/l concentrations of each. Callus obtained on this medium gave maximum G I valve – 19.88

4. Callus of E. littorale responded to range of different concentrations of Kin and 2, 4-D. However the variation in the appearance with different concentration was clearly noticed. Callus at lower concentrations of hormones was pale yellow and compact and that at higher concentrations was green and compact. Callus could also be obtained on the basal MS without hormones but the time taken to start the callus growth was more than a month. Maximum G I value -26.8 was obtained with medium containing 1mg/l kin and 1mg/l 2, 4-D.

5. Biochemical analysis of in vivo and in vitro (2, 4, 6 and week callus) of C.phlomidis showed that protein, sugar and amino acid were notable higher in leaf while starch and phenols were higher in callus. Enzyme activities like peroxidase, invertase, protease and polyphenol oxidase were higher in cytoplasmic as well wall- bound fraction of callus whereas amylase, enzyme protein, IAA oxidase and catalase were higher in the cytoplasmic fraction of the leaf.
6. Biochemical analysis of in vivo and in vitro (2, 4, 6 and week callus) of T. coronaria showed negligible variations in the amount of protein, starch and amino acid while sugar and phenols were higher in leaf. Estimation of enzyme activities like peroxidase, invertase, protease, total amylase and polyphenol oxidase were higher in cytoplasmic as well wall-bound fraction of callus whereas IAA oxidase. Enzyme protein and catalase were lower in the callus.

7. Biochemical analysis of in vivo and in vitro (2, 4, 6 and week callus) of B. spectabilis showed that protein and sugar were higher in leaf whereas amino acids, starch and phenols were higher in callus. Enzyme estimation showed that peroxidase, invertase, enzyme protein and IAA oxidase were higher in cytoplasmic as well wall-bound fraction of callus whereas amylase, polyphenol oxidase and catalase did not showed much variations.

8. Biochemical analysis of in vivo and in vitro (2, 4, 6 and week callus) of E. littorale showed that protein starch and sugar were higher in leaf whereas amino acids and phenols were higher in callus. Enzyme estimation showed that peroxidase, invertase, enzyme protein, polyphenol oxidase and were higher in cytoplasmic as well wall-bound fraction of callus whereas amylase, IAA oxidase and catalase did not showed much variations.

9. Phytochemical analysis of alkaloids through different chromatographic techniques showed that numbers of alkaloids were more in 2 week old callus in comparison to its leaves in C.phlomidis. 6 week old callus of T. coronaria showed higher amount of alkaloids than its leaves. In B. spectabilis the number of alkaloids in 8 week old callus were double than that of its leaf. While callus of E. littorale showed less amount of alkaloids in comparison to on in vivo material.

10. Phytochemical analysis of flavonoids through different chromatographic techniques showed that numbers of Flavonoids were more in 8 week old callus in comparison to its leaves in C.phlomidis. 8 week old callus of T. coronaria showed higher amount of flavonoids than its leaves. In B. spectabilis the number of alkaloids was similar in callus as well as its leaves. While 4 week old callus of E. littorale showed more higher amount of flavonoids in comparison to on in vivo material.

11. Phytochemical analysis of steroids through different chromatographic techniques showed that numbers of steroid increases gradually with increase in the age of callus and maximum amount was noted in 8 week old callus in comparison to its leaves in C.phlomidis. No significant difference was noted among steroidal content in both the conditions in T. coronaria. In B. spectabilis the number of steroids was higher in 8 week old callus. While 8 week old callus of E. littorale showed more higher amount of steroids in comparison to its in vivo material.
SALIENT FEATURES

Important conclusions from the above studies are as follows:

1. Morphological variations are observed in callus grown on media supplemented with different combinations of PGRs and notable variation observed in *Enicostema littorale* and *Tabernaemontana coronaria*.

2. *Clerodendron phlomidis* L. and *Tabernaemontana coronaria* L. gives quick response in callus regeneration and callus starts growing within week.

3. Steroids are produced maximum in *in vitro* material in comparison to *in vivo* material.

4. Biochemical activities are good marker in terms of primary metabolites and enzyme activities.

5. Spot tests, TLC and HPTLC are better techniques for quick identification and analysis of secondary metabolites like alkaloids, flavonoids and steroids of *in vivo* and *in vitro* grown plant materials.

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