Chapter

SUMMARY & CONCLUSION
Diabetes, aptly called the ‘silent killer’, has claimed a huge number of lives the world over, as its symptoms are neglected. Being a metabolic disease with heterogeneity, there is need for appropriate therapy. Apart from the conventional oral hypoglycemic drugs and insulin, traditional plant medicines are being used throughout the world for not only treating diabetes but also its associated complications.

Out of 25 million species of plants worldwide, only 20,000 have so far been listed as being of known medicinal value and nearly 80% of the world’s population use plants as drugs. Botanical drugs are fully accepted and widely prescribed in China, Japan, India and other Asian and African countries. Some botanical drugs are under clinical development in the USA. Due to the gargantuan market for medicinal plant based drugs and the increasing anxiety among scientists to explore the enigmatic potential of phytochemicals in disease prevention and treatment, much of the research focuses on medicinal plants.

In India, Ayurveda has been using these botanicals for various ailments including diabetes. India has the highest number of persons with diabetes in the world with about 19 million clinically diagnosed people. Treatments of diabetes with conventional oral hypoglycemic agents, which are derivatives of sulfonylurea, have a limited effect. There are evidences indicating the long-term harmful side effects, the major side effects being hypoglycemia, allergic skin reactions, headache, fatigue, indigestion, nausea and vomiting and liver damage. In comparison the botanical medicines are known to have no or fewer side effects making their studies imperative for the treatment of diabetes.

In view of the increasing inclination and acceptance of herbal drugs and products by the people as well as practitioners for the treatment of various ailments, and the alarming rise in the number of diabetics, our study involves a search for various plants having hypoglycemic activity.

**The thesis has been divided into following chapters:**

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B) Phytochemical studies
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Objectives of our study

This study has been taken up:

- To selectively study plants used as an alternative medicine for treatment of diabetes with the view to ascertaining their hypoglycemic activity. One of the plants was deliberately selected such that its hypoglycemic activity was reported and promoted so that it could serve as a reference plant and make a comparative study was possible.
- To check the capability of the selected plants to reduce blood sugar in experimental diabetic rats.
• To standardize protocols for the micropropagation of the plants having significant hypoglycemic activity with the view of ascertaining means of reducing depletion of source material and enhancing their hypoglycemic potential.

• To evaluate regenerated plants in vitro or callus culture for enhanced anti-diabetic activity.

• To initiate cell suspension studies of the plant exhibiting maximum hypoglycemic activity with the aim of inducing production of the bioactive principle in vitro.

• Standardizing culture conditions for optimizing secondary metabolite production in vitro for setting up of possible bioreactors for commercial scale-up of the product.

A. Elucidation of Hypoglycemic Potential in Gymnema sylvestre R.Br, Catharanthus roseus L., Taberneamontana divaricata and Ocimum basilicum var Thai Queen

Oral Glucose Tolerance tests were carried out to verify the diabetic state of male, adult, Charles Foster strain albino rats (200-250gms), that were administered alloxan subcutaneously (150mg/kg body weight) in three injections at 48-hour intervals, after overnight fasting.

The animals were divided into normal and diabetic controls and experimental rats treated with the plant extracts. For the treatment, 10% extracts of fresh leaves and dried leaves were prepared. The dried leaves were extracted with different organic solvents like petroleum ether, chloroform, benzene and ethanol. These fractions were checked for hypoglycemic activity. The extracts were administered orally (0.5ml/oral, 1.0ml/oral and 2.0ml/oral) once a day for three weeks. The animals fasted for 18hrs were checked for hypoglycemic activity.

OGTT was carried out to find out the effect of the treatment. Cultures of Gymnema sylvestre R.Br. and C. roseus grown in vitro were checked for hypoglycemic activity. The experimental rats were monitored for their general disposition and body weight.
Findings are summarized:

1. Of the four plants selected *Gymnema sylvestre* R. Br. aqueous extracts showed a −40–45 % lowering of blood glucose followed by aqueous extracts of *Catharanthus roseus* L. (~18 – 20%) while the least was found with *Ocimum basilicum var Thai Queen* (~10 – 12%). *Tabernaemontana divaricata* did not exhibit any hypoglycemic activity, but the blood glucose level did not increase during treatment. Hence it may be considered that it has anti-hyperglycemic effect.

2. The extracts using the dried leaf powders of these plants had less hypoglycemic potential than the extracts of fresh leaves of these plants. Thus, on drying some of the activity was lost.

3. The ethanol extract of *G. sylvestre* had a higher potential for lowering blood glucose than the aqueous extract. The ethanol extract showed dose-dependent lowering of blood glucose. Saponins are known to be extracted with ethanol/methanol and water and hence, the ethanol extract contained saponins, which are the reported active principles that lower the blood glucose levels. These experimental rats treated with *G. sylvestre* lost body weight and showed much better physical activity than the untreated diabetic rats.

4. The *in vitro* callus culture extract of *G. sylvestre* R. Br. also showed glycemic activity (~ 3-5%). These results therefore indicated that the cells in the callus were able to synthesize saponins responsible for the anti-sweet property of *G. sylvestre* R. Br. Thus, to induce production of *Gymnema* saponins *in vitro*, plant cell culture studies became the main focus in this study.

5. *C. roseus* with white flowers showed higher hypoglycemic potential than the other variety with pink flowers. The lowering of blood glucose by *C. roseus* was also dose-dependent. The *in vitro* grown shoot cultures of *C. roseus* also exhibited hypoglycemic potential. The rats treated with these extracts however were less active (lethargic) and lost weight.

6. The chloroform and benzene extracts of *C. roseus* showed lowering of blood glucose in the experimental rats, which was however less than the hypoglycemic activity exhibited by the aqueous extract. Thus, alkaloids
may have the bioactive principle as these phytochemicals get extracted with chloroform and benzene.

7. *C. roseus* extracts had hypoglycemic activity in both normal rats and diabetic rats, while *G. sylvestre* and *O. basilicum var Thai Queen* extracts did not lower blood glucose levels in normal rats.

8. *O. basilicum var Thai Queen* apart from its aqueous extract did not show hypoglycemic potential in the other extracts. The bioactive principles may have got denatured during the process of extraction.

B. Plant tissue culture studies of *Gymnema sylvestre R. Br*, *Catharanthus roseus L. and Ocimum basilicum var Thai Queen*

Tissue culture studies of all the four plants were initiated simultaneously with the elucidation of their hypoglycemic activity. Studies with *T. divaricata* were however discontinued, as the plant did not exhibit hypoglycemic potential.

Various explants (Nodes, internodes and leaves etc) were inoculated in nutrient media (modifications of Murashige and Skoog’s media) fortified with various hormones like IAA, NAA, IBA, 2,4-D, BA and Kinetin singly and in combinations. Certain media constituents were also modified along with the plant growth regulators. The carbon source concentration (Sucrose) was also varied.

Findings are summarized:

1. *Gymnema sylvestre R.Br.*
   i) The nodes and leaves were found to be better explants than the internodes, shoot tips and axillary buds. Of the two, the nodes showed a better percent explant establishment than leaves.

   ii) About 50–70% of the cultures showed bacterial growth in a week’s time. Fungal contamination was remarkable during the rainy season. Thus, several surface sterilization procedures were tried. The combination treatments of 30s 70% Ethanol + 20% NaOCl (20') + Teepol (20') + 0.1% Bavistin (10-20') + 0.1% HgCl₂ (10') and Running Tap Water (1 hr) + 50% Ethanol (1') + 0.1% HgCl₂ with 0.1% SDS (4-5') was found to be effective.
It was found that the plant had endogenous bacterial contamination. The isolated Gram-negative bacteria showed maximum sensitivity to Ampicillin/Sulbactam and the Gram-positive bacteria were sensitive to both Ampicillin/Sulbactam (20µg) and Cefotaxime (30µg).

iii) Callus was initiated in all the different trials with various hormones individually and in combination. In media containing only auxins, the callus initiation was faster than in media containing only cytokinins.

iv) The rate of proliferation of callus was best in MS (modified) media containing an auxin to cytokinin ratio of 1.

v) An increase in temperature and extended duration of subculturing led to browning of the callus cultures. Thus, maintenance of callus cultures needed controlled temperature conditions as well as frequent subcultures.

vi) MS (modified) media containing cytokinins singly and in combination with any auxin led to initiation of callus, which was initially off white and friable and later turned chlorogenic and nodular. This was an indication of organogenic development.

vii) Microscopic studies of greenish, nodular callus growing in MS (modified) media fortified with NAA and BA showed structures similar to adventitious shoot buds, which however did not differentiate into shoots.

viii) Callus initiated in high concentrations of auxins, when transferred to media containing no auxins or very low amount of auxins and cytokinin turned highly nodular, which could be possible proembryonic clusters. These callus cultures when transferred to media formulations prescribed for somatic embryo formation did not differentiate into somatic embryos.

ix) Increased sucrose concentrations, addition of reduced nitrogen (Glutamine, Tyrosine), addition of activated charcoal and ascorbic acid, increased nitrate concentration, alternative carbon...
source (glucose) etc. in the somatic embryo induction media were not successful in inducing formation of somatic embryos. This could be because of possible presence of high endogenous levels of auxins that affected both formation of adventitious shoots and somatic embryos.

x) Axillary bud formation and subsequent bud break was seen in our trials with media containing MS inorganic constituents and B5 organic constituents fortified with high concentration of BA and low concentration of NAA. The shoots however fell off from the explants instead of sustaining and growing. Callus initiation was also observed in this media after a period of two to three weeks.

2. *Catharanthus roseus L.*

i) Direct shooting (1-2 shoots per explant) was obtained from nodes in MS (modified) media fortified with 4.52 and 10.95μM 2,4-D. The shoots were slender, elongated with small sized leaves. Shoots did not proliferate or multiply on subculturing.

ii) Callus was initiated in both leaves and nodes in media fortified with 2,4-D and NAA. The callus was non-organogenic, brownish and soft.

iii) In MS (modified) media containing BA, axillary shoots were formed with healthy and large sized leaves and multiplied on further subculturing, although the multiplication rate was slow.

iv) The percent explant establishment for shooting from nodes was better in media containing BA and Kinetin than in media containing 2,4-D. The best results were with nodes in media containing BA as mentioned above.

v) Rhizogenesis was not seen in these shoot cultures and hardening studies with *ex vitro* rooting were not successful.

vi) These *in vitro* studies also encountered very high bacterial contamination indicating possible presence of endogenous bacteria originating from the soil. Surface sterilization of the
explants with 1% mercuric chloride for a period of 10 minutes increased explant establishment.

3. Ocimum basilicum var Thai Queen

i) Explants including nodes, internodes, leaves, inflorescences were used for regeneration of Ocimum basilicum var. Thai Queen. The best response was obtained in nodal explants – 80 to 100% wherein it was 20-30% in case of leaves.

ii) Marked seasonal variation was seen in establishment percentage, especially during monsoon. Most of the cultures were lost to fungal contamination due to humidity.

iii) Callus was initiated from both nodes and leaves inoculated in media containing IAA. The callus, which was generally obtained from the cut margins of the leaf explants, was off white, soft and moderately hard. The rate of proliferation of the callus from the leaves was not good. The callus was initially friable and off-white and over a period of time developed a pink to purple colour that eventually turned blackish as the culture aged. The rate of proliferation of the callus was initially good but as the callus started forming pigments its proliferation decreased. The callus however did not have any organogenic development.

iv) Callus was also induced at the base of the nodal explants established on solid MS media supplemented IAA and BA. There was no callus initiation in cultures established on MS media supplemented with low concentration of BA alone, till a period of 30-45 days, after which in some of the cultures there was slight callus initiation. This could be due to the media getting depleted of the supplemented cytokinin. Callus induction was slower and lesser as the cytokinin concentration in the media increased. Callus induction from nodes was better than from leaves. The callus proliferation was however, not good probably because of the accumulation of pigments in the cells.
v) Axillary buds developed within 7-10 days of inoculation and the buds sprouted in another 7-10 days time in media containing IAA and BA. The presence of the auxin restricted the shoot initiated per explant (1-2 per explant) and also initiated callus at the base of the nodal explant.

vi) Multiple shoots (10 – 15 shoots / explant) were obtained in modified MS supplemented with 26.5 μM BA. The shoots in this media grew to a height of 2 – 3 inches in 45 – 60 days followed by development of rooting in 90 days in the same culture vessels.

vii) Roots were formed on each shoot in the culture, which enabled hardening studies and also minimized a step in the micropropagation protocol, making it a simple and rapid method.

viii) Approximately 50% of the rooted shoots survived on hardening till the polyhouse stage. Further studies are needed for efficient hardening of the plants.

C. Establishment of cell suspensions using callus cultures of Gymnema sylvestre R. Br. and optimization of conditions for saponin production.

Saponins are glycosides of steroids, steroid alkaloids or triterpenes found in plants. A mixture of triterpenoid saponins referred to as ‘gymnemic acids’ is found in the leaves of Gymnema sylvestre R. Br. They are of closely related structure and are one of the active principles and they are anti-sweet saponins. It would be very beneficial if saponins can be directly obtained from the cell suspension cultures of Gymnema in a bioreactor on a large-scale. Also, along with a potential solution to the problem of diabetes, Gymnema has several other medicinal properties to cure ailments. Since diabetes is a worldwide problem having limited treatment, this study aims to provide possible solution to the problems by establishing possibilities regarding large scale production of saponins from Gymnema using a bioreactor and its other valuable medicinal properties.
The cell suspensions were initiated by inoculating callus grown in different initiation media fortified with different PGRs into liquid MS media fortified with different PGRs individually and in combination. The cells were separated by filtration and the filtrate was processed further for phytochemical analysis. For saponins, the aqueous solution (filtered) was directly used for chromatographic analysis.

Saponins in the cell suspensions were identified by Thin Layer Chromatography using the solvent system of chloroform: methanol: water (65:50:10). The saponins were visualized using Vanillin-sulphuric acid. The plates were heated to 100°C for a few minutes. Development of blue-violet indicated presence of saponins. The absorbance of the saponin in the eluent was measured at 214nm using UV spectrophotometer.

Various chemical factors (plant growth regulators, carbon sources, pH, additives, modifications in the nutrients of the media etc) and physical factors (temperature, age of inoculum, number of subcultures etc) were optimized for enhanced saponin production.

Findings are summarized:

1. Initiation of callus was successfully done in different media formulations and some cell lines showed certain degree of differentiation. Saponins were detected in the cell suspensions. The saponins synthesized by these cell lines accumulated in the media. Thus, the filtered media could be directly used to check for the presence of saponins in TLC plates. Spots were eluted with methanol and absorbance was measured at 207–214nm, the range for Gymnema saponins.

2. The saponin production was highest in cell suspensions established in liquid media fortified with 0.88μM BA and 1.07μM NAA from callus growing in MS (modified) media containing 0.88μM BA, 1.14μM IAA and 10.7μM NAA, and MS modified media containing 0.88μM BA, 1.14μM IAA and 10.95μM 2,4-D.
3. The cell suspensions established in liquid media containing high auxin concentrations resulted in very low saponin production. The saponin production was less in those formulations of hormones, where the auxin concentration was higher than 0.2ppm. Thus, high concentration of auxins are not conducive for the production of saponins from Gymnema cell cultures. The growth rate of cells in suspension was however less at 0.2ppm auxin concentration.

4. The cytokinin level was also preferable at a low concentration of 0.2ppm as the cells in culture clump together at higher concentrations of BA.

5. The saponin content in the media was seen to increase after 4 weeks of inoculation and the absorbance was high in the sixth week. Thus, the presence of saponin and the absorbance was checked in the sixth week of culture.

6. The cell lines obtained from callus initiated in both leaves and nodes showed an almost similar absorbance (214nm). This indicates that the source of the explant did not affect the production of saponins in culture.

7. The cell suspensions established in MS liquid media containing 0.88μM BA and 1.07μM NAA from callus initiated in MSB media fortified with 0.88μM BA, 1.14μM IAA and 10.7μM NAA and 0.88μM BA, 1.14μM IAA and 10.95μM 2,4-D, the production of saponin was higher than from callus generated in MS (modified) media. Thus, in media without glycine and high concentration of thiamin, there was enhancement in saponin accumulation in the media.

8. Increased concentration of sucrose also enhanced high saponin production; however, the growth of the cells in culture was affected, as there was more clumping of cells in culture. Thus, 3% sucrose was observed to be optimum for saponin accumulation as well as the growth of the cell sin suspension.

9. On adding maltose along with sucrose (maintaining 3% carbon source) and proline in the media, the saponin production was higher than in media containing only sucrose.

10. The increase in nitrate in the form of potassium nitrate and also addition of proline and glutamine led to an increase in the saponin production in the cell suspensions.
11. The cell suspension culture when maintained at a temperature of 40±2°C led to production of a very high accumulation of saponin in the media. The callus used as an inoculum in the established cell culture (MS modified fortified with 0.88μM BA and 1.07μM NAA) was initiated in MSB fortified with 0.88μM BA, 1.14μM IAA and 10.95μM 2,4-D. This cell line showed high levels of saponin production and the increase in the temperature led to a further increase in saponin accumulation.

12. When the callus used as an inoculum was taken after two to three subcultures rather than from an initiation stage, the saponin production in the cell suspension was higher.

13. The cells recycled during these studies also showed that more the number of passages in the cell suspensions more were the production of the saponins in the respective cell culture. In other words with every subculture during the cell suspension studies, there was an increase in the accumulation of saponins in the media.

14. The cells maintained in the cell suspensions showed less tendency of browning than the cells maintained in solid media.

D. Pilot study to scale up saponin production using a bioreactor

Though suspension cultures have a higher growth rate than cultures on a solid medium, large-scale industrial production of metabolites is not possible through limited volumes of cultures just maintained in Erlenmeyer flasks kept on rotary shaker. So, bioreactors are the practical solution for large-scale production of secondary metabolites through plant cell cultures.

A pilot study was initiated in a 2L bioreactor (Sartorius, India). A fresh inoculum (0.1ml per 100ml) of cell suspension was fed into the stirred tank bioreactor. The cell line selected for inoculation in the bioreactor was a suspension established using callus of nodal culture induced on media containing 0.88μM BA, 1.14μM IAA and 10.95μM 2,4-D with 2mg/l Glycine (MS modified). The media used was Basal MS supplemented with fortifications.
Chapter V

Summary & Conclusion

Findings are summarized:

1. The proliferation of the cells in the bioreactors was faster and uniform in comparison to suspensions maintained in individual flasks.
2. The absorbance at 214 nm per 0.1 ml of the filtrate obtained from the harvest was 1.24 Au. This absorbance when compared to 0.54 Au for 0.1 ml of filtrate from a cell suspension in an Erlenmeyer flask (See Cell suspension studies) indicated that the production of saponins has almost doubled in the bioreactor.
3. Harvesting was carried out only to check the presence and amount of saponin.

Conclusions

Ethanol extracts of *Gymnema sylvestre* R.Br. exhibited the best hypoglycemic activity when compared to the hypoglycemic potential of *Catharanthus roseus*, *Tabernaemontana divaricata* and *Ocimum basilicum var Thai Queen*. The ethanol extracts of *Gymnema sylvestre* R.Br. contained the saponins, which are reported be the bioactive principle and thus justifies its high hypoglycemic potential.

The fresh juices of the plants had a higher potential of lowering blood glucose levels than the dried powders of the leaves. However, the hypoglycemic activity of dried powders of leaves was not completely lost. Hence their use can be considered.

White variety of *C. roseus* exhibited greater hypoglycemic potential than its pink variety. The bioactive principle in *C. roseus* could be alkaloids as the benzene and the chloroform extracts of the plant showed lowering of blood glucose. The presence of activity in more than one extracts indicated that the hypoglycemic activity could be because of a complex mixture.

Callus was initiated in all the trials of *Gymnema sylvestre* R. Br. with both modified MS media and MSB fortified with various hormones singly and in combination. There was chlorogenesis and high nodularity in several cultures depending upon the auxin to cytokinin ratio.
The saponin production and growth of the cells were seen to be greater in the controlled conditions of the bioreactor. Consequently, the saponin amount was doubled in the bioreactor, thus showing a scale up in the production. This opens up further prospect of large scale production of saponins for future pharmaceutical formulation in order to combat this fatal global disease.

**Future Plan of Work**

The results obtained in the present studies opens up further investigative scope to substantiate use of these plants for treatment of diabetes.

1. There are several other factors that need to be optimized for enhancing the production of *Gymnema* saponins. These factors include pH, light intensity and type of light, oxygen tension, biotransformation, elicitation and immobilization.
2. Quantification of saponins by HPLC method would give an accurate analysis of the cost effectiveness of the product and enable its commercial production in the future.
3. Cytological studies of the cell lines giving optimum saponin production may help further modifications in the cell lines to increase production in the cell cultures.
4. The success of the pilot study in the bioreactor necessitates further studies to make commercial ventures feasible. Isolation and purification studies of saponins from these cultures will need to be standardized.
5. Studies can be initiated to elucidate the better hypoglycemic potential of the white variety of *C. roseus* and identify the bioactive principle. The *in vitro* shoot cultures, which had hypoglycemic potential, should be studied thoroughly to find out whether these had more potential than the field grown plants.
6. Further studies are needed to identify the blood glucose lowering complex or molecule in *Ocimum basilicum var Thai Queen*. And also ascertain whether the tissue-cultured plants had a higher hypoglycemic potential.
7. Studies need to be initiated to ascertain whether *T. divaricata* did have anti-hyperglycemic activity.
8. The mode of actions of the bioactive principles of these plants needs to be studied along with its toxicity studies.

9. *In vitro* micropropagation from mature *Gymnema* plants growing in hot and dry conditions needs to be standardized.

10. Rooting and hardening studies of the shoot cultures of *C. roseus* need to be standardized.

11. Studies also need to be carried out to check contamination *in vitro* as these results in a loss of cultures and decrease the cost effectiveness of the method. The use of broad-spectrum anti-fungal and anti-microbial agents needs to be carried out to combat this menace that plagues tissue culture studies.