Chapter 6

SUMMARY AND CONCLUSIONS

6.1 SUMMARY

BACKGROUND

★ In India, indigenous remedies have been used in the treatment of diabetes mellitus since the time of Charaka and Sushruta. India is endowed with a rich tradition of herbal medicines as is evident from the fact that the Sushruta Samhita differentiated between genetically and acquired forms of diabetes and recommended many herbal medicines in different oral formulation for the treatment of disease.

★ Pharmacological treatment of Diabetes mellitus is based on oral hypoglycemic agents and insulin. Though insulin therapy affords effective glycemic control, it has number of disadvantages such as short shelf life, constant refrigeration, and ineffectiveness on oral administration and fatal hypoglycemia in case of excess dosage. The synthetic hypoglycemic agents such as sulphonylurea, biguanides and glitazones can produce serious side effects and are toxic to liver and kidney.

★ Phytochemicals identified from traditional medicinal plants are presenting an exciting opportunity for the development of new types
of therapeutics. This has accelerated the global effort to harness and harvest those medicinal plants that bear substantial amount of potential phytochemicals showing multiple beneficial effects in combating diabetes and diabetes related complications.

The resolution of the thirty-first WHO Assembly requested a complete inventory, evaluation of the efficacy and safety and standardization of medicinal plants. The WHO has also recommended the evaluation of the traditional method of treatment for Diabetes mellitus. Therefore, as the disease is progressing unabated, there is an urgent need of identifying indigenous natural resources in order to procure them, and study in detail, their potential on different newly identified targets in order to develop them as new therapeutics.

**Hypothesis**

The medicinal plants in the Indian system of medicine are capable of normalizing all the biochemical parameters related to the pathobiochemistry of diabetes and prevent its complications. The plant medicines can reverse the disease to a degree rather than just palliate glycemia.

**Plant chosen for the investigation**

*Terminalia bellerica* Roxb. (Combretaceae) is a large deciduous tree which occurs widely in the moist valleys of India and its fruits are most commonly used in Indian traditional systems of medicine. The fruit rind is used in different preparations, for example, as an ingredient in the popular Ayurvedic formula known as ‘Triphala’ (three fruits), used for the treatment of fever, cough, diarrhea, dysentery, skin diseases and liver disorders. The fruit is reported to have hepatoprotective, hypotensive, anti-mutagenic, antimicrobial and anti-HIV-1 activity. The plant is known to lower the levels of lipid in hypercholesterolemic animals. Triphala and *T. bellerica*
crude extracts were found to reduce serum glucose level and have marked antioxidant properties in alloxan-induced diabetic rats.

The overall tonic effects of the fruit of *Terminalia bellerica* has been known for thousands of years in India and other Asian countries and even now, Ayurvedic practitioners recommend the dry fruits of *T. bellerica* as a daily and preventive supplement to diabetic patients alone or in the triphala formula. But there is no detailed study on the beneficial role of this plant in preventing diabetes mellitus and its secondary complications. Also, there are no reports on the bioassay-guided fractionation and isolation of the active principle responsible for its antihyperglycemic and hypolipidemic effects. Therefore the present study was undertaken to evaluate the anti diabetic potential of *T. bellerica* fruits, identify the antihyperglycemic principle(s) and analyze the therapeutic potential and mode of action of the active compounds.

**Objectives**

- To assess the hypoglycemic effect of hexane, ethyl acetate and methanol crude extracts of fruit rind of *Terminalia bellerica* (TB) in STZ induced diabetic Wistar rats.

- To evaluate the effect of crude extracts of *Terminalia bellerica* fruits on biochemical parameters such as insulin, C-peptide, lipid profile, LFT and KFT and histology of pancreas, liver and, kidney of STZ-rats and choose the best extract.

- To isolate the hypoglycemic active principle(s) from the most bioactive fraction through Bioassay guided fractionation.

- To identify the structure of the active compound(s) by spectral analyses (NMR, IR, EIMS, etc.,).
To analyze the therapeutic potential and mode of action of the active compound(s) in preventing Diabetes mellitus and its complications in STZ induced rats by biochemical, molecular and histological studies.

To assess the effect of the active compound(s) on glucose transport by analyzing GLUT 4 mRNA and protein expression in the diabetic rats.

To find the toxic effects, if any, of the plant and its compounds in tissues like liver, kidney and pancreas.

To predict the binding of active principles to the active ligand binding domain of receptor proteins [1IRK and/or PPAR-γ] through docking studies by using Discovery Studio (Version 2.1).

**Methodologies**

- Biochemical parameters such as Blood glucose, glycosylated hemoglobin and hemoglobin were determined adopting appropriate methods.

- The levels of plasma insulin and C-peptide were analyzed adopting RIA method.

- Biochemical parameters of the serum with respect to lipid metabolism, such as total cholesterol, triglycerides, LDL and HDL cholesterol were also determined.

- Liver and muscle glycogen were determined using appropriate methods.

- Biochemical parameters with respect to kidney function such as serum urea, creatinine and uric acid were also estimated.

- Biochemical parameters with respect to Liver function such as plasma total protein, albumin and enzyme markers of liver damage such as serum AST, ALT and ALP were determined using appropriate methods.
Bioassay guided Fractionation and Isolation of Active Principles were made using column chromatography, HPTLC and TLC techniques, adopting appropriate solvent system.

Identification of Active Principles by spectral analyses namely, $^{13}$C and $^1$H NMR, EIMS and IR were made.

Activities of the enzymes involved in carbohydrate metabolism such as glucokinase, glucose-6-phosphatase, fructose-1,6-bisphosphatase, glycogen phosphorylase and glycogen synthase were determined.

Lipid peroxidation, enzymatic and non-enzymatic antioxidants of liver and kidney tissues were studied.

Light microscopic studies of Pancreas, Liver and kidney tissues were made.

GLUT 4 mRNA expression was analyzed in the skeletal muscle tissue using techniques such as Reverse Transcriptase - Polymerase Chain Reaction and Agarose Gel Electrophoresis.

GLUT 4 protein expression was also determined using Western blot analysis.

Docking of Active compounds to the ligand binding domain of Insulin receptor Tyrosine Kinase (1IRK) and PPAR-γ were done using Discovery Studio (Version 2.1).

**Salient Features of the Results**

**Isolation and Identification of Active Principles**

A pure pale yellow compound TB1 eluted from the most active fraction (F11) of ethyl acetate soluble part, of methanol extract was subjected to spectral analyses for structural determination. The IR spectrum
showed peak for hydroxyl, acid carboxyl and aromatic system. In EIMS- the single fragment ion peak of gallic acid was seen at m/z 171(M+H).

The $^1$H NMR spectrum in CD$_3$OD showed a single peak at 7.06. $^{13}$C NMR spectrums in CD$_3$OD also confirmed the structure to be gallic acid (3, 4, 5-Trihydroxybenzoic acid).

A pure compound isolated from the active fraction (FII) of ethyl acetate insoluble part of methanol extract was subjected to spectral analyses for structural determination. The IR band indicated the presence of - OH group, carboxyl group of ester moiety, $-C-O-C-$ vibration and benzene ring. Mass spectrum indicated the presence of long chain alkane moiety, $-COOCH_2-$ moiety and aromatic moiety with 3 - hydroxyl groups.

The $^1$H - NMR spectrum exhibited the presence of methyl protons, $-CH_2-$ proton, methylene protons and OH proton. The proton decoupled $^{13}$C - NMR spectrum showed NMR assignments for aromatic groups, carboxyl ester group, methylene carbon adjacent to oxygen, methyl carbon and the remaining methylene carbons, also confirmed the structure of the compound, n-octyl-3, 4, 5-trihydroxybenzoate.

**Biochemical changes**

Induction of diabetes mellitus by streptozotocin in Wistar albino rats increased plasma glucose level and altered all the biochemical parameters related to the patho-biochemistry of diabetes mellitus.

Oral administration of extracts of *Terminalia bellerica* fruit rind (TBFE) and the isolated compounds Gallic acid (GA) and Octyl gallate (OG) significantly restored the altered parameters to near normal range.
* The increased levels of plasma glucose in STZ-induced diabetic rats were lowered by the administration of fruit extracts of *T. bellerica*. Significant reduction was observed in methanol extract treated diabetic rats followed by ethyl acetate and hexane extract treated rats.

* The increased levels of plasma glucose in STZ-induced diabetic rats were lowered by the oral administration of gallic acid and octyl gallate in a dose-dependent manner.

* Dose-dependent fall in blood sugar levels were quite similar in both the standard GA treated and GA isolated from *Terminalia bellerica* fruit extract treated diabetic rats and were homogenously significant.

* Plasma insulin and C-peptide levels significantly increased.

* Glucose tolerance level was greatly increased.

* Body weight, serum total protein and albumin markedly increased.

* Glycogen content in Liver and skeletal muscle tissue increased.

* Serum levels of total cholesterol, triglycerides and LDL cholesterol decreased whereas HDL cholesterol increased.

* Glycosylated hemoglobin level was brought down.

* Serum urea, creatinine and uric acid levels were near normal.

* Significant reduction in the activities of serum AST, ALT and ALP were observed.

* TBARS level in the liver and kidney of treated diabetic rats decreased with an increase in enzymatic and non-enzymatic antioxidant levels.

* The activities of the enzymes GK & GS increased and Glu-6-Pase, Fru-1,6-2Pase & glycogen phosphorylase decreased.
Dose dependent increase in the GLUT 4 mRNA and protein expression was observed in the skeletal muscle tissues of GA and OG treated diabetic rats.

Based on the LibDock values, number of hydrogen bonds and other interactions, gallic acid has good binding affinity towards 1IRK and octyl gallate has good affinity towards both 1IRK and PPAR-γ.

**Histopathological studies**

- In STZ induced diabetic rats, islets were small and shrunken and showed degeneration and general fibrosis. Liver and kidney sections showed congestion and necrosis.

- Diabetic rats treated with *T. bellerica* fruit extracts, GA and OG showed the restoration of the normal architecture of islets of Langerhans, with well formed islets and the near normal architecture of islet histology indicates the regeneration of beta cells.

- *Terminalia bellerica* fruit extract and compound treated diabetic rats also revealed the normal architecture of hepatocytes, glomerular and tubular cells without any inflammation, congestion and necrosis.

**The study leads to the following inferences**

- Oral administration of various crude extracts of *Terminalia bellerica* for 60 days decreased the blood glucose level in STZ-induced diabetic rats. The decrease in blood glucose was greater in the methanol extract treated diabetic rats indicating the presence of active principles in the methanol extract.

- The increased level of insulin and C-peptide in TBFE and compound treated diabetic rats indicate that the normoglycemic action of TBFE,
GA and OG might be due to regeneration of beta cells and potentiation of insulin release from the existing beta cells of islets of Langerhans.

- Administration of TBFE and the active compounds GA and OG brought back the levels of serum lipids to near normal values which indicates the dyslipidemic property of the extract and compounds. This effect is due to an increase in insulin secretion that inhibits lipolysis and decreases the mobilization of free fatty acids from adipose tissue.

- The observed decrease in glycosylated hemoglobin indicates better glycemic control in TBFE and compound treated diabetic rats.

- Significant increase in the body weight, liver and muscle glycogen, plasma total protein and albumin might be due to an improvement in insulin secretion and glycemic control in fruit extract and compounds treated rats.

- Significant reduction in the activities of serum AST, ALT and ALP in STZ-induced diabetic rats indicate the hepatoprotective role of the fruit extract and compounds.

- TBFE, GA and OG lowered all the three non-protein nitrogenous substances, urea, uric acid and creatinine in diabetic rats suggesting the renoprotective role of the compounds in preventing diabetic nephropathy.

- The increase in the activity of GK & GS and decrease in the activity of Glu-6-Pase, Fru-1,6-P2ase & glycogen phosphorylase reflects the improvement in carbohydrate metabolism and the insulin secretory effect of active principle(s).

- Significant reduction in TBARS level and increase in GSH, GPx, SOD and CAT activity in diabetic rats reveal the antioxidant property of
the plant extract and active principles.

- Treatment with the plant extract and its bioactive compounds, GA and OG ameliorated and reversed the pancreatic lesions induced by streptozotocin. The normal histoarchitecture of islet of Langerhans with well formed islets indicates the regeneration of β cells by the active compounds.

- TBFE and the bioactive compounds GA and OG reduced the incidence of liver lesions and repaired the necrosis induced by STZ. Therapeutic hepatoprotective effect of the compounds might be due to their antioxidant and membrane stabilizing activity.

- Diabetic rats treated with TBFE, GA and OG revealed normal architecture of glomerular and tubular cells without any inflammation and congestion. The renoprotective effect might be due to their anti-inflammatory and antioxidant activity that prevents the oxidative damages to the microstructure of the kidney caused by STZ.

- Dose dependant increase in the GLUT 4 mRNA and protein expression in the skeletal muscle tissues and increased glucose tolerance indicate enhancement in the glucose uptake, utilization and better glycemic control in GA and OG treated diabetic rats.

- As per in silico studies, good docking interactions and Lib Dock score indicate that GA has good binding affinity towards the insulin receptor (1IRK) and OG has good affinity to both 1IRK and PPAR-γ.

- Normal levels of all the above biochemical parameters and the normal histoarchitecture of pancreatic acinar and β cells, hepatocytes, glomerular and tubular cells without any inflammation in the GA and OG treated normal rats indicate the non toxic nature of the compounds.
6.2 CONCLUSIONS

☆ The present study for the first time demonstrated that gallic acid (GA) and octyl gallate (OG) present in the methanol extract of *Terminalia bellerica* fruits are responsible for the antihyperglycemic activity as evident from the biochemical, histopathological and molecular studies.

☆ Oral administration of extracts of *Terminalia bellerica* fruit rind and the isolated compounds Gallic acid and Octyl gallate significantly increased plasma insulin and C-peptide and restored the altered biochemical parameters to near normal range.

☆ Dose dependant increase in the GLUT 4 mRNA and protein expression in active compound treated diabetic rats indicate enhancement in the glucose uptake and better glycemic control.

☆ Treatment with the plant extracts and the compounds GA and OG ameliorated the lesions induced by streptozotocin in pancreas, liver and kidney and significantly improved the regenerative capacity of the β cells of pancreas and other vital organs.

☆ The plant extracts and compounds under study did not exhibit any toxicity to the animal model.

☆ In *in silico* studies, GA has good binding affinity towards the receptor, 1IRK and OG has good affinity towards both 1IRK and PPAR-γ.

☆ The compounds GA and OG satisfy Lipinski’s rule of five and can be developed into valuable drugs with mutiple therapeutic potential to treat diabetes and its complications.