CHAPTER 8

SUMMARY

&

CONCLUSIONS
Diabetes mellitus is a progressive metabolic disorder associated with hyperglycemia due to insulin deficiency or insulin resistance. Chronic hyperglycemia is associated with disturbances in lipid metabolism and increased generation of free radicals leading to oxidative stress. In addition to allopathic treatment, traditional health care system and medicinal plants offer an exciting opportunity for the development of alternative therapies in DM. Several plants have been screened which have shown multiple beneficial effects in combating diabetes (Shukla et al. 2000). Pharmacological significance of *W. coagulans* fruit has also been reported in ayurvedic system of medicine.

Few studies have reported the antihyperglycemic and antihyperlipidemic effects of water extract of fruit of *W. coagulans* by using high doses i.e. 750 – 1,000 mg/kg bw (Khodaei et al. 2012). Water soluble hypoglycemic compounds have also been isolated from fruit of *W. coagulans* (Maurya et al. 2008). However, to the best of our knowledge, no studies have been carried out using low doses of aqWC, moreover, mechanism of action of active antihyperglycemic compounds isolated from *W. coagulans* has not been studied in diabetes mellitus. Therefore, this work was planned to study the antihyperglycemic effect of water extract of *W. coagulans* fruit and the isolated active antihyperglycemic component (aqWCFIId), and study its mechanism of action. Toxicity studies have also been performed to establish safety of this component. The results are as follows:

**AIM 1: ANTIHYPERGLYCEMIC AND HYPOLIPIDEMIC EFFECTS OF AQUEOUS EXTRACT OF *WITHANIA COAGULANS* FRUIT (aqWC) IN DIABETES MELLITUS**

**Antihyperglycemic effects:**
- Preliminary work was conducted with water extract of fruit of *W. coagulans* (aqWC) to study its antihyperglycemic effects in mild (FPG = 126 -200 mg/dl) and severe diabetic rats (FPG > 200 mg/dl) by using three different doses i.e. 125, 250 and 500 mg/kg bw given orally for one month.

- After single treatment with three different doses of aqWC, glucose lowering effect in MD & SD-rats was observed after 2 hours of administration, however maximum decrease in FPG and PPPG levels was observed with 250 and 500 mg/kg bw of aqWC-treatment as compared to diabetic-untreated rats.
- Fifteen & 30 days treatment with all three doses of aqWC produced significant decrease in FPG and PPPG in MD & SD-treated rats \( (p<0.0001) \), which were brought down to near normal levels in MD-treated rats.

- HbA1c was significantly increased and insulin & C-peptide levels were significantly decreased in MD & SD-untreated rats as compared to healthy controls \( (p<0.001) \). After 30 days of treatment with different doses of aqWC, HbA1c was significantly decreased and insulin and C-peptide levels were significantly increased in MD & SD-treated rats \( (p<0.01) \).

- These above effects were comparable with glibenclamide-treated rats, which also showed similar glucose lowering effects \( (p<0.0001) \).

- The above results revealed, that doses 250 and 500 mg/kg of aqWC produced almost similar percentage decrease in FPG and PPPG levels.

- Since, no extra benefit was observed with higher dose of aqWC i.e. 500mg/kg bw, therefore, 250 mg/kg of aqWC was considered as most effective dose (MED) and used for further experiments.

**Hypolipidemic effects:**

- Diabetic-rats showed significantly increased TC, TAG and LDL-C and decreased HDL-C levels as compared to healthy control rats \( (p<0.001) \).

- These lipid parameters were suitably improved in MD & SD-rats after 30 days of treatment with aqWC i.e. significantly decreased TC, TAG and LDL levels, and increased HDL levels as compared to diabetic-untreated rats \( (p<0.01) \).

- Body weight of diabetic animals were significantly lower as compared to healthy controls \( (p<0.01) \) and there was significant improvement in body weight of MD & SD-rats treated with aqWC \( (p<0.05) \), however these were still lower than healthy controls.
Antioxidant effects:

- To determine the antioxidant potential of aqWC, MDA, GSH, SOD and FRAP levels were measured in MD and SD-rats.

- In diabetic-untreated rats, MDA levels were significantly increased whereas GSH, FRAP and SOD activity were significantly decreased in blood and tissues as compared to healthy controls (p<0.001).

- Diabetic-rats treated with aqWC showed significantly decreased MDA and increased GSH, SOD & FRAP as compared to diabetic-untreated rats (p<0.01).

Antihyperlipidemic and antioxidant effect of aqWC in rabbits fed high cholesterol-diet:

- Body weight of cholesterol fed rabbits was significantly increased as compared to healthy controls (p<0.001). However, animals receiving cholesterol along with aqWC showed significantly lower body weight as compared to only cholesterol fed animals (p<0.01), however it was still higher than healthy controls.

- Cholesterol-fed rabbits showed significantly increased TC, TG and LDL levels and decreased HDL levels after 6 weeks (p<0.001), whereas rabbits receiving aqWC along with cholesterol showed significantly decreased TC, TG, LDL-C and increased HDL-C levels as compared to only cholesterol-fed rabbits (p<0.01), thus showing the beneficial effects of aqWC on lipid profile parameters.

- Cholesterol-fed rabbits also showed significantly increased MDA and decreased GSH, SOD and FRAP activity in blood and tissues (liver & heart) as compared to healthy controls (p<0.001), whereas rabbits receiving cholesterol + aqWC showed that MDA was significantly decreased and GSH, SOD and FRAP were significantly increased in blood and tissues as compared to only cholesterol-fed rabbits (p<0.01).

- Thus, these observations showed that cholesterol feeding led to hypercholesterolemia and oxidative stress; which were counteracted by simultaneous administration of aqWC.
AIM 2: ISOLATION AND CHARACTERIZATION OF ANTIHYPERGLYCEMIC COMPONENT FROM AQUEOUS EXTRACT OF *WITHANIA COAGULANS* FRUIT (aqWC)

- Phytochemical tests for aqWC revealed the presence of bioactive molecules viz carbohydrates, glycosides, saponins, phenolics, tannins, alkaloids, terpenoids and flavanoids.

- Water extract of fruit of *W. coagulans* was subjected to purification by column chromatography (Sephadex G75) and thin layer chromatography. Four different fractions were eluted from column chromatography which were designated as FI, FII, FIII & FIV and fed to diabetic rats for 15 days. The maximum effect were observed with fraction FII i.e. 27% decrease in FPG and 30% decrease in PPPG levels as compared to diabetic-untreated rats (p<0.001).

- This fraction (FII) was further purified by thin layer chromatography (TLC) on silica gel plates. Four different fractions of Rf value 0.96, 0.82, 0.75 and 0.63 named as FIIa, FIIb, FIIc and FIId were obtained, which were fed to diabetic rats. Fraction FIId showed maximum glucose lowering effect i.e. FPG and PPPG decreased by 30% and 32% respectively (p<0.001). FIId was designated as aqWCFIId and subjected to HPLC.

**HPLC analysis:**

- The most active fraction i.e. aqWCFIId was applied on HPLC and eluted with mobile phase [MeOH : H2O (90:10)] and obtained single peak on spectra. Purified fraction was dried and fed to diabetic-rats for 15 days, which showed that FPG and PPPG were significantly decreased i.e. 28% and 32% respectively as compared to 0 days (p<0.001). This active fraction was subjected to structural analysis.

**LC-MS analysis of active fraction isolated from HPLC:**

- Isolated fractions from HPLC were subjected to LC-MS and spectra of M⁺ ion peak with m/z 671 was observed.
NMR of isolated active antihyperglycemic compound(s) from HPLC:

- $^{13}$C (carbon) and $^1$H (proton) NMR of aqWCFIIId was done to elucidate the number of hydrogen ($^1$H) and carbon ($^{13}$C) which may be 48 and 35 respectively according to NMR.

Anti-hyperglycemic and anti-hyperlipidemic effect of active compound aqWCFIIId isolated from the fruit of *W. coagulans*:

- The effect of single treatment with three different doses of aqWCFIIId i.e. 25, 50 & 100 mg/kg bw was studied in MD & SD-rats. The MD & SD-untreated which rats showed significantly increased FPG & PPPG levels as compared to healthy controls ($p<0.0001$). These were decreased in MD & SD-treated rats with all three doses of aqWCFIIId. However, the decrease in plasma glucose levels was similar with 50 and 100 mg/kg bw of aqWCFIIId after single treatment, therefore 50 mg/kg bw was considered as effective dose (MED) and further work was carried out with this dose.

- FPG and PPPG were significantly decreased in MD & SD-rats treated with aqWCFIIId for 30 days, which were brought down to near normal levels in MD-rats ($p<0.0001$).

- HbA1c was significantly increased in MD and SD-untreated rats ($p<0.001$), which was significantly decreased after treatment with aqWCFIIId ($p<0.01$).

- Upon treatment with aqWCFIIId for 30 days, MD and SD-rats showed significant improvement in lipid profile parameters i.e. decreased TC, TG and LDL levels and increased HDL level as compared to diabetic-untreated animals ($p<0.01$).

- The body weight of MD & SD-rats were significantly decreased ($p<0.01$), whereas the same increased to near normal in MD-rats treated with aqWCFIIId for 30 days ($p<0.05$), however it was still lower than healthy controls.
AIM 3: MECHANISM OF ACTION OF ANTIHYPERGLYCEMIC COMPOUND i.e. aqWCFIId IN DIABETES MELLITUS

To get an insight into the mechanism of action of aqWCFIId, following parameters were measured in diabetic rats:

Effect at pancreatic level:

1. Effect of aqWCFIId on plasma insulin and C-peptide levels (In vivo):
   - MD & SD-untreated rats showed significantly decreased insulin and C-peptide levels as compared to healthy controls (p<0.001). Treatment with aqWCFIId to diabetic rats showed significantly increased insulin and C-peptide levels as compared to diabetic-untreated rats (p<0.01).

2. Release of insulin from β-cell islets of pancreas (In vitro):
   2.1. Effect of aqWCFIId on insulin release from isolated pancreatic islets of healthy and diabetic rats:
      - Glucose induced insulin secretion from pancreatic islets of healthy & diabetic rats after 1 hr and 2 hrs of incubation with 11mM glucose was studied.
      - Addition of aqWCFIId to the β-islets in incubation mixture, markedly increased glucose-induced release of insulin from islets of healthy and diabetic rats (p<0.01).

   2.2. Release of insulin from pancreatic islets of diabetic rats fed aqWCFIId for 30 days:
      - Pancreatic islets isolated from aqWCFIId treated diabetic rats were incubated with glucose at 3 and 11mM concentration.
      - Release of insulin was significantly increased after 2 hrs as compared to respective islets from diabetic untreated rats (p<0.001).
      - In addition, islets from aqWCFIId treated rats were further incubated with glucose (3 and 11mM) + aqWCFIId; these showed significantly increased insulin release after 2 hrs as compared to only glucose treated islets (p<0.05).
3. Histopathological examination of pancreas of MD and SD-rats:

- Histopathological examination of pancreas showed that most of the islets of diabetic-rats were damaged and / or degenerated. After 30 days aqWCFIId treatment, MD & SD-rats revealed increase in size and number of β-cell islets suggesting regeneration of β-cells along with few lymphocytes.

- Histological findings suggested that, aqWCFIId treatment resulted in improvement in morphology of pancreatic islets in diabetic animals, prevented β-cell cytotoxicity therefore, resulting in increased secretion of insulin from β-cells.

Extra pancreatic actions:

1. Autophosphorylation of insulin receptors by tyrosine kinase:

- The autophosphorylation of insulin receptors by integral tyrosine kinase is an important mechanism of insulin action. Tyrosine kinase activity was significantly decreased in erythrocytes and hepatocytes of MD & SD-untreated rats as compared to healthy control rats (p<0.001). After, 30 days treatment with aqWCFIId, the tyrosine kinase activity was significantly increased in erythrocytes and hepatocytes of MD & SD-rats (p<0.01).

2. Glucose transporter 4 (GLUT4) expression:

- Expression of GLUT4 was studied in muscle tissue of diabetic rats by immunohistochemistry (IHC) and Western blotting. Immunostaining of skeletal muscles of diabetic-untreated rats showed decreased staining for GLUT4 (less brown colour), which suggested there was reduction in GLUT4 expression in cytoplasm and plasma membrane as compared to healthy controls. MD and SD-treated with aqWCFIId showed increased staining for GLUT4 by immunohistochemistry as compared to diabetic-untreated rats.

- GLUT4 expression was further confirmed by Western blot analysis, SD-untreated rats showed decreased expression of GLUT4, which was improved in aqWCFIId-treated SD-rats.
3. Regulatory enzymes of glucose and lipid homeostasis:

These were assayed in liver of aqWCFIId treated MD & SD-rats:

- Glucokinase (GK), phosphofructokinase (PFK) and glucose-6-phosphatase (G-6-Pase) are important regulatory enzymes of glucose homeostasis. The activities of GK and PFK were significantly decreased and G-6-Pase was significantly increased in liver of MD & SD-untreated rats as compared to healthy controls (p<0.001), whereas, 30 days treatment with aqWCFIId to MD & SD-rats showed significantly increased activity of GK & PFK and decreased G-6-Pase activity in liver as compared to diabetic-untreated rats (p<0.01).

- Activity of HMGCR, which is the regulatory enzyme of cholesterol synthesis, was significantly increased in diabetic-untreated rats whereas after 30 days of treatment with aqWCFIId, diabetic-rats showed significantly decreased HMGCR activity in liver (p<0.001). ACC activity, which is regulatory enzyme of fatty acid biosynthesis, was significantly decreased in diabetic-untreated rats whereas after 30 days treatment with aqWCFIId, it was significantly increased in liver of MD and SD-rats as compared to diabetic-untreated rats (p<0.01).

4. Effect of aqWCFIId treatment on glycogen and lipid content in MD & SD-rats:

- Glycogen content in liver and muscle of MD & SD-untreated rats was significantly decreased as compared to healthy controls (p<0.001), which was restored after 30 days treatment with aqWCFIId (p<0.01).

- MD & SD-treated with aqWCFIId showed significantly decreased lipid content in liver and heart tissue as compared to diabetic-untreated rats (p<0.01).

AIM 4: TOXICITY STUDIES

- Acute toxicity study indicated that aqWCFIId at a dose 1 g/kg bw did not produce any apparent signs of toxicity or mortality in rats and revealed normal physiological functions

- In sub-chronic toxicity, feeding effective dose (ED) and 5 times of ED of aqWCFIId to rats for 28 days did not show any significant adverse change in
biochemical parameters i.e. FPG, liver and kidney function tests and hematological parameters (Hb, total leukocytes, differential leukocytes etc).

- Histological examination of liver, heart and kidney also did not reveal any pathological changes in treated animals as compared to controls.

CONCLUSIONS

Our study has demonstrated that aqueous extract of *W. coagulans* fruit has significant antihyperglycemic effect in diabetes mellitus at low doses (250 mg/kg bw) as compared to high doses reported in literature. Treatment with aqWC for 30 days normalized the fasting and postprandial glucose levels in mild diabetes and it was also effective in severe diabetes. AqWC has also shown significant hypolipidemic and antioxidant effects in diabetes mellitus in rats, as well as in hypercholesterolemic rabbits. The active principle isolated from *W. coagulans* fruits by conventional Sephadex G-75 chromatography and TLC, it has a molecular weight is 671. This purified component designated as aqWCFIId, has shown significant glucose lowering effects in diabetic rats. Upon 30 days treatment, fasting and postprandial plasma glucose levels in mild diabetes were normalized whereas these were significantly decreased in severe diabetes. The mechanism of action of this compound also been studied and it seems to possess both pancreatic and extrapancreatic effects. Feeding aqWCFIId increased plasma insulin levels (*in vivo*) and enhanced insulin release from pancreatic islets (*in vitro*) even at low concentration of glucose. Treatment with active component to diabetic rats also improved the morphology of β cells islets as confirmed by histology of pancreas. Tyrosine kinase activity of insulin receptors in erythrocytes and hepatocytes and GLUT4 expression in muscle was also suitably improved, which may help in glucose utilization. In addition, treatment with aqWCFIId has shown beneficial effects on glucose and lipid homeostasis as evidenced by favorable changes in the activity of respective enzymes and glycogen and lipid content in tissues.

In view of above studies, active component isolated from aqWC has potential to be developed as a drug for the treatment of diabetes mellitus.