CHAPTER 6
DISCUSSION OF RESULTS

Type 2 diabetes mellitus is a complex heterogenous metabolic disorder, characterized by hyperglyemia mainly due to altered insulin secretion and sensitivity of insulin on tissues. This occurs due to abnormal production of glucose, impairment in the lipid and protein metabolism. It is an emerging global health problem of the 21st century. It affects nearly 10% of the population in all age groups in various geographical regions [6].

The major and commonly observed long term complications of type 2 DM are renal failure, hypertension, retinopathy, slow healing wounds, neuropathy [129]. Evidence from current research studies shows that insulin receptors are present in hippocampus, hypothalamus, cerebral cortex and olfactory bulb and play a important role in cognition, energy homeostasis and mood behavior in the CNS [130]. Several studies have shown that there is a link between IR and cognitive impairment. Hyperinsulinemia in IR causes downregulation of insulin-degrading enzyme (IDE) levels in the brain leading to accumulation of amyloid plagues similar to Alzheimer’s disease [64]. Studies have shown that IR induced hyperglycemia in CNS inudces ROS generation and inflammation that might contribute to cognitive impairment [131]. Lack of management strategies to combat the cognition impairment caused due to DM, establishes rationale for research in this area.

In the present study an attempt was made to evaluate the potential of three traditionally used medicinal plants, *Casearia elliptica* roots (Family- Flacourtiaceae), whole plant of *Cissus quadrangularis Linn* (Family- Vitaceae) and root tubers of *Cyperus rotundus* (Family- Cyperaceae), for their potential against type-2 diabetes mellitus and its associated cognitive impairment. The plants selected for the current study were not completely new. Few earlier reports and the ethnobotanical claims mention their antidiabetic activity [132-134]. Intentionally, such plants were selected as the objective of the study was to develop a synergestic combination of herbs which can combat diabetes mellitus and associated cognitive impairment.
The ethanolic extracts of *Casearia elliptica*, *Cissus quadrangularis* and *Cyperus rotundus* and PHF were screened for phytochemical analysis as per standard procedures and mentioned in the chapter 4. The preliminary analysis revealed the presence of active phytoconstituents like alkaloids, flavonoids, saponins carbohydrates, glycosides, phenolic compounds, tannins and terpenoids.

The major objective of the treatment in type 2 diabetes mellitus is to lower the blood glucose levels. Conventional oral hypoglycemic agents reduces blood glucose levels but vowing to the side effects associated with them, compliance of the patients to the treatment was affected. This contributes to the development of complications in diabetic patients [135,136] and dragged the attention of the people towards use of herbal medicines since they exhibit fewer side effects. Currently more research is emphasized in the area of folklore medicines to develop a lead molecule for remedy of DM.

Oral Glucose Tolerance Test is a general procedure used for evaluation of normal hypoglycemic activity in normal animals in which the drug is administered prior to glucose administration and periodically samples were collected at different time intervals 0, 30, 60 & 120 min to estimate glucose levels [104]. The extracts and PHF were initially screened individually for their hypoglycemic activity in OGTT model in rats. Since this model is simple and reliable test for studying oral hypoglycemic effect of the test compounds. In the current study, OGTT in overnight fasted rats revealed that the EECE, EECQ and EECR showed that the blood glucose levels were decreased at 60 minutes but the maximum effect was observed at 90 minutes. This indicates that EECE, EECQ and EECR are having significant effect on hyperglycemia.

To confirm the antihyperglycemic effect of the studied plants, diabetes mellitus was induced in rats by administration of (STZ 40 mg/kg/i.p) [137, 138]. Several studies have shown that STZ injection causes destruction of pancreatic beta cells of islets of langerhans. STZ acts on pancreatic beta cells via glucose transporter-GLUT-2 and causes alkylation of DNA, as a result pancreatic beta cells are destroyed by necrosis. This is characterized by hyperglycemia and decreased insulin secretion. STZ injection at low dose induces diabetes in animals similar to type 2 diabetes mellitus in humans [106, 139, 140].
DM is a metabolic syndrome showing marked impairment of carbohydrate, protein and lipid metabolism. The major sign of DM was noticed as reduced body weight. Similar decrease in body weights were noticed in the DC group after STZ injection. The rats treated with EECE, EECQ, EECR significantly reversed the STZ induced changes and prevented the loss of body weight which can be considered as a major hint for the diabetic activity of the selected plants.

The next parameter focused in the study was blood glucose levels, hyperglycemia is considered as the hallmark feature of DM. Diabetic control rats showed marked raise in the blood glucose levels which were reduced on treatment with EECE, EECQ and EECR. The effect produced by the selected plants was observed similar to standard glibenclamide at high dose.

The secondary complication of type-2 diabetes mellitus is dyslipidemia. Mobilization of fat from adipose tissue occurs because of insulin deficiency [141]. The diabetic rats exhibited increased levels TC, TG, LDL-c, and VLDL-c and decreased HDL-c. Administration of EECE, EECQ and EECR in rats significantly lowered the levels of TC, TG, LDL-c, VLDL-c and markedly improved the levels of HDL-c in the blood. This suggests that the possible mechanism of action might be due to elevation of insulin levels and decreased mobilization of fat from the adipose tissue.

Hyperglycemia induced by STZ leads to depletion of liver glycogen stores. Similarly, in the diabetic rats the glycogen content in the liver was markedly decreased. The results of our study showed that the glycogen content levels were significantly increased in diabetic rats after treatment with EECE, EECQ and EECR for 14 days. This effect might be due to stimulation of insulin secretion and increased uptake of glucose by the liver.

Histopathological studies of pancreas in diabetic rats showed marked degradation of beta islets of pancreas after STZ injection in diabetic rats, confirming that STZ is cytotoxic to pancreatic cells. In diabetic rats, treated with EECE, EECQ and EECR, there is a marked regeneration of pancreatic beta cells, indicating that the extracts have potential antidiabetic and protective effect on pancreas.
After confirmation of the antidiabetic effect of individual plants, a PHF was prepared based on the EC\textsubscript{50} values obtained from the OGTT studies of the individual plants. The PHF was once again evaluated for the hypoglycemic effect in OGTT and antidiabetic activity in the STZ model. The results of those studies confirmed that PHF possessed better hypoglycemic effect and antihyperglycemic effect when compared to the individual plant extracts. The effect of PHF in OGTT, blood glucose levels, lipid profile, liver glycogen content and histopathology of pancreas attested the synergistic effect of the plants used in the PHF.

There is a line of evidence that type 2 diabetic patients are more prone to cognitive decline. The exact mechanism for cognitive impairment in diabetes was not clear. Mild cognitive impairment (MCI) occurs in normal aging but the prevalence of MCI is more in diabetic patients and it has increased upto 32.7% [142]. Peripheral hyperinsulinism is associated with decreased levels of insulin in the brain. This alters the metabolic rate of glucose in CNS and disrupts the transport and signalling mechanism of insulin in brain contributing to impaired glucose degradation. Hyperglycemia in brain results in free radical generation and cause damage to the brain [131, 143]. Several studies support that insulin resistance is a risk factor for cognitive impairment and alzheimer's disease (AD). It was clinically proven that cognitively impaired elderly patients exhibit insulin resistance. Studies have found that the insulin signaling mechanism is impaired in AD patients with low insulin levels in brain and cerebrospinal fluid and hyperinsulinemia in plasma, which was further supported in AD brain post-mortem [144 -148].

Prolonged hyperglycemia and increased free fatty acids in blood induces insulin resistance. Insulin resistance was characterized by hyperinsulinemia associated with hyperglycemia, they play a major role in cognitive decline and dementia in diabetic patients [38]. Dexamethasone induces hyperglycemia by inhibiting the ability of insulin to activate glycogen synthase and decreases insulin-stimulated protein kinase B (PKB), and glycogen synthase kinase 3 (GSK-3) phosphorylation [149]. Glucocorticoids induces insulin resistance by increasing the hepatic glucose production and decreasing the peripheral utilization of glucose. Studies have shown that dexamethasone also induces oxidative stress that might cause damage to pancreas since pancreatic β cells are more
vulnerable and susceptible to ROS. Dexamethasone treated rats showed hyperglycemia, hypoinsulinemia and peripheral insulin resistance as observed in type 2 DM. Hence it was considered as suitable experimental model for screening insulin resistance [150, 151]. In the present study, insulin resistance was induced in rats by administrating dexamethasone (10 mg/kg/s.c) for 10 days.

Cognitive impairment in diabetes might occur in early stages of diabetic patients and also in insulin resistance patients without diabetes. IR interferes with hippocampus plasticity, metabolism of amyloid precursor protein (APP) and tau protein, and inflammation reaction. Several studies have reported that hyperglycemia induced ROS plays an important role in brain neuronal damage. Peripheral insulin resistance also alters the brain insulin sensitivity leading hyperglycemia. Long term hyperinsulinemia reduces the brain insulin uptake, causes neurodegeneration and impairs the memory [152, 153].

Scopolamine, a cholinergic antagonist is commonly used to induce memory impairment in experimental animals [154, 155]. Studies have shown that scopolamine induced cognitive changes were similar to the clinical features observed in Alzheimer’s disease. Recent studies have shown that the scopolamine might be considered as a psychopharmacological model for Alzheimer’s disease, since it alters the brain network and disrupts the frontal cortex function as in AD. Muscarinic antagonists significantly reverse the changes induced by scopolamine which provides further supports the scopolamine induced cognitive impairment [156]. A low dose of dexamethasone and scopolamine significantly impaired the memory retrieval in animals [108].

Combination of dexamethasone and diazepam was also tried in the present study to enhance the memory impairment induced by dexamethasone, as it is well documented that the sedative effect of benzodiazepines contributes to cognitive impairment [157]. Hence in the study, scopolamine and diazepam were used as a challenging agents at the end of dexamethasone administration to exacerbate the effects of insulin resistance induced cognitive impairment.

In the investigation undertaken, it is to reported that PHF significantly decreased the hyperinsulinemia induced by dexamethasone and improved the insulin sensitivity (HOMA-IR). Studies have shown that insulin resistance in CNS was indicated by
decreased motor coordination, impairment in memory retrieval and energy homeostasis [158]. In the study dexamethasone in combination with scopolamine/diazepam induced behavioral changes were significantly reversed by PHF.

PHF treated rats increased the inflexion ratio in elevated plus maze, decreased the escape latency in Morris water maze and increased the motor coordination on rota rod compared to dexamethasone treated group. The improvement in the behavioural parameters or cognition can be correlated with the serum insulin levels and HOMA-IR index. Our study, thus confirm that IR plays a major role in decline of cognitive function and the prepared PHF was able to improve the cognition.

The link between cholinergic neurons and insulin resistance has been extensively studied. Insulin and Insulin-like Growth Factor (IGF)-1 stimulates the expression of Choline Acetyltransferase (ChAT) in brain especially hippocampus which is mainly involved in the synthesis of acetylcholine and these expressions were found to have decreased Alzheimer’s disease. Insulin resistance impairs the signaling pathway that decreases the acetylcholine levels in hippocampus [159]. Dexamethasone in combination with scopolamine/diazepam groups showed elevated levels of AchE in hippocampus and cerebral cortex. PHF treated groups significantly decreased the levels of AchE compared to dexamethasone treated group, which indicates that PHF has protective effect on acetylcholine and increased the acetylcholine levels. The findings of the study was further supported with histopathological studies of hippocampus. In hippocampus the neuronal degeneration induced by dexamethasone was reversed by PHF by increasing the number of pyramidal cells and regeneration of the neurons in CA3 region [159]. The histopathological findings and AchE levels also support the improvement of cognition caused by PHF, which can be attributed to its ability to reduce IR.

The dexamethasone study protocol doesn’t include any standard drug for comparison as no such standard drug was available in the literature in the STZ and Dexamethasone induced IR model. The traditional standard drugs used in antidiabetic studies viz., glibenclamide and metformin have not been proven to improve cognitive decline in the above mentioned models. Hence, the study was compared with disease control group.
Several studies have shown that the indigenous plants exhibit their action by stimulating the pancreatic β cells thereby increasing the insulin secretion, preventing the absorption of carbohydrates from intestine, decreasing the glucose production by interfering with the enzymes involved in synthesis and inhibiting the glucose transport to the tissues [160-164]. This contributes to the efficacy of ethnomedicinal plants for their use in diabetes mellitus.

In this context few in vitro studies were carried out to explore the probable mechanism of action of selected plants and PHF. Alpha glucosidase and alpha amylase are carbohydrate hydrolyzing enzymes which helps in absorption of glucose from the gut. Postprandial induced hyperglycaemia is responsible for the complications of macro and micro vascular structures in diabetic patients. The drugs which inhibit these enzymes were clinically found to be good on postprandial hyperglycaemia, but gastrointestinal side effects like abdominal pain, flatulence, and diarrhoea are commonly observed. These side effects are inconvenient, embarrassing to the patients and moreover their prices are high. Screening of natural drugs on alpha glucosidase and alpha amylase are gaining potential importance in Ayurvedic medicine [165, 166].

In the study, polyherbal formulation showed appreciable inhibitory activity on these enzymes. Several studies have shown that the inhibitors of these enzymes interfere with the digestion of carbohydrate, their absorption into systemic circulation and delay the postprandial hyperglycemia. Numerous studies support that there exists a positive correlation between total phenol and flavonoid content, and their inhibitory activity on alpha glucosidase and alpha amylase enzymes [167]. The same is also confirmed from the current study as the IC$_{50}$ values in the enzyme inhibition studies were directly proportional to their total flavonoid and total phenolic content. In connection with the earlier reports the PHF might be good in the management of postprandial hyperglycemia.

Extensive studies have shown that acetylcholine plays an important role in learning and memory. Dysregulation in insulin signaling pathway alters cognition and impairs the memory processes. Insulin releases a variety of enzymes involved in glucose metabolism, including choline acetyltransferase (ChAT). The underlying mechanism for insulin resistance and cognitive function is unclear but it interferes mainly with the
hippocampal plasticity. Insulin resistance was characterized by hyperinsulinemia associated with hyperglycemia [108]. Numerous studies have reported that hyperglycemia induces ROS generation by direct glucose oxidation in the neurons which leads to increased production of reactive oxygen species and plays an important role in brain neuronal damage [168]. Hence, in vitro studies were carried out to understand the effect of individual plants and PHF on ROS generation.

Hyperglycemia induced ROS was evaluated in SH-SY5Y neuronal cells. Preclinical SH-SY5Y findings serves as an alternative cellular and animal model for screening several neurodegenerative diseases [169, 170]. Hence in the study, use of the SH-SY5Y cell line is considered. In the present investigation, PHF showed protective effect in MTT assay induced cytotoxicity and cell viability in SH-SY5Y neuronal cells. Several studies revealed that high blood glucose levels by direct glucose oxidation in the neurons produces reactive oxygen species and also facilitates the amyloid beta (Aβ) production by inhibiting amyloid precursor protein (APP) degradation [108, 171].

In the present study glucose at 250 mM concentration induced ROS generation in SH-SY5Y neuronal cells. PHF dose dependently reversed the hyperglycaemia induced ROS. PHF at dose 100µM showed significant effect at 6 hrs compared to control. It shows that PHF has protective role in SH-SY5Y neuronal cells and prevents the damage induced by hyperglycaemia. The in vitro studies confirms that PHF was better than the individual extracts in ameliorating the ROS generated by glucose. These in vivo findings are in correlation with our in vitro studies on SH-SY5Y neuronal cells indicating the protective role of PHF in diabetes associated cognitive impairment.

The present study provides systematic scientific evidence for the antidiabetic effect of traditionally claimed individual plants and their PHF. For the first time we have attempted to study the effect of selected plants and PHF on cognitive impairment associated with diabetes. The study though cannot explain the exact molecular targets of action for PHF, it can be understood that the PHF possessed the antidiabetic activity and also ameliorated the cognitive decline associated with IR by acting at multiple sites. Undoubtedly, the antioxidant mechanism is the prime cause for the pharmacological benefit shown by the PHF. Additionally, the PHF also possess acarbose like mechanism
of action. Hence it was concluded that the polyherbal formulation designed in the current study can be a promising agent for the development as a drug to manage diabetes and associated cognitive impairment.