Diabetes mellitus is reaching as epidemic proportion across the globe, a metabolic disorder characterized by hyperglycemia resulting from defect in insulin secretion, insulin action or both (ADA, 2009). Currently, there are 40 million people with diabetes in India and estimated to rise almost 70 million by 2025 (IDF, 2006). Hyperglycemia is the most important factor in the onset and progress of diabetic complications mainly by producing oxidative stress (Giugliano et al., 1996). Altered cellular metabolism caused by hyperglycemia has been suggested to play an important role in increasing the risk of cardiovascular, renal, ophthalmic and neurological complications of DM (ADA, 2009). In DM, beside hyperglycemia, abnormalities in lipid level which is directly correlated with accelerated atherosclerosis and subsequent CVD is the major cause of death in the world. It has been found that the chronic hyperglycemia is associated with increase production of free radicals and several hypotheses for their genesis have been reported that include oxidation of glucose, constant increase in the formation of glucose derived AGEs and degradation of glycated protein. Membrane lipid peroxidation and protein oxidation are significantly increased in diabetic condition clearly signifying augmented free radical production (Gallou et al., 1993). Increased free radical levels or inefficient free radical scavenging leads to tissue damage that is assessed by the measurement of lipid peroxide and protein carbonyl level (Maxwell et al., 1997). Moreover, disturbance of antioxidant defense system lead to alteration in enzymatic and non-enzymatic antioxidants like impaired glutathione metabolism (McLennan et al., 1991) that are the major cause of both microvascular and macrovascular complications including accelerated form of atherosclerosis due to endothelial dysfunction and microangiopathy of retinal vessels (Foyer and Noctor., 2005). It has been found that in the developing countries where the resources are meager, the best source for the management
of DM is green medicine (Bnouham et al., 2006). Despite of the availability of known antidiabetic medicine in the pharmaceutical sector, diabetes and its related snag continues to be the major medicinal problem. All oral hypoglycemic agents are accompanied with a number of serious, undesirable effects. Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new hypoglycemic, hypolipidemic drug coupled with antioxidant property because of the unmatched availability of chemical diversity (Moller, 2001). The attributed antihyperglycemic effects of these plants are due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or decrease in the intestinal absorption of glucose (Pari and Saravanan, 2004). In addition, hyperglycemia induced oxidative complications are treated and managed by supportive therapies that include the use of antioxidants and natural products (Garg and Bansal, 2000). In the last few decades, plants of genus Phyllanthus (Euphorbiaceae) came in focus due to their wide distribution, diversity in the genus, broad therapeutic potential, and variety in the secondary metabolites (Calixto et al., 1998). This family includes several plant species in which; P. amarus, P. urinaria, P. niruri, P. maderaspatensis, P. virgatus, and P. fraternus are the most popular ones due to their antioxidant properties as well as their extensive use in the treatment of disease related to kidney, liver, urinary bladder, intestinal infection, cancer, and diabetes (Okoli et al., 2009; Mbagwu et al., 2011).

Phyllantus virgatus is rich in polyphenols (Calixto et al., 1998) and is known traditionally for its antioxidant (Kumaran and Karunakaran, 2007), antimicrobial, antiseptic, anti-inflammatory agent (Hemendra and Chouhan, 2011) and anticancer activity (Poompachee and Chudapongse, 2011). The antidiabetic properties of various Phyllanthus species have been investigated in experimental models (Shabeer et al., 2009, Raphael et al., 2002), while
only one study speculated the antidiabetic property of *P. virgatus* (Shabeer *et al.*, 2009). Similarly, another member of this family i.e. *P. maderaspatensis* demonstrated chemoprotective, hepatoprotective and choleretic agent (Sharma *et al.*, 2011; Asha *et al.*, 2004). The extract of this plant possess antioxidant (Raja *et al.*, 2011) and antibacterial (Karthikeyan *et al.*, 2011) activity and also taken as dietary supplement in India.

The present study explores the antihyperglycemic, hypolipidemic and antioxidant potential of *P. maderaspatensis* L. and *P. virgatus* Frost extracts and their possible bioactive constituents via *in vitro*, *in silico* and *in vivo* study. The first part of this thesis intends to investigate and correlate the antioxidant, genoprotective protective, α-amylase and α-glucosidase inhibitory activity as well as glucose uptake property of various sequentially extracted fractions of *P. virgatus* and *P. maderaspatensis*. The fraction which exhibited most potent therapeutic activity was processed further for identification of biochemical compounds as well as investigation of their hypoglycemic and antioxidant potential.

In the second part of the thesis the bioactive compound responsible for the aforesaid *in vitro* antidiabetic and antioxidative property has been isolated and identified via GC-MS analysis. In addition the therapeutic role of *P. virgatus* extract and its partially purified fraction in alleviating the condition of STZ-induced diabetes, diabetes linked hyperlipidemia and oxidative stress in rats has also been delineated. Moreover, the preventive role of these fractions in ameliorating the lipid per oxidation by products, antioxidant enzyme activity as well as in protecting the retinopathy and nephropathy in STZ-induced diabetic stressed rats has been studied.

In our study, a sequential extraction, involving the solvent of decreasing polarity to extract the bioactive compounds was used because the nature, polarity and hence the solubility of the bioactive compounds in *P.*
virgatus and P. maderaspatensis were unknown. The results initially indicated that among all the sequentially extracted fraction of P. virgatus and P. maderaspatensis the methanol extract of both the plant showed significant presence of polyphenol and also exhibited total phenol content. The results are in agreement with previous reports that demonstrate marked presence of polyphenols and TPC content in P. virgatus and P. maderaspatensis methanol extract (Sharma et al., 2011). It is noted that a solvent system for extraction is selected according to the purpose of extraction such as preparation or analysis. Also, it was chosen according to the nature of interested components, the physicochemical properties of the matrix, the availability of reagents and equipments, cost, and safety concerns. Absolute ethanol and 50 % acetone have been used to prepare antioxidant extracts and 70 % methanol is widely accepted solvents for extracting phenolic compounds (Yu et al., 2002). Table 4.4 illustrated significantly higher FRAP value in the methanol extract of P. virgatus in comparison to P. maderaspatensis, which is even greater than the standard ascorbic acid. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity and generally correlates with the presence of reductones, which have been shown to exert antioxidant activity by breaking the free radical chain and donating a hydrogen atom (Narasimhudu and Raju, 2011). Phenolic compounds have been said to account for most of the antioxidant activities of plant extracts (Dai and Mumper, 2010) and thus antioxidant activity of methanol extract would be granted to these polyphenolic compounds. Among the various fraction of both the plant extracts, our results observed high linear correlation ($R^2 =0.942$) between the FRAP value and TPC of various extracts of P. virgatus indicating that the antioxidant activity of the extract is mainly due to its phenolic content. It is well known that compounds capable of scavenging free radicals can delay, inhibit, or prevent the oxidation of various
biomacromolecules and diminish the oxidative stress, which play major role in the development of several diseases like diabetes (Haslam, 1996; Gutteridge 1994; Singh et al., 2009). Our results demonstrated that methanol extract of *P. virgatus* and *P. maderaspatensis* exhibited strong DPPH radical scavenging activity with respect to other extracts (Table 4.6, 4.7), which in turn signifies their potent antioxidant activity. These results are in concordance with earlier studies where *P. virgatus* and *P. maderaspatensis* methanol extract showed higher DPPH radical scavenging activity (Raphael et al., 2002; Sampath et al., 2012). A great number of *in vitro* experiments showed that hydroxyl radical, the most reactive among ROS, has the capacity to damage DNA which appears to represent the major target involved in mutagenesis, carcinogenesis, diabetes, and so forth (Finkel and Holbrook, 2000; Singh et al., 2009). Thus, on the basis of above data it has been concluded that methanol and water extracts of both the plants showed significant antioxidant activity when compared to other fractions. Therefore, we initially evaluated the role of methanol and water extract of *P. virgatus* and *P. maderaspatensis* in directly scavenging and in protecting the DNA damage caused by hydroxyl radicals. The degradation of deoxyribose to TBARS by hydroxyl radicals was markedly decreased by methanol and water extract. The observed IC_{50} value of *P. virgatus* methanol extract indicates that this extract is a better hydroxyl radical scavenger than standard mannitol. There are several reports indicating that various antioxidants present in plant are good scavengers of hydroxyl radicals (Zhang et al., 1996), including only one report from Phyllanthus sp., that is *P. maderaspatensis* (Asha et al., 2004). Damage of plasmid DNA due to hydroxyl radical resulted in single- and double-strand break. Studies have identified potent antioxidants from plants that are effective against DNA damage (Singh et al., 2009). *P. virgatus* was also known for its potent antioxidant property (Poompachee and
Chudapongse, 2011), and our preliminary result showed that the DNA damage induced by hydroxyl radical was significantly ameliorated by *P. virgatus* methanol extract (Figure 4.6) and is almost comparable with standard mannitol. In contrast, methanol extract of *P. maderaspatensis* observed partial protection against oxidative DNA damage. These observations are well correlated with the reports of Kumaran and Karunakaran 2007 that also depicted DNA damage protective property of polar fractions of *P. virgatus* and *P. maderaspatensis*. The above genoprotective action of *P. virgatus* methanol extract was maybe due to its strong antioxidant activity, which prevents the reaction of Fe$^{2+}$ ions with H$_2$O$_2$ or through mechanism including quenching of ROS by donating H atom. The result illustrating the potent oxidative DNA damage protective activity of methanol extract is well correlated with results of our various antioxidant parameters, illustrating the greater ROS-quenching capacity by methanol extract, which in turn indicates that this extract may be used as therapeutic agent in treating ROS-related pathological conditions including T2DM.

There are several therapeutic approaches to decrease postprandial hyperglycemia one of which is retarding the absorption of glucose through inhibition of carbohydrate hydrolyzing enzymes either α-amylase or α-glucosidase (Laar *et al.*, 2008; Cheng and Fantus, 2005). Alpha-amylase is involved in the breakdown of long chain of carbohydrates and α-glucosidase breaks down starch and disaccharides to glucose. They serve as the major digestive enzymes and help in intestinal and hepatic portal vein absorption. Alpha-amylase and α-glucosidase inhibitors are the potential targets in the development of lead compounds for the treatment of diabetes (Smith, 2005). Presently used oral antihyperglycemic agents have however a risk of inducing hypoglycemia and lose their efficacy over time, and they have prominent side effects and fail to significantly alter the course of diabetic complications.
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(Cheng et al., 2005). The management of diabetes without any side effects is still a challenge; therefore, WHO has recommended research and use of complementary medicines from plants for the treatment and management of this disease (Tiwari, 2005). Our investigation provides the first evidence of \( \alpha \)-amylase and \( \alpha \)-glucosidase inhibitory property of sequentially extracted \textit{P. virgatus} and \textit{P. maderasptensis} fractions. The initial screening of all extracts of \textit{P. virgatus} and \textit{P. maderasptensis} demonstrated some \( \alpha \)-amylase inhibitory activity with maximum activity exhibited by \textit{P. virgatus} methanol extract. In addition, this extract also exhibited a concentration-dependent increase in percent inhibition of \( \alpha \)-amylase activity with an IC\(_{50}\) value of 33.20±0.556 \( \mu \)g/ml, which is quite better than the IC\(_{50}\) value of standard acarbose 76.88±0.277 \( \mu \)g/ml. This observation suggests that porcine pancreatic \( \alpha \)-amylase is inhibited by more polar constituents of \textit{P. virgatus}, which is in agreement with other studies that reported \( \alpha \)-amylase inhibitory activity in the more polar extracts of plant materials (Bhandari et al., 2008; Jung et al., 2006). Thus, the enzyme inhibitory activity of methanol extracts could be due to the presence of polyphenols, flavonoids, and their glycosides, which are known to be soluble in more polar solvents. Enzymes inhibitors obeying Michaelis-Menten kinetics are often characterized in terms of their effects on the kinetic constants, \( K_m \) and \( V_{\text{max}} \), using either Lineweaver-Burk plots or Dixon secondary plots. In the current study, \textit{P. virgatus} methanol extracts demonstrated non-covalent type of noncompetitive (\( V_{\text{max}} \) decreased whereas \( K_m \) remained the same) mode of inhibition against porcine pancreatic \( \alpha \)-amylase, whereas acarbose was competitive in nature. These observations might suggest that the \( \alpha \)-amylase inhibitory components of methanol extracts do not resemble the normal substrates of the enzymes in structure (Mogale et al., 2012). The \( \alpha \)-amylase is one of the major secretory products of the pancreas and salivary glands, playing a role in digestion of
starch and glycogen and can be found in microorganisms, plants and higher organisms (Kandra, 2003). This enzyme catalyses the initial step in hydrolysis of starch to mixture of oligosaccharides consisting of maltose, maltotriose, and branched oligosaccharides of 6–8 glucose units that contain both α-1,4 and α-1,6 linkages. These are further degraded to glucose by α-glycosidase which on absorption enters bloodstream. Further, the mechanism of inhibition of acarbose seems to be due to the unsaturated cyclohexene ring and the glycosidic nitrogen linkage that mimics the transition state for the enzymatic cleavage of glycosidic linkages (Piparo et al., 2008). It has been previously shown that acarbose is a competitive inhibitor of α-amylase, which is in strong agreement with our results (Mogale et al., 2012).

Similarly, initial screening of *P. virgatus* and *P. maderasptensis* extracts for α-glucosidase enzyme inhibitory activity demonstrated that among all the fraction of both the plants *P. virgatus* methanol extract showed better enzyme inhibitory activity with an IC$_{50}$ value of 22.36±0.78 µg/ml in comparison to the standard acarbose (IC$_{50}$ 15.18±1.21 µg/ml). On the other hand the mode of inhibition of *P. virgatus* methanol extract against α-glucosidase was also determined by the Lineweaver-Burk plot shown in Figure 4.15. The plant extract showed non competitive inhibition (apparent Km is constant and apparent Vmax is decreased) which suggest that the compounds present in the methanol extract of *P. virgatus* do not resemble with the enzyme substrate in structure, while acarbose showed competitive inhibition against α-glucosidase (Bischoff, 1995). Based on the observations it was found that α-glucosidase is inhibited mostly by the polar or medium polarity chemical components which is well in agreement with other reports that depicted the same findings (Jung et al., 2006; Andrade-Cetto and Wieldifield 2004).
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Postprandial blood glucose level is known to be regulated by glucose uptake, a rate limiting step for glucose metabolism. In the present study, we used differentiated 3T3-L1 cell lines because it was previously established that glucose uptake was higher in these cells than in undifferentiated one, which is probably due to the presence of GLUT4 in their expression (Eldar-Finkelman and Krebs, 1997). Our result, for the first time, speculated that *P. virgatus* methanol extract possesses the ability to improve glucose uptake in the adipose tissue. The positive controls chosen for glucose uptake in 3T3-L1 cells due to their antidiabetic activity were metformin and insulin, as they are known specifically for the affirmative effect on the translocation of GLUT4 to the cell surface thereby promoting glucose uptake. Insulin and metformin significantly promote the glucose uptake alone, and there was also significant synergistic effect when given in combination (in presence or absence of extract). On this basis, a mechanism of action of *P. virgatus* may be hypothesized, which could be linked to insulin mediated glucose transport transduction pathway in which a series of proteins (phosphatidylinositol-3 kinase, protein kinase C, and PPAR) are involved (Eldar-Finkelman and Krebs, 1997; Zierath and Wallberg-Henriksson, 2002). This may perhaps lead to the translocation of GLUT4 to the plasma membrane to facilitate the uptake of glucose from the bloodstream into the cells. Thus, the occurrence of polyphenolic compounds in methanol extract may be responsible for the activation of these signaling proteins (Yang *et al.*, 2003) and might therefore also account for their up regulation of these proteins, which in turn is responsible for its glucose uptake activity. Further, the methanol extract of *P. virgatus* was subjected to MTT assay on 3T3-L1 preadipocytes cell line and it was found that the methanol extract of *P. virgatus* was noncytotoxic at various concentrations.
In a search for the source of bioactive compounds responsible for the above actions of *P. virgatus* methanol extract, preliminary GC-MS analysis was performed. For the first time, it was noted that the sequentially extracted *P. virgatus* methanol fraction contains phthalic acid, asarone, acrylic acid, palmitic acid, linoleic acid, 11-octadecenoic acid, and 6-octadecynoic acid (Table 5). Various species of Phyllanthus reported the presence of these compounds with antioxidant, antidiabetic activity and anticancer (Calixto *et al.*, 1998; Rajeshkumar *et al.*, 2002).

From the above data, it may be concluded that these bioactive compounds of *P. virgatus* methanol extract alone or in combination possess significant antioxidant activity, which could be responsible in ameliorating all the above oxidative damages, including inhibition of glucose metabolizing enzyme (α-amylase, α-glucosidase) activity. Moreover, our *in silico* investigation is a novel approach to identify the molecular targets involved in inhibition of α-amylase and α–glucosidase activity by this extract. Protein-ligand interaction is comparable to the lock-and-key principle, in which the lock encodes the protein and the key is grouped with the ligand. The major driving force for binding appears to be hydrophobic interaction (Kubinyi, 1998). *In silico* techniques helps in identifying the drug target via bioinformatics tools. They can also be used to explore the target structures for possible active sites, generate candidate molecules, dock these molecules with the target, rank them according to their binding affinities, and further optimize the molecules to improve binding characteristics (Amuthalakshmi and Anton, 2013). Similarly, an initiative was taken to study the binding pattern and mode of inhibition of the compounds against α-amylase and α-glucosidase enzymes via *in silico* study. We previously revealed the implication of molecular docking studies in elucidating the mechanistic aspect of natural products against different enzymes (Iqbal *et al.*, 2014a, Iqbal *et al.*, 2014b;
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Molecular simulation study is considered to be an important vehicle to investigate the mode of interaction of ligand against its target protein that also makes us understand their binding or inhibition mechanism. Validation of docking protocol was performed by redocking co-crystallized acarbose into its respective binding site within porcine pancreatic α-amylase and α-glucosidase. As already discussed, these are the key enzymes of dietary carbohydrate digestion in humans, inhibitors of these enzymes may be effective in retarding carbohydrate digestion and glucose absorption to suppress post prandial hyperglycemia (Kandra, 2003). α-glucosidase is an attractive target for treatment of T2DM and obesity.

Redocked inhibitor was found to interact with the same amino acids of the active site of α-amylase and α–glucosidase as were in the crystal structure with RMSD of 1.58 °A and 1.55 °A respectively, between the conformations (Figure 4.19, 4.21). Our results demonstrated that 9,12-octadecadienoic acid (linoleic acid) was the most potent inhibitor of pancreatic α-amylase, whereas 11-octadecenoic acid, 2,4,5-trimethoxy propenyl (asarone), and tridecyl ester also showed good inhibitory activity in terms of their binding energy. These results are well supported by wet lab studies where asarone, linoleic acid and acrylic acid have been reported to exhibit the antidiabetic property (Ryder et al., 2001). However, results for α-glucosidase demonstrated that phthalic acid, hexadecanoic acid, and asarone were strongly interacted with the enzyme and inhibited its activity, as clearly shown in Figure 4.22, in accordance with their binding energy. Among all the seven compounds, phthalic acid and hexadecanoic acid are known antidiabetic compound (Sivajothi et al., 2007) and gave the high binding activation energy as shown in Table 4.13. Figure 4.20 illustrated that among all the compounds 9,12-octadecadienoic acid with binding energy of -6.11 Kcal/mol was anchored at the non catalytical site, whereas 11-octadecenoic acid and asarone were found very well postioned
with catalytical site (Asp197 and Glu233) of the α-amylase enzyme. Hence the data consensus with the *in vitro* kinetic study results, signifying 9,12 octadecadienoic acid to be the compound responsible for non competitive inhibition.

Similarly, Figure 4.22 clearly showed catalytical and non-catalytical binding pattern of the chemical compounds, identified via GC/MS analysis, against α-glucosidase with functional residues Asp 542 and His 600. Among all the seven compounds phethalic acid with highest binding energy (-4.64 Kcal/mol) was found anchored with the non catalytic site of the enzyme which may be the major compound responsible for non competitive inhibition, while as hexadecinoic acid and asaron, were found anchored with the catalytical binding site as standard acarbose this might explain why the enzymatic activity of α-glucosidase were successfully blocked. Thus, it is very difficult to name a single compound responsible for the whole activity. Therefore, based on our *in vitro* and *in silico* results, we suggest that the α-amylase and α-glucosidase inhibitory activity of *P. virgatus* methanol extract might be because of the synergistic effect of these compounds against both the enzymes.

Thus, on the basis of above discussion, it has been concluded, till now, that among all the sequentially extracted various fraction of *P. virgatus*, methanol extract showed the most potent antioxidant, genoprotective, α-glucosidase and α-amylase inhibitory activity as well as the extract also illustrated enhanced glucose uptake in pre-adipose 3T3-L1 cell line and was found to be non cytotoxic. In addition, the preliminary GC-MS analysis of this extract revealed several bioactive compounds and their inhibitory mechanism was well validated via *in silico* analysis (Hashim *et al.*, 2013).

In continuation, the second part of the thesis aims to identify and isolate the partially purified fraction as well as to examine their role in STZ-induced
diabetes and diabetes linked hyperlipidemia in rats. *P. virgatus* methanol extract was subjected to repetitive preparatory TLC in array to isolate partially purified bioactive fraction. Previous study also demonstrated the use of TLC mediated isolation of partially purified fraction for *in vivo* study (Gaytri *et al*., 2010). Our TLC data illustrated the presence of six prominent bands and the preliminary screening of these bands against α-amylase and α-glucosidase inhibitory activity showed that the 4th band has marked inhibitory property with an IC$_{50}$ value of 48 µg/ml and 25.96 µg/ml, respectively, which depicted that this fraction might be responsible for the antidiabetic activity of sequentially extracted methanol fraction. Our initial screening for the identification of bioactive compound in this fraction, via GC-MS analysis, showed the presence of benzoic acid, hexadecanoic acid, 9-octadecanoic acid and octadecanoic acid compounds, which are in agreement with the previously published reports indicating the presence of these compounds with antioxidant and antidiabetic properties (Kumar *et al*., 2010). The occurrence of these chemical compounds in partially purified fraction is in agreement with the compound present in *P. virgatus* methanol extract, that also illustrated the existence of these compounds as discussed above. Among these compound hexadecanoic acid has already been known to play important role in biological process (Aleryani *et al*., 2005).

Streptozotocin is known to induce diabetes in animal models along with hyperlipidemia/atherosclerosis, diabetic nephropathy, retinopathy, and neuropathy (Ebara *et al*., 1994). Streptozotocin has been chosen as an agent that induces hyperglycemia due to its selective destruction of pancreatic β-cell and DNA alkylation by entering into β-cell via GLUT-2 transporter, thus induces the activation of poly ADP-ribosylation which in turn leads to depletion of cellular NAD$^+$ and ATP. Therefore, increased dephosphorylation of ATP supplies substrate for xanthine oxidase resulting in generation of free
radicals. Thus, STZ action results in destruction of β-cells by necrosis and consequence in characteristic alteration in blood insulin and glucose level which in turn leads to hyperglycemia.

Hyperglycemia has been known to depress natural antioxidant system and enhanced oxidative stress that has been well documented in experimental and human DM (Baynes, 1991). Lipid peroxidation has been co-existed with chronic hyperglycemia due to increased carbonyl stress (Baynes and Thorpe 1997) which in turn increases peroxy radical and hydroxyl radical formation. Thus, oxidative stress induced by lipid peroxidation is one of the characteristic features of chronic uncontrolled diabetes. Since, DM is related with increased propensity to accelerated atherogenesis, and is directly correlated with abnormalities in lipid profile, which are one of the major contributing causes of micro and macrovascular complications in diabetic patients (Giugliano et al., 1996). Oxidative stress also plays an important role in diabetes that results in increased glycation and increased oxidation of lipoprotein (Bowie et al., 1993), particularly LDL, which could be one of the mechanisms for an early development of atherosclerosis in diabetes.

It has been previously reported that STZ selectively destroy the pancreatic cells that secrete insulin and results in substantial hyperglycemia as well as also result in weight loss, ketosis and a high rate of mortality (Arulmozhi et al., 2004; Szkudelski, 2001). Since, it has been shown that hyperglycemia is directly associated with decreased body weight of diabetic animals (Zafar and Naqvi, 2010), our data is in agreement with this study and also demonstrated a decline in the body weight of diabetic rats, that could be due to muscle destruction or degeneration of structural proteins (Salahuddin and Jalalpure, 2010). Repeated oral administration of methanol extract and its partially isolated fraction showed gain in body weight. Among the treated group, CT-1 group showed maximum gain in body weight followed by CT-2 > PET-1 >
PET-2 groups which may be due to the reversal of hepatic gluconeogenesis accompanied with the suppression of lipolysis of adipose tissue (Robertson, 2007; Postic et al., 2001). The present data revealed that fasting plasma glucose level was significantly increased and insulin level was decreased in STZ-induced diabetic rats in comparison to rats in NC group. Among all the treatment groups, the partially isolated fraction (CT-1 group) of plant extract at a dose of 0.5 mg/rat/day for 28 days showed a significant decrease in the plasma glucose level and increase in insulin level. The plant extract and partially purified fraction being a potent free radical scavenger and inhibitor of lipid peroxidation (Hashim et al., 2013), might prevents STZ-induced oxidative stress and protects or regenerate insulin producing cells resulting in increased insulin secretion and decreased blood glucose levels. At present, the finding of this study is in agreement with previously published data which illustrated the antioxidant and antihyperglycemic effect of P. simplex crude methanol extract (Shabeer et al., 2009). The extent of hemoglobin glycation is currently used as a cumulative index of glycemia over the previous few weeks in the clinical management of diabetes (Saudek and Rastogi, 2004). In diabetes, there is a formation of AGEs products when glucose interact with various proteins such as hemoglobin, albumin, collagen, LDL, or crystalline proteins to form labile Schiff bases, which further undergoes modification to form Amadori products (Klein, 1995; Singh et al., 2001; Ahmad et al., 2013). Evidence showed that glycation itself may induce the formation of oxygen-derived free radicals in diabetic condition, and the level of HbA1c is considered as one of the markers of degree of oxidative stress in DM (Bravi et al., 2006). Therefore, the measurement of HbA1c is supposed to be a very sensitive index for glycemic control. Our results demonstrated a substantial decrease in hemoglobin and a significant increase in HbA1c levels in diabetic rats, which is in agreement with earlier finding (Nathan et al., 2007). In the
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Present investigation, rats in DC group showed higher levels of HbA1c, compared with those of control group. After 28 days of treatment with P. virgatus methanol extract and its partially isolated fraction, both Hb and HbA1c levels were significantly ameliorated close to normal control values with a marked restoration in CT-1 group (54 & 66 %), which could be due to an improvement in hyperglycemia. Present findings bear a resemblance with previously published data that showed the treatment of Phyllantus sellowianus (Hnatyszyn et al., 1997) and Phyllanthus emblica ethanolic (Krishnaveni et al., 2010) extracts to STZ-induced diabetic rats significantly ameliorated the glycated hemoglobin and blood glucose.

Another biochemical parameter that is affected due to altered insulin level is hepatic glycogen, which is a storage form of glucose and its activity is a direct reflection of insulin level. As insulin level promotes intracellular glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase. Previous studies also demonstrated the decrease in hepatic glycogen activity and increase insulin level in STZ-induced diabetic rats (Whitton and Hems, 1975). During diabetes the glucose synthesis in rat liver is altered which indicate the diminished hepatic glycogen content in diabetes. The uptake and storage of glucose in liver is regulated via glycogenesis and the glucose is release by activating glycogenolysis and gluconeogenesis (Begum and Rajini, 2011). Consistent with these reports, our data also exhibited marked decrease in hepatic glycogen level in STZ-induced diabetic rats. Oral administration of plant extract and its partially purified fraction to STZ-induced diabetic rats significantly increased the liver glycogen content by stimulating the remnant β-cells to release insulin. The ability of CT-1, followed by CT-2, PET-1 and PET-2, to ameliorate glycogen level suggests that it has the potential to be a lead antidiabetic agent.
In diabetes there was major alteration in carbohydrate, lipid and protein metabolism such as protein glycation, dyslipidimia and lipid peroxidation (Bakker et al., 2000). Our result indicating a decline in TPC in STZ-induced diabetic rats which might be due to microproteinuria, which reflect as a significant systemic indicator of diabetic nephropathy, or to increased protein catabolism (Daisya, 2010). *P. virgatus* methanol extract and its partially purified fraction significantly increase the total protein content in liver tissue of diabetic rats which is almost comparable or better than the restoration exhibited by glibencalimide. Moreover, another important carbohydrate metabolic and insulin dependent enzyme i.e. hexokinase was found to be decrease in STZ-induced diabetic rats which is in agreement by the reports of other workers (Gandhi and Sasikumar, 2012; Bera et al., 2013). Simultaneous administration of *P. virgatus* methanol extract and its partially purified fraction (CT-1) resulted in a significant recovery in the activity of this enzyme that may be correlate another possible way of its antidiabetogenic activity.

Diabetes mellitus is also related with increased propensity to accelerated atherogenesis, and is directly correlated with abnormalities in lipid profile, which are one of the major contributing causes of micro and macrovascular complications in diabetic patients (Ono et al., 1998; Giugliano et al., 1996). Dyslipidemia that include elevated serum TC, LDL-C and TG as well as low level of HDL-C is one of the most common complication of DM that are mediated through derangement in various regulatory process, especially insulin deficiency, which make diabetic patients more prone to hyperglycemia and hypertriglycerideridemia (Begum and Rajini, 2012). Oxidative stress also plays an important role in diabetes that results in increased glycation and increased oxidation of lipoprotein (Bowie et al., 1993), particularly LDL, which could be one of the mechanisms for an early development of atherosclerosis in diabetes. Thus, it is worthwhile to evaluate
the role of this plant and their bioactive fraction in the prevention of hyperlipidemia, which is directly related with STZ-induced diabetes in rats. As expected, our data showed a marked increase in TG, TC, and non-HDL-C levels in diabetic-hyperlipidemic rats when compared to normal control rats. The observed increase in cholesterol level of diabetes was may be due to increase in 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase activity and is in concordant with several reports that showed increase HMG-CoA reductase activity in diabetic rats (Prince and Kannan, 2006; Rydgren and Sandler, 2009). In addition, this increase in cholesterol level has also been shown to be associated with the deficiency in insulin which can be due to enhanced mobilization of lipid from adipose tissue to plasma (Karthikesan et al., 2010). After 28 days of treatment with plant extract and partially purified fraction (at different doses), a significant reduction was observed in TG, TC and non-HDL-C level. Similarly, VLDL-C, LDL-C and HDL-C levels were significantly increased in diabetic rats, which were significantly reduced after treatment with plant extract, partially purified compound or glibenclamide. Our data showing an increase in plasma HDL-C level in DC group is consistent with other reports, where a significant increase in HDL-C level in diabetic rats has been reported (Ebara et al., 1994; Kobayashi et al., 2000). Further, glibenclamide treated group also depicted reduction in plasma lipid and lipoprotein level and is consistent with earlier findings (Tamai et al., 1981), but the reduction was much less as compared to CT-1 & CT-2 group. Moreover glibenclamide is associated with severe side effects like mental status, seizure, coma and death (Burge et al., 1998; Sills et al., 1997).

It has been established that LDL-C/HDL-C and HDL-C/TC ratios are good predictors for the presence and severity of CAD (Drexel et al., 1992). In addition to LDL/C/ HDL-C and HDL-C/TC ratios, we have also presented the ratios of HDL-C/LDL-C and TC/HDL-C, which were markedly increased in
DC rats, respectively, in comparison to the corresponding ratios in NC rats, as well as these data are in resemblance with previous reports (Heidarian and Soofiniya, 2011). Treatment with plant extract and partially purified fraction resulted in a significant improvement in these ratios, indicating normalization of above lipid and lipoprotein parameters. The hypolipidemic activity of plant extract and partially purified fraction might be attributed to the decrease in HMG-CoA reductase activity which could be due to an enhance in insulin level along with an associated amelioration of both glucose and lipid levels. There are several in vitro and in vivo reports which indicated that reduction in the enzymatic activity of HMG-CoA reductase by plant extract is directly associated with decrease in plasma lipid and lipoprotein levels (Iqbal et al., 2014a, b; Khan et al., 2011). Here, it is interesting to mention that same fraction (CT-1) which illustrated most potent hypoglycemic activity, also showed remarkable hypolipidemic property in vivo. These finding are in concordant with previously published data which illustrated the hypolipidemic activity of various species of Phyllanthus in STZ-induced diabetic rats (Okoli et al., 2010; Mbagwu et al., 2011). Thus, in addition to glycaemic control, extract of this plant may further reduce mortality from complications of the disease by ameliorating diabetes-induced hyperlipidemia. This profound activity of CT-1 group might be attributed to the synergistic effect of bioactive compounds viz. hexadecanoic acid and octadecanoic acid, present in this fraction. From the data presented above, it has been extracted that methanol fraction of this plant and their partially purified compound exert potent hypoglycemic and hypolipidemic activity. Moreover, the methanol extract at higher dose (50 mg/rat/day) gave good response, while the partially purified fractions at a dose comparable to standard (0.5 mg/ rat/day and 0.1 mg/rat/day) illustrated improved
hypoglycemic and hypolipidemic activity than the glibenclamide treated diabetic rats.

Hyperglycemia induced metabolic disorder leads to oxidative stress which is known to be a major contributor of ROS production that in turn promote and lead to the lipid peroxidation in the structure of cell membrane which finally affects its functions. Due to increase oxidative stress, that leads to enhanced generation of free radicals in DM results in sharp reduction of antioxidant defense system (Ahmed and Goldstein, 2006). The enhanced oxidative stress is also found to be involved in the pathogenesis and progression of diabetic issues. Since, the role of *P. virgatus* methanol extract and its partially isolated fraction in alleviating the condition of hyperglycemia in STZ-induced diabetic rats, was discussed above, this part of thesis was intended to evaluate the protective role of these fraction in STZ-induced oxidative stressed-diabetic rats.

Elevated glucose level has been known to depress natural antioxidant system and enhanced oxidative stress that has been well documented in experimental and human DM (Baynes, 1991). Lipid peroxidation has been co-existed with chronic hyperglycemia due to increased carbonyl stress (Baynes and Thrope, 1997) which in turn increased peroxy radical and hydroxyl radical formation. Thus, oxidative stress induced by lipid peroxidation is one of the characteristic features of chronic uncontrolled diabetes. When the lipid peroxides formed and gathered to a certain degree, they escape from the organ or tissue in to the blood stream and increase the lipid peroxide level in the blood. Thus, the increased blood lipid peroxide level obviously indicates the occurrence of some membrane damage in cells of some organ or tissue provoked by diabetes. Accordingly, the blood lipid peroxide level also indicates the severity of the disease (Yagi, 1987). Concomitant with these reports, our data also showed an increase in plasma conjugated diene, lipid
hydroperoxide and MDA level in STZ-induced diabetic-stressed rats. The increase in plasma lipid peroxidation product is closely associated with significant decline in plasma total antioxidant level. Administration of *P. virgatus* methanol extract and its partially purified fraction to diabetic stressed rats significantly decreases the level of lipid peroxidation byproducts and increased the total antioxidant power which could be due to improve glycemic control. Our results are in agreement with previously published reports which indicated that oxidative stress in diabetes coexists with a reduction in the antioxidant power (Seghrouchi *et al.*, 2002).

Erythrocytes of diabetic patients are exposed to continuous oxidative stress because oxygen radicals are continuously generated by auto-oxidation of hemoglobin (Ahmet *et al.*, 2001). It is known that the oxygen radicals formed in excess of detoxifying capacity of erythrocytes will in turn attack the polyunsaturated fatty acid of lipid membrane, which results in MDA accumulation. Our results show a greater susceptibility of hydrogen peroxide induced lipid peroxidation in erythrocytes of diabetic rats than those from normal rats indicating massive oxidative stress in STZ-induced rats. These results are consistent with previously published reports indicating that erythrocytes of diabetics are more prone to lipid peroxidation when treated with \( \text{H}_2\text{O}_2 \) (Rajeswari *et al.*, 1991). Furthermore, *in vitro* MDA release from erythrocytes were significantly blocked in all the treated group, with the maximum reduction observed in CT-1 group followed by CT-2, PET-1 and PET-2 group. Similar to increase in plasma lipid peroxidation products, liver and kidney lipid peroxidation products were also significantly increased in STZ-induced diabetic-stressed rats. These results illustrated that treatment with partially purified fraction at higher dose (CT-1) causes significant reduction in the level of MDA, conjugated dienes and hydroperoxides formation in liver and kidney tissues followed by CT-2, PET-1 and PET-2
groups. *P. virgatus* is known previously for its antioxidant property (Hashim *et al*., 2013; Shabeer *et al*., 2009; Kirtikar and Basu, 1933; Calixto *et al*., 1998; Kumaran and Karunakaran, 2007) including antiperoxidative effect of partially purified compounds in CT-1 which might be due to presence of hexadecanoic acid, octadecinoic acid and linolenic acid (Basu *et al*., 2013; Hashim *et al*., 2013). In agreement with these reports the decrease level of lipid per oxidation product in this study was apparently due to increase consumption of antioxidant. Moreover, our results showed inverse association between lipid peroxidation product and total antioxidant level in various treated group (CT-1, CT-1, PET-1, PET-2), which further supports the potent antioxidative role of *P. virgatus* methanol extract and its partially purified fraction. Shabeer *et al*., (2009) also showed the similar results indicating the role of crude *P. virgatus* methanol extract in ameliorating the oxidative stress condition induced by STZ in rats.

Our results indicating the erythrocytes membrane and tissue damage of STZ-induced diabetic rats, are in consensus with previously published reports (Gutteridge, 1994; Kolanjiappan *et al*., 2002). However, the exact mechanism by which increased blood glucose leads to lipid peroxidation of erythrocytes and tissues of diabetic subject is not well known. Though based on our results it seems plausible that free radicals formed over and above the detoxification capacity can attack the membrane phospholipid and causes lipid peroxidation. This free radical mediated peroxidation of membrane fatty acid is significantly blocked in STZ-induced diabetic rats treated with antioxidant *viz* *P. virgatus* and its bioactive compounds.

The function of ROS in causing cell injury or death is increasingly recognized. The mechanism that relates diabetes and ROS generation is the increase concentration of glucose resulting in oxidative stress including autoxidation of glucose, the non enzymatic glycation of proteins, and the
increased production of glucose derived from AGEs and activation of polyol pathways. In the polyol pathway the NADPH necessary co-factor for GPx activity, is utilized causing increase concentration of NADH, which is essential for the activation of enzyme NADH oxidase that causes oxidative stress (Lorenzi, 2007). Vital organs are capable for antioxidant defence mechanism by their concerted action of both antioxidant enzyme and non enzymatic antioxidants. The role of non enzymatic antioxidant, glutathione, is to protect the cellular system against deleterious effects of lipid peroxidation and involved in the repair of free radical mediated biological damage. The level of reduced glutathione was decreased in diabetic condition and may alter antioxidant enzymes (Baynes and Thorpe, 1997). Consensus with these reports, we observed a decrease in level of enzymatic (SOD, CAT, and GPx) and non-enzymatic (GSH) antioxidants in STZ-induced diabetic-stressed rats with the subsequent increase in lipid peroxide level of liver and kidney tissue. These changes might be attributed due to glucose oxidation, free radical generation and nitric oxide donor property of STZ (Daisya, et al., 2010).

In diabetes, the activity of GPx is significantly decreased by generation of superoxide radical as well as by glycation reactions. GPx along with GSH catalyzed the reduction of H$_2$O$_2$ into nontoxic metabolites. Moreover, the reduced GSH concentration decreased the GPx activity. As NADPH necessary for GSH regeneration was utilized by the polyol pathway which is prominent in chronic hyperglycemic conditions, there occurs a depletion of GSH resulting in lowered GPx activity (Lorenz, 2007). Similarly, the activity of GST activity was decreased due to depletion in GSH because it acts as a substrate for the GST activity (Rathore et al., 2000). In addition, SOD and CAT plays a significant role in eliminating the toxic free radicals induced by STZ. The reduced activity of hepatic and renal SOD and CAT in diabetic rats is in agreement with previous report (Srinivasan and Pari, 2012). The removal
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of hydroxyl radical is the most effective defense mechanism against diseases. SOD is known to converts the superoxide radicals into H₂O₂ and molecular oxygen, while CAT protected the tissues from highly reactive hydroxyl radical through catalyzing the reduction of hydrogen peroxides. The results indicating reduced activities of enzymatic antioxidants during diabetes might result in a number of other deleterious effects due to the accumulation of free radicals. Enzymatic activities of hepatic and renal GPx, Gred, GST, CAT, and SOD level as well as hepatic GSH content were maximally increased after treatment with CT-1 in STZ-induced diabetic rats followed by CT-2, PET-1 and PET-2 groups, respectively. These results revealed that CT-1 prevent pathological alteration caused by hydroxyl radicals which is due to free radical scavenging activity and are in concordant with previously published report (Shabeer et al., 2009). Thus among all the treated groups CT-1 significantly ameliorate the enzymatic and non-enzymatic antioxidant levels.

Previously published report clearly indicates the role of free radical scavengers in alleviating the altered level of antioxidant enzymes including GSH in diabetic rats (Jones, 2002).

Long term damage, dysfunction and kidney failure are the major secondary complication of DM that results in end stage renal diseases. On the other hand hyperglycemic condition lead to oxidative stress which is one of the causative factors for diabetes associated nephropathy and retinopathy (Onozato et al., 2002). Our study was carried out in STZ-induced diabetic rats that are seen to have similar early stage human diabetic–associated disorders of kidney (Kiran et al., 2012). However the histological evaluation of present study shows significant messengial cell proliferation, reduction in bowman capsule spacing and thinning of membrane of bowman capsule in DC group as compared to NC group. The primary function of the glomeruli is to help in ultrafilteration of the plasma and thus helping in maintenance of fluid and
electrolyte homeostasis. These results are in concordant with the report of Kiran et al., 2012 that exhibited similar finding. Administration of *P. virgatus* methanol extract and its partially purified fraction improves the histoarchitecture of kidney and helps restore its functionality. The group administered with CT-1 at a dose of 0.5 mg/rat/day demonstrated a distinct regenerative capacity over the other treated groups.

Angiogenesis is the key player in vision dysfunction during the process of diabetes retinopathy (Yoshida et al., 2012). Moreover, corneal section of diabetic rats revealed intense degenerative changes eg; epithelial cells, stromal spacing, degradation of its collagen fibers, increased corneal thickness. Our data demonstrated that the stromal cells of the diabetic cornea are having darkly stained nuclei and vacuolated cytoplasm which is in agreement with the previous record of other scientists who declared that in diabetes, formation of AGEs lead to proteins cross linking that caused destruction of intracellular organelles. Along with it, stromal vascularization and retinal detachment was also detected in diabetic animals. Corneal neovascularization in diabetes was recorded by some investigators who suggested that the presence of stromal edema might allow blood vessels into compact stroma (Chang et al., 2001). Treatment for over 28 days with *P. virgatus* methanol extract and its partially isolated fraction (CT-1) showed significant restoration of the histological features of the cornea and there was decrease in the thickness of the corneal layer in the entire treated group. Although glibenclamide, a known antidiabetic agents, which is identified to exert a host of side effects (Yamamoto et al., 1990) also showed some protection against oxidative stress induced by STZ but are not comparable to protection exhibited by *P. virgatus* methanol extracts and its bioactive fraction.
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The collective in vitro, in silico and in vivo results demonstrated in the thesis provide strong evidence in support of the use of P. virgatus methanol extract as a natural source of antioxidants for disease prevention and/or general health promotion through improved nutrition. The biological activity of all the sequentially extracted fractions of P. virgatus and P. maderaspatensis observed in this study strongly indicated the potential role of P. virgatus methanol extract as an antioxidant, antidiabetic and antidyslipidemic agent. The data from in vitro studies strongly suggest that P. virgatus methanol extract contain more amount of polyphenols, possess significant antioxidant, genoprotective, α-glucosidase and α-amylase inhibitory activity as well as the extract also illustrated enhanced glucose uptake in pre-adipose 3T3-L1 cell line and was found to be non cytotoxic. In addition, the preliminary GC-MS analysis of this extract revealed several bioactive compounds and their inhibitory mechanism was well validated via in silico analysis. In contrast, aforesaid activity of various sequentially extracted fractions of P. maderaspatensis was not comparable to the data exhibited by P. virgatus extract. Thus, it is a good approach to manage T2DM as a whole with these compounds/extracts, which showed good enzyme inhibitory and antioxidant activities. Based on the above scientific rational bioactive compounds from P. virgatus methanol extract responsible for the aforesaid activity has been isolated and identified.

In addition, this extract or its partially purified fraction effectively prevented the STZ-induced oxidative stress and adverse alteration in diabetic rats and ameliorated/normalized all the diabetes related parameters including diabetes linked hyperlipidemia parameters evaluated in present study. From our in vivo data, it has been extracted that methanol fraction of this plant and their partially purified fraction exert potent hypoglycemic and hypolipidemic activity. Moreover, the methanol extract at higher dose (50 mg/rat/day) gave
good response, while the partially purified fraction at dose comparable to standard (0.5 mg/rat/day and 0.1 mg/rat/day) illustrated improved hypoglycemic and hypolipidemic activity than the glibenclamide treated diabetic rats. Further purification of this fraction and specific structure elucidation is necessary for development of hypoglycemic and hypolipidemic drugs. Moreover, our results also demonstrate significant protection against STZ-induced oxidative stress, nephropathy and retinopathy condition by *P. virgatus* methanol extract and its bioactive compound which can be due to its profound antioxidant activity. However, in view of several toxic effects exhibited by glibenclamide and its inadequacy to exhibit pleiotropic therapeutic effect, use of *P. virgatus* methanol extract or its partially purified fraction could be preferred that will be both efficacious and cost effective. The combined results have undoubtedly provide scientific confirmation and evidence for the use of *P. virgatus* methanol extract and its partially purified fraction and demonstrated that strong hypoglycemic, hypolipidemic and antidiabetic impacts of this plant coupled with their potent antioxidative property, can provide additional benefits in inhibition of oxidative stress and hence in the prevention and treatment of nephropathy, retinopathy and diabetes linked hyperlipidemia with and without CVD.