DISCUSSION

The present study was an effort to reinstate the anti-hyperglycemic property and uncover the anti-cataract, anti-oxidant, hypolipidemic property and anti-inflammatory potentials of two plant extracts, dry fruit aqueous extract of *T.terrestris* (TT) and heartwood aqueous extract of *P.marsupium* (PM). Study was divided into two phases to determine the efficacy of plant extracts in prevention and reversion of diabetic complications.

Phase-1 study:

Plant extract treatment with TT and PM in diabetic rats for 16 weeks effectively reduced the progression of diabetic complications. Treatment successfully inhibited development of diabetic cataract, diabetes associated inflammation and improved learning and memory in diabetic animals.

Body weight: STZ induced diabetes is characterized by severe loss in body weight(259) and the same was seen in the present study. The decrease in body weight observed in diabetic rats might be the result of protein wasting due to unavailability of carbohydrate for utilization as an energy source(260,261). Plant extract and standard drugs improved the body weights of the diabetic rats and it was comparable to the weight of normal control rats. Many of the previous reports suggest that increase in weight gain is due to comprehensive control of DM and its complications by the plant extracts(262,263). Body weight of the non-diabetic rats treated with the TT showed significant increase in body weight when compared to normal control. Some commercially available preparations claimed that TT increases lean muscle, strength and performance by increasing luteinizing hormone levels and testosterone.(264)

Effect on glycemic profile and histology of pancreas:

Glycemic profile was well controlled upon 16 weeks of treatment with the TT, PM and Glibenclamide(Glib) in diabetic rats. Diabetic rats showed increased RBG, HbA1c and insulin levels. Elevated insulin levels in diabetic controls represents the T2DM insulin resistance in rats. TT showed dose dependent reduction in blood glucose, HbA1c and insulin levels. In diabetes, blood glucose and HbA1c are interlinked. Excess glucose present in the blood reacts non-enzymatically with haemoglobin to form HbA1c. Other proteins in the body like haemoglobin, albumin,

collagen, and crystalline proteins also exhibit the same process(265). Glycation rate depends on the concentration of blood glucose present in the body(266) and levels of HbA1c formed parallels the chronicity of diabetes. Studies also reported that glycation itself may induce the formation of oxygen-derived free radicals in diabetic condition(267). Therefore, the measurement of HbA1c is apparently a very sensitive index for glycemic control(268). TT and PM effectively controlled the blood glucose and HbA1c levels in the present study. Glib treatment in diabetes rats steadily reduced blood glucose, HbA1c levels, endocrine depletion and significantly improved insulin levels. Earlier studies also observed regeneration of pancreatic β-cells on glibenclamide treatment.(269)

Earlier short studies also reported the efficacy of TT and PM in diabetic animals. TT at a dose of 50mg/kg for three weeks significantly decreased the blood glucose and HbA1c levels in diabetic rats(185). In another, 30 days treatment with TT reduced the fasting blood glucose and HbA1c levels. Same study also reported that 60% α-glucosidase inhibition was observed with TT at a dose of 30µg/ml concentration in in vitro (186). Gandhi *et al.,* proved that treatment with TT in STZ induced diabetic animals promoted β-cell regeneration in pancreas and also improved insulin levels. It also increased target cell insulin sensitivity which enhances peripheral utilization of glucose in the muscles and adipose tissues(178). Lim et al., reported that hypoglecemic effect of TT is due to the presence of triterpene glycosides i.e. saponins in the plant extract. Saponin treatment in alloxan induced diabetes mice significantly reduced the blood glucose levels and improved the insulin levels(179). TT in the present study, reduced the excessive insulin levels in diabetic animals and improved the insulin sensitivity by increasing the peripheral utilization of glucose.

Marsupin and pterostilbene, constituents derived from heartwood of PM caused reduction in blood glucose in experimental diabetic rats(270). Tannates present in the PM extract could also be responsible for the observed hypoglycemic activity in diabetic rats. Flavonoid fraction from PM has been exhibited to cause pancreatic beta cell regeneration(212). Hence, it can be said that presence of pterostilbenes, tannins and flavonoid parts in PM heartwood are responsible for antihyperglycemic action in diabetic rats which is evident by the decrease in blood glucose and HbA1c levels in the present study. Epicatechin, isolated compound from *P.marsupium* has shown dose dependent increase in cAMP content and insulin release from pancreatic β-cells. It

been shown to enhance the conversion of pro-insulin to insulin and stimulate cAMP content in the islets of Langerhans in vitro(214). In the present study, PM enhanced the release of insulin and increased glucose utilization which was evident by increased insulin levels and decreased blood glucose levels.

Histopathology of untreated diabetic rats pancreas showed inflammation and reduction in endocrine part and treatment with TT and PM increased the β-cells density and reduced the lymphocyte infiltration. This impressive finding shows that, in addition to the antidiabetic effect, β-cell regeneration is also possible with these plant extracts. Recent animal studies suggested that β-cell regeneration (Neogenesis) is possible with some hormones like Glucagon- like peptide (GLP-1) and gastrin, which protects from cell apoptosis and promotes β-cell proliferation and differentiation. Many companies are now developing and testing the GLP-1 analogues in this regard in T1DM and T2DM patients(271). Hence plausible mechanism for β-cell neogenesis in the present study could be due to upholding the actions of above hormones by the plant extracts.

In addition, the plant extracts also significantly reduced α-glucosidase activity *in vitro*. 37.73% and 46.76% inhibition was seen at 30µg/ml and 50µg/ml concentrations with TT and PM respectively. Lamba *et al.,* reported that *T.terrestris* at 30µg/ml concentration reduced α-glucosidase enzyme activity by 60% (186). α-glucosidase is a key enzyme in carbohydrate digestion which is present in the brush-border surface membrane of small intestine(272). Therefore, α-glucosidase inhibitors could delay the absorption of glucose (273,274) and reduce the stress on β-cells of the pancreas. Thus, they have become the most common oral agents used to improve postprandial hyperglycemia since their introduction.(275,276)

Acarbose, a synthetic α-glucosidase inhibitor, delays absorption of carbohydrates, suppresses postprandial hyperglycemia and contributes to a decrease in HbA1c. Attenuation of HbA1c could reduce the occurrence of long-term vascular complications in diabetic subjects. The solitary reason which is limiting the usage of synthetic analogues of α-glucosidase inhibitors is its displeasing side effects, such as flatulence, diarrhea, and abdominal cramping(273). Several in vitro studies have reported the presence of potential α-glucosidase inhibitors in various food components and plants like cranberry(277), *Cuscuta reflexa* (278), pepper(279), soy

bean extracts (280) etc. It has been shown that flavonoid compounds are potent α-glucosidase inhibitors which check on postprandial hyperglycemia(281). In the present study both plant extracts showed potent *in vitro* α-glucosidase enzyme inhibition. However, *in vitro* inhibitory activity could not be directly related to the *in vivo* activity of a particular plant extract. Thus proof of model needs to be demonstrated in preclinical animal studies to confirm the safety and efficacy of plant extract.

Effect on renal histology and renal function tests:

In diabetes, albumin appears in the urine due to accelerated protein catabolism and increased glomerular permeability(282). In the present study, immunoturbidimetric method was used to detect albumin levels in urine. In diabetic rats, the levels were remarkably higher after 16 weeks of onset of disease(283). Microalbuminuria, is a powerful predictor of both renal failure and cardiovascular disease in patients with T2DM(284). Microalbuminuria or pauci-albuminuria refers to the excretion of 30-300mg albumin/day in urine, whereas values more than 300mg/day referred to as albuminuria, is an indicator of overt diabetic nephropathy (285). Clinically, diabetic nephropathy is characterized by a progressive proteinuria and declining GFR.

Angiotensin-II (AT-II), Aldose reductase (AR) enzyme and AGEs play a major role in nephropathy. Angiotensin-II (AT-II) preferentially constricts the efferent arteriole in the glomerulus, leading to higher glomerular capillary pressure. AT-II also stimulates renal hyperplasia and fibrosis and upregulates the TGF-β and other growth factors. Studies have suggested that angiotensin converting enzyme (ACE) inhibition may delay the development of nephropathy in diabetes patients with microalbuminuria(286). Many in vivo and in vitro studies have shown that, oxidative stress is one of the major mechanisms involved in the pathogenesis of nephropathy in DM(287). Excess glucose in diabetes irreversibly binds to proteins in the extra cellular fluid to form AGEs. The formed AGEs contribute to renal damage by amplifying the release of growth factors such as platelet-derived growth factor and transforming growth factor-β (TGF-β). TGF-β plays a role in expansion and later fibrosis of glomerular mesangium via the stimulation of collagen and fibronectin(288). Short and long-term studies have suggested that AGEs inhibition may delay the development of overt nephropathy in diabetes patients with

microalbuminuria (288,289). Evidences derived from other experimental diabetes studies indicate that aldose reductase (AR) also may initiate a process favoring both glycoxidative and lipoxidative changes that may be damaging to renal microvascular, glomerular and tubular cells and AR inhibition can delay or prevent many early changes(290).

Serum urea levels were elevated in diabetic rats when compared to normal controls. Treatment with TT and PM for a period of 16 weeks significantly reduced these levels in diabetic rats. The increase in renal mass due to diabetes induced hydropic changes, also decreased on treatment. Diabetic rats treated with extracts showed reduced glomerular necrosis, hypercellularity and edema. While, the non-diabetic rats which received the extracts also showed trivial congestion which were inconsequential. Thus, the plant extracts acted differently in diabetic and non-diabetic rats. Previous studies have reported that TT administration in rats resulted in toxic effects to major organs of the body. Ren et al., reported that steroidal saponins accumulates in liver, kidney and heart, thereby causing damage(291). Another study findings articulated that unhydrolyzed saponins from TT extract produced the unexpected kidney damage in rats(178). Currently nephrotoxic effects (hypercellularity and congestion of glomeruli) by TT in non-diabetic rats could be due to prolonged duration of administration and accumulation of active compounds in the rat kidney.

Creatinine, values rise only when there is extensive loss of renal functioning. In this study diabetes group had no much increase in creatinine. Treatment with plant extract also did not significantly reduce the creatinine levels. Surprisingly, creatinine levels were slightly raised in non-diabetic rats treated with the plant extracts and it also correlated with the histopathological findings.

Glib treatment in diabetic rats showed a significant improvement in urine microalbumin, serum urea and creatinine. There was minimal hypercellularity and edema in treated diabetes rats which was confirmed by the microscopic analysis of kidney.

Effect on Liver and histopathology:

Liver is the central organ for most metabolic processes. It is also the site for detoxification. There is extensive involvement of the liver in diabetes with regards to glucose/glycogen metabolism and lipid metabolism. Fatty liver, steatohepatitis and cirrhosis of the liver are frequently found in diabetic patients. AST and ALT are the intracellular enzymes involved in transamination of amino acids in the liver. Elevated levels of these enzymes indicate hepatocellular damage. GGT is secreted by the liver and involved in the synthesis of glutathione via transfer of gamma glutamyl residues to substrate(285). Moderately elevated GGT is an indicator for infective hepatitis and highly elevated levels for biliary obstruction. Chemicals and drugs which induce hepatic microsomal enzymes are known to increase GGT levels(292). In diabetes, there is increase in lipolysis and FFA production. FFAs can directly hepatocytes damage and lead to obstruction in later stages. The associated insulin resistance in diabetes provoke the release of proinflammatory cytokines such as TNF-α and IL-6 which further worsen the hepatocellular damage. These histopathological changes leads to increase in transaminases(293). STZ is known to have harmful effects on pancreas, liver and kidneys(294). One of the preceding study has documented that organelle degeneration and hepatocyte vacuolization in STZ-treated animals were prevented by insulin administration, indicating that these effects were caused by insulin deficiency and not by STZ toxicity(294).

Although some studies demonstrated the beneficial effects with TT and PM on liver(210,218,221), in the current study, there was no optimization in the levels of liver enzymes, especially ALT and GGT. Moreover, previous studies reported that these plants can cause liver damage on account of accumulation of unhydrolyzed saponins(291). Microscopy of liver showed dilatation of blood vessels, lymphocyte infiltration and tissue necrosis in TT and PM treated diabetic animals. Surprisingly, non-diabetic rats treated with TT and PM also had inflammation (lymphocyte infiltration) and dilated blood vessels in the liver. The above observations suggest that TT and PM extracts have deleterious effect on liver.

The contradictory findings in the present study could be due to the duration of the study (16 weeks) which is much longer than most of the previous studies. The effect on liver is possibly due to difference in the nature and concentration of the active principles in different plants. The anti-oxidant effect of plant extracts might have protected the liver from further toxic damage. Thus it may be concluded that the plant

extracts used in the present study showed a mild toxic effect on hepatocytes of rats. On the other hand, diabetic rats treated with Glib showed significant reduction in all hepatic enzymes in serum samples and mild inflammation was seen under microscopic examination.

Glycogen in liver:

Glycogen in the liver was significantly decreased in diabetic control group. Glucose is a major fuel of the tissues of animals and it is converted into glycogen by complexing with proteins for storage in liver. Insulin is considered to be the main regulator of glycogenesis in liver and muscle. Hence, the decrease in hepatic glycogen observed in this study is due to altered action of insulin and glycogen synthase. The decrease in hepatic and skeletal muscle glycogen contents in diabetic rats have been observed earlier(226). Improper regulation of lipid metabolism and pathophysiological changes in liver is again linked to compromised insulin action and reduction of glycogen synthesis in diabetic rats(295). It is documented that administration of herbs increases the response of insulin and stimulates the conversion of the inactive form of glycogen synthase to the active form and enhances the glycogenesis(296,297). These plant extracts act by stimulating the glycogenesis and/or inhibiting glycogenolysis in the diabetic rat liver(298). Ahmad *et al.,* demonstrated that epicatechin from PM mimic the actions of insulin and promotes glycogen content of diabetic rat in a dose-dependent manner(214). In a study by Gupta *et al., P.marsupium* treatment for 14 days (300mg/kg/day) restored the liver glycogen towards normal range in comparison to untreated diabetic group(218). Grover *et al.,* reported that administration of PM aqueous extract for 30 days to diabetic rats significantly increased the activity of carbohydrate enzymes and partially improved the glycogen storage in liver(226). Lamba *et al.,* estimated the glycogen levels in *T.terrestris* treated diabetic rats and confirmed that it increases the liver glycogen storage upon 30 days treatment(186). In the current study Glib treatment also significantly increased the glycogen storage in liver. The significant accumulation of glycogen in the liver with the plant extracts and Glib could be possible due to either enhanced insulin release from β-cells or due to insulinomimetic activity resulting in direct peripheral glucose uptake or combination of the two. Furthermore, insulin sensitization by various constituents of the plant extracts cannot be ruled out.

Lipid profile:

In the present study, treatment with both the plant extracts for a period of 16 weeks reduced the TG levels in diabetic rats. The reduction in TG levels may be due to extracts such marsupin, pterosupin and liquiritigenin from *P.marsupium* and saponins from *T.terrestris*. Jahromi *et al.,* tested the effects of flavinoids, liquiritigenin and pterosupin on dyslipidemic rats. Fourteen days treatment produced convincing reduction in serum cholesterol levels, LDL and atherogenic index(225). Some authors detailed that Vijayasar (*P.marsupium*) bark extract substantially prevented the increase in TC, TGs and insulin in rats with fructose-induced metabolic disorder and in alloxan induced diabetes rats(221,226). Singh *et al.,* tested a dose of 250mg/kg methanolic extract of *P.marsupium* for hypolipidemic activity in alloxan induced diabetic adult female Wistar rats. *P.marsupium* treatment effectively controlled TC and TG in dyslipidemia rats(222). Mishra *et al.,* reported that 100mg/kg dose of ethanolic extract of *P.marsupium* exhibited antidyslipidemic effects in high fat diet fed Syrian golden hamsters. In the same study, *P.marsupium* 28 days treatment normalized the altered renal and hepatic function markers and serum insulin levels of high fat diet fed-low dosed STZ diabetic rats. Study concluded that phytoconstituent phenolic-C-glycosides in *P.marsupium* heartwood are responsible for the favourable actions(224).

Chronic hyperlipidemia is considered as one of the causative factor for development of atherosclerosis. Its an established fact that in addition to conventional anti-lipidemic drugs, traditional medicinal plants too have remedial effects in hyperlipidemia and atherosclerosis (299–301). Several authors evaluated preventive and therapeutic effects of saponin from *T.terrestris* in diet-induced hyperlipidemic diabetic animals. Saponin treatment significantly reduced the levels of serum TC, LDL-c and TG(179,191,193). Study by Tantawy *et al.,* reported that three weeks treatment with 50mg/kg dose of *T.terrestris* significantly reduced the serum LDL, TC and TG in STZ induced diabetes rats(185).

Contrary to the above studies, in the present study *T.terrestris* and *P.marsupium* could effectively control only TG levels in diabetic rats and showed very mild effect on TC and HDL. Similar results were seen in a previous study which was conducted on rabbits, where 12 weeks treatment with TT noticeably reduced the serum lipids, TC

and LDL levels still remained higher in test group(302). Hence, TT alone may not be sufficient to treat hyperlipidemia. Glib treatment also did not show much encouraging outcomes. Glucotoxicity and lipotoxicity are the two accepted mechanisms for β-cell loss. They are also strong contributors to coronary artery disease. Managing lipid levels is always a challenge in diabetes. TT and PM treatment do not offer much respite in this regard.

Effects on retinopathy

AR enzyme activity in lens and sciatic nerve:

In the present study, diabetic rats treated with plant extracts showed reduced AR enzyme activity in lens and sciatic nerve. AR activity was significantly reduced in positive control group rats treated with glibenclamide. Normal rats treated with plant extract did not show much change in AR enzyme activity.

AR is a rate limiting enzyme in the polyol pathway and its over activity leads to accumulation sorbitol in lens fibers, osmotic hydration, osmotic stress and finally lens opacification(303). Hence the inhibition of AR activity by developing AR inhibitors is one of the possible approaches in the management diabetic complications including diabetic cataract(304).

AR causes intracellular glucose toxicity in neural, glial and vascular cells of retina. Polyol pathway is considered as a major contributor to oxidative stress in lens as well as in neurons of diabetic animal. Few reports mention that aldose reductase inhibitor reduces oxidative stress in diabetes cataract(305). Treatment with antioxidants also keeps AR in reduced form and thus prevent drug resistance that develops due to AR modification under conditions of chronic hyperglycemia(306). Diabetic retinopathy and macular edema are the two common microvascular complications seen in diabetes patients and it may have impact on visual acuity and finally leading to blindness. While, Diabetic retinopathy is characterized by the formation of new blood vessels to supply oxygenated blood to ischemic area, on other hand macular edema is retinal thickening in the macular area. Many studies have demonstrated the early lesions in retinal vessels in experimental diabetic animal models(307,308). AR inhibitors are known to prevent capillary basement membrane thickening, the early structural lesion observed in the retina of diabetes animals.(308)

Diabetes associated phenomena such as increased retinal AR activity, oxidative stress and increased vascular permeability are interrelated. AR triggers the whole cascade by causing oxidative stress, which in turn leads to overexpression of vascular endothelial growth factor and increased vascular permeability(305). Indeed, all three components of this cascade have been successfully blocked by AR inhibitors treatment(309). Cortical cataract in rats occur mainly due to liquefaction of the subepithelial and posterior subcapsular fibers. The damaged epithelial cells and their abnormal progeny show endosmosis in most areas of the cortical cataract. Previous studies have stated that, regained cortical transparency can be due to following approaches (1) processing and removal of damaged tissue products from the lens; and (2) restoration of normal epithelium (3) resumption of normal fiber-genesis and, possibly (4) repair of minimally damaged fibers(309,310). In the present study, TT and PM by virtue of their AR inhibitory property showed an improvement of lens opacity. One or more of the above mentioned proceedings would have played a major role in this outcome.

Though polyol pathway causes oxidative stress in both lens and nerves, its role in the development of lesions in these two tissues seemed to be different. While in diabetic cataract, it is mainly due to osmotic stress from accumulation of sorbitol. In diabetic neuropathy, it is due to hyperglycemia induced metabolic derangements causing neurochemical alteration in diabetic nerves(311).

In a previous study, treatment with an AR inhibitor, sorbinil, in diabetic rats was found to protect against the loss of Myo-inositol(MI), taurine and other amino acids which are required to maintain the nerve homeostasis(312). Studies have reported that depletion of MI, is a key factor in causing neuronal homeostatic disturbances(313,314). Greene *et al.,* conducted a study on rabbit endoneurial preparation and reported that glucose competitively inhibits MI uptake. Loss of MI and other substances is due to increase in membrane permeability caused by osmotic swelling of the nerve. MI loss also affects the Na^+K^+-ATPase activity, leads to a gain in sodium ions which accounts for nerve conduction abnormalities(315).

In the present study, observed beneficial effects noted with the use of PM could be possible due to presence of poly phenolic compounds namely catechin, epicatechin(316) and pterostilbene(317,318). Previous studies have reported that epicatechin is a potent AR inhibitor with IC50 value of 79 µM(319). It also prevents

N-methyl-N-nitrosourea-induced cataracts and lens epithelial cell apoptosis in rats(320).

Lamba *et al.,* demonstrated that *T.terrestris* inhibits the AR enzyme completely and improves the insulin sensitivity by increasing the utilization of glucose in diabetic animals(186). Various research works have reported that effective blood glucose control with insulin therapy, supplemented with AR inhibitors help in completely preventing capillary basement membrane thickening in retina (308). Saponins present in TT may be responsible for the favourable effects in the present study.

In diabetic rats, there was minimal increase in lens density, but significant rise in sciatic nerve mass. Treatment with TT significantly reduced the lens and sciatic nerve weight in diabetes animals whereas PM brought down the weight of sciatic nerve. The decrease in the weight of lens would be an indicator of decrease in the hydropic changes in the lens.

Effects on Antioxidants:

Oxidative stress has been suggested as the most common underlying mechanism of diabetic cataract and augmentation of the antioxidant defenses of the lens prevent or delay cataract and neuropathy in diabetes animals(321). The present study showed antioxidant effect of TT and PM, indicated by a significant increase in GSH in lens homogenate *in vivo* and DPPH free radical scavenging capacity *in vitro* (322).

GSH levels were hugely reduced in diabetic rat lens when compared to normal control. In a normal cell there is an appropriate pro-oxidant and antioxidant balance. This balance can be shifted towards the pro-oxidant when production of reactive oxygen species is increased or when levels of antioxidants are diminished. The increased glucose in the blood undergoes auto-oxidation to release free radicals. Glycosylated proteins are also other source for free radical generation (321). Reactive oxygen species (ROS) such as hydroxyl radicals, superoxide anions and nitric oxide inactivate antioxidant enzymes and increase lipid peroxidation leading to damage of cellular organelles. Antioxidants are agents which offer resistance against the oxidative stress by scavenging the free radicals and inhibiting lipid peroxidation (323).

In diabetes, glucose undergoes polyol pathway, where AR converts glucose to sorbitol by utilizing NADPH. Since NADPH is also required for the generation of antioxidant (GSH), Excessive depletion of NADPH by AR in polyol pathway leads to impairment in intracellular antioxidant defence(324). Antioxidants are responsible for protection of lens epithelial cells against oxidative injury. Thus, inhibition of AR pathway and increase in GSH in lens could be one of the strategies to prevent diabetic complications especially cataract.

Literatures have revealed that cataract maturation can be prevented by using plant extracts which are high in flavonoids and phenols. These compounds reduce glucose level, inhibit polyol pathway and AR enzyme activity(316) . In the present study, use of TT and PM extracts for 16 weeks in diabetes rats improved the lens GSH levels. Natural antioxidants have beneficial implications for management of DM. Diabetic complications can be prevented or retarded by administration of appropriate antioxidants, in addition to traditional therapeutic medications(325). Previous studies showed that majority of the plasma antioxidants are depleted in Type 2 diabetic patients.(326,327)

Phenolic compounds are known for their scavenging potential due to the presence of hydroxyl groups. Several phenolic antioxidants such as flavanoids, tannins, coumarins, xanthones and procyanidins scavenge radicals in a dose dependent manner. Therefore they are considered as promising therapeutic agents for free radical pathologies (328). In the present study both the plant extracts showed significant in vitro free radical scavenging activity. Among them, aqueous extract of PM heartwood exhibited higher effect when compared to TT fruit aqueous extract. Presence of high phenolic compounds like catechin, epicatechin and pterostilbene might be responsible for its potent action.

Effects on cognitive function:

In the present study diabetic rats displayed significant increased escape latencies during learning phase in Morris water maze (MWM) test. These alterations are symbolic of cognitive decline in diabetic animals. Chronic hyperglycemia and insulin resistance associated cerebrovascular alterations cause these changes in diabetes animals. Insulin is best known for its involvement in the regulation of numerous brain functions including cognition, memory and synaptic plasticity through complex

insulin/insulin receptor signalling pathways. Insulin resistance in diabetes impairs signalling pathways of brain leading to various biochemical and histopathological changes. Biessels *et al.,* proved that insulin treatment successfully prevented the cognitive deficits in streptozotocin-diabetic rats using MWM test(329). Antidiabetic plant extracts also seems to be equally effective in improving cognitive activities. In a study conducted by Chauhan *et al.,* reported that oral administration of methanolic extract of *P.marsupium* showed improvement on cognitive impairment in rats by using MWM test(241). Many studies have supported the fact that *T.terrestris* has positive effects on cognitive function in diabetic rats. A study by Prabhu *et al.,* explains the dose dependent beneficial effect of the aqueous extract of TT on learning and memory in rats. 14 days treatment with TT at 200m/kg showed beneficial effects in diabetic rats(189). Study by Roghani *et al.,* documented oral feeding of TT improved learning and memory in STZ induced diabetic rats by suppressing lipid peroxidation in hippocampus(188).

Supporting the previous studies, in the present study TT and PM aqueous extracts administration to diabetic animals showed improvement in learning, memory retention and adaptive capability(330,331). Both plant extracts contain some saponins and flavonoids and that could be responsible for the observed positive effects in the present study. It is known that saponins have nootropic activities(332). In addition, studies have also confirmed that AR enzyme inhibitors ameliorate the slowing of nerve conduction in diabetic subjects(333). In the present study possible reason for improvement in cognition in diabetic rats could be due to reduced AR activity in nerve and improved nerve conduction. Hence the beneficial effects of the plant extract can also be attributed to its potent AR inhibitory activity and antioxidant properties of the plant extract.

Effect on inflammation:

Although hyperglycemia and insulin resistance/insensitivity are the central pathologies in diabetes, the selective and extensive involvement of certain tissues was difficult to understand. That was when the role of inflammation in the pathogenesis was elicited. It is now widely accepted that almost all the complications of diabetes share an inflammatory basis. This has been particularly appreciated in coronary artery

disease and nephropathy. Hence targeting the inflammation pathway would reduce the burden of complications.

IL-6 in serum and kidney was elevated in the diabetic control group. IL-6 is one of the earliest cytokines which is upregulated in many infectious conditions. Cytokines such as TNF-α and IL-6 in diabetes, increases ROS production and causes activation of Ikkβ. Activation of Ikkβ results in IRS-1 serine phosphorylation on Ser307 and inhibits insulin action (334). Overexpression of IL-6 in mice increases β-cell inflammation(335). IL-6 and IL-8 levels are usually elevated in insulin-resistant states such as obesity, impaired glucose tolerance, T1DM and T2DM (336,337). Anti-IL-6 therapy significantly prevents the inflammatory process in rats. When diabetic rats treated with plant extracts having anti-inflammatory property, there was decrease in circulating levels of proinflammatory cytokines, improvement in insulin sensitivity and glucose metabolism(338). Similarly, in the present study, pro-inflammatory cytokine IL-6 in serum and kidney was significantly decreased in the plant extract treated groups compared to untreated diabetic control group.

Expression of renal IL-6 is directly related to mesangial proliferation, tubular atrophy and interstitial infiltration in various models of renal disease and causing further progression of the disease(339,340). Adiponectin is said to have anti-inflammatory and antioxidative properties. Subjects with obesity, T2DM and coronary artery disease have low levels of adiponectin(38,39). In vitro studies have reported that recombinant adiponectin suppresses the cytokine-induced expression of endothelial adhesion molecules. It also selectively increases the expression of tissue inhibitor of metalloproteinases-1(TIMP-1). In addition, they protect the vascular wall from plaque rupture by inducing the release of anti-inflammatory cytokine, IL-10 (47,341). Hence, possible mechanism for observed beneficial effects in the present study with *T.terrestris* and *P.marsupium* could be due to potentiated action of adiponectin and inhibition of inflammatory cytokines expression in diabetic rats.

Although diabetic retinopathy is not widely considered to be an inflammatory disease, the pathogenesis in microvasculature is similar to atherosclerosis and diabetic nephropathy. In Beaver Dam Eye Study (342), a cross-sectional study done in Wiscosin on 396 diabetic subjects between the age of 43 to 84, found that retinal arteriole:venule ratio (AVR), an index of arteriolar narrowing, correlates with increase

in systemic inflammatory substances like IL-6, C-reactive protein and amyloid A. Suppression of these inflammatory cytokines decreased the incidence of retinal AVR. It is possible that *T.terrestris* and *P.marsupium* with AR inhibitory activity, restrains the inflammatory cytokines production and reduces the complications of diabetes.

On the other hand, plasma levels of anti-inflammatory cytokine (IL-10) is positively correlated to insulin sensitivity in healthy subjects and are reduced in obese and diabetic subjects(343). In the present study, renal IL-10 levels in diabetic rats drastically reduced. Severe inflammation to diabetic kidneys could be a reason for depleted IL-10 levels. However, serum IL- 10 levels was significantly increased in diabetic control group. A study reported that increased levels of IL-6 induces the release of IL-10 release from T helper cells via transforming growth factor-β (TGF-β) (344). This could be one of the reasons for observed elevated levels of serum IL-10 in diabetic rats in present study.

IL-10 has inhibitory action on IL-6 release. Prior studies have shown that IL-10 treatment completely prevented IL-6 induced insulin resistance (345). The protective effects of IL-10 were associated with normalization of insulin signalling and intramuscular fatty acyl-CoA levels(346). Interestingly, acute IL-6 treatment caused skeletal muscle and hepatic insulin resistance in vivo and IL-10 co-treatment prevented IL-6 and lipid-induced insulin resistance(345). IL-10 levels in serum and kidney homogenate was significantly elevated in the plant extract treated diabetic rats. Hence, it can be said that low IL-6 levels in the treated groups could be due to IL-10 mediated suppression of IL-6.

Whereas diabetic rats treated with glibenclamide showed significant improvement in IL-10 and effectively controlled the IL-6 in serum and kidney. A previous study has reported that glibenclamide exhibits anti-inflammatory action by effectively reducing the circulatory cytokines levels in diabetic patients.(137)

Phase-2 study:

Although the protective effects of plant extracts are the common mode of studies, if the results are to be extrapolated to humans, mimicking the human conditions is better to understand the efficacy. In clinical practice, treatment of diabetes begins only after

clinical diabetes set in. Since its a chronic disorder, patients are often not aware of the condition and present with complications at the time of diagnosis. Hence this phase of the study was expected to test the actual clinical usefulness of these plants in optimization of hyperglycemia and its associated disorders. Literature search has not resulted in similar reported studies with the particular plant extracts chosen for this study. Hence comparison was made with available study results. Dose selection for the phase-2 was made based on the phase-1 study results. Among two doses of plant extracts, low doses of both plant extracts i.e. 150mg/kg of TT and 250mg/kg of PM was found effective in the phase-1 study and the same was selected for the phase-2 study.

Treatment with TT and PM extracts alone in diabetic rats for 30 days did not effectively reduce the progression of diabetic complications. Whereas the combination of glibenclamide and plant extracts treatment successfully controlled the glycemic profile as well as the production of inflammatory cytokines.

Body weight:

There was a significant increase in body weights of experimental groups. Administration of TT plant extract to diabetic animals significantly increased the body weight compared to all other groups. Even though body weight of diabetic control rats reduced, it was not statically significant when compared to normal controls. The decrease in body weight observed in diabetic rats might be the result of protein wasting due to unavailability of carbohydrate for utilization as an energy source(260,261). Plant extract and standard drugs improved the body weights of the diabetic rats and it was comparable to the weight of diabetic control rats. The improvement in body weight of rats with plant extracts and Glib treatment could be the result of improved glycemic control. Some commercially available preparations claimed that TT has anabolic effect thereby it increases lean muscle, strength and performance by increasing luteinizing hormone levels and testosterone (264).

Effect on glycemic profile and histopathology of pancreas:

Increase in blood glucose, HbA1c and insulin levels following NA-STZ injection was observed in the present study was supported by previous work (347,348). Elevated insulin levels in diabetic controls represents the T2DM insulin resistance condition in

rats. Treatment with combination of Glibenclamide and TT (Glib+TT) and Glibenclamide and PM (Glib+PM) successfully controlled the blood glucose levels and HbA1c. Glib+PM treatment had effect on HbA1c levels too. According to previous literature, most of oral hypoglycemic drugs reduce the HbA1c by 0.5 to 1.25% and TZDs and sulfonylureas lowers HbA1c by 1.0-1.25% (349). In the present study *P.marsupium* and glibenclamide combination efficacy was equal to the TZDs and sulfonylureas, where it reduced the HbA1c by ~1%. Standard drug glibenclamide treatment returned blood glucose, HbA1c and insulin levels closer to normal range in diabetic rats. The present study results are in accordance with the previous study, Solanki *et al.*, reported that 4 weeks after development of diabetes in rats, administration of *Ficus racemose* for 4 weeks exhibited protective effect by reducing complications of diabetes by preventing a rise in glucose, HbA1c and reduced oxidative stress and early inflammation(350). In the present study, histopathological studies of pancreas in diabetic control rats showed exocrine damage, endocrine depletion and inflammation. β-cell regeneration and reduced lymphocyte infiltration was seen in standard drug group and Glib+TT and Glib+PM groups. Whereas in the rats treated with only plant extracts showed depleted islet cells and inflammation of pancreas. These findings indicates that combination of plant extract and glibenclamide works superior than plant extracts alone. Presence of antidiabetic compounds like saponins and flavonoids in plants extracts may add up to the hypoglycemic effect of glibenclamide. Hence, TT and PM can be used as adjuvants along with the effective oral hypoglycemic drug glibenclamide to improve the glycemic profile and restrict the progression of complications in diabetes.

Effect on renal profile and histopathology of kidney:

In the present study, diabetic control group and groups treated with only plant extract TT and PM showed high serum urea, creatinine levels. Histopathology of kidney showed hypercellularity, lymphocyte infiltration and congestion. Creatinine level was significantly reduced in Glib+TT and Glib+PM combination treated diabetic rats. Mild inflammation was seen in combination therapy groups. Supporting the present study results, Poplawski *et al.*, has reported that use of ketone 3-beta-hydroxybutyric acid (3-OHB) for 8 weeks in T1DM and T2DM animal model reversed nephropathy by reducing the albumin/creatinine ratios(351). However, the microscopic changes of

nephropathy was only partly reversed. Therefore, these studies demonstrate that diabetic nephropathy once developed, can only be partly reversed with treatment.

Effect on liver enzymes and histopathology:

AST, ALT and GGT levels are significantly elevated in diabetic control group. Although Baquer *et al.,* reported that antidiabetic plants (*Momordica charantia* and *Trigonella foenum graecum* seed powder) improve the diabetic rat tissues by reversing the altered liver enzymes and lipogenic enzymes(352), in the current study treatment with plant extract did not satisfactorily reduce liver enzyme levels and it could be correlated with hepatotoxic effects of the plant extracts in phase-1 study. Histology of all treated groups showed inflammation which was evident by lymphocyte infiltration, necrosis, dilated sinusoids and portal triaditis. However, Glibenclamide treatment alone in diabetic rats reduced the AST and ALT levels and reduced the hepatic damage under microscopic examination. Treatment with test and standard drugs did not improve glycogen storage in diabetic rat liver. Overall liver findings suggest plant extracts and glibenclamide have very negligible hepatoprotective actions in phase-2 study.

Effect on lipid profile:

Levels of TC and TG were strikingly elevated and HDL levels lowered in untreated diabetic control rats. It is well known that in diabetic rats, the rise in blood glucose level is accompanied by an increase in the serum cholesterol and triglyceride level. The level of glycaemic control is the major determinant of serum triglyceride level(353). Glib+TT and Glib+PM groups showed reduced TG and improved HDL levels. Treatment with glibenclamide alone also showed similar response in diabetic animals. TC was not significantly reduced in any of the treated groups. Previous investigations have confirmed that normalization of the blood glucose levels results in reduction in the levels of TC and free fatty acids. In consideration to the above statement, in the present work, plant extract treatment did not effectively control the blood glucose and this might be the reason for failure to normalise TC even after treatment.

Effect on AR activity and GSH:

AR activity was high in lens and sciatic nerve of untreated diabetic control groups. Combination therapy and TT treatment did not effectively reduce the AR enzyme activity. On the contrary, Diabetic rats treated with glibenclamide and PM displayed significant reduction in AR activity in lens. GSH level was low in diabetic control group and treatment with plant extracts and standard drug did not improve the GSH in lens. These results indicate that plant extracts play no role in reversing retinal complication of diabetes.

Effect on inflammation:

Levels of both pro-inflammatory cytokine IL-6 as well as anti-inflammatory cytokine IL-10 were elevated in diabetic rat serum. Whereas in kidney, IL-6 was increased and IL-10 was reduced. Treatment with only TT extract improved the IL-10 levels in kidney and its combination with glibenclamide effectively reduced the IL-6 levels of kidney. Treatment with only PM extract reduced the kidney IL-6 levels and its combination with glibenclamide improved the IL-10 levels in diabetic rat kidneys. Group treated with glibenclamide alone did not effectively control the inflammation markers.

There was no added advantage of combining plant extracts with glibenclamide except on the levels of IL-6 and IL-10. Since inflammation plays a key role in progression of diabetes, through this mechanism they may be expected to serve as adjuvants to standard drug glibenclamide.

To conclude, singular administration of both the plant extracts seems to be inadequate in reverting the complications. When they are combined with glibenclamide, beneficial effects were seen in controlling the blood glucose and inflammatory profile. Combination therapy showed β-cell regeneration in affected diabetic pancreas. *P.marsupium* combination with glibenclamide showed better results than *T.terrestris* and glibenclamide combination. The exact role of plant extracts in the reversion of diabetic complications can be predicted by monitoring the other antioxidants status and levels of inflammatory markers. However more research needs to be done pertaining to this aspect.