Chapter 5

Discussion
5. DISCUSSION

DM is a most common serious metabolic disorder and is considered to be one of the five leading causes of death in the world (Ugochukwu and Babady, 2003; Devendra et al., 2004). All over the world, several therapeutic approaches based on the medicines of various origins are practiced for DM. Although many kinds of antidiabetic medicines have been developed, none has been reported as safer drug. The treatment of diabetes with medicines of plant origin that proved much safer than synthetic drugs is an integral part of many cultures throughout the world and gained importance in recent years. India has a rich history of using various potent herbs and herbal components for treating various diseases including diabetes (Craig, 1999; Ryan et al., 2001). Many Indian plants have been investigated for their beneficial use in different types of diabetes (Grover et al., 2002). Gymnema species are traditionally used to treat disorders such as diabetes, high cholesterol, wounds, inflammation and gastrointestinal ailments. *G. montanum* H. is one of such species, endemic to India found mainly in the Shola forests of Western Ghats, The Nilgiris. Since some members of the Gymnema species (*G. sylvestre, G. inodorum* and *G. yunnanense*) have been reported to possess antidiabetic properties, it is expected that *G. montanum* also possess antidiabetic property which has not been explored so far. Hence this study was conducted to assess the antidiabetic property of the *G. montanum* leaf extract using alloxan-induced diabetic rats. As metabolism of both carbohydrate and lipids are deregulated in diabetes, to understand its mode of action, the effect of *G. montanum* on the levels of blood glucose, plasma insulin, carbohydrate metabolic enzymes, lipid profile, fatty acid composition, lipid peroxidation and endogenous antioxidants were studied. Further the chemical composition of the extract was also elucidated.

5.1. EFFECT OF GLEt ON CARBOHYDRATE METABOLISM

This study was undertaken to assess the antihyperglycemic property of ethanol extract of *G. montanum* (GLEt) by measuring the levels of blood glucose, plasma insulin, glycoproteins and carbohydrate metabolic enzymes in alloxan-induced diabetic rats.

5.1.1. Blood glucose, plasma insulin and urine sugar

Administration of GLEt might enhance glucose utilization because it significantly reduces blood glucose level in diabetic rats. From the data obtained with the OGTT, it is clear that blood glucose levels reached a peak and returned to fasting values after 2 h in both normal and GLEt treated diabetic rats.
(diabetic + GLEt). In untreated diabetic rats, blood glucose levels remained high even after 2 h. Administration of GLEt effectively prevented the increase in blood glucose without causing a hypoglycemic state. In this context, other antidiabetic medicinal plants such as *Coccinia indica*, *Cassia auriculata*, *Phaseolus vulgaris* and *Scoparia dulcis* have also been reported to have similar effect (Singh et al., 1985; Latha et al., 2003; Venkateswaran and Par, 2002a; Latha et al., 2004).

Reports from previous studies conducted on other members of this genus indicated that administration of the extract of *G. sylvestre* reduced the blood glucose and increased the plasma insulin levels in experimental diabetic rats (Persaud et al., 1999). Similarly, in this study also it is clearly evident that the antihyperglycemic effect of GLEt as indicated by decrease in blood glucose levels is dose dependent and this is probably an indicative of the presence of active principle(s) in the plant extract. Moreover, increase in plasma insulin levels observed following GLEt treatment indirectly indicates that the antihyperglycemic activity of this plant, at least in part, is through stimulating the release of insulin from the pancreas. However, administration of GLEt to normal rats did not cause any significant alteration neither in blood glucose nor in plasma insulin levels. Hence, the observed rise in plasma insulin levels in GLEt administered diabetic rats may be due to its insulin stimulatory effects rather than insulin-mimetic effects.

5.1.2. Haemoglobin and glycated haemoglobin

Glycated proteins are formed post-translationally by the slow, non-enzymic reaction between glucose and amino groups on proteins (Wu and Monnier, 2003). As Hb is highly susceptible to non-enzymic glycation, the rate of synthesis of HbA1c depends on the concentration of glucose to which the erythrocytes are exposed. In diabetic condition the excess glucose present in the blood reacts with Hb to form HbA1c, which has altered affinity for oxygen and this may be a cause for tissue anoxia observed in diabetes (Yiping et al., 2004). In this experiment also the HbA1c was significantly increased in alloxan-induced diabetic rats, and this increase is directly proportional to fasting blood glucose levels as reported earlier (Latha and Par, 2003; Par and Saravanan, 2002). However, GLEt treatment caused a significant decrease in the level of HbA1c and rise in Hb level in diabetic rats. This may be due to antihyperglycemic action of GLEt which reduced the blood glucose to normoglycemic levels in diabetic rats. The reduction in glycation of Hb observed after GLEt treatment may help to reduce diabetes associated tissue damage and chronic complications associated with the eyes, kidneys, nerves and cardiovascular system.
5.1.3. Hepatic glycogen

As the primary storage form of glucose in mammals, glycogen is a key factor in the maintenance of glucose homeostasis. As in earlier reports (Chakrabarti et al., 2003), in this study also hepatic glycogen content was reduced significantly in diabetic control rats as compared to the normal rats. This decrease in the liver glycogen content is probably due to lack of insulin in the diabetic state, which results in the inactivation of glycogen synthase and increase in glycogen phosphorylase activity (Panneerselvam and Govindaswamy, 2002). GLEt treatment to alloxan-induced diabetic rats prevented reduction in liver glycogen content which is possibly due to its stimulatory action on insulin release from existing β-cells and subsequent enhanced glycolysis (Lolitkar and Rao, 1996).

5.1.4. Glycolytic and gluconeogenic enzymes

In diabetic condition, deficiency of insulin has been shown to change the activities of many carbohydrate metabolic enzymes (Beutler, 1988). One of the important enzymes in the catabolism of glucose is hexokinase, which phosphorylates glucose and converts it into glucose-6-phosphate (Laakso et al., 1995). Hexokinase activity has been reported to decrease in diabetic rats (Dyer et al., 1993), which leads to decreased glycolysis and decreased utilization of glucose for energy production (Gancedo and Gancedo, 1971). Administration of GLEt to diabetic rats resulted in significant increase and normalization of hexokinase activity which may help to improve glycolysis and glucose utilization.

Similarly glucose-6-phosphate dehydrogenase activity was significantly decreased in alloxan-induced diabetic rats. A decrease in the activity of glucose-6-phosphate dehydrogenase slows down the HMP-shunt pathway in diabetic condition (Abdel-Rahim et al., 1992). A significant increase in the enzyme activity caused by GLEt treatment suggests restoration of the hydrogen shuttle system and the redox state of the cells, which leads to increased utilization of glucose by lipogenesis (Baquer et al., 1998).

Insulin decreases gluconeogenesis by decreasing the activities of key enzymes, such as glucose-6-phosphatase, fructose-1,6-bisphosphatase, phosphoenolpyruvate carboxykinase and pyruvate carboxykinase (Zhang and Moller, 2000; Panneerselvam and Govindaswamy, 2002). Glucose 6-phosphatase plays an important role in the homeostasis of blood glucose (Foster and Nordlie, 2002). Fructose-1,6-bisphosphatase is also one of the important enzymes of gluconeogenic pathway (Tillmann et al., 2002). Activities of these gluconeogenic
enzymes, glucose-6-phosphatase and fructose-1,6-bisphosphatase were increased significantly in the liver and kidney of diabetic rats (Nordlie and Sukalski, 1985) as a result of insulin deficiency. The increased activities of these enzymes in the liver may be due to the activation or increased synthesis of them contributing to the increased glucose production during diabetes. The GLEt administration significantly decreased the activity of these gluconeogenic enzymes in diabetic rats. Since GLEt administration to diabetic rats caused a significant increase in plasma insulin levels, it may be inferred that the suppression of gluconeogenic enzyme activity observed in GLEt treated diabetic rats may be a consequence of inhibitory effect of insulin over these enzymes. The magnitude of reduction in these enzymes’ activity can result in the decreased level of blood glucose.

A sequential metabolic correlation between increased glycolysis, decreased gluconeogenesis and restoration of normoglycemic condition in GLEt treated alloxan-induced diabetic rats suggests the possible antihyperglycemic effect of GLEt in experimental diabetes. However, as GLEt treatment significantly increased the plasma insulin levels in diabetic animals, the observed effect of GLEt on carbohydrate metabolism and consequent restoration of normoglycemic condition might be secondary to its insulin secretory action.

5.1.5. Glycoprotein metabolism

Glycoproteins are carbohydrate linked protein macromolecules found in the cell surface, which form the principal components of animal cells. Abnormal levels of glycoproteins are important in the pathogenesis of diabetes related complications of liver and kidney. Generalized abnormalities in glycoprotein metabolism are observed in both naturally occurring and experimental diabetes (Gavella et al., 2003).

Previous reports suggest that serum concentrations of glycoproteins are significantly increased in diabetes (McMillan, 1972; Jonsson and Wales, 1976). This increase in plasma glycoproteins has been reported to be associated with the severity and duration of diabetes. Glycoproteins found in a variety of tissues including the arterial wall are very similar in structure and composition to those in plasma. Therefore, diabetes related vascular complications that involve complex protein-carbohydrate molecules could contribute to an increase in plasma glycoproteins.
In hyperglycemia, free amino groups of proteins react slowly with the carbonyl groups of reducing sugars such as glucose, to yield a Schiff's-base intermediate (Maillard reaction). The Amadori product further undergoes a series of reactions through dicarbonyl intermediates to form advanced glycated endproducts (AGE). Formation of some AGE combines both the glycation and oxidative steps in a process termed glycoxidation. Glycation of the extracellular matrix produces changes in macromolecular structure affecting cell–cell and cell–matrix interactions associated with decreased elasticity and increased fluid filtration across arterial wall and endothelial cell adhesion (Thornalley, 2002).

When the concentration of AGE increased above a critical level, cell surface AGE receptors become activated. This is associated with increased expression of extracellular matrix proteins, vascular adhesion molecules, cytokines and growth factors. Depending on the cell type and concurrent signaling this is associated with chemotaxis, angiogenesis, oxidative stress and cell proliferation or apoptosis (Vlassara and Palace, 2002), all processes that are thought to contribute to disease mechanisms associated with the development of diabetic complications (Guillot et al., 1994).

The hyperglycemia in diabetic rats leads to decreased utilization of glucose, thereby enhancing the formation of hexose, hexosamine and fucose for the accumulation of glycoproteins (Youngren et al., 1996). At the cell surface or inside the cells, there are also carbohydrates such as fucose and sialic acid which form specific structure, called glycanic chains, covalently linked to lipids or proteins. An increase in the biosynthesis or a decrease in the metabolism of glycoprotein could be related to the deposition of these materials in the basement membrane (Rasch et al., 1995), leading to basement membrane thickening which may be influenced by insulin deficiency. Thickening of capillary basement membrane is accompanied by the disruption of glycemic control in the DM (Roth et al., 1993). Alterations of those functional glycoconjugates could induce abnormal cellular behavior, as it is frequently described in diabetic microvascular complications (Schiller and Dorfman, 1957). The decrease in the sialic acid content in tissues may be due to its utilization for the synthesis of fibronectin, which contains sialic acid residues in the core structure. The synthesis of fibronectin was also reported to increase significantly in various tissues of diabetic patients (Blum and Fridovich, 1985).

Administration of GLEt to diabetic rats significantly reversed these changes to near normal levels. The antihyperglycemic action of GLEt, which is mediated via
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an enhancement of insulin action as evidenced by the increased level of insulin in GLEt treated diabetic rats, may be responsible for the reversal of glycoprotein changes associated with diabetes.

Results of these studies conducted on alloxan-induced diabetic rats indicate that diabetic related impairments in the glucose metabolizing enzymes in liver have been corrected by the GLEt treatment which may be due to its insulin secretary effect. Further enhanced activity of insulin by GLEt integrates hepatic carbohydrate metabolism by normalizing the activities of enzymes of glycolysis, glycogenesis, pentose oxidative pathway and gluconeogenesis. As the insulin secretary effect of GLEt was dose dependent, presence of phytoactive compound(s) may be postulated. Murakami et al. (1996) reported that, G. sylvestre was found to be rich in gymnemagenin and gymnemic acids that are responsible for its antihyperglycemic effect. Since, the test plant also belongs to the same genus; the presence of such active constituents in G. montanum also may be envisaged.

5.2. LIPID METABOLISM

Abnormalities in lipid profile are one of the most common complications in DM, leading to much of the diabetes associated morbidity and mortality. One of the major pathogenesis of disturbances in lipid metabolism during diabetes is the increased mobilization of fatty acids from adipose tissue and secondary elevation of free fatty acid level in the blood (Bierman et al., 1975). In animals, the administration of diabetogenic doses of alloxan induces hyperlipidemia (Velminsky et al., 1970). The results of the present study clearly show that GLEt has a lowering action on serum TG, TC, VLDL-C, LDL-C, FFA and PL levels. There is substantial evidence that lowering the TC and serum lipids will lead to a reduction in the incidence of coronary heart disease (CHD), which is still a leading cause of death in diabetic patients (Casiglia and Palatini, 1998). Lower VLDL levels in serum observed in GLEt treated diabetic animals may be due to (a) suppression of hepatic synthesis of VLDL (b) elevation of fatty acid oxidation and/or (c) inhibition of VLDL secretion from the liver (McEneny et al., 2000). As there is a close relationship between elevated serum TC level and the occurrence of atherosclerosis, the ability of GLEt in selective reduction of TC through the reduction of VLDL and LDL components could be beneficial in preventing atherosclerotic conditions and thereby could reduce the possibility of CHD.

Excess of free fatty acids in serum produced by the alloxan lowers the glucose utilization and promotes conversion of excess fatty acids into phopholipids
and cholesterol in liver, which in turn discharged them into blood along with excess triglycerides in the form of lipoprotein (Bopanna et al., 1997). Both increased hepatic production of triglycerides and decreased peripheral removal demonstrated in diabetes, is one of the primary factors in the development of atherosclerosis (Pushparaj et al., 2000; Milagro and Martinez, 2000). The marked hyperlipidemia that characterizes the diabetic state may therefore be regarded as a consequence of the uninhibited actions of lipolytic hormones on the fat depot (Goodman and Gilman, 1985).

Accumulation of TG in the body is one of the risk factors in CHD. The significant increase in the level of TG in liver and kidney of diabetic control rats may be due to deregulation of lipid metabolism associated with insulin deficiency in DM, as under normal condition, insulin activates the enzyme lipoprotein lipase that hydrolysis TG (Frayn, 1993). The administration of GLEt reduces TG in serum and tissues of alloxan-induced diabetic rats and hence may prevent the progression of CHD.

The activity of glucose-6-phosphatase was significantly increased while the hexokinase activity was decreased in the liver of diabetic rats. The higher activity of glucose-6-phosphatase provides H+ to form NADPH from NADP+ and thus is helpful in the synthesis of fats from carbohydrates. Even when glycolysis slows down, the pentose phosphate pathway still remains active in the liver causing continuous breakdown of glucose and supply NADPH. However, administration of GLEt to alloxan-induced diabetic rats resulted in normalizing the activities of hexokinase and glucose-6-phosphatase. This suggests that GLEt reduces plasma lipid content in diabetic rats possibly through its effect on carbohydrate metabolizing enzyme.

HMG-CoA reductase catalyzes the rate-limiting step in cholesterol biosynthesis and its activity correlates closely with the rate of tissue cholesterol synthesis (Magni et al., 1992). Decreased HMG-CoA/Mevalonate ratio indicates increased HMG-CoA reductase activity of the enzyme. In this study, a significant increase in the HMG-CoA reductase levels was observed in diabetic rats as reported in previous studies (Babu et al., 1997; Lagor et al., 2005). Following GLEt treatment, the level of HMG-CoA reductase was found to be reduced. This indicates that GLEt treatment can prevent the complications of diabetes associated changes in lipid profile.
Hyperglycemia has been increasingly linked to alteration in free fatty acid composition. The fatty acid composition of cell membranes can influence membrane-associated phenomena such as the interaction between insulin and its receptor (Stubbs and Smith, 1984). Further, a marked alteration in the fatty acid composition of total lipids was observed in the serum, liver and kidney tissues. There was an increase in the level of saturated fatty acid such as palmitic acid and stearic acid in plasma and tissues of diabetic rats. This observation coincides with the previous findings, which show that there is a preferential synthesis of stearic acid and total saturated fatty acids in diabetic patients (Tilvis et al., 1986; Vessby, 2000). Administration of GLEt to diabetic rats significantly decreased the concentration of saturated fatty acids in the plasma and tissues. In diabetic rats, the concentration of oleic acid was found to be increased significantly. This observation was correlated well with the earlier study that shows an increased concentration of oleic acid in the cell membrane of both type 1 and type 2 diabetic patients (Seigneur et al., 1994). In addition, several reports demonstrated that, the fatty acid compositions of various tissues have been altered in both experimental and human diabetes (Pari and Venkateswaran, 2004).

The linoleic acid (n-6) and/or α-linolenic acid (n-3) are further metabolized by a series of desaturation and elongation steps to produce several polyunsaturated fatty acids, including arachidonic acid (n-6) and eicosapentaenoic acid (n-3), which are major precursors of prostanoids, leukotrienes and other mediators. Diabetes reduces the rate limiting desaturation steps, particularly delta-6-desaturation that converts linoleic acid to γ-linolenic acid and α-linolenic acid to stearidonic acid. Thus, the reduced availability of essential fatty acid intermediates in diabetes is further exacerbated by increased destruction due to elevated free radicals (Cameron and Cotter, 1999). In the present study, the levels of linoleic acid (18:2) and linolenic acid (18:3) were found to be low in diabetic rats.

The administration of GLEt modified the fatty acid composition and the analysis of fatty acids showed that there was an increase in the concentrations of polyunsaturated fatty acids (PUFA) and a decrease in saturated fatty acid (SFA) content in the serum, liver and kidney of diabetic rats. The PUFAs are known to decrease risk of thrombosis and atherosclerosis and lower the incidence of cardiovascular disease (Demaison et al., 1994). This effect of GLEt may also be due to improved glycemic control and increased plasma insulin that allows the diabetic rats treated with plant extract to maintain tissue fatty acid composition at a normal level.
Epidemiologic data supports a positive association between intake of SFA and risk of impaired glucose tolerance, insulin resistance and diabetes (West and Kalbfleisch, 1971). Wang et al. (2003) clearly demonstrated that high proportion of SFAs in plasma was associated with an increased incidence of type 2 diabetes. An increase in the SFA content of cell membranes leads to decreased membrane fluidity, decreased insulin receptor affinity and an increased number of low-affinity receptors (Ginsberg et al., 1981). The results of the present study are consistent with the previous findings and the treatment with GLEt suppressed the disease associated elevation of SFA into near normal level in diabetic rats.

Further, lipid peroxidation markers: TBARS and hydroperoxides contributing to oxidative stress were found to be increased in alloxan-induced diabetic rats. Administration of GLEt to alloxan-induced diabetic rats increased the glutathione content in tissues and decreased the lipid peroxidation. GLEt treatment may lead to high NADP⁺ production which results in down-regulation of lipogenesis, lower risk of the tissues for oxidative stress and high resistance for diabetes.

Apart from the regulation of carbohydrate metabolism, insulin is a potent inhibitor of lipolysis and has direct role in the metabolism of lipids. Therefore, the increase in the plasma lipid levels observed in diabetic rats is mainly due to the lack of insulin. During diabetes, enhanced activity of lipases increases lipolysis and releases more free fatty acids in to the circulation, which raises the cholesterol and other lipids. Insulin inhibits the hormone sensitive lipases in adipose tissue and suppresses the release of free fatty acids. Therefore, it may be suggested that GLEt decreases plasma lipid content in alloxan-induced diabetic rats through its insulin stimulatory effect.

The hyperglycemia coupled with hyperlipidemia increases the risk for cardiovascular diseases in diabetes. Since, GLEt significantly reduces the levels of serum and tissue lipids in alloxan-induced diabetic rats, the antihyperlipidemic effect exhibited by GLEt could represent a protective mechanism against the development of DM associated atherosclerosis.

5.3. **OXIDATIVE STRESS METABOLISM**

Involvement of free radicals in diabetes and diabetes associated lipid peroxidation and the antioxidant defense system in countering their effect have been studied extensively. Several mechanisms such as oxidation of –SH groups,
inhibition of glucokinase, generation of free radicals and disturbances in calcium homeostasis have been proposed as the cause for alloxan-induced diabetes (Szkudelski et al., 2001). In pancreatic β-cells, alloxan was reduced in the presence of reducing agents such as GSH, cysteine and protein-bound sulfhydryl groups or NADH (Lenzen and Munday, 1991; Donnini et al., 1996; Szkudelski et al., 1998). Reduced form of alloxan generates dialuric acid which can be re-oxidized back to alloxan, forming a redox cycle for the generation of $O_2^*$ radicals in the presence of reducing agents. These radicals and their products can damage proteins, lipids and DNA leading to cellular injury and cell death. Initiation of lipid peroxidation is a process solely carried out by free radicals such as $O_2^*$, OH$^*$ and H$_2$O$_2$ causing cellular injury (Halliwell and Gutteridge, 1989). Elevated levels of lipid peroxidation in tissues and plasma is one of the characteristic features of chronic diabetes (Prince and Menon, 2001; Feillet et al., 1999; Venkateswaran et al., 2002). Therefore inhibition of free radical generation and oxidative damage could be considered as an important strategy in the management of diabetes. Hence, this study was undertaken to assess the antiperoxidative and antioxidant properties of *G. montanum* leaf extract in alloxan-induced diabetic rats.

Increase in OH$^*$ formation in alloxan-induced diabetic rats may be elucidated by two biochemical mechanisms. One mechanism is the increased production of activated oxygen species such as $O_2^*$ or H$_2$O$_2$. OH$^*$ radicals are generated from $O_2^*$ or from H$_2$O$_2$ by the iron catalyzed Haber-weiss reaction and Fenton reaction respectively. Another mechanism is inhibiting the activity of antioxidative enzymes (SOD, CAT and glutathione dependent enzymes) that scavenge the activated oxygen species (Chiou and Tzeng, 2000).

### 5.3.1. Lipid peroxidation

It has been reported that ROS, $O_2^*$, and OH$^*$ enhance the lipid peroxidation that damages the cell membrane and its physiological functions leading to disturbances in membrane integrity (Datta et al., 2000). Lipid peroxide-mediated tissue damage has been observed in the development of type 1 and type 2 diabetes. Increased concentrations of lipid peroxidation markers: TBARS and hydroperoxides were observed in plasma and tissues of alloxan-induced diabetic rats. Previous studies have reported increased lipid peroxidation in the liver and kidney tissues of diabetic animals (Sundaram et al., 1996; El-Missiry and El-Gindy, 2000). This increased level of lipid peroxidation could be associated to increase in free radicals generation in diabetes caused primarily due to high blood glucose
levels, which upon autoxidation generates free radicals and secondarily due to the effects of diabetogenic agents like alloxan (Ivorra et al., 1989). Administration of GLEt to alloxan-induced diabetic rats tends to bring the plasma and tissue peroxides back to near normal levels. This indicates that GLEt helps to mitigate oxidative stress mediated complication in the system.

The erythrocytes and erythrocyte membrane of the diabetic animals are found to be more susceptible to H$_2$O$_2$ attack and consequently release higher amount of MDA when compared to the cells obtained from normal rats. In this context, it is suggested that incubation of the RBCs isolated from diabetic rats with sodium azide, an inhibitor of CAT, results in release of higher amounts of TBARS reflecting oxidative stress induced damage in the erythrocytes of diabetic rats. The enhanced release of lipid peroxides from erythrocytes of diabetic rats might be attributed to impairment of antioxidant protection and loss of functional integrity of membrane. The fluidity of erythrocyte membrane derived from diabetic rats is lower and the membrane was easily susceptible against hemolysis induced by peroxyl radicals (Selvam and Anuradha, 1988). Administration of GLEt has effectively reduced the RBC lipid peroxidation in alloxan-induced diabetic rats that suggest the protective property of GLEt on the cell membrane composition and cellular antioxidant potential.

5.3.2. Antioxidants

Associated with changes in lipid peroxidation, the diabetic tissue showed decreased activity of the key antioxidants SOD, CAT, GPx, GST and GSH, which are important in scavenging the toxic intermediates. A decrease in the activity of these cellular antioxidants can lead to an excess availability of O$_2^-$ and H$_2$O$_2$ in biological systems, which in turn generate OH$^*$ resulting in initiation and propagation of lipid peroxidation (Kumuhekar and Katyane, 1992).

SOD and CAT are the two major scavenging enzymes that remove toxic free radicals in vivo. SODs are metalloproteins catalyzing the dismutation of O$_2^-$ to H$_2$O$_2$ and O$_2$ (Sies, 1993). Catalase is a hemoprotein that catalyses the reduction of H$_2$O$_2$ and protects the tissues from highly reactive OH$^*$. It is well documented that the activity of SOD is reduced in DM (Vucic et al., 1997; Feillet-Coudray et al., 1999). Reduced activities of SOD and CAT in liver and kidney as observed in the present study could lead to a number of deleterious effects due to the accumulation of O$_2^-$ and H$_2$O$_2$ (Searle and Wilson, 1980). Therefore, removing O$_2^-$ and OH$^*$ is probably one of the most effective defenses against diseases such as DM, cancer, etc.
Administration of GLEt significantly increased the activities of SOD and CAT in alloxan-induced diabetic rats. The result of increased activities of SOD and CAT suggest that GLEt contains a free radical scavenging activity, which could exert a beneficial effect against pathological alterations caused by the presence of $O_2^*$ and $OH^*$. The activities of GPx and GST were observed to decrease significantly in diabetic rats. These enzymes catalyze the reduction of $H_2O_2$ and hydroperoxides to non-toxic products (Bruce et al., 1982). Depletion of these enzymes may result in deleterious oxidative changes due to the accumulation of toxic products. It has been proposed that GPx is responsible for the detoxification of $H_2O_2$ in low concentrations whereas CAT comes into play when GPx pathway is reaching saturation with the substrate (Salahudeen, 1995). Furthermore, the decreased lipid peroxidation is correlated well in accordance with the induction of antioxidant enzymes. In this context, few reports also described a decrease in the activities of these antioxidant enzymes in the liver and kidney of diabetic rats (Yu, 1994; Farombi and Ige, 2007).

The second line of defense consists of the non-enzymic scavengers such as GSH, vit-C and vit-E, which scavenge residual free radicals escaping from decomposition process mediated by the antioxidant enzymes. As enzymic antioxidants are saturated by the excessive levels of free radicals, the presence of non-enzymic antioxidants is presumably essential for the removal of these radicals (Allen, 1991).

GSH functions as a free radical scavenger and involves in the repair of free radical caused biological damages. A significant decrease in GSH levels was observed in plasma, liver and kidney during diabetes, possibly due to increased utilization of it due to excessive oxidative stress (Sharma et al., 2004). Administration of GLEt increased the GSH content in both plasma and tissues of diabetic rats. The elevated level of GSH protects cellular proteins against oxidation through glutathione redox cycle and also directly detoxifies ROS generation (Yu, 1994).

Reduction in lipid peroxidation observed after administration of GLEt in diabetic rats suggests the protective property of it on the cell membrane composition and cellular antioxidant potential.
5.4. HISTOLOGICAL STUDIES

Oxidative stress is produced as a result of diabetic conditions and possibly causes a variety of tissue damage in patients with diabetes (Baynes and Thrope, 1999). The liver and kidney exhibits numerous morphological and functional alterations during diabetes (Sochar et al., 1985). In this study, pathological examination of diabetic rat liver showed fatty changes and inflammatory cell infiltration around the portal triad. In diabetic rats, collagen deposition was increased in the portal triad with some signs of hepatocyte necrosis (El-Soud et al., 2007). Treatment with GLEt caused marked reduction in fatty changes and inflammatory cell infiltration in the liver. Further diabetic pancreas showed shrinkage of islet cells and growth of adipose tissue in the pancreas. It showed almost complete destruction of β- cells, which may be due to alloxan used in this study. Treatment with GLEt reduced the intensity of these changes in the pancreas, which supports the biochemical analysis. Histopathology of diabetic kidney showed large area of hemorrhage, lymphocyte infiltration and fatty infiltration, which upon treatment with GLEt reduced markedly. The present results indicated that treatment of diabetic rats with GLEt significantly prevented the alteration in liver, kidney and pancreas pathology with the return to their normal texture.

5.5. PROTEOMICS

Proteomic approaches succeed in assessing protein profiles in cells, tissues and body fluids under different circumstances. Disease specific variations in protein expression profiles are identified in different disease states, including various types of cancers, diabetes and inflammatory diseases (Anderson and Anderson, 2002). It is documented that levels of several proteins are altered in serum (Zhang et al., 2004), cell lines and tissues of animal models of diabetes (Korc, 2003). Thus, those proteins, in themselves, either over expressed or suppressed are considered as the markers of diabetes and can also be used as targets for the treatment of diabetes. In the present study, the differentially regulated proteins were studied by monitoring changes in protein expression during the GLEt treatment in serum of alloxan-induced diabetic rats.

In the present study, in 2-D gel analyses, out of 14 differently expressed protein spots, 12 spots were found to be down-regulated and 2 spots were up-regulated in the diabetic rats when compared with control rat serum. Among them, seven proteins were restored - either increased or decreased - to normal level by GLEt treatment. Whereas, no change was observed in the levels of 3 protein spots.
(kallikerin binding protein, haptoglobin-β and α2-HS glycoprotein) in diabetic rat serum when compared with control rat serum.

In accordance with previous report (Manuel et al., 1971), in the present study also, the serum level of hemopexin (Hpx) was found to be increased in alloxan-induced diabetic rats. Hpx is a heme-binding serum glycoprotein produced in the liver and an essential heme scavenger whose primary role is to bind free heme and transport it to the liver (Delanghe and Langlois, 2001). Hpx markedly inhibits heme-catalyzed oxygen radical formation and is important in preventing oxidative damage to lipids and proteins (Hunt et al., 1996; Miller et al., 1996; Chen et al., 1998). Hepatic Hpx gene expression is induced by heme, lead acetate, several chemical carcinogens, endotoxin, hyperoxia and partial hepatectomy (Nikkila et al., 1991; Albrecht et al., 1994; Immenschuh et al., 1995; Wenger et al., 1995). Increased Hpx concentrations have been found in some malignancies, especially malignant melanoma (Manuel et al., 1971) and breast cancer but not in benign breast fibroadenoma (Coombes et al., 1977). In adult epileptics, long-term treatment with phenobarbital resulted in a significant increase in Hpx concentration (Tutor et al., 1982). In the present study, Hpx level was restored by the administration of GLEt in diabetic rats.

The term "acute phase proteins" is used to denote proteins, secreted by the liver, whose levels have been shown to be modulated in both acute and chronic medical disorders (Koh, 1974). One of the major proteins of hepatic origin involved in the acute phase reaction is α₁-anti-trypsin (AAT). The AAT, the major serum serine-protease inhibitor, inhibits the enzymatic activity of neutrophil elastase, cathepsin G, proteinase, thrombin, trypsin and chymotrypsin. AAT facilitates the survival of islet transplants in engrafted patients, because it prevents inflammatory cytokine production, blocks immune cell infiltration and function, inhibits complement activation and delays the development of diabetes in non-obese diabetic mice (Daemen et al., 2000; Lewis et al., 2005). AAT has been shown to have anti-apoptotic and anti-inflammatory effects leads to the delayed type of protection associated with ischemic preconditioning in the kidney during an ischemic / reperfusion injury (Petrache et al., 2006; Janciauskiene et al., 2007). AAT is synthesized primarily in the liver as well as by neutrophils and monocyte/macrophages (Ghavami et al., 2005; Hashemi et al., 2005). The mechanism by which AAT prolongs islet graft survival was demonstrated by Lewis et al. (2005) and reported that (i) AAT reduces the degree of inflammation, (ii) AAT promotes the viability of islets in the presence of inflammatory agents, (iii) AAT decreases
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islet immunogenecity in the form of low MHC class II expression and (iv) AAT reduces infiltration of immune cells elicited by a variety of inducers, including those that are independent of MHC recognition. AAT reported to suppress nuclear factor-κB translocation and increase inhibitor of NF-κB levels in vivo (Churg et al., 2001). Recent report suggested that AAT protects β-cells against apoptosis through inhibition of caspase-3 activity (Zhang et al., 2007). In the present study, the diabetic rats showed decreased levels of this protein and coincided with recent report by Hashemi et al. (2007) that the impaired activity of serum α1-antitrypsin in DM. The administration of GLEt restored this protein into normal level.

Concentrations of serum transport proteins such as albumin (Ab), transferrin (Tf), transthyretin (TTR) and retinol-binding protein (RBP) were found to be decreased in diabetic rat serum but were almost restored to normal value by the GLEt treatment.

TTR is a thyroid hormone-binding protein, most abundant in the choroid plexus but also present in the liver. It probably transports thyroxine from the bloodstream to the brain. In plasma TTR binds RBP and normally circulates as a 1:1 molar RBP-TTR complex. Recently, Refai et al. (2005) found that the total concentration of TTR was decreased in type 1 diabetes. Kemp and Frindik (1991) suggested that TTR might be clinically useful as an indicator of fuel utilization in patients with diabetes, since an increase in TTR was closely related with improved fuel utilization. There are several reports mentioning a significant decrease in the serum TTR concentration in type 1 diabetic patients compared to that in normal subjects (Itoh et al., 1992; Refai et al., 2005). In the present study, it was observed that the concentration of TTR was decreased about 14 fold in diabetic control rats and it was remarkably increased by GLEt treatment and restored to the normal level.

The expression of oxidative stress protein, Tf was down-regulated in the alloxan-induced diabetic rats. Tf is responsible for the transport of iron from sites of absorption and heme degradation to the storage area and found to be down-regulated in liver and serum of diabetic rats (Welch, 1992). Serum Tf may also have a further role in stimulating cell proliferation. One of the most important mechanisms of antioxidant defense is the sequestration of iron in a redox-inactive form by Tf. Lower Tf concentration and its glycation would, by enhancing the prooxidant effects of iron, contribute to the increased lipid peroxidation observed in diabetes (van Campenhout et al., 2003). Tf levels were found significantly lower in
the serum of diabetic patients than those of the controls (Memisogullari and Bakan, 2004). With antioxidant activity to inhibit lipid autoxidation, Tf appeared decreased in the diabetes, suggesting that a defective serum antioxidant status contributes to the increased oxidative stress in IDDM (Asayama et al., 1993). The concentration of this protein was also almost restored by GLEt administration.

Serum Ab, the main protein of plasma/serum, has a good binding capacity for water, Ca, Na, K, fatty acids, hormones, bilirubin and drugs. Its main function is the regulation of the colloidal osmotic pressure of blood. In the present study, the albumin bands were not detected in the diabetic rat serum. It has been found in diabetic animals that hepatic synthesis of albumin was reduced as shown by decreased mRNA levels (Peavy et al., 1978). Unlike other transport proteins, serum albumin levels were not restored even after the administration of GLEt. The lower serum albumin concentration may be due to excess renal excretion in diabetic rats.

Serum RBP is secreted by liver and adipocytes and is implicated in systemic insulin resistance in rodents and humans. RBP delivers retinol from the liver stores to the peripheral tissues (Laurent et al., 1985; Sundelin et al., 1985). RBP normally binds to the larger TTR homotetramer, forming a protein complex that reduces renal clearance of RBP. It has been reported that decreased clearance of serum RBP in insulin-resistant ob/ob mice (Mody et al., 2008). Few studies have shown that insulin-dependent diabetic patients have decreased concentrations of both plasma retinol and its carrier protein when compared with non-diabetic subjects (Basu et al., 1989; Krempf et al., 1991; Martinoli et al., 1993). IDDM patients have also increased urinary excretion of RBP (Holm et al. 1987; Rowe et al. 1987). The results of the studies involving patients with IDDM showed that the plasma RBP as well as TTR concentrations were found to be significantly reduced (Basu et al., 1989; Kemp and Frindik, 1991). In this study also, though the RBP levels were found to be reduced in diabetes, it was normalized by the GLEt administration.

Melanin-concentrating hormone (MCH) is an important mediator of energy homeostasis via regulation of food intake, energy expenditure and locomotor activity (Segal-Lieberman et al., 2006). Mice lacking MCH or its receptor are lean and have an increased metabolic rate (Marsh et al., 2002, Shimada et al., 1998). Segal-Lieberman et al. (2006) reported that MCH exert part of its effects on energy balance via direct pituitary hormone regulation. In alloxan-induced diabetic rats,
MCH level was found to be decreased and restored to the normal level by GLEt administration.

Complement C3, the third complement component, is a cytokine produced by activated macrophages (Zimmer et al., 1982), which are the cells mostly implicated in the formation of atherosclerotic plaques (Libby et al., 1995). Moreover, C3 is an acute phase reactant secreted by adipocytes and the liver (Alper et al., 1969) in response to interleukin-1 which, in turn, is secreted by the activated macrophages in the inflammation sites (Baumann and Gauldie, 1994). Serum C3 is reported as a powerful indicator of the risk of myocardial infarction (MI) (Muscari et al., 2000). The relationship between C3 and MI seems to be specific for MI, since no prospective association was demonstrated with diabetes (Muscari et al., 2000). Few lines of evidences suggested an inverse association of C3 with insulin sensitivity (Muscari et al., 2007). C3 split products were demonstrable in a high percentage in the blood plasma manifested in type 1 diabetic as well as in type 2 diabetic persons (Krantz et al., 1988). Moreover, the levels of C3 have been shown to be reduced by treatment with glibenclamide (Carter et al., 2005). In the present study, although the protein was not detected in the diabetic rat serum, it was completely restored after the administration of GLEt.

α1-macroglobulin (α1-M) and fetuin-β which have no known association with diabetes were found to be decreased significantly in alloxan-induced diabetic rats’ serum. Expressions of these proteins were also restored after GLEt treatment.

It was demonstrated that α2-HS regulates insulin action and plays a pivotal role in regulating postprandial glucose disposal, insulin sensitivity, weight gain and fat accumulation. Thus, this protein considered as a novel therapeutic target in the treatment of type 2 DM and other insulin-resistant conditions (Srinivas et al., 1993; Mathews et al., 2002). In this study, no significant change in this protein level was observed in diabetic rats whereas the administration of GLEt slightly elevated this protein level.

Kallikerin-binding protein (KBP) binds and inhibits kallikenn and also inhibits trypsin (Chao et al., 1990; Pages et al., 1990). Studies in diabetic animal models and patients with diabetes showed potential involvement of the tissue kallikerin-kinin system in DM (Margolius, 1989). In STZ-induced diabetic rats, tissue kallikerin levels in the submandibular gland, kidney and plasma were reduced
(Jaffa et al., 1987; 1992). In the present study, the level of serum KBP was not altered significantly both in alloxan-treated rats as well as in GLEt administered diabetic rats.

The haptoglobin-β (Hp) combines with free plasma Hb, preventing loss of iron through the kidneys and protecting the kidneys from damage by Hb, while making the Hb accessible to degradative enzymes. It is a tetramer of two α and two β chains. Although the β chain is clearly related to serine proteases, Hp has no enzymic activity. In this study, no significant difference was observed in the levels of Hp in the diabetic groups and in GLEt administered group.

5.6. ANTIAPOPTOTIC EFFECT OF GLEt

Alloxan is selectively toxic to pancreatic β-cells and has direct effect on islet cell permeability (Weaver et al., 1978b; Boquist et al., 1983). Morphologic abnormalities in alloxan-treated cells have suggested the disruption of β-cell membrane (Kliber et al., 1996). All the deleterious effects of alloxan on membrane permeability, transport, intracellular energy generating pathways and insulin secretion are mediated through the formation of free radical. Increase in cytosolic Ca$^{2+}$ also plays an important role in the diabetogenic action of alloxan (Nelson and Boquist, 1982; Lenzen et al., 1992). It was reported that alloxan causes DNA strand breaks, activation of PARP, depletion of cellular NAD$^+$ content and ultimately β-cell death (Seckin et al., 1993). Numerous evidences clearly demonstrated the importance of medicinal plants in the treatment of oxidative stress induced apoptosis or β-cell death in experimental diabetes (Tapsell et al., 2006; Jung et al., 2006a).

In this study, it is clearly indicated that GLEt prevented alloxan-induced cell death in RINm5F cells as assessed by MTT assay (Ferrari et al., 1990). In order to assess the protective effect of GLEt against the cytotoxicity of alloxan in RINm5F cells, three different modes of treatments were adapted: pre-, co- and post-alloxan exposure. The results of these studies showed that GLEt alone was not toxic to RINm5F cells at the tested concentrations. Among the three different modes of exposures, pre-treatment of GLEt showed higher protective effect. This may be possibly due to the free radical scavenging activity of the extract.

Lipid peroxidation is the most extensively investigated process induced by free radicals. Possible sources of oxidative stress in diabetes include an increased production of ROS, especially from enhanced glycation and decreased enzymic or
Discussion

Non-enzymic antioxidant defense systems (Halliwell, 1999). ROS participate in the toxic actions that lead to necrosis or apoptosis of the insulin-producing cells. In the present study, increased formation of lipid peroxides was observed in alloxan-treated RINm5F cells. However, pre-treatment of RINm5F cells with GLEt resulted in a decrease in the formation of lipid peroxides indicating that oxidative stress related damage was much less in GLEt-treated cells.

Pancreatic \( \beta \)-cells have low levels of antioxidant enzymes, in particular GPx and catalase (Grankvist et al., 1981). In the present study, alloxan-treated RINm5F cells showed decreased activity of antioxidant enzymes. This decrease may be due to increased oxidative stress induced by alloxan. GLEt pre-treatment caused an increase in the activities of these enzymes in RINm5F cells indicating GLEt was able to reduce alloxan-induced oxidative stress.

Further, flow cytometric studies indicate that GLEt treatment has significantly lowered the proportion of apoptotic cell death caused by alloxan as shown by sub-G1 DNA content. The cells exposed to alloxan exhibited the distinct morphological features of apoptosis, such as increased population of annexin-V positive cells and an increase in the sub-G1 hypo diploid cells (Sakurai et al., 2001). The cell cycle analysis data clearly revealed the protective effects of GLEt on apoptotic and growth inhibitory effects of alloxan in RINm5F cells.

Results of this study indicate that GLEt treatment can decrease oxidative stress by reducing lipid peroxidation and stimulating antioxidant enzymes. It has been shown that elevated extracellular and intracellular glucose levels intensify oxidative stress (Bonnefont-Rousselot et al., 2000), since glucose plays an important role in the prooxidant/antioxidant balance as well as in the antioxidant metabolism. The capacity of GLEt to reduce lipid peroxidation may be due to its function as a preventive antioxidant to scavenge initiating radicals. In conclusion, GLEt treatment reduces damage caused by oxidative stress and protects pancreatic \( \beta \)-cell integrity.

5.7. OXIDATIVE DNA DAMAGE

Oxidative stress induced by reactive oxygen intermediates including \( \text{O}_2^* \) and \( \text{H}_2\text{O}_2 \) is known to cause apoptotic cell death in the pathogenesis of diverse human diseases including cancer, diabetes and neural and endocrine disorders (Halliwell, 1996; Ceriello, 2006). The toxic effects of the free radicals are partially reduced by antioxidants, thereby reduces the oxidative related complications (Sweetman et
There are several intracellular antioxidant mechanisms including scavenging of free radicals, enzymatic inactivation of ROS and metal chelation (Halliwell, 1999) which protect different biological macromolecules including proteins, lipids and nucleic acids from ROS induced damages (Bandyopadhyay et al., 1999). In addition, activation of DNA repair enzymes is also important to maintain the low baseline levels of DNA damage under the normal conditions of continuous oxidant challenge (Szeto et al., 2002). Numerous antioxidants have been tested for their protective property against oxidative stress mediated DNA damage (Zinoveva and Spasov, 2004). Due to the beneficial effects associated with antioxidants, the search for natural antioxidants has greatly increased in recent years. In this context, numerous medicinal plants/plant products have been examined for their antioxidant potential (Vichnevetskaia and Roy, 2001).

DNA in human lymphocytes has become more resistant to oxidative stress following antioxidant pre-treatment and/or after dietary supplementation with antioxidants (Davies, 2000; Scalbert et al., 2005). In this context, several antioxidant rich plants have been reported for their protective property against DNA damage in human lymphocytes (Rani et al., 2005; Santos et al., 2006). Duthie et al. (1997) reported that the dietary flavanoids, quercetin and myrecetin, reduced DNA damage in human lymphocytes. In the present study, the antigenotoxic property of GLEt in human peripheral blood lymphocytes was evaluated. In this experiment, two genotoxic agents (H$_2$O$_2$ and MMS) that cause DNA damage by different mechanisms were used. It is believed that H$_2$O$_2$ causes DNA damage by the generation of the hydroxyl radicals close to DNA molecule via Fenton reaction, whereas MMS is a direct acting methylating agent that produces a base alkali labile site (Petzold and Swenberg, 1978).

While GLEt itself did not induce any DNA damage in human lymphocytes, it confers significant protection against oxidative DNA damage caused by H$_2$O$_2$. However, it didn’t exhibit any protection against MMS induced DNA damage. Similarly GLEt treatment caused a more marked recovery from the H$_2$O$_2$-induced damage. Further, the cells treated with different concentrations of GLEt have significantly lowered the proportion of apoptotic cell death caused by H$_2$O$_2$ as shown by sub-G1 DNA content of the cell population. The cells exposed to H$_2$O$_2$ exhibited the distinct morphological features of apoptosis, such as nuclear fragmentation and an increase in the sub-G1 hypo diploid cells (Pervaiz and Climent, 2002). Lymphocytes treated with H$_2$O$_2$ showed typical features of early and late apoptotic cells. However treatment with GLEt and ascorbic acid prior to
H$_2$O$_2$ treatment greatly reduced the level of early apoptosis suggesting the protective effect of GLEt and ascorbic acid towards the free radical attack.

The free radical (such as OH\textsuperscript{\bullet}, O$_2$\textsuperscript{\bullet} etc) scavenging properties of GLEt might account for its antioxidant capabilities. On the other hand, GLEt showed no evidence of protection against MMS induced DNA damage which is caused by base alkylation. This further confirms the mechanism of protective effect of the GLEt through oxidative pathway and also due to its antioxidant potential.

5.8. ISOLATION AND CHARACTERIZATION OF THE ACTIVE PRINCIPLE

A dark yellowish brown compound was isolated from the ethanol extract of *G. montanum* using silica gel chromatography, using benzene and methanol (95:5) as an eluting mixture. The yield was found to be 45 mg from 500 g of the air dried leaves and the melting point of the compound was found to be 214°C. The isolated compound was characterized using spectral analysis. In its IR spectrum (KBr) ($\nu_{\text{max}}$ Cm$^{-1}$) bands at 3384 (-OH, hydroxyl), 2952, 2921, 2852 (-CH$_3$) methyl and (-CH$_2$-) methylene, 1456 and 1377 (alkenyl -C=C-) showed the corresponding functional groups possibly present in the compound.

The $^1$H and $^{13}$C NMR analysis yielded the complete skeleton of the isolated compound, a broad singlet at $\delta$ 5.12 was observed for OH proton and a multiplet at $\delta$ 3.7-3.9 for a single proton integration showed the presence of -C-H bonded with O, triplet at $\delta$ 0.84 shows 3 proton integration for CH$_2$-CH$_3$-H; doublet at $\delta$ 0.98 for nine proton integrations showed the presence of C$_{30}$-(CH$_3$)$_2$-H and C$_4$-CH$_3$-H; a singlet at $\delta$ 1.25 for six proton integration shows the presence of C$_{10}$, C$_{13}$-CH$_3$; a three proton integration singlet at $\delta$ 1.55 showed the signal of C$_{19}$-CH$_3$; three proton singlet at $\delta$ 1.67 for C$_{20}$-CH$_3$ and multiplet at $\delta$ 2.03-2.34 for twenty six proton for C$_1$, C$_2$, C$_4$, C$_5$, C$_6$, C$_7$, C$_8$, C$_9$, C$_{11}$, C$_{12}$, C$_{14}$, C$_{15}$ and C$_{27}$H-H showed the possible reason of characterizing the compound as a sterol.

In $^{13}$C NMR 72.17 shows carbon which was bonded with oxygen and the four shifts at 125.1, 127.91, 130.97 and 135.07 showed the presence of four carbons which were bonded by two alkenyl double bonds. In $^1$H NMR no signals were observed for alkenyl protons which were clearly confirmed that the alkenyl bonded carbons were bonded with carbons, not with hydrogens.

The molecular weight was indicated by its molecular ion peak at m/z 452.40 and the elemental analysis showed the molecular formula C$_{32}$H$_{52}$O. Successive
Elimination of different ions showed the peaks at 406.1, 341 and 280.9 and the resulting fragments were shown.

All the above spectral analyses confirmed that it possesses methyl substituted dihydrostigmasterol moiety. The above spectral and analytical data
lead to the structure of compound as 3, 6a, 8a, 9, 10-pentamethyl-11-(2-methylpentan-3-yl)-2, 2a, 3, 4, 5, 6, 6a, 6b, 7, 8, 8a, 11, 12,13,13a,13b-hexadecahydro-1H-indeno[2,1-a]phenanthren-4-ol. Further refined techniques like Correlation spectroscopy (COSY), NOESY and X-RD are needed for the stereochemical study of the proposed structure.

3, 6a, 8a, 9, 10-pentamethyl-11-(2-methylpentan-3-yl)-2, 2a, 3, 4, 5, 6, 6a, 6b, 7, 8, 8a, 11, 12, 13, 13a, 13b-hexadecahydro-1H-indeno[2,1-a]phenanthren-4-ol

Stigmasterol- (Z)-17-(5-ethyl-3, 6-dimethylhept-2-en-2-yl)-10, 13-dimethyl-2, 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol.

So far, no studies have been carried out on the phytochemistry of G. montanum leaves. This is the first report deals with isolation and characterization of the active compound(s) from this plant. In the present study, a sterol compound was isolated and found that it is closely related to stigmasterol, which is an important unsaturated phytosterol with a molecular weight of 412.691 and a formula of C_{29}H_{48}O. The isolated compound has been designated as GPS throughout the study.
Literature search has indicated that stigmasterol is useful in prevention of certain cancers, including ovarian, prostate, breast and colon cancers (Normén et al., 2001). Studies have also indicated that a diet high in phytosterols may inhibit the absorption of cholesterol and lower serum cholesterol levels by competing for intestinal absorption. Studies with laboratory animals fed with stigmasterol found that both cholesterol and sitosterol absorption decreased 23% and 30% respectively over a 6 week period (Ashok et al., 2006). Results from previous experiments have shown that plant sterols are capable of reducing serum total cholesterol and LDL-cholesterol levels (Miettinen et al., 1995; Hallikainen et al., 2000; Hendriks et al., 1999; Jones et al., 2000). Phytosterol and its derivatives are widely applied in pharmaceutical, food and cosmetic industry due to their special biological-activity, physical and chemical properties.

The isolated compound was tested for its antidiabetic activity using alloxan-induced diabetic rats. GPS administered at 500 µg/kg b.w for 7 days showed a significant decrease in blood glucose and increase in plasma insulin level in diabetic rats. From this data, it is clear that GPS might enhance glucose utilization since it significantly reduces blood glucose in diabetic rats. GPS exhibited antihyperglycemic activity and the effect was comparable with that of reference drug, glibenclamide.

The protective effect of GPS was studied against alloxan-induced cell damage in RINm5F cell line in vitro. Numerous evidences reported that alloxan toxicity is mainly through the generation of free radicals (Szkudelski, 2001; Lenzen, 2008). In the present study, GPS pre-treatment at doses of 2 and 5 µg/ml showed dose-dependent protection against alloxan-induced cell death in RINm5F cells. Flow cytometric analyses were carried out to determine the mode of cell death, cell cycle analysis and annexin-V-assay. In this experiment, the cells were treated with alloxan, alloxan + GPS (2 µg/ml) and alloxan + GPS (5 µg/ml). There was a marked increase in the sub-G1 phase in alloxan-treated cells as compared with control cells that further confirm the occurrence of apoptosis. This marked increase in the sub-G1 peak in alloxan-treated cells was reduced by GPS treatment. The annexin-V positive cells were markedly increased in alloxan-treated group, which was reduced by GPS treatment. These findings showed the protective property of GPS against alloxan mediated cell death. The role of free radicals in the alloxan mediated cell death have been confirmed by the increase in the levels of TBARS and decline in the levels of antioxidants, which were normalized by the GPS.
treatment. These findings indicate that the cytoprotective property of GPS is possibly due to the scavenging property of free radicals.

Data obtained by these experiments revealed that GPS has beneficial effects in diabetic animals, which is exerted through its antihyperglycemic, antioxidant, antiapoptotic and cytoprotective action. Thus this study supports the notion that treatment with GPS would help in achieving glycemic control by offering protection and preserving the residual β-cell mass without further loss.