Chapter -IV

DISCUSSION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Chronic hyperglycemia leads to the development of micro and macro vascular complications which in turn leads to increased morbidity and mortality (Hoogwerf *et al.*, 2005). Several pharmaco therapeutic agents, including sulfonylureas, biguanides, thioazolidinediones and α- glycosidase inhibitors are widely used for the management of diabetes mellitus which directly or indirectly regularize blood glucose levels. These moieties are associated with characteristic profiles of side effects (Chandramohan *et al.*, 2008). World health organization committee on diabetes recommended that, plant derived bioactive constituents possessing hypoglycemic activity may provide a complementary utilization source for the development of new oral antihyperglycemic drugs by pharmaceutical entities (WHO-1980). Hence drug discovery shifted its focus on novel type of antidiabetic medications from plant origin, having minimal/ no side effects which made an expanding request by patients to utilize the natural products having antihyperglycemic activity (Kameswara Rao & Appa Rao, 2001).

Plant derived active compounds have been effective in preventing and managing of diabetes (Marles & Fransworth, 1995). In recent years, several researchers have studied the worth of different medicinal plants in controlling the DM and delaying the long term effects of DM. Several studies describe the effects of polyphenols in insulin secretion. Flavonoids exhibit various biological activities such as antioxidant, anti-inflammatory, antiallergic, vasodilatory and anticarcinogenic properties (Rice-Evans *et al.*, 1996). Due to the presence of phenolic group in structure flavonoids possess antioxidant activity which emphasizes potential health promoting and disease preventing nature (Fernandez *et al.*, 2006). In various traditional ethnic remedies flavonoids have been reported as antidiabetic components (Jung *et al.*, 2006) widely used for the management of diabetes mellitus by minimizing the blood glucose levels (Ruter *et al.*, 2010). Kolaviron, a biflavonoid complex isolated from *Garcinia kola*, has hypoglycaemic and hypolipidemic activities (Adaramoye & Adeyemi, 2006).

In the traditional system of medicine *A. echioides* is used as folk medicine for the treatment of dysentery, swellings, jaundice (Nadkarni, 2005) besides possessing hepato protective and antioxidant properties (Basu *et al.*, 2009). Phytochemical analysis of *A. echioides* revealed the presence of various flavonoids, which support the plant as a rich source of flavonoid constituents (Yang Shen *et al.*, 2013). This focused our interest towards isolation of total oligomeric flavonoid (AETOF) fraction from leaves of *A. echioides* and to evaluate its antihyperglycemic activity and to identify the phytochemicals present in it. Hence the present study was taken up to investigate the antihyperglycemic property of AETOF fraction in STZ induced diabetic rats. In our study Streptozotocin (STZ) was used to induce diabetes mellitus in rats. At low dose, STZ (50 mg/kg bw) partially destruct the beta cells, which secrete insufficient insulin causing type 2 diabetes (Gomes *et al.*, 2001). It is widely accepted animal model and reported to resemble human hyperglycemic non ketotic diabetes mellitus (Weir *et al.*, 1981) in STZ-induced diabetic rats. A significant decrement in the FBG levels after treatment with glibenclamide, an oral hypoglycemic agent gave an evidence for the presence of remnant β-cells in STZ induced rats (Vinay Kumar *et al.*, 2010).

In the present study, administration of CASAE to STZ induced diabetic rats caused a decrease of 63 % in their FBG levels confirming the antihyperglycemic activity of the leaves of *A. echioides*. Based on the earlier reports (Govindachari *et al.*, 1965; Jayaprakasam *et al.*, 2001), we have isolated total oligomeric flavonoid fraction from leaves of *A. echioides*. The flavonoid content present in the AETOF fraction was quantitatively found to be 174 ± 9.4 µg quercetin equivalents (QE)/mg of AETOF fraction.

LC-ESI-MS/MS analysis is one of the hyphenated techniques for metabolite profiling of biological and chemical analysis for qualitative purpose (Jemal, 2000). The AETOF fraction when subjected to LC-ESI-MS/MS analysis resulted in the presence of 32 phytochemical constituents which were identified and confirmed by mass fragmentation analysis (Table. 7). These results confirmed that AETOF fraction is a flavonoid rich fraction which could be responsible for the antidiabetic activity. Literature revealed that flavonoids and polyphenols are being used for the management of diabetes (Martinello *et al.*, 2006).

The results of our study on the antihyperglycemic activity of the AETOF fraction demonstrated that oral administration of AETOF fraction in the diabetic rats at a dose of 30, 50 and 70 mg/kg bw produced a significant reduction (57.2%, 68.4% and 83.8% respectively) of glycemic levels after 6h of treatment in a dose dependent manner. All the results were compared with those of standard oral hypoglycemic agent glibenclamide. None of the doses of the AETOF fraction caused any hypoglycemia in normal treated rats. But the dosage of AETOF fraction (70 mg/kg bw) caused hypoglycemia in diabetic rats after 4 hours of treatment, but same dosage did not produce any hypoglycemic condition in normal rats. This may be due to the efficient activity of the counter regulatory hormones to insulin and this mechanism was lacking in the STZ induced diabetic rats (Swapna *et al.*, 2013). The possible mechanism of antihyperglycemic activity of AETOF fraction could be due to increase in the pancreatic secretion of insulin from remnant β-cells. There is an evidence that more than one flavonoid like Naringin and Hesperidin combine to perform an insulin release activity for the management of diabetes in a synergistic manner (Mahmoud *et al.*, 2012) and poly flavonoids isolated from *Acacia auriculiformis* like gallic acid, caffeic acid, catechin, rutin, quercetin, myricetin and kaempferol were shown to have combined activity for the treatment of diabetes (Sathya *et al.*, 2012). The oral glucose tolerance test (OGTT) in diabetic rats also showed that the AETOF fraction possess blood glucose lowering activity. The AETOF fraction enhanced the glucose utilization, so blood glucose levels were significantly decreased in glucose loaded rats. The OGTT study also has confirmed 50 mg/kg bw to be the most effective dose. Thus, the present study shows the antihyperglycemic property of the AETOF fraction in diabetic rats without any hypoglycemic action in normal rats. No evidence of hypoglycemia in normal rats after the administration of the AETOF fraction makes it to be safer than glibenclamide. Flavonols present in fruit of *Diospyros peregrina* were shown to be responsible for antidiabetic activity (Saikat Dewanjee *et al*, 2009). Flavonoids are known to be bioactive antidiabetic principles (Oliver- Bever, 1986; Ivorra *et al.*, 1989; Atta-Ur-Rhemann and Khurshid Zaman, 1989; Kameswara Rao *et al.*, 1997). Flavonoid rich fraction of *Pilea microphylla* (L.) is an effective antidiabetic agent in high fat diet/streptozotocin-induced diabetes in mice (Bansal *et al.*, 2012). Ragunathan and Sulochana (1994) showed a significant hypoglycaemic activity of a new flavonol bioside from the flowers of *Hibiscus vitifolius* (L.), in glucose-induced hyperglycaemic rats.

Chronic diabetes leads to increased hepatic glucose output. In this condition liver glycogen stores are mobilised and then hepatic gluconeogenesis is used to produce glucose. Decreased levels of insulin impair glucose transport in to the tissues. Due to deficiency of insulin, nonhepatic utilization of glucose in particular muscle and adipose tissue is markedly reduced. The combination of increased hepatic glucose production and reduced peripheral tissue metabolism leads to elevated plasma glucose levels. Severe hyperglycemia in untreated diabetic rats results from abnormal persistence of hepatic glucose production due to impairment in insulin's suppressive effect on gluconeogenesis.

In the long term study, the maximum 71.7% decrease in FBG levels were observed at a dose of 50 mg of AETOF/kg bw and no significant effect was observed on increasing the dose further. Therefore long term treatment of diabetic rats was carried out with this dose of AETOF fraction. In the present study, elevated fasting blood glucose and depleted plasma insulin levels were observed in diabetic untreated rats compared to normal rats. Oral administration of AETOF fraction at a dosage of 50 mg /kg bw for 40 days resulted in a significant reduction in fasting blood glucose and significant rise in insulin levels in STZ induced diabetic rats. The possible mechanism by which the AETOF fraction exhibiting the antihyperglycemic action in diabetic rats could be due to its stimulating effect on the remnant β-cells of islets of Langerhans, increasing insulin secretion and thereby increasing the oxidation of glucose in various tissues (Prakasam *et al.*, 2002). The effect of AETOF fraction seems to be similar to that of glibenclamide which induces insulin exocytosis from β-cells (Tian *et al.*, 1998). Glibenclamide has been used for many years to treat diabetes, to stimulate insulin secretion from pancreatic β-cells (Babu *et al.*, 2007). There are many scientific reports showing flavonoids possessing antidiabetic activities. The quercetin glycoside, rutin, when orally administered (25-100 mg/Kg bw) to STZ-induced diabetic rats for 45 days induced an increase in insulin secretion (Kamalakkannan & Prince., 2006). Flavone, apigenin glycoside (Apigenin-6-C-(2''-O-rhamnosyl)-fucoside) was found to have an acute effect on blood glucose lowering when administered orally (50 mg/Kg bw) to diabetic rats and stimulated glucose-induced insulin secretion after oral treatment in hyperglycemic rats (Cazarolli *et al.*, 2009). Isovitexin and swertisin are two C-glycosylflavones admistered orally at the dose of 15 mg/Kg bw, showed antihyperglycemic effect in hyperglycemic rats as well as stimulated significantly insulin secretion in vivo (Folador *et al.*, 2010).

The insulin secretagogue activity of AETOF fraction is more pronounced than that of glibenclamide. In this context, a number of flavonoids isolated from other plants have been reported to have antihyperglycemic activity with a stimulatory effect on insulin release. Naringin and Hesperidin combine to perform an insulin release activity for the management of diabetes in a synergistic manner (Mahmoud *et al.*, 2012). Apigenin-6-C-β-L-fucopyranoside a flavonoid isolated from *Averrhoa carambola* enhanced glycogen synthesis and insulin secretion in hyperglycemic rats. Results on the insulin release from pancreas directly indicates that antihyperglycemic activity of the AETOF fraction is through the release of insulin from the pancreas i.e. it exerts a direct insulinotropic effect.

In streptozotocin induced diabetic rats, loss of body weight is due to the increased muscle wasting and loss of tissue proteins (Swanston Flat *et.al.*, 1990; Chatterjee *et al.*, 2002). Hakim, (1997) has stated that decreased body weight in diabetic rats could be due to dehydration and catabolism of fats and proteins. Increased catabolic reactions leading to muscle wasting might also be the cause for the reduced weight gain by diabetic rats (Rajkumar *et al.*, 1991). The results obtained in this study have shown that, administration of AETOF fraction to diabetic rats for a period of 40 days, caused a significant gain in body weights in diabetic treated rats and the results were comparable with that of the standard oral hypoglycemic agent glibenclamide. The body weight gain could be due to improved insulin secretion and glycemic control in the treated diabetic rats. Rutin administration to diabetic rats for a period of 45 days significantly improved the body weight and this could be due to a better control of the hyperglycaemic state in the diabetic rats (Kamalakkanan *et al.*, 2006). Decreased levels of blood glucose could improve body weight in streptozotocin-diabetic rats (Kamalakkanan *et al.*, 2003).

In diabetic rats, the levels of HbA1c are increased due to the persistent hyperglycemia which results in glycation of haemoglobin. HbA1c is used as a marker for estimating the degree of protein glycation in diabetes mellitus. HbA1c was found to increase in patients with diabetes mellitus and the amount of increase is directly proportional to the fasting blood glucose level (Al-yassin & Ibrahim, 1981). In diabetic condition, the excess glucose present in the blood reacts with haemoglobin to form HbA1c (Koening *et al.*, 1976). Hence HbA1c levels were elevated and total haemoglobin levels were depleted in untreated diabetic rats. HbA1c levels were well regulated near to normal levels in AETOF fraction treated diabetic group, this could be due to an improvement in insulin secretion and also insulin action. In this

context a number of other plants such as *Cucumis trigonus, Musa sapientum, Aegle marmelos, Aloe vera, Annona squamosa, Coscinium fenestratum* have also been observed to have similar effects on glycosylated haemoglobin (Salahuddin *et al.*, 2010; Pari & Umamaheswari., 2000; Narendhirakannan *et al.*, 2006; Tanaka *et al.*, 2006; Gupta *et al.*, 2005). Improvement in fasting blood glucose decreased the levels of HbA1c with simultaneous increase in the levels of haemoglobin in diabetic rats treated with AETOF fraction indicating its effect on carbohydrate metabolism.

During the diabetes, the decrease in total protein might be due to increased protein catabolism due to insulin deficiency (Almdal *et al.*, 1998). In our study STZ induced diabetes resulted in decreased levels of total plasma, liver and kidney proteins. But after the oral administration of AETOF fraction for 40 days, there was a significant improvement in the plasma and tissue proteins. Glycogen levels in liver were low in diabetic animals. Liver is regarded as the central metabolic organ in the body, with an important role in glucose and lipid homeostasis (Saravanan & Pari ., 2003). During diabetes, there is a decrease in liver weight due to enhanced catabolic processes such as glycogenolysis, lipolysis, proteolysis (Umesh *et al.*, 2005) and therefore the quantification of glycogen, the primary intracellular storage form of glucose in liver can be considered as an important indicator of diabetes mellitus. Glycogen levels in various tissues especially in liver and muscles indicate direct reflection of insulin activity since it regulates glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase. Since STZ causes selective destruction of β-cells of islets of Langerhans resulting in marked decrease in insulin levels, it could be predicted that glycogen levels in tissues (muscle and liver) decrease as the influx of glucose in the liver is inhibited in the absence of insulin and recovers on insulin treatment (Golden *et al.*, 1979; Weber *et al.*, 1966; Vats *et al.*, 2004). Our results showed that in diabetic rats after treatment with AETOF fraction for 40 days there was significant elevation in muscle, kidney and hepatic glycogen content. Flavonoid rich extract of *Eugenia jambolana* seeds (Bhavna *et al.*, 2008) and *Gymnema montanum* (Kunga Mohan Ramkumar *et al.*, 2011) also effectively increased both hepatic and skeletal muscle glycogen concentration in STZ induced diabetic rats.

The insulin dependent hexokinase and glucose-6-phosphate dehydrogenase are key enzymes for maintaining the glucose homeostasis (Ugochukwu & Babady., 2003; Mayes, 2000). Both of these enzyme activities were decreased in STZ induced diabetic rats, which is in agreement with our previous observations (Vinay Kumar *et al.*, 2010; Ramesh Babu *et al.*,

2010). The decreased levels of HK and G-6-PD may be due to the impairment in glycolytic and pentose phosphate pathways in diabetic state respectively (Saraswathi Pannerselvam *et al.*, 2002). The insulin secretagogue action of AETOF fraction may be the cause for maintaining HK and G-6-PD activities to near normal levels in diabetic treated rats. Hexokinase is the key enzyme catalyzing the conversion of glucose to glucose-6-phosphate and also hexokinase activity is severely impaired during diabetes (Hua Zhou *et al.*, 2007). In the present study both hepatic and kidney HK activities were decreased in diabetic rats compared to those in normal rats, which may be due to insulin deficiency. Decreased activity of hepatic HK in diabetic animals was previously shown by several researchers (Sheela & Augusti., 1992; Bopanna *et al.*, 1997). Administration of AETOF fraction to diabetic rats enhanced the hexokinase activity in liver and kidney, which could be due to increased insulin secretion. The increased activity of hexokinase inturn increases glycolysis and increased utilization of glucose for energy production leading to reduced blood glucose levels

Glucose-6-phosphate dehydrogenase (G-6-PDH) is the key enzyme to maintain normal blood sugar level (Mayes, 2000). In streptozotocin induced diabetic rats, there is reduction of G-6-PDH activity in liver which obstruct glucose utilization through pentose phosphate pathway as this enzyme activity is controlled by insulin (Ugochukwu & Babady., 2003). Insulin is reported to increase the activity of G-6PDH in a dose dependent manner (Pannerselvam & Govindaswamy., 2002). Administration of AETOF fraction to diabetic rats increased the activity of G-6PDH considerably. This may be attributed to the insulin stimulatory effect of AETOF fraction. Similar results were observed with *Catharanthus roseus* (Som Nath *et al.*, 2001), *Coccinia indica* leaves, *Momordica charantia* (Shibib *et al.*, 1993).

Glucose-6-phosphatase (GP) and Fructose-1,6 bisphosphatase (FB) are the key enzymes in gluconeogenesis. The enzyme levels were observed to be increased in diabetic rats (Arathi *et al.*, 2003). The increased activity may be due to insulin insufficiency. In tigogenin treated rats, the activities of glucose-6-phosphatase and fructose-1,6 bisphosphatase were found to be decreased and it may be due to the modulation and regulation of the activities of these two gluconeogenic enzymes either through regulation of cAMP or inhibition of gluconeogenesis (Gupta *et al.*, 1999). In the present study an attempt was made to gain an insight into the underlying biochemical mechanisms in the action of AETOF fraction, we assayed the key enzymes of gluconeogenesis GP and FB from liver and kidney of diabetic rats. GP, one of the key enzymes in the homeostatic regulation of blood glucose levels,

catalyzes the terminal step in both gluconeogenesis and glycogenolysis (Béaudet., 1991). The enzyme is mainly found in the gluconeogenic tissues liver and kidney (Gupta *et al.*, 1999), where it plays a major role in homeostasis of glucose and inorganic phosphate. FB catalyzes one of the irreversible steps in gluconeogenesis and serves as a site for the regulation of the process (Tillmann *et al.*, 2002). The flavonoid components like catechin, hesperitin 5-O-glucoside, pruning present in the methanolic extract of *Prunus davidiana* stem exhibited significant hypolipidemic and also hypoglycemic effects (Okazawa and Uro., 1991). Administration of Diosmin, a citrus flavonoid to STZ-Nicotinamide-induced diabetic rats altered the plasma and tissue levels of lipids and lipid metabolizing enzymes to near normal levels thus minimizing further diabetic complications (Srinivasan and Pari., 2013). Epicatechin, a constituent of the stem bark of *Pterocarpus marsupium*, is believed to be the principle responsible for antihyperglycemic and antihyperlipidemic activity (Farboodniay Jahromi *et al.*, 1993).

The activities of GP and FB were increased in the present study in both liver and kidney of diabetic rats compared to those of normal rats and these enzyme activities were decreased to near normal levels after the treatment with AETOF fraction. The increased activity of hepatic and or kidney gluconeogenic enzymes (GP and or FB) in diabetes were reported by several other researchers (Sunitha Kumari *et al.*, 2012; Ananda Prabu *et al.*, 2012; Prince *et al.*, 1997). The activities of these enzymes were also reverted back in diabetic animals treated with glibenclamide.

The lipid changes associated with diabetes mellitus are attributed to increased free fatty acid flux secondary to insulin resistance (Arshag., 2009). Abnormalities in lipid profile are one of the most common complications in diabetes mellitus. It is associated with profound alterations in the plasma lipid, triglycerides and lipoprotein profile and with an increased risk of coronary heart disease (Maghrani *et al.*, 2004). Insulin deficiency causes an increase in free fatty acid mobilization from adipose tissue which results in increased production of cholesterol rich LDL particle and dyslipidemia. The increased production of cholesterol rich LDL particle is mainly due to elevated levels of HMG-CoA reductase, a key rate-limiting enzyme responsible for the metabolism of cholesterol rich LDL particles. Insulin has inhibitory action on HMG-CoA reductase. In the present study treatment with the AETOF fraction for 40 days resulted in significant reduction in the ratio of HMG-CoA and mevalonate, which is an indicator of HMG-CoA reductase activity in diabetic rats.

STZ-induced diabetes also developed hyperlipidemia which is in agreement with our previous observations (Sireesha *et al.*, 2011; Sameena Fatima *et al.*, 2010). In the present study, the treatment with AETOF fraction produced marked decrease in serum TC, TG, LDL-C and VLDL-C levels and a significant increase in HDL-C. There is substantial evidence that lowering the TC, particularly the LDL-C level will lead to a reduction in the incidence of coronary heart disease, which is still a leading cause of death in diabetic patients. Lower VLDL-C levels in serum of diabetic rats treated with AETOF fraction may be due to (i) repression of hepatic synthesis of VLDL-C (ii) elevation of fatty acid oxidation and/or (iii) inhibition of VLDL-C secretion from the liver. As there is a close relationship between elevated serum TC level and the occurrence of atherosclerosis, the ability of the AETOF fraction in selective reduction of TC through the reduction of VLDL-C and LDL-C components could be beneficial in preventing atherosclerotic conditions and thereby reduce the possibility of coronary heart disease. However, it is interesting to find that in the present study the AETOF fraction at a dose of 50 mg/kg bw not only lowered the TC, TG, and LDL levels but also enhanced the cardio protective lipid HDL after 40 days treatment. In the present study the AETOF fraction could reverse the metabolic derangements of lipoproteins in diabetic treated rats. The treatment with AETOF fraction reduced the levels of total cholesterol, TG, LDL, VLDL-C and improved HDL-C. This would definitely reduce the incidence of coronary events (Lipid Research Clinics Programs., 1984), which is the major cause of morbidity and deaths in diabetic subjects (Baynes., 1991). Earlier studies from our laboratory (Kameswara Rao *et al.*, 2003; Sireesha *et al.*, 2011, Sameena Fatima *et al.*, 2010; Nabi *et al.*, 2012, Prasad *et al.*, 2012) and several other groups have reported the antihyperlipidemic activities of medicinal plants in experimental diabetic animals.

Diabetes is marked by increased production of free radicals or impaired antioxidant defenses. The generation of superoxide anion radicals by glucose oxidization and its dismutation to hydrogen peroxide leads to the formation of reactive hydroxyl radicals (Jiang *et al.*, 1990; Wolff *at al.*, 1987). In diabetes, it is thought that hypoinsulinemia increases the activity of the enzyme, fatty acyl coenzyme A oxidase, which initiates β-oxidation of fatty acids, resulting in lipid peroxidation (LPO) (Rahimi *et al.*, 2005). Increased LPO impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors (Soon & Tan., 2002).

The significant effect of LPO products that was measured as thiobarbituric acid reactive substances (TBARS) has been reported in diabetes (Pari & Latha., 2005; Rajasekaran *et al.*, 2005). In the present study, we observed a significant increase in lipid peroxide levels (TBARS) in plasma and tissues (liver and kidney) of diabetic rats compared to normal rats. Administration of AETOF fraction or glibenclamide potentially abrogated the levels of TBARS in plasma and tissues of diabetic rats. This shows that AETOF fraction might protect the plasma and tissues against the cytotoxic action and oxidative stress of Streptozotocin. The reduction in lipid peroxidation could be due to the improvement of the glycemic control and increased insulin secretion as insulin decrease the activity of fatty acyl coenzyme A oxidase. A number of other plants and their extracts such as methanolic extract of flowers of *Pterospermum acerifolium* (Papiya Mitra Mazumder *et al.*, 2011), methanolic extract of gum resins of *Commiphora mukul* (Ramesh *et al.*, 2011), hydroalcoholic seed extract of *Nymphaea nouchali* (Parimala *et al.*, 2013), ethanolic extract of *Punica granatum* flowers (Manoharan *et al.*, 2009), have also been observed to have similar effects on lipid peroxidation.

Implication of oxidative stress in the pathogenesis of diabetes mellitus is suggested not only by oxygen free radical generation but also due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired antioxidant enzyme, and formation of peroxides (Vincent *et al.*, 2004). DM is associated with increased formation of free radicals and decrease in antioxidant potential. . Due these events, the balance normally present in cells between radical formation and protection against them is disturbed (Nazirogilu & Butterworth., 2005). An imbalance of oxidant/antioxidant defense systems results in alterations in the activity of antioxidant enzymes, such as SOD, CAT, GR, GPx, and impaired glutathione metabolism (Maritim *et al.*, 2003). The present data indicates that STZ-induced diabetes disrupts actions of Plasma and tissue (liver and kidney) antioxidant enzymes. The decreased activities of these enzymes in pancreas may be due to the production of ROS such as superoxide (O^{-}_{2}), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH) that reduces the activity of these enzymes (Kaleem *et al.*, 2006).

SOD has been postulated as one of the most important enzymes in the enzymatic antioxidant defense system which catalyses the dismutation of superoxide radicals to produce H_2O_2 and molecular oxygen, hence diminishing the toxic effects caused by their radical. The H_2O_2 produced by SOD is excreted as H_2O based on the activity of glutathione peroxidase and catalase (Punitha *et al.*, 2005), therefore protecting the body from oxygen toxicity. However,

this process can cause lipid peroxidation if H_2O_2 is not decomposed immediately. Moreover, CAT (a hemoprotein) is known to be involved in detoxification of high H_2O_2 concentrations, whereas GPx (a selenium containing enzyme) is sensitive to lower concentrations of H_2O_2.

In our study, the activities of SOD, GPx and GST were decreased in diabetic rats compared to normal rats, which could be due to free radical-induced inactivation and glycation of the enzymes in diabetic state (Al-Azzawie & Alhamdani, 2006). Long-term treatment of diabetic rats with AETOF fraction had reverted the activities of these enzymatic antioxidants, this means that the flavonoid fraction can reduce the potential glycation of enzymes or they may reduce reactive oxygen free radicals and improve the activities of antioxidant enzymes.

In our study the activity of CAT was significantly increased in liver and kidney of diabetic untreated rats. The possible explanation for the increase in catalase activity is that it could be a compensatory mechanism to prevent tissue damage by the increased levels of H_2O_2 and decreased levels of GPx. In diabetes, it is thought that hypoinsulinemia increases the activity of the enzyme, fatty acyl coenzyme A oxidase, which initiates β-oxidation of fatty acids, resulting in increased levels of H_2O_2. The CAT activity was restored to near normal in diabetic rats treated with AETOF fraction, which might be due to decreased LPO levels and increased insulin secretion.

Various studies in the past reported conflicting results regarding the status of antioxidant enzymes in diabetes (Selvam & Anuradha., 1988; Kaji *et al.*, 1985; Matkovics *et al.*, 1982). Majority of authors reported the decreased enzymatic antioxidant activities (SOD, CAT, GPx and GST) in tissues of diabetic rats (Selvan *et al.*,2008). A few authors had shown an increased activity of catalase in diabetic erythrocytes (Matkovics *et al.*, 1977; Kesavulu *et al.*, 2001) or tissues (Matkovics *et al.*, 1977; cekic *et al.*, 1999) while others could not detect any change. A few studies have reported increased GPx activity (Rema *et al.*, 1995). Some authors reported increased SOD activity (Matkovics *et al.*, 1977) in diabetes mellitus. Hazem *et al.*, (2007) reported elevated levels of enzymic antioxidants SOD and reduced CAT in diabetic rats.

GSTs are a super family of detoxifying enzymes that have broad substrate specificity (Hayes & Pulford., 1995). Five families of cytosolic GSTs have been identified in humans, of which four have been thoroughly characterized: Alpha (α), Mu (μ), Pi (π) and Theta (τ). The GSTs conjugate GSH with compounds containing an electrophilic centre thereby provide

critical protection against xenobiotics and products of oxidative stress. Since the GSH-conjugate is transported out of the cell, intracellular GSH is consumed irreversibly in the conjugation and thus maintenance of intracellular GSH levels is essential for the optimal function of GSTs. Many GST enzymes possess GPx activity as well. Many of the substrates of GSTs also induce the expression of the GST genes, suggesting an adaptive response to chemical stress. Carcinogens and alkylating agents may induce GST (Zhang *et al.*, 1998).

GSH has a multifactorial role in antioxidant defense. It is a direct scavenger of free radicals as well as a cosubstrate for peroxide detoxification by glutathione peroxidases (Wohaieb *et al.*, 1987). Loven *et al.*, (1986) suggested that the decrease in tissue GSH could be the result of decreased synthesis or increased degradation of GSH by oxidative stress in diabetes. Increased oxidative stress, resulting from significant increase in aldehydic products of lipid peroxidation has probably decreased hepatic GSH content. In the present study, the elevation of GSH levels in liver and kidney was observed in the AETOF fraction treated and glibenclamide-treated diabetic rat. This indicates that the AETOF fraction and glibenclamide can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH, or have both effects. Reduced Glutathione, a non-enzymatic antioxidant is known to accord protection against reactive O_2 species by effectively scavenging free radicals and other ROS directly and indirectly through enzymatic reactions. The glutathione levels in the cell are maintained by activities of GR, GST and G6PDH. In diabetic untreated rats the activity of G6PDH is markedly reduced due to insulin deficiency resulting in impaired flow of glucose through hexose monophosphate shunt. Hence the supply of NADPH is reduced. The decreased availability of NADPH in diabetes could be due to either reduced synthesis of NADPH through HMP shunt or increased utilization of NADPH through polyol pathway. The lowered NADPH level decreases the activity of glutathione reductase which in turn affects the production of GSH. Low levels of GR and GSH further influence activity of GST, a multifunctional enzyme requiring GSH. Deficiency of GSH may lead to various complications like neuropathy, myopathy and cataract in diabetic rats (Amin et al., 2006).

Vitamin C also plays an important role in detoxification of reactive intermediates produced by Cytochrome P450, which detoxify xenobiotics (Prakasam *et al.*, 2005). Vitamin C is one of the most powerful natural antioxidant (Bendich *et al.*, 1986), capable of regenerating α-tocopherol from tocopheroxyl radical that is formed upon the inhibition of lipid peroxidation by vitamin E (Niki *et al.*, 1982). Vitamin C levels are reduced in plasma from

patients with NIDDM (Sundaram *et al.*, 1996) and metabolic syndrome (Ford *et al.*, 2003), and in the plasma, liver, and kidneys of STZ-induced diabetic rats (Obrosova *et al.*, 2003). The amount of Vitamin C present in the plasma is inversely related to the duration of NIDDM (Sundaram *et al.*, 1996). Treatment of diabetic rats with the antioxidant α-lipoic acid (Obrosova *et al.*, 2003) or insulin (Kashiba *et al.*, 2002) partially suppresses the loss of Vitamin C in the plasma, liver, and kidney, indicating the importance of overall antioxidant status and glycemic control in the maintenance of vitamin C levels. In the present study, Vitamin C levels were decreased in STZ induced diabetic rats compared to those in normals.

In diabetes the decrease in ascorbic acid level in plasma and tissues (liver and kidney) may be due to increased utilization for scavenging the free radicals and decreased GSH level, which is required for recycling of vitamin C. Administration of AETOF fraction enhanced the ascorbic acid levels by promoting phytoconstituent mediated scavenging of free radicals and thus sparing vitamin C from over utilization. Increased GSH levels upon treatment also helps in the recycling of vitamin C. Prince and Menon (1999) reported that the antioxidant activity of *Tinosporia cardifolia* in experimental diabetes involves elevation in vitamin C and GSH levels.

Vitamin E (α-tocopherol) is a lipophilic, most important antioxidant in the cell membranes and inhibits lipid peroxidation by scavenging lipid peroxyl radicals to yield lipid hydroperoxides and the α-tocpheroxyl radicals (Stahl & Sies., 1997), hence it protects the cell structures against lipid peroxidation mediated damage (Garg *et al.*, 1996). In our study, the levels of vitamin-E was increased both in liver and kidney of diabetic rats as reported earlier (Prince & Menon., 1999; Santhakumari *et al.*, 2003), which could be due to increased membrane damage by ROS and the subsequent release of membrane bound α-tocopherol from the damaged cell membrane since it is water insoluble (Niehius & Samuelsson., 1968). Treatment with AETOF fraction brought the vitamin- E to near normal levels which could be as a result of decreased membrane damage as evidenced by decreased lipid peroxidation. Takenaka. (1991) reported increased levels of the α-tocopherol in the liver of diabetic rats inspite of increased susceptibility to oxidation. A decreased level of Vitamin-E in plasma of STZ induced diabetic rats was also reported by other authors (Ananthan *et al.*, 2003; Punitha *et al.*, 2006; Chandramohan *et al.*, 2009). Vitamin E levels are depleted in the plasma of NIDDM (Sundaram *et al.*, 1996) and IDDM patients (Martin-Gallan *et al.*, 2003). The amount of plasma Vitamin E is inversely correlated with the existence of diabetic complications in

IDDM (Martin-Gallan *et al.*, 2003) and with the duration of NIDDM (Sundaram *et al.*, 1996). Supplementation of Vitamin E prevents glucose-induced lipid peroxidation in rat mesangial cells (Trachtman et al., 1994), the renal cortex of STZ-induced diabetic rats (Jachec *et al.*, 2002), and in plasma of IDDM patients (Jain *et al.*, 1998).

There are conflicting results regarding the levels of Vitamin-C and Vitamin-E in diabetic rats. Prakasam. (2005) reported that *Casearia Esculenta* root aqueous extract remarkably improved ascorbic acid (Vitamin-C) and Vitamin-E in liver and kidney of the STZ-diabetic rats. Cemek et al., (2008) reported no significant change in the levels of ascorbic acid in Streptozotocin induced diabetic rats as well as diabetic rats treated with different doses of *Matricaria chamomilla* extract compared to normal rats. Punitha et al., (2006) reported the declined levels of vitamin-C and vitamin-E in the diabetic rats and significantly restored upon treatment with berberine, a benzyl tetra isoquinoline alkaloid. Pavana et al., (2007) reported significant restoration of ascorbic acid and vitamin-E upon treatment with *tephrosia purpurea* seed extract in diabetic rats. Recently Chandramohan et al., (2009) reported decreased levels of Vitamin-C and Vitamin-E in plasma, erythrocytes and liver of diabetic rats. The restoration of Vitamin-C was observed upon treatment with the 3-hydroxymethyl xylitol in Streptozotocin induced diabetic rats.

Serum enzymes including AST, ALT and ALP are used in the evaluation of hepatic disorders. An increase in these enzyme activities reflects active liver damage (Gautam et al., 2004). Inflammatory hepatocellular disorders result in extremely elevated transaminase levels (Hultcrantz *et al.*, 1986). ALT is an important enzyme present in hepatocytes (liver cells). When liver is damaged, it leaks this enzyme into the blood, where it is measured. ALT rises dramatically in acute liver damage such as viral hepatitis or paracetamol (acetaminophen) overdose. The rise in the activity of ALT in hepatocellular damage is usually accompanied by a rise in AST. AST is similar to ALT in that it is another enzyme associated with liver parenchymal cells. It is raised in acute liver damage, but is also present in red blood corpuscles, cardiac and skeletal muscle and is therefore not specific to the liver. The ratio of AST to ALT is sometimes useful in differentiating between causes of liver damage. According to Tanaka *et al.*, (1988) and Nanbara *et al.*, (1990) AST had more activity than ALT in the liver of diabetic rats. Elevated AST levels are not specific for liver damage, and AST has also been used as a cardiac marker. ALP is an enzyme in the cells lining the biliary ducts of the liver. ALP levels in plasma will rise with large bile duct obstruction or infiltrative diseases of

the liver. ALP is also present in bone and placental tissue. The mechanism by which alkaline phosphatase reaches the circulation is still uncertain; leakage from the bile canaliculi into hepatic sinusoids may result from leaky tight junctions and the other hypothesis is that the damaged liver fails to excrete alkaline phosphatase made in the bone, intestine and the liver (Gautam *et al.*, 2004). Elevated activities of serum aminotransferases are a common sign of kidney damage and are observed more frequently among people with diabetes than in general population (McAnuff-Harding *et al.*, 2006; Ohaeri, 2001). Measurement of enzymatic activities of aminotransferases (AST and ALT) and ALP is of clinical and toxicological importance, as changes in their activities are indicative of tissue damage by toxicants or in disease conditions (Singh *et al.* 2001). Jafar *et al.*, (2010) and Ahmed *et al.*, (2005) demonstrated that the administration of several herbal extracts could restore the changes in the activities of serum enzymes, like alkaline phosphatase (ALP), acid phosphatase and transaminases, aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Serum ALT, AST and ALP levels were determined to evaluate the hepatic functions (Degirmenchi *et al.*, 2002). The activities of serum ALT, AST and ALP were increased in diabetic rats compared to the treated rats. Administration of AETOF fraction lowered the serum AST, ALT and ALP activities in diabetic rats. Glibenclamide treated diabetic rats also have shown decreased levels of ALT, AST and ALP after long term treatment. Our findings are in agreement with those of Prakasam *et al.*, (2004). AETOF fraction has protective effect against liver toxicity caused by STZ. Treatment with AETOF fraction in normal rats for 40 days did not produce any hepatotoxicity. Flavonoids present in amla fruit were shown to have hepatoprotective effect (Jose and Kuttan., 2000). Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hepatoprotective activities (Tapas *et al.*, 2008). Silymarin, flavonolignan from "milk thistle" (*Silybum marianum*) plant is used exclusively for hepatoprotection (Pradhan and Girish., 2006).

Blood urea and creatinine are considered as significant markers of renal function (Almdal and Vilstrup, 1988). The plasma levels of urea and creatinine were measured, as DM also causes renal damage due to abnormal glucose regulation, including elevated glucose and glycosylated protein levels, haemodynamic changes within the kidney tissue, and increased oxidative stress. In this study elevated levels of blood urea and creatinine were observed in diabetic untreated rats, which are considered as significant markers of renal dysfunction (Bethesda et al., 2001). In diabetic subjects negative nitrogen balance with enhanced tissue

proteolysis and decreased protein synthesis can contribute to increased serum urea and creatinine levels, indicating impaired renal functions in diabetic animals (Jensen et al., 1981). After the treatment with AETOF fraction a significant reduction in the levels of urea and creatinine were observed in the diabetic treated rats. It indicates that AETOF fraction is preventing the renal damage in diabetic rats. These results are in agreement with other previous studies on the *sorghum* phenolic extracts (Ill-Min Chung et al., 2011), *Merremia emarginata Burm.* F. (G Rajiv Gandhi *et al.*, 2012) and mesocarp extract of *B. aegyptiaca*, (Mansour & Newairy, 2000). The urea and creatinine levels were also brought down to near normal after treatment with the standard drug glibenclamide.

***In silico* studies**

The K^+-ATP channel in β-cells is comprised of two subunits. One subunit contains the cytoplasmic binding sites for both sulfonylureas and ATP, and is designated as the sulfonylurea receptor type 1 (SUR1). The other subunit is the potassium channel, which acts as the pore-forming subunit (Inagaki *et al.*, 1995). Either an increase in the ATP/ADP ratio or ligand binding to SUR1 results in the closure of the K^+-ATP channel and cause insulin secretion. However, Sur1 receptor has not been crystallized yet and limited number of crystal structures of these family like Na^+/K^+ channels are available in the Protein Data Bank. So far, Sur1 receptor is a huge complex structure that possesses 12 transmembrane helices which are aligned in the lipid bilayer and two ATP binding domains were located at the cytoplasmic region. Two ATP binding pockets of Sur 1 were built and stabilized by molecular dynamics simulation. In order to identify the best binding lead molecules, docking of 32 phytoconstituents of AETOF fraction with ATP I and ATP II domains revealed that three flavonoids viz. Skullcap flavone I, Echioidin and Echiodinin have shown highest binding affinity with ATP II binding pocket in comparison with standard drug Glibenclamide and its natural substrate Adenosine triphosphate.

HISTOPATHOLOGICAL STUDIES

The histological sections of the pancreas, liver and kidney tissues were studied to know the effect of AETOF fraction on theses tissues in normal and diabetic rats. This was done to observe any protective or harmful effect of AETOF fraction in non-diabetic and diabetic rats.

STZ selectively destroys the pancreatic β-cells, which causes the inhibition of synthesis and release of insulin thereby leading to the onset of DM (Balkis Budin *et al.*, 2009). The decrease in cellularity within islets of Langerhans observed in diabetic rats in the present study reflects the cytotoxity of streptozotocin (Szudelski, 2001). Streptozotocin destroys β-cells selectively and a single adequate dose produces lasting hyperglycemia and insulin deficiency. Previous studies have reported that streptozotocin enters the beta cells via a glucose transporter (GLUT2) and causes alkylation of DNA. DNA damage induces activation of poly ADP- ribosylation, a process that is more important for the diabetogenecity of streptozotocin than DNA damage itself. Poly ADP-ribosylation leads to depletion of NAD+ and ATP. Enhanced ATP dephosphorylation after streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of super oxide radicals. Consequently, hydrogen peroxide and hydroxyl radicals are generated. Furthermore, streptozotocin liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of the streptozotocin action, β-cells undergo destruction by necrosis (Szudelski 2001). Other studies indicated that cytotoxic effects of streptozotocin are dependent upon DNA alkylation by site-specific action with DNA bases (Benneth & Pegg, 1981) and by free-radical generation during streptozotocin metabolism (Bolzan & Bianchi, 2002).

In the present study partial restoration of the pancreatic islets cells after treatment with the AETOF fraction indicate that the possible mechanism by which the AETOF fraction reduced blood glucose concentration of the diabetic rats may be by increasing the pancreatic secretion of insulin from the islets of Langerhans. Liver is the main organ affected during the diabetic condition. The changes in the liver in diabetic rabbits induced by streptozotocin have been reported earlier (Mitra *et al*, 1996). The diabetic liver showed degeneration and congestion. In diabetes, degradation of liver glycogen and gluconeogenesis are increased while glucose utilization is inhibited. Glucose 6-phosphatase increases in the liver, facilitating glucose release into the blood. The opposing enzymes which phosphorylate glucose is hexokinase, which is unaffected by insulin and glucokinase, which decrease in diabetes. As a result, the liver continues to produce glucose even with severe hyperglycemia. Under these circumstances the normal liver would shut off and deposit glycogen (Sherlock & Dooley, 1993). AETOF fraction when administered to streptozotocin-induced diabetic Wistar rats, reverted the degenerative changes associated with diabetes mellitus by reducing fatty change, necrosis, hepatocyte vacoulation, connective tissues derangement and restoration of the cyto-

architecture of the liver. Thus it can be concluded that AETOF fraction possess hepato-protective activity as evidenced by liver tissue regeneration and significantly reduced liver enzyme activities in streptozotocin-induced diabetic rats.

The kidney of streptozotocin induced diabetes control rats showed vascular degeneration in some tubular epithelial cells and cell debris scattered in tubular lumina. Moreover, it has been reported that streptozotocin does not possess any significant nephrotoxic potential (Floretto *et al.*, 1998). All structural changes in kidneys resulting from STZ administration in rats can thus be attributed to altered metabolism in diabetes (Rasch, 1980). In a study reported by Bolkent *et al.*, (2004) in the neonatal (100 mg/kg bw) STZ-induced type-I diabetes, alteration in the structural integrity of the apical membrane of proximal tubules of the kidney tissue in the diabetic rats was observed. Normoglycaemia in diabetic rats with AETOF fraction treatment in this study could ameliorate the glomerular and tubular lesions that characterise diabetic nephropathy. The improvement of renal morphology and function in STZ diabetic rats after treatment with AETOF fraction in the present investigation could be attributed to its antidiabetic action resulting in alleviation of altered metabolic status in animals. However, the excellent recovery of renal function expected with treatment of AETOF fraction can be explained by the regenerative capability of the renal tubules (Kissane, 1985). Kidney sections of healthy rats treated with AETOF fraction showed no pathological changes and were comparable to those of normal control rats. Our study on the histology of the kidney damage could help in better understanding of the damage caused in the kidneys in DM and highlight the protective action of AETOF fraction.

The results obtained from this study conducted with the AETOF fraction in STZ induced diabetic rats and normal rats suggested that the leaves of *A. echioides* have antidiabetic, anti hyperlipidimic and antioxidant activities mediated through their effects on insulin secretion, β-cell regeneration and insulin action on various enzymes involved in the Carbohydrate, lipid and protein metabolisms and also reducing the generation of free radicals.

These activities could be due to the flavonoids such as Echioidin, Echioidinin and Skullcap flavone I etc, present in the AETOF fraction obtained from the leaves.