Chapter II

4. EFFECT OF ASIATIC ACID ON THE ACTIVITIES OF KEY ENZYMES OF CARBOHYDRATE METABOLISM IN STREPTOZOTOUCIN INDUCED DIABETIC RATS

4.1. INTRODUCTION

Liver is an insulin-sensitive tissue, plays a major role in maintaining glucose homeostasis by regulating the interaction between the glucose utilization and gluconeogenesis (Ferre et al., 1996). Hepatocytes express dozens of enzymes that are alternatively turned on or off depending on whether blood glucose levels are either rising or falling out of the normal range.

The net glucose release is the result of two simultaneous ongoing pathways that are tightly regulated. Indeed, liver produces glucose by breaking down glycogen (glycogenolysis) and by de novo synthesis of glucose (gluconeogenesis) from non-carbohydrate precursors such as lactate, amino acids and glycerol (Nordlie et al., 1999; Saltiel and Kahn, 2001). The exact contribution of each of these two processes to glucose production remains however controversial. Glycogenolysis occurs within 2-6 h after a meal in humans, and gluconeogenesis has a greater importance with prolonged fasting. The rate of gluconeogenesis is controlled principally by the activities of unidirectional enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), fructose-1, 6-bisphosphatase (FP2ase) and glucose-6-phosphatase (G6Pase). PEPCK catalyzes one of the rate limiting steps of gluconeogenesis, the conversion of oxaloacetate to phosphoenolpyruvate (PEP), while G6Pase catalyzes
the final step of gluconeogenesis, the production of free glucose from glucose-6-phosphate (G6P). The genes of these gluconeogenic enzymes are controlled at the transcriptional level by hormones, mainly insulin, glucagon and glucocorticoids. Insulin inhibits gluconeogenesis by suppressing the expression of PEPCK and G6Pase, whereas, glucagon and glucocorticoids stimulate hepatic glucose production by inducing these genes (O’Brien and Granner, 1996). In both type 1 and type 2 diabetes, excessive hepatic glucose production is a major contributor of both fasting and postprandial hyperglycemia (Taylor, 1999).

In addition, the simultaneous overproduction of glucose and fatty acids in liver further stimulates the secretion of insulin by the pancreatic β-cells and exacerbates peripheral insulin resistance thereby establishing a vicious circle (McGarry, 1992). The key role of the liver in controlling both carbohydrate and lipid homeostasis in vivo has been confirmed by recently generated transgenic and knockout models (Postic et al., 2004).

Enzymes that have high control strength on hepatic glucose metabolism are potential targets for controlling hepatic glucose balance and thereby glucose levels in type 2 diabetes (Agius et al., 1996). One strong candidate in that respect is hepatic glucokinase (GK), which catalyzes the conversion of glucose to glucose-6-phosphate, the first step of glucose metabolism. Hepatic GK, by virtue of kinetic characteristics that distinguish it from other hexokinases (HK) allows, for rapid and efficient phosphorylation of glucose in glucose 6-phosphate (G6P), a key step of glycolysis, glycogen synthesis and pentose phosphate pathway (Girard, 1997;
Wilson, 2003). Ferre et al. (1996) had reported that the streptozotocin-induced increase in glucose concentrations is normalized in GK over expressing mice. In addition, the recent finding that allosteric activators of hepatic GK improve glucose tolerance and increase hepatic glucose uptake makes of hepatic GK a feasible gene therapy-based approach to reduce hyperglycemia in diabetes (Grimsby et al., 2003; Brocklehurst et al., 2004). In fact, as revealed by a series of in vivo analysis, hepatic GK is crucial for glucose uptake and glycogen synthesis in liver, as well (Postic et al., 2004).

The synthesis and degradation of glycogen in the liver are important mechanisms in the control of blood glucose homeostasis (Barf, 2004; Taguchi et al., 2005). The inhibition of enzymes involved in glycogenolysis constitutes an alternative approach to suppressing hepatic glucose production and lowering blood glucose levels (Jung et al., 2006; Agius, 2007). Hepatic glycogen phosphorylase (GP) and glucose-6-phosphatase (G6Pase) are two key enzymes in glycogenolysis. GP catalyzes the first step of the breakdown of glycogen to yield glucose-1-phosphate, whereas G6Pase catalyzes the final reaction in hepatic glucose production (Onda et al., 2008).

\[
\text{Glycogen phosphorylase} \\
(\text{Glucosyl})_n + \text{Pi} \rightarrow \text{Glucose-1-phosphate} + (\text{glucosyl})_{n+1} \\
\text{Glucose} \quad \downarrow \quad \downarrow \\
\text{Glucose - 6 - phosphatase} \\
\text{Glucose - 6 - phosphate}
\]

Both enzymes have been proposed as potential targets for antihyperglycemic drugs for diabetes (Kurukulasuriya et al., 2003; Agius, 2007).
Medicinal plants continue to be a powerful source for new drugs, now contributing about 90% of the newly discovered pharmaceuticals (Mosh, 2005). Recently there has been a growing interest in hypoglycemic agents from natural products, especially those derived from plants because, plant source are usually considered to be non-toxic, with fewer side effects than synthetic sources (Sabu et al., 2002). There are more than 200 compounds from plant sources that have been reported to show blood glucose lowering effect. The wide variety of chemical classes indicates that a variety of mechanisms of action are likely to be involved in lowering blood glucose level (Kim et al., 2008). Naringin (4’, 5, 7-trihydroxy flavonone 7-rhamno-glucoside) is a bioflavonoid derivative of grape fruit and related citrus species. A research study on the influence of naringin on blood glucose concentration and the activities of hepatic key enzymes in normal and streptozotocin-nicotinamide induced diabetic rats revealed that treatment with naringin resulted in a significant elevation of hepatic glucokinase activity and glucose-6-phosphate dehydrogenase activities, whereas, the activities of glucose-6-phosphatase and fructose 1, 6 bisphosphatase were decreased. The results suggested that naringin can play an important role in preventing the progression of hyperglycemia, partly by increasing hepatic glycolysis and glycogen concentration (Pari, 2010).

There is a growing interest in several biological properties of phenolic compounds in addition to their antioxidant effects, and the evidence suggests that certain dietary polyphenolic compounds may result in an altered glucose metabolism (Scalbert et al., 2005). Among various phenolic compounds, caffeic acid [3, 4-di
(OH) - cinnamate], found in many types of fruit and coffee in high concentrations. It is known to have an antidiabetic effect in streptozotocin-induced diabetic rats (Okutan et al., 2005; Park and Min, 2006). Jung et al. (2006) investigated the protective effect of caffeic acid on the oxidative damage induced by diabetes and their possible role in ameliorating the development of diabetes. Caffeic acid significantly elevated hepatic GK activity compared with the control group by approximately 28%. In contrast, G6Pase and PEPCK activities were markedly lower in the caffeic acid group by 29 and 19%, respectively. The hepatic glycogen concentration was significantly higher in the caffeic acid group.

Hepatic glycogen phosphorylase (GP) and glucose-6-phosphatase (G6Pase) are important in control of blood glucose homeostasis, and are considered to be potential targets for antidiabetic drugs. Astragaloside IV is a glycoside of cycloartane-type triterpene isolated from the *Astragalus memebranaceus* (Fisch), has been reported to have a hypoglycemic effect. In an experimental study by Lv et al. (2010), type 2 diabetic mice were treated with astragaloside IV for 2 weeks. The results indicated that Astragaloside IV at 25 and 50 mg/kg significantly decreased the blood glucose, TG and insulin levels, and inhibited the mRNA and protein expression as well as enzyme activity of GP and G6Pase in diabetic mice.

In a similar study by Pari and Rajarajeswari (2009), the antidiabetic effect of coumarin on carbohydrate metabolic key enzymes streptozotocin (STZ)-nicotinamide (NA)-induced diabetic rats were evaluated. Oral administration of coumarin at a dose of 100 mg/kg bw/day to diabetic rats for 45 days resulted in a
significant reduction in the levels of plasma glucose, glycosylated haemoglobin (HbA\textsubscript{1c}) and increase in the levels of insulin and haemoglobin. Administration of coumarin caused a significant increase in the levels of glycolytic enzyme (hexokinase) and hepatic shunt enzyme (glucose-6-phosphate dehydrogenase), whereas significant decrease in the levels of gluconeogenic enzymes (glucose-6-phosphatase and fructose-1, 6-bisphosphatase) in diabetic treated rats. Further, the action of diosmin on hepatic key enzymes of carbohydrate metabolism in streptozotocin-nicotinamide-induced diabetic rats was assessed (Pari and Srinivasan, 2010). Oral administration of diosmin (100 mg/kg bw) significantly decreased glycosylated haemoglobin and increased haemoglobin and plasma insulin. The activities of the hepatic key enzymes such as hexokinase and glucose-6-phosphate dehydrogenase were significantly increased, whereas glucose-6-phosphatase and fructose-1, 6-bisphosphatase were significantly decreased.

Diabetes is associated with impaired glucose clearance by the liver in the postprandial state, and with elevated glucose production in the post-absorptive state. New targets within the liver are currently being investigated for development of antihyperglycemic drugs for diabetes. They include glucokinase, which catalyses the first step in glucose metabolism, the glucagon receptor, and enzymes of gluconeogenesis and/or glycogenolysis such as glucose 6-phosphatase, fructose 1,6-bisphosphatase and glycogen phosphorylase. Preclinical studies with candidate drugs on animal models or cell-based assays suggest that these targets have the potential for pharmacological glycemic control (Agius, 2007).
Xiaoan et al. (2005) described the semi-synthesis, *in vitro* and *in vivo* biological evaluation of the naturally occurring pentacyclic triterpenes such as corosolic acid and maslinic acid and reported that they represent a new class of inhibitors of glycogen phosphorylases. Both inhibited the increase of fasted plasma glucose of diabetic mice induced by adrenaline. It is therefore proposed that naturally occurring pentacyclic triterpenes corosolic acid and maslinic acid might reduce blood glucose, at least in part, through inhibiting hepatic glycogen degradation. Further studies on synthesis and biological evaluation of maslinic acid derivatives revealed that a series of maslinic acid derivatives are inhibitors of rabbit muscle glycogen phosphorylase (Xiaoan et al., 2006). Similarly a series of 23-hydroxybetulinic acid derivatives were prepared and tested *in vitro* as a new class of inhibitors of glycogen phosphorylase (Peiqing et al., 2009).

The effects of plant derived oleanolic acid bioactive compound on glycogenic enzymes in streptozotocin-induced diabetic rats were studied (Musabayane et al., 2010). The study evidenced that the anti-hyperglycemic effects of Syzygium cordatum leaf triterpene mixtures (oleanolic acid (OA) and ursolic acid) in streptozotocin (STZ)-induced diabetic rats are mediated in part via increased hepatic glycogen synthesis. To further elucidate the mechanism(s) of the hypoglycemic effects of the triterpene, this study investigated the effects of plant derived OA on glycogenic enzyme activities in STZ-induced diabetic rats after 5 weeks of twice-daily treatment with OA (60 mg/kg. p.o). The study indicated that OA increased hepatic and muscle glycogen concentrations of both non-diabetic and STZ-induced
diabetic rats as did standard drugs, metformin and insulin. OA and insulin significantly increased muscle hexokinase activity in the non diabetic animals. OA and insulin significantly increased hepatic hexokinase and glucokinase activities in STZ-induced diabetic rats. These findings suggest that the plant-derived OA increases glycogen synthesis, partly by increasing the activities of the enzymes hexokinase and glucokinase in the muscle and liver, respectively thereby reducing blood glucose levels.

Asiatic acid, the triterpene compound used for the present study is also reported to be inhibitors of glycogen phosphorylase (GP) (Zhang et al., 2009). Twenty-four Asiatic acid derivatives have been synthesized and biologically evaluated as inhibitors of glycogen phosphorylase (GP). Within this series of compounds, Asiatic acid benzyl ester exhibited more potent activity than its parent compound. SAR Analysis showed that Asiatic acid possessing a 2α-OH function exhibited more potent GP inhibitory activity than eriantic acid B which possesses a 2β-OH function. Apart from the studies on glycogen phosphorylase, no other studies were undertaken so far to explain the effect of Asiatic acid on the key enzymes of carbohydrate metabolism in diabetes. Moreover, the results presented in the previous chapter showed antihyperglycemic effect of Asiatic acid. Therefore, inorder to understand how Asiatic acid causes hypoglycemic effect, the present study was targeted on the effect of Asiatic acid on the activity of key enzymes of carbohydrate metabolism in streptozotocin induced diabetic rats.
4.2. MATERIALS AND METHODS

4.2.1. Study design

The liver samples of the control and experimental groups of rats treated with AA and glibenclamide at appropriate doses orally for 60 days were used for the present study (details presented in Chapter 2). The liver samples were homogenised at appropriate pH in cold condition and were centrifuged. A known volume of the separated supernatant was used for the determination of glycogen. The activities of key enzymes such as glycogen phosphorylase, hexokinase, glucose 6-phosphatase and fructose 1, 6-bisphosphatase were determined.

4.2.2. Estimation of liver glycogen

Liver glycogen was estimated by the method of Carroll et al. (1956). 250 mg of the liver tissue was homogenized with 1ml of 30% KOH. It was then kept in ice. To this 0.5 ml of saturated sodium sulphate and 1 ml of 95% ethanol were added. It was then centrifuged at 2000 rpm for 10 min. The supernatant was discarded, redissolved in 2 ml distilled water and 0.1 ml aliquot was used for the assay of glycogen. Assay system containing 0.1 ml of glycogen source and 2.5 ml of anthrone reagent were kept in boiling water bath for 10 min at 90°C and the green colour formed was measured at 660 nm colorimetrically against a reagent blank containing 0.1 ml distilled water and 2.5 ml of anthrone reagent. For the standard, tubes containing 0.5 to 2.5 ml of glucose working standard of concentrations 0.025 – 0.125 mg were treated in the similar manner.
**Calculation**

Glycogen (mg/100g tissue) = \( \frac{\text{mg. glucose}}{\text{Vol. of enzyme extract}} \times \frac{\text{Total volume of enzyme extract}}{\text{weight of tissue used (g)}} \times 100 \)

**4.2.3. Assay of glycogen phosphorylase**

Glycogen phosphorylase was assayed by the method of Sutherland *et al.* (1957). 250 mg of chilled liver tissue was homogenized at 0°C with 5 ml citrate buffer (pH 6), centrifuged at 3000 rpm for 10 minutes at 0°C and 0.5 ml of the supernatant was used for the assay. Assay medium containing 0.6 ml of sodium fluoride, 0.2 ml of glucose-1-phosphate, 0.6 ml of citrate buffer, 0.1 ml of 4% glycogen and 0.5 ml of the enzyme source was kept for 30 min at 30°C. Then 1 ml of 10% TCA was added, diluted to 10 ml using 7 ml of distilled water. It was then centrifuged and 1 ml of supernatant was used for phosphate estimation by the method described by Fiske and Subbarow (1925). To 1 ml of the supernatant 0.6 ml of distilled water, 1 ml of ammonium molybdate and 0.4 ml ANSA reagent were added. The blue colour developed after 20 min. was then read against a reagent blank at 620 nm. Tubes containing 0.5 to 2.5 ml of the working standard of concentrations 4 to 20 \( \mu \)g were also treated in the same way as the test.

**Calculation**

Glycogen phosphorylase (\( \mu \) moles of phosphorus liberated/h/mg.protein) =

\[
\frac{\mu \text{ moles of phosphorous liberated}}{\text{Vol. of supernatant}} \times \frac{\text{Total Volume}}{\text{Vol. of enzyme extract}} \times \frac{1}{\text{Incubation time}} \times \frac{60}{\text{mg. protein}}
\]
4.2.4. Assay of hexokinase

Hexokinase was assayed by the method of Brandstrup et al. (1957). 250 mg of the chilled liver tissue was homogenized at 0°C with 0.01M Tris-HCl buffer at pH 8 (1:1 w/v), centrifuged at 3000 rpm for 10 minutes at 0°C and 1.0 ml of the supernatant was used for the assay. The reaction mixture in a total volume of 5 ml contained the following: 1 ml of glucose solution, 0.5 ml of magnesium chloride, 0.5 ml of dipotassium hydrogen phosphate solution, 0.4 ml of potassium chloride, 0.1 ml of sodium fluoride solution and 2.5 ml of Tris-HCl buffer (pH 8). The mixture was pre incubated at 37°C for 5 min. The reaction was initiated by the addition of 1 ml of enzyme source. Immediately (zero time), 1.0 ml aliquot of the reaction mixture was taken to a tube containing 1 ml of 10% TCA. A second aliquot was removed after 30 min of incubation at 37 °C and added to a tube containing 1 ml of 10% TCA. The precipitated protein was removed by centrifugation and the residual glucose in the supernatant was estimated by the o-toluidine method of Sasaki and Senae (1972). The supernatant was mixed with 4 ml of O-toluidine reagent and was kept in boiling water bath for 15 min. The green colour developed was read colorimetrically at 620 nm. A reagent blank was run with each test. The amount of glucose phosphorylated was given by the difference between the two values.

**Calculation**

Hexokinase (µ moles of glucose phosphorylated /h/mg protein) =

\[
\frac{\mu \text{ moles of glucose phosphorylated}}{\text{Vol. of aliquot used}} \times \frac{\text{Total volume of aliquot}}{\text{Incubation time}} \times \frac{60}{	ext{mg. protein}}
\]
4.2.5. Assay of glucose 6-phosphatase

Glucose 6-phosphatase was assayed by the method of Koide and Oda (1959). 250 mg of the chilled liver tissue was homogenized at 0°C with 0.1 M citrate buffer at pH 6 (1:1 w/v), centrifuged at 3000 rpm for 10 minutes at 0°C and 0.2 ml of the supernatant was used for the assay. Pipetted out 0.3 ml of citrate buffer, 0.5 ml of glucose6- phosphate and 0.2 ml of tissue homogenate in a test tube and incubated at 37 °C for 1 h. 1 ml of 10% TCA was added to the tube to terminate the enzyme activity and then centrifuged. The phosphate content of the supernatant was then estimated by Fiske and Subbarow (1925) method. To 1 ml of the aliquot of supernatant, 1 ml of ammonium molybdate and 0.4 ml of ANSA reagent were added. The blue colour developed was read after 20 min at 620 nm. A tube devoid of the enzyme served as control. A series of standards containing 8-40 µg of phosphorous was treated similarly along with a blank containing only the reagent.

**Calculation**

Glucose – 6 – phosphatase (µ moles of inorganic phosphate liberated /min/mg.protein) =

\[
\frac{\text{µ moles inorganic phosphate liberated}}{\text{Vol. of tissue homogenate}} \times \frac{1}{60} \times \frac{1}{\text{mg. protein}}
\]

4.2.6. Assay of fructose 1, 6-bisphosphatase

Fructose 1, 6-bis phosphatase was assayed by the method of Gancedo and Gancedo (1971). 250 mg of the chilled liver tissue was homogenized at 0°C with 0.1 M Tris-HCl buffer at pH 7 (1:1 w/v), centrifuged at 3000 rpm for 10 minutes at 0°C
and 0.2 ml of the supernatant was used for the assay. The assay medium in a final volume of 2 ml contained 1 ml of Tris-HCl buffer, 0.4 ml of substrate, 0.1 ml of magnesium chloride, 0.2 ml of potassium chloride, 0.1 ml of EDTA and 0.2 ml of enzyme source. The tube was incubated at 37°C for 15 min. The reaction was then terminated by the addition of 1 ml of 10% TCA. The suspension was centrifuged and the phosphorous content of the supernatant was estimated according to the method described by Fiske and Subbarow (1925). To 1 ml of an aliquot of the supernatant, 0.3 ml of distilled water and 0.5 ml of ammonium molybdate were added. After 10 min, 0.2 ml of ANSA reagent was added. The tubes were shaken well, kept aside for 20 min and the blue colour developed was read at 620 nm. Tubes containing 0.5 to 2.5 ml of the working standard of concentrations 4 to 20 µg were also treated in the same way as the test.

**Calculation**

Fructose 1, 6- bisphosphatase (µ moles of inorganic phosphorous/h/mg. protein) =

\[
\frac{\text{µ moles of inorganic phosphorous liberated}}{\text{Vol. of supernatant}} \times \frac{\text{Total Volume}}{\text{Vol. of enzyme extract}} \times \frac{1}{\text{Incubation time}} \times \frac{60}{\text{mg. protein}}
\]

**4.2.7. Estimation of protein in liver tissue**

Protein content of the liver tissue was determined by the method of Lowry et al. (1951). Extraction of protein was carried out with appropriate buffers used for the enzyme assay. 250 mg of the liver tissue was ground well with a pestle and mortar in cold condition (pH 6-8) to give 20% homogenate (w/v). The homogenate was
centrifuged at 3000 rpm for 10 min at 0°C in a refrigerated centrifuge. 0.5 ml of the tissue homogenate was mixed with 0.5 ml of 10% TCA and centrifuged for 10 min. The precipitate was dissolved in 1 ml of 0.1 N NaOH. From this, 0.5 ml was taken, mixed with 4.5 ml of alkaline copper reagent and allowed to stand at room temperature for 10 min. 0.5 ml of Folin’s phenol reagent was added and the blue colour developed was read after 20 min at 640 nm. A standard curve was obtained with standard bovine albumin and was used to assay the tissue protein level.

**Calculation**

\[
\text{Protein (mg)} = \left( \frac{\text{OD of the test}}{\text{OD of the std.}} \right) \times \left( \frac{\text{Conc. of the standard}}{\text{Vol. of sample}} \right)
\]
4.3. RESULTS

4.3.1. Effect of Asiatic acid on glycogen content and the activity of glycogen phosphorylase in the liver samples of STZ-diabetic and control rats

The results on the effect of Asiatic acid on hepatic glycogen and the activity of glycogen phosphorylase in the liver samples of STZ-diabetic and control rats are given in Table 4.1.

4.3.1.1. Glycogen

The glycogen content of normal control rats was 71.06 ± 2.03 mg/100g of liver, likewise it was more or less similar (72.88 ± 1.83 mg/100g of liver) in the control rats treated with AA. The glycogen content in STZ-induced diabetic rats was very much reduced to 32.17 ± 1.32 mg glycogen/100g of liver. But in the case of different concentrations of AA treated diabetic rats, the value was varied between 42.14 ± 1.51 and 63.42 ± 0.723 mg/100g of liver. However, the diabetic rats treated with glibenclamide at a dose of 600 µg/kg, it was found to be 64.62 ± 2.75 mg glycogen/100g of liver. STZ-induced diabetic control rats showed 54.83% reduction in the level of hepatic glycogen content than that of the normal control. The percentage reduction in glycogen content of AA treated groups was 40.77, 21.17 and 10.77 in 25, 50 and 100 mg/kg of AA treated groups, respectively. Administration of glibenclamide in STZ-induced diabetic rats displayed 9.08% reduction in glycogen level. But the normal rats treated with AA (50 mg/kg) did not show significant (p>0.05) difference in the liver glycogen content (Fig.4.1). The statistical one way ANOVA revealed that the liver glycogen content between different groups of
experimental and control rats was highly significant ($F = 473.105; \ p<0.0001$) (Table 4.3).

4.3.1.2. Glycogen phosphorylase

The glycogen phosphorylase activity in the normal control rats was $95.00 \pm 3.46\ U/mg$ protein, whereas the glycogen phosphorylase activity of control rats treated with AA had $91.66 \pm 3.01\ U/mg$ protein. At the same time, in diabetic control rats, it was comparatively higher ($130.66 \pm 4.32\ U/mg$ protein). But the STZ-induced diabetic rats treated with different concentration of AA showed $114.83\ (25\ mg/kg\ AA), 108.33\ (50\ mg/kg\ AA)$ and $99.83\ (100\ mg/kg\ AA) \ U/mg$ protein. However, the diabetic rats treated with glibenclamide recorded the glycogen phosphorylase level of $98.16 \pm 2.31 U/mg$ protein.

The mean percentage difference of glycogen phosphorylase level in the liver samples of control rats treated with AA was reduced to $3.51\%$ from the normal group, which however was not significant ($p>0.05$). At the same time it was increased to the maximum ($37.44\%$) in the diabetic control rats. But in the case of diabetic rats treated with different concentrations of AA showed much reduction in the mean percentage difference and was in the order of $20.82, 14.39$ and $5.07\%$ respectively in $25, 50$ and $100\ mg/kg\ AA$ treated rats. However, the diabetic rats received glibenclamide displayed only $3.32\%$ increase of glycogen phosphorylase when compared with normal control rats (Fig.4.1). The one way ANOVA revealed that the glycogen phosphorylase activity between different groups of experimental
and control rats was statistically more significant ($F = 103.197; p<0.0001$) (Table 4.3).

4.3.2. Effect of Asiatic acid on the activities of hexokinase, glucose-6-phosphatase and fructose 1, 6-bis phosphatase in the liver samples of STZ-diabetic and control rats.

The results on the effect of AA on the activities of hexokinase, glucose-6-phosphatase and fructose 1, 6-bis phosphatase in the liver samples of different groups of rats are represented in Table 4.2.

4.3.2.1. Hexokinase

The hexokinase activity in normal control rats was 0.480 ± 0.001 U/mg of protein, while that of Asiatic acid control rats had slightly higher level (0.489 ± 0.002 U/mg of protein). But the activity of hexokinase in STZ-induced diabetic control rats was decreased to 0.184 ± 0.003 U/mg of protein. Oral administration of AA at the doses of 25, 50 and 100 mg/kg in STZ-induced diabetic rats significantly ($p<0.0001$) increased the activity of hexokinase to 0.312 ± 0.003, 0.362 ± 0.003 and 0.421 ± 0.003 U/mg of protein, respectively. The hexokinase activity of glibenclamide treated rats was 0.435 ± 0.003 U/mg of protein. When compared to the normal control group, the percentage reduction in the activity of hexokinase in STZ-induced diabetic control group was 61.66%, while the AA treated groups showed a reduction between 12.29 and 34.99%. Diabetic group administered with glibenclamide showed 9.37% reduction in hexokinase activity from the normal group. Normal rats treated with AA (50 mg/kg) did not show significant ($p>0.05$)
difference in the activity of hexokinase (Fig.4.2). The statistical one way ANOVA revealed that the activity of hepatic hexokinase activity between different groups of experimental and control rats was highly significant (F = 6260.052; p<0.0001) (Table 4.3).

4.3.2.2. Glucose 6-phosphatase

The activity of glucose 6-phosphatase in normal control rats was 0.182 ± 0.003 U/mg protein, whereas that of AA control was 0.179 ± 0.002 U/mg protein. The activity was considerably increased to 0.420 ± 0.011 U/mg protein in STZ-induced diabetic rats, while the AA treated rats showed an increase in the range between 0.233 ± 0.002 and 0.292 ± 0.004 U/mg protein. The mean glucose 6-phosphatase activity of glibenclamide treated rats was 0.221 ± 0.002 U/mg protein. In comparison to the normal control group, the percentage elevation in the activity of glucose 6-phosphatase in STZ-induced diabetic control group was 130 ± 76%, while the AA treated groups showed an elevation between 28.02 and 60.44%. Diabetic group administered with glibenclamide showed 21.43% elevation in glucose 6-phosphatase activity from the normal group. But the normal rats treated with AA (50 mg/kg) did not show significant (p>0.05) difference in the activity of glucose 6-phosphatase (Fig.4.2). The one way ANOVA revealed that the hepatic glucose 6-phosphatase activity between different groups of experimental and control rats was statistically more significant (F = 1442.161; p<0.0001) (Table 4.3).
4.3.2.3. Fructose 1, 6 - bis phosphatase

The mean activity of fructose 1, 6 - bis phosphatase in normal control rats was 0.431 ± 0.002 U/mg protein, whereas that of AA control rats was 0.413 ± 0.004 U/mg protein. The activity was high as 0.698 ± 0.006 U/mg protein in STZ-induced diabetic control rats, while the mean activities of 0.614 ± 0.006, 0.587 ± 0.002 and 0.534 ± 0.003 U/mg protein were recorded for the rats treated with the dosage of 25, 50 and 100 mg/kg of AA, respectively. The mean fructose 1, 6 - bis phosphatase activity of glibenclamide treated rats was 0.520 ± 0.001 U/mg protein. When compared to the normal control rats, the STZ-induced diabetic rats showed a significant (p<0.0001) elevation (61.95%) in the activity of fructose1, 6- bisphosphatase, while different concentrations of AA treated rats showed an elevation ranging from 23.89 to 42.46%. The effect produced by oral administration of 100 mg/kg AA in STZ-induced diabetic rats was comparable to that of glibenclamide (20.65%) treated diabetic rats. The effect of AA (50 mg/kg) on normal rats did not show significant (p>0.05) difference in the activity of fructose 1, 6- bisphosphatase (Fig.4.2). The statistical one way ANOVA revealed that the activity of fructose 1, 6 - bis phosphatase in liver samples between different groups of experimental and control rats was highly significant (F = 2935.913; p<0.0001) (Table 4.3).
4.4. DISCUSSION

Liver plays a vital role in regulation of blood glucose level and hence it is of interest to study the role of Asiatic acid on key enzymes of carbohydrate metabolism in liver. Liver is primarily responsible for maintaining blood glucose homeostasis through its ability to store glucose as glycogen and to produce glucose from glycogen breakdown or from gluconeogenic precursors (Roden and Bernroider, 2003). Glycogen level in various tissues especially in liver and skeletal muscle indicates direct reflection of insulin activity since it causes glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase (Kasetti et al., 2010). It has been previously reported that glycogen deposition from glucose is impaired in diabetic animals (Bollen et al., 1998). During diabetes, there is a decrease in liver weight due to enhanced catabolic process such as glycogenolysis, lipolysis and proteolysis (Yadav et al., 2005). Therefore the quantification of glycogen, the primary intracellular storage form of glucose in liver can be considered as an important indicator of diabetes mellitus.

In the present study, a marked reduction (54.83%) in the level of liver glycogen and increased activity of glycogen phosphorylase were observed in diabetic control rats. Treatment with Asiatic acid for 60 days significantly increased the hepatic glycogen levels in STZ diabetic rats in a dose dependent manner. Treatment with 25 mg/kg Asiatic acid in diabetic rats resulted in elevation of liver glycogen to 42.14 ± 1.51 mg/100g tissue with 40.77% decrease from the normal rats, while that of 50 mg/kg Asiatic acid elevated glycogen to 56.04 ± 0.926 mg/100g tissue with
21.17% decrease from the normal rats. Maximum elevation was elicited by 100 mg/kg Asiatic acid for which the glycogen content recorded was 63.42 ± 0.723 mg/100g tissue with just 10.77% reduction from the normal. Glibenclamide also exhibited elevation of liver glycogen (64.62 ± 2.75 mg/100g tissue) similar to 100 mg/kg AA treated rats. This dose dependent elevation of glycogen content in liver indicates the insulinotropic activity of Asiatic acid. In a study, Stalmans et al. (1997) reported that, conversion of glucose to glycogen in the liver cells is dependent on the extracellular glucose concentration and the availability of insulin which stimulate glycogenesis over a wide range of glucose concentration. The reduction of glycogen in diabetic rats has been attributed to increased activity of glycogen phosphorylase (Roesler and Khandelwal, 1986). According to Vats et al. (2004), glycogen levels in tissues (muscle and liver) decreases as the flux of glucose in the liver is inhibited in the absence of insulin and recovers on insulin. This is in agreement with the present findings that the reduced liver glycogen content and increased activity of glycogen phosphorylase in diabetic rats were reversed by Asiatic acid treatment which is attributed to the insulinotropic effect. The activity of glycogen phosphorylase at different doses of Asiatic acid showed significant inhibition of glycogen phosphorylase activity in liver which ranged from 99.83 ± 2.31 to 108.33 ± 3.26 U/mg protein. The present findings are in agreement with numerous other reports on the influence of pentacyclic triterpenes including Asiatic acid on glycogen phosphorylase action in different animal models and in vitro study. For eg. Xiaoan et al. (2005) reported that naturally occurring pentacyclic triterpenes such as corosolic acid and maslinic acid inhibited the increase of fasting plasma
glucose level in adrenaline induced diabetic mice through inhibition of glycogen phosphorylase. Therefore, they proposed that corosolic acid and maslinic acid might reduce blood glucose, at least in part, through inhibiting hepatic glycogen degradation. Further studies by Xiaoan et al. (2008) on synthesis and biological evaluation of pentacyclic triterpenes revealed that a series of derivatives including Asiatic acid inhibited glycogen phosphorylase of rabbit muscle. It was found that Asiatic acid exhibited the inhibitory action by binding on to the allosteric activator site of the enzyme, where the physiological activator AMP binds. They further concluded that pentacyclic triterpenes represent a promising class of multiple-target antidiabetic agents that exert hypoglycemic effects, at least in part, through glycogen phosphorylase inhibition. Similar reports were published by Zhang et al. (2009) stating that, of the twenty-four Asiatic acid derivatives evaluated as inhibitors of glycogen phosphorylase, Asiatic acid benzyl ester exhibited more potent activity than Asiatic acid which exhibited more potent GP inhibitory activity than eriantic acid B.

Hexokinase is an insulin dependent key enzyme in the glycolytic pathway and is crucial for the glucose homeostasis. The enzyme catalyzes the phosphorylation of glucose to glucose-6-phosphate thus playing crucial function in tissue intermediary metabolism. Insulin increases hepatic glycolysis by increasing the activity of hexokinase. The increased hexokinase activity causes the increase in glycolysis and glucose utilization for energy production (Kondeti et al., 2010). But during diabetes, hexokinase activity is decreased due to insulin deficiency and this
result in decreased influx of glucose into the cells, decreased utilization of glucose by various tissues and increased production of glucose by gluconeogenesis by the liver (Granner, 1993).

In the present study, the hexokinase activity was found to be decreased significantly in the diabetic rats as compared to the normal control rats, which may be due to insulin deficiency during diabetes. Decreased enzymatic activity of hexokinase has also been reported in diabetic animals, resulting in depletion of liver and muscle glycogen (Murray et al., 2000). Administration of the Asiatic acid at the doses of 25, 50 and 100 mg/kg to diabetic rats enhanced the hepatic hexokinase activity to normal, which may be due stimulation of insulin secretion from pancreatic β cell. The activity is more pronounced in the diabetic rats which are treated with Asiatic acid at a dose of 100 mg/kg and was 0.421 ± 0.003 U/mg protein with a percentage decrease of 12.29% from the normal rats. Glibenclamide also showed enhanced activity of the enzyme (0.435 ± 0.003 U/mg protein) with a percentage decrease of 9.37% from the normal. The present findings are supported by an earlier work by Musabayane et al. (2010), who reported that oleanolic acid (OA) a triterpene extracted from the plant Syzygium cordatum increased hepatic and muscle glycogen concentrations of both non-diabetic and STZ-induced diabetic rats similar to that of standard drugs like metformin and insulin. Further, OA significantly increased muscle hexokinase activity in the non diabetic animals and hepatic hexokinase and glucokinase activities in STZ-induced diabetic rats.
Glucose-6-phosphatase and fructose-1, 6-bis phosphatase are the key enzymes in gluconeogenesis. Glucose-6-phosphatase is a key enzyme involved in the maintenance of blood glucose homeostasis and it catalyzes the terminal step in gluconeogenesis and glycogenolysis and fructose-1, 6-bis phosphatase is a hepatic enzyme that converts fructose-1, 6-bis phosphate to fructose-6-phosphate in the gluconeogenic pathway (Mithievre et al., 1996).

In the present study, the hepatic gluconeogenic enzymes, glucose-6-phosphatase and fructose-1, 6-bis phosphatase were found to be significantly higher in diabetic rats as compared to the normal control rats. Earlier reports have also shown the increased levels of these enzymes in alloxan induced male Wistar diabetic control rats (Ananthi et al., 2003; Guruvayoorappan and Sudha, 2008). Increased levels of the gluconeogenic enzymes may be due to a state of insulin deficiency, because under normal conditions, insulin functions as a suppressor of gluconeogenic enzymes (Pushparaj et al., 2001). In the present study, the activities of glucose-6-phosphatase and fructose-1, 6-bisphosphatase were found to be decreased significantly in the liver of Asiatic acid treated diabetic rats. According to Gupta et al. (1999) this elevated activities 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. Previously, several phytochemical substances such as pterostilbene (Pari and Satheesh, 2006), coumarin (Pari and Rajarajeswari, 2009), diosmin (Pari and Srinivasan, 2010), were used in antidiabetic studies based on carbohydrate metabolic enzymes and all of them have
reported to exhibit stimulatory effect on glucose-6-phosphatase and fructose-1, 6-bis phosphatase. Yet another, supportive work to the present study is that Astragaloside IV, a glycoside of cycloartane-type triterpene isolated from the *Astragalus memebranaceus* (Fisch), at a dose of 25 and 50 mg/kg significantly decreased the blood glucose and inhibited enzyme activity of Glucose-6-phosphatase in diabetic mice (Lv *et al.*, 2010).

The above findings suggest that Asiatic acid by its modulating effect on the activities of the key enzymes of carbohydrate metabolism in liver elicits an antidiabetic effect.