International Journal of Pharmaceutical Sciences and Drug Analysis

E-ISSN: 2788-9254
P-ISSN: 2788-9246
IJPSDA 2023; 3(1): 01-06
www.pharmacyjournal.info
Received: 01-10-2022
Accepted: 03-11-2022

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Evaluation of *in vivo* anti-hyperglycaemic properties of Asiatic acid in male wistar rats (*Rattus norvegicus*)

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Abstract
Asiatic acid is a major triterpene obtained from plant C. asiatica. Asiatic acid has been reported to have antihyperglycemic activity in animal models. However, detailed and elaborated study depicting mode of action of this chemical has not been adequately described. The present study was designed to examine the antidiabetic effect of Asiatic acid (AA) in streptozotocin (STZ) induced diabetic rats. Diabetes was induced in male Wistar rats by a single intraperitoneal injection of STZ (40 mg/kg body weight). Diabetic rats show increased plasma glucose, reduced insulin level, relatively high amount of glycosylated haemoglobin and relatively less amount of plasma protein content. The antihyperglycemic effect of AA was compared with glibenclamide, a well-known antihyperglycemic drug. In conclusion, this study indicates that AA showed antihyperglycemic effect in experimental diabetic models.

Keywords: Asiatic acid, streptozotocin, diabetes, wistar rats

Introduction
Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both (1). Diabetic complications arise partly from glycosylation damage to structural and functional proteins and reflect chronic failure to maintain blood glucose homeostasis. Other complications such as diabetic nephropathy, diabetic retinopathy, diabetic neuropathy and diabetic cardiomyopathy prevail as a result of hyperglycemia (NK Rao and Nammi, 2006) [2]. Inspite of the presence of known antidiabetic medicine in the pharmaceutical market, remedies from medicinal plants are used with success to treat this disease (Bhattaram *et al*., 2002) [3]. Plant drugs (Bailey and Day, 1989) [4] and herbal formulations (Bhattacharya *et al*., 1997) [5] are frequently considered to be less toxic and free from side effects than synthetic drugs. In recent years, several authors evaluated and identified the antidiabetic potential of traditionally used Indian medicinal plants using experimental animals (Kameswara Rao *et al*., 1997) [6]. Although a large number of medicinal plants have been already tested for their antidiabetic effects, these effects remain to be investigated in several other Indian medicinal plants (Punitha and Manoharan, 2006) [7]. The attributed antihyperglycemic effects of these plants are due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin dependent processes. Hence treatment with herbal drugs has an effect on protecting β-cells and smoothing out fluctuation in glucose levels (Jia *et al*., 2003) [8].
Asiatic acid is one of most important triterpenes obtained from C. asiatica plant demonstrated potential to lower plasma glucose. However, scientific investigation has not so far been conducted to systematically analyze the antihyperglycemic activity of Asiatic acid and protective effect of AA in prevention of diabetic complications. Therefore, in the light of the above, the present investigation is aimed to evaluate the antihyperglycemic activity of the triterpene Asiatic acid in streptozotocin induced diabetic adult male Wistar rats.

Materials and Methods
Study Design
The control and experimental rats were treated with Asiatic acid (AA) and glibenclamide at appropriate doses for 60 days. The treatment was then stopped and observations were made. The rats were fasted for 12 h and sacrificed by cervical dislocation. Blood was then collected and processed. In the processed blood samples of control and experimental groups of rats, the following blood parameters such as plasma glucose level, plasma insulin level, glycosylated haemoglobin level, blood urea level, serum protein level, were estimated.

Estimation of Plasma Glucose

Glucose was estimated by the method of Trinder (1969) [9] using reagent kit. To 0.01 ml each of plasma, standard and distilled water (blank) in to three separate tubes, 1 ml each of the enzyme reagent was added, mixed well and kept at 37 °C for 15 minutes. The color developed was read at 510 nm in a spectrophotometer against reagent blank.

Estimation of Plasma Insulin

Plasma insulin was assayed by the solid phase system amplified sensitivity immunoassay using reagent kits obtained from Medgenix-INS-ELISA, Biosource, Europe S.A., Belgium (Burgi *et al*., 1988). Standards or samples containing insulin react with capture antibodies coated on a plastic well and with monoclonal antiobodies labelled with horseradish peroxidase (HRP). Selected sufficient strips to accommodate standards, controls and all test samples. Then fitted the strips into the holding frame. 50 µl of each standard, control or samples were dispensed into the appropriate wells. Time between distribution of first standard and last sample was kept minimum. 50 µl of antiserum HRP conjugate was dispended into all wells and incubated for 30 min at room temperature on a horizontal shaker set at 700 rpm. The plates were washed after aspirating the liquid from the well. Then 0.4 ml of washing solution was dispensed into each well and the contents were aspirated. This was repeated twice for complete washing. 200 µl of the freshly prepared revelation solution was added into each well 15 min after washing. Then the plate was incubated for 15 min on a horizontal shaker set at 700 rpm at room temperature, avoiding direct sunlight and 50 µl of arresting reagent was added into each well. The absorbance was read within one hour at 450 nm in a spectrophotometer.

Estimation of Glycosylated Haemoglobin (HbA1c)

Glycosylated haemoglobin in the blood samples was estimated by the method of Sudhakar and Pattabiraman (1981) [11]. 0.5 ml of saline washed erythrocytes was lysed with 5.5 ml of water, mixed and incubated at 37 °C for 15 min. The contents were centrifuged and the supernatant was discarded, then 0.5 ml of saline was added, mixed and processed for estimation. To 0.2 ml of aliquot, 4 ml of oxalate 42 hydrochloric solution was added and mixed. The contents were heated at 100 °C for 4 h, cooled and precipitated with 2 ml of 40% TCA. The mixture was centrifuged and to 0.5 ml of supernatant, 0.05 ml of 80% phenol and 3.0 ml of concentrated sulphuric acid were added. The colour developed was read at 480 nm in a spectrophotometer after 30 min.

Estimation of Protein in Serum

Protein in serum was determined by the method of Lowry *et al*. (1951) [12]. 0.5 ml of serum was added to 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 color developed was read after 20 min at 640 nm against a reagent blank. For a standard curve, 0.2-1.0 ml of standard bovine serum albumin was treated in the similar way as the test.

Results

Blood Glucose

The mean fasting plasma glucose level of control rats was not varied much from day 3 after STZ administration to the end of the experimental period. At the beginning of the experiment, the glucose level of control group was 70.33±4.27 mg/dl and it was 71.66±3.32 mg/dl in control group treated with 50 mg/kg AA. After the experimental period of 60 days, the glucose level was not changed (70.83±4.35 mg/dl) in the control rats. Normal rats treated with 50 mg/kg AA did not show significant (p<0.05). Variation in glucose level after the experimental period and it was 69.83±3.65 mg/dl. Significant increase (p<0.05) difference in the liver glycogen content (Fig.1.1). The statistical one-way ANOVA revealed that the liver glycogen content between different groups of experimental and control rats was highly significant (p<0.05).

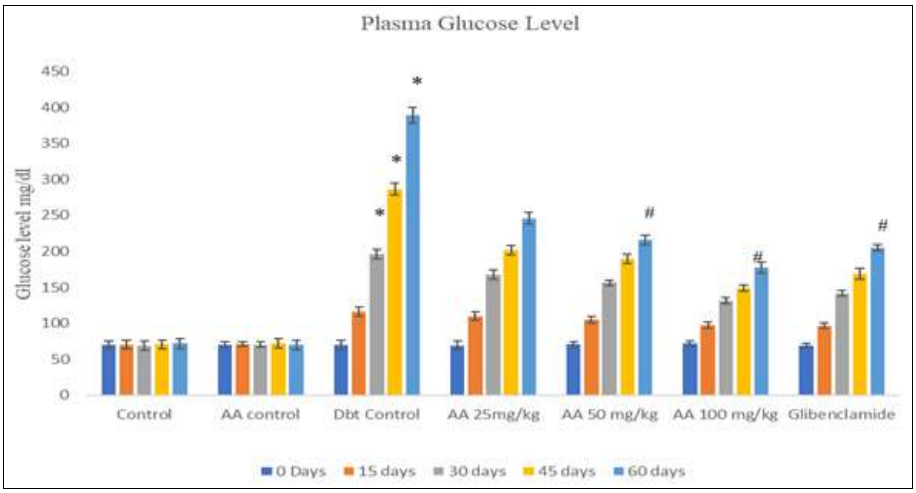

Fig 1: Determination of plasma glucose level in rat following treatment. At 0 day no group demonstrated any significant rise in plasma glucose level. At 15 or 30 or 45 or 60 days glucose level increased in streptozotocin treated rats. Increased concentrations of Asiatic acid were capable of ameliorating the plasma glucose level. Here * and # shows significant changes with respect to control and diabetic group respectively (p<.0.05).

Plasma Insulin

The mean plasma insulin level recorded after the experimental period in normal control rats was 14.90±0.493 µU/ml, while a mild increase (14.94±0.299 µU/ml) in mean level of insulin was noticed in AA control rats, which however was not significant. When compared to normal

control rats, the diabetic control rats showed a significant decrease in insulin level (6.99±0.151 µU/ml). The insulin level in rats treated with 25 mg/kg AA was 9.52±0.492 µU/ml and that of 50 mg/kg 50 AA was 12.10±0.304 µU/ml. But in the diabetic rats treated with 100 mg/kg AA, the insulin level was restored to normal (13.94±0.299 µU/ml) and it was comparable to that of the diabetic rats treated with 600 µg/kg of glibenclamide (14.13±0.236 µU/ml). When compared to the normal control rats, 53.08% decrease of insulin level was noticed in diabetic rats, while AA treatment in diabetic rats reversed the effect in a dose dependent manner and the mean percentage difference was in the order of 36.10%, 18.78% and 6.44% for the rats received a dose of 25, 50 and 100 mg/kg of AA, respectively. But the normal control rats treated with 50 mg/kg AA did not show significant variation in insulin level from the untreated normal control rats (Fig.1.2). The one-way ANOVA revealed that the plasma insulin level between different groups of experimental and control rats was statistically more significant (p<0.05).

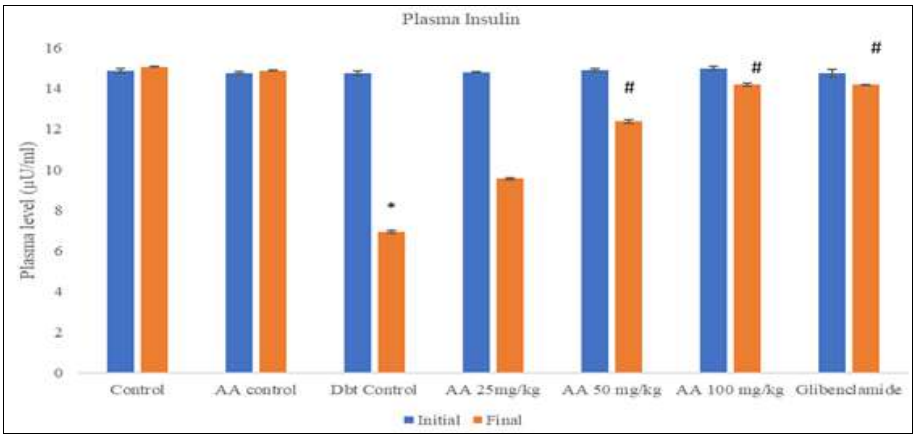

Fig 2: Measurement of plasma insulin level was in different rat groups. In streptozotocin treated group plasma insulin was declined to half. In AA treated groups a gradual restoration of insulin level was observed. Here * and # shows significant changes with respect to control and diabetic groups respectively (p<.0.05)

Glycosylated Haemoglobin

The mean level of glycosylated haemoglobin in normal control rats was 3.70±0.095 mg/g of Hb, whereas in Asiatic acid treated control rats, it was slightly decreased to 3.60±0.182 mg/g of Hb.

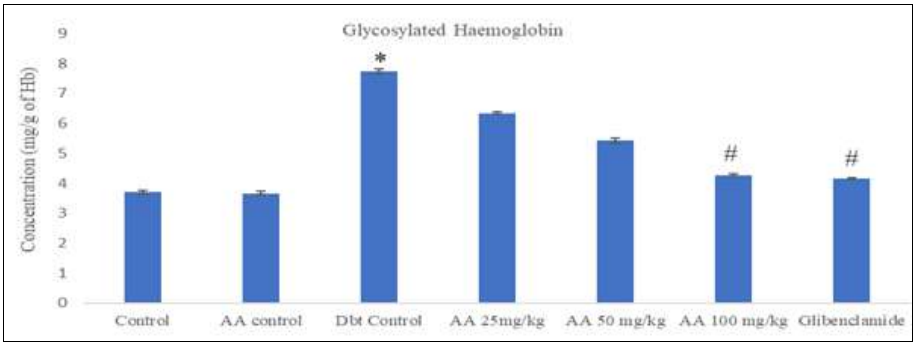

Fig 3: Haemoglobin glycosylation is marked with increased concentration of glucose. In streptozotocin treated diabetic control group more than 2 times elevation in glycosylated haemoglobin concentration was observed in comparison to control or 20 µM Asiatic acid treated rat group. An increasing dose of Asiatic acid along with streptozotocin reduce the concentration of glycosylated haemoglobin. A dose of 100 mg/kg bring the glycosylated haemoglobin level down to normal which is comparable to control group. Here * and # shows significant changes with respect to control and diabetic group (p<.0.05)

The level of glycosylated haemoglobin in diabetic control rats was increased to 7.62±0.409 mg/g of Hb. But in different concentrations of AA treated diabetic rats, the glycosylated haemoglobin level was gradually decreased to normal and it was in the order of 6.32±0.339, 5.40±0.343 and 4.26±0.305 mg/g of Hb. Similarly, the level of glycosylated haemoglobin in glibenclamide treated diabetic rats decreased to 4.14±0.358 mg/g of Hb Fig 1.3. When compared to the control rats, there was a significant change ((p<0.05).

Total Protein

The total protein in the serum samples of normal control rats was 6.68 ± 0.147 g/dl, while that of AA treated control rats was 6.81 ± 0.117 g/dl which was not significantly different. But the total protein content in the serum samples was significantly different (p<0.05). But the total protein content in serum decreased significantly (p<0.05) (4.55±0.104 g/dl) in diabetic control rats. and the value decreased to 5.28±0.213 g/dl in diabetic rats treated with 25mg/kg AA. But it was restored to normal (5.78±0.116 g/dl) in 50 mg/kg AA treated diabetic rats and the level was high (6.41±0.116 g/dl) in diabetic rats treated with 100 mg/kg AA. However, the protein level in the serum samples of glibenclamide treated diabetic rats was 6.45±0.104 g/dl. The one-way ANOVA revealed that the total protein level between different groups of experimental and control rats was statistically more significant (p<0.05).

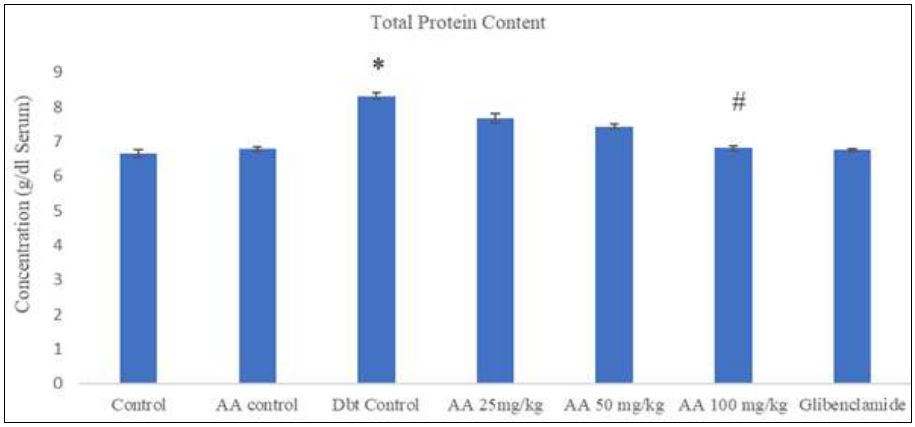

Fig 4: Measurement of total protein content in serum of experimental rats. An elevation of protein content was observed as a response to hyperglycemic conditions arise due to injection of streptozotocin in diabetic control rats. However, the impact was subsided with increasing dose of Asiatic acid. Here * and # shows significant changes with respect to control and diabetic group (p<.0.05)

Discussion

Streptozotocin is an antibiotic and anticancer agent, selectively destroys the pancreatic insulin secreting β-cells, producing less active cell and resulting in diabetic state (Szkudelski, 2001) [13]. It breaks the nuclear strand of the islet cells and brings an increase in blood glucose levels (Takasu *et al*., 1991) [14].

Glibenclamide is often used as a standard antidiabetic drug in streptozotocin induced diabetic rats to compare the efficacy of variety of hypoglycemic compounds (Paredes *et al*., 2001) [31]. Several drugs such as biguanides and sulfonylureas are presently available to reduce hyperglycemia in diabetes mellitus, but these drugs have side effects, therefore search of new class of compounds are essential to overcome diabetic problems (Coldren 2003) [32].

Asiatic acid is a pentacyclic triterpene derived initially from the plant Centella asiatica and is used as a medicine in tropical regions (Coldren *et al*., 2003) [32]. In addition to several medicinal properties, it is also reported to possess inhibitory action on rabbit muscle glycogen phosphorylase activity, which is one of the regulatory enzymes in the liver responsible for the control of blood glucose level. In this background, alternate to the current antidiabetic 60 medicines, the present study was undertaken to assess the antihyperglycemic activity of Asiatic acid (AA) in streptozotocin induced diabetic rats.

In the present study, the level of fasting blood glucose was increased in streptozotocin injected diabetic rats as expected, since streptozotocin causes a massive reduction in insulin release, by the destruction of the β-cells of the islets of langerhans and thereby induces hyperglycemia (Schein *et al*., 1973) [17]. After 60 days of AA treatment, the entire AA treated diabetic rats showed significant (p<0.05) reduction in fasting blood glucose level. Maximum reduction in glucose level (46.01%) was elicited by diabetic rats treated with a dose of 100 mg/kg AA, which is better than that of diabetic rats treated with 600 µg/kg glibenclamide (36.14%). Results of the present study indicated that the antihyperglycemic activity exhibited by AA was in a dose dependent manner. Hypoglycemic activities of terpenoids such as oleanolic acid, ursolic acid (Liu, 1995) [18] and dehydrotrametenolic acid (Sato *et al*., 2002) have been previously described. As reported by Tzu-Hsuan *et al*. (2011), triterpenes of Poria cocos dehydrotumulosic acid effectively reduced blood glucose level in STZ-diabetic mice, while other triterpenes of the same plant such as dehydrotrametenolic acid and pachymic acid had shown anti-hyperglycemic effect to a lesser extent. In another study, treatment with a triterpenoid, 3β, 19 α-dihydroxyurs-12, 20 (21)-diene-28-oic acid (30 mg/kg) showed significant decrease in blood glucose level in normal and STZ induced diabetic mice (Pe´rez Gutie´rrez *et al*., 2009).

In this study, no significant reduction in fasting glucose level was observed in the normal rats treated with AA at a dose of 50 mg/kg. This suggested that AA does not exhibit hypoglycemic activity. Besides, reduction in glucose level in Asiatic acid treated diabetic rats, a concomitant rise in insulin level was observed. Optimum level of insulin (13.94±0.299 µU/ml) was found in 100 mg/kg AA treated diabetic rats and it was comparable to that of the glibenclamide treated diabetic rats (14.13±0.236 µU/ml). In diabetes mellitus, insulin is not or insufficiently synthesized, developing hyperglycemia with biochemical changes in glucose, and lipid metabolism leading to an increased production of reactive oxygen species (ROS) (Rajasekaran *et al*., 2006) [37].

Insulin influences the intracellular utilization of glucose in a number of ways. Studies suggested that insulin is essential to maintain the glucose homeostasis by enhancing the glycolysis and glycogen synthesis in skeletal muscle (Mandarino *et al*., 1987) [21] with the concomitant decrease in glycogenolysis in liver and skeletal muscles (Shimazu, 1987) [22] also, insulin regulates the GLUT4 gene expression (Jones and Dohm, 1997) [23]. It has been reported that flavanoids, glycosides (Hii and Howell, 1985) [24] and terpenoids stimulate the secretion of insulin in β-cells of pancreas. In the present study, increase in serum insulin level in AA treated groups indicated that AA might have stimulated insulin secretion from regenerated β-cells of pancreas. The decrease in blood glucose in diabetic rats treated with AA might be due to the stimulation of β-cells for elevated secretion of insulin, thereby increasing the oxidation of glucose in various tissues (Prakasam *et al*., 2002) [25]. AA might have exerted its effect by preventing the death of β-cells and/or may have helped in the rejuvenation or recovery of partially destroyed β-cells (Ahmed *et al*., 1998). The findings of the present study are supported by previous studies, Chauhan *et al*. (2010) [33] reported that the ethanolic and methanolic extracts of C. asiatica (the plant from which Asiatic acid is isolated) at a dose of 250 mg/kg each have shown significant reduction (69% and 51%, respectively) in blood glucose levels in both

glucose loaded and alloxan induced diabetic rats. The ethanolic extract produced maximum antidiabetic activity and was higher than the hypoglycemic activity of glibenclamide in the diabetic rats. In a recent study, Liu *et al*. (2010) [34] reported the reduction of glucose level and elevation of insulin level in streptozotocin induced diabetic rats upon treatment with 25 mg/kg AA for 2 weeks. Blood glucose level was reduced to less than 10 mmol/l and insulin level was increased to 8 ng/ml by AA treatment. This is in agreement of the present study. In uncontrolled or poorly controlled diabetes, there is an increased glycosylation of number of proteins including haemoglobin and β-crystalline lens (Asgary *et al*., 2002) [26], which is directly proportional to the fasting blood glucose level (Goodarzi *et al*., 2006) [27, 29]. In the present study, total haemoglobin level of diabetic rats was significantly decreased, which may be due to increased formation of HbA1c. A previous report has indicated that protein synthesis is decreased in all tissues of diabetic albino Wistar rats, which is due to the relative deficiency of insulin and depressed synthesis of haemoglobin. HbA1c consist of 3.4 to 5.8% of the haemoglobin in normal human red cells, but it is increased in patients with overt diabetes mellitus and has been found to increase over a long period of time in diabetes mellitus with decrease in haemoglobin level (Bunn *et al*., 1978) [35]. The present study has shown a significant increase in the level of glycosylated haemoglobin in diabetic rats. Increased HbA1c levels and decreased haemoglobin levels in experimental diabetes have been previously reported by Muruganandan *et al*. (2002). In the present study treatment of diabetic rats with Asiatic acid had reduced glycosylated haemoglobin levels from 6.32±0.339 to 4.26±0.305 mg/g of Hb. The effect shown by AA at adose of 100 mg/kg in diabetic rats was comparable to that of diabetic rats treated with 600 µg/kg of glibenclamide, which elevated haemoglobin to 13.78±0.470 g/dl and reduced glycosylated haemoglobin level to 4.14±0.358 mg/g of Hb. These effects may be due to improvement of glycemic control and plasma insulin levels by AA. In support of the present study, a recent study by Ramdas *et al*. (2011) [36] suggested that administration of aquous extract of S. sesban to normal and STZ-induced diabetic rats at the doses of 250 and 500 mg/kg body weight per day orally for 30 days showed a significant decrease in glycosylated haemoglobin level as compared to normal groups. Preliminary phytochemical screening of the extract revealed the presence of triterpenoids, carbohydrates, tannins, saponins, glycosides and steroids which were reported to possess antihyperglycemic property. Results of another recent study evidenced that, administration of methanolic extract of Costus igneus rhizome (100 to 200 mg/kg.bw/day) on STZ-diabetic rats has lowered the elevated HbA1c levels to near normal level after 30 days of treatment and the active fraction was reported to contain tannins, saponins, flavonoids, terpenoids, cardiac glycosides and naturally occurring phenolic compound like quercetine (Pazhanichamy *et al*., 2011) [28]. In diabetes, protein synthesis ceases and protein catabolism increases.

In the present study, the total protein level in streptozotocin induced diabetic rats was found to be decreased. It may be due to absolute or relative deficiency of insulin (Ananthi *et al*., 2003) [30]. But the total protein level was found to be normal in 50 and 100 mg/kg AA treated diabetic rats and it was in the order of 5.78±0.116 and 6.41±0.116 g/dl, respectively. The results thus indicated an effective insulin secretion and or action in the diabetic rats due to AA treatment.

Therefore, the present study reveals that, AA causes anti-hyperglycemic effect as evident by reduction in plasma glucose level and reduced plasma HbA1c level whereas increasing glycosylated hemoglobin. It is clear from the results on that Asiatic acid causes insulin secretion as indicated by the increase of plasma insulin. From the results of the hepatic enzymes, serum protein and urea, it is evident that Asiatic acid reduces toxicity in STZ diabetic rats and nontoxic by itself.

Conclusion

The marked decrease in plasma glucose level upon administration of Asiatic acid in dose dependent manner establishes the triterpene a potential hyperglycemic agent. This has been supported by increased insulin level decreased glycosylated hemoglobin when Asiatic acid was administered in a dose dependent manner.

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