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Original article

Antihyperglycemic effect of diosmin on hepatic key enzymes of carbohydrate metabolism in streptozotocin-nicotinamide-induced diabetic rats

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ABSTRACT

The purpose of this study was to investigate the effect of diosmin on hepatic key enzymes of carbohydrate metabolism in streptozotocin-nicotinamide-induced diabetic rats. Diosmin was administered to streptozotocin-induced (45 mg/kg b.w) diabetic rats at different doses (25, 50, 100 mg/kg b.w) for 45 days to assess its effect on fasting plasma glucose, insulin, glycosylated hemoglobin, hemoglobin and carbohydrate metabolic enzymes, it was found that plasma glucose was significantly reduced in a dose-dependent manner when compared to the diabetic control. In addition, oral administration of diosmin (100 mg/kg b.w) significantly decreased glycosylated hemoglobin and increased hemoglobin 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. Furthermore, protection against body weight loss of diabetic animals was also observed. These results showed that diosmin has potential antihyperglycemic activity in streptozotocin-nicotinamide-induced diabetic rats.

1. Introduction

Diabetes mellitus is a chronic metabolic disease with the highest rates of prevalence and mortality worldwide that is caused by an absolute or relative lack of insulin and/or reduced insulin activity, which results in hyperglycemy and abnormalities in carbohydrate, protein, and fat metabolism [1,2]. It can be associated with serious complications and premature death, but people with diabetes should take steps to control the disease and lower the risk of complications [3], in spite of the use of many oral hypoglycemic agents such as sulphonylureas and biguanides. All of these pharmacological modalities also show limited efficacy and certain adverse effects such as liver toxicity, lactic acidosis, diarrhoea and attenuation of response after prolonged use and are expensive particularly for developing countries like India and China. Comparatively very less side effects and low cost of phytochemicals from natural resources open new avenues for the treatment of various diseases including diabetes [4]. Therefore, there is a need for phytochemicals that have antihyperglycemic potential, which are cost-effective and also safe without long-term side effects.

Diosmin (diosmetin 7-O-rutinoside; Fig. 1), a natural flavone glycoside therapeutically used to improve the symptoms of venous and lymphatic vessel insufficiency [5]. Diosmin is readily obtained by dehydrogenation of the corresponding flavanone glycoside, hesperidin that is abundant in the pericarp of various citrus [6]. It is considered to be a vascular-protecting agent used to treat hemorrhoids, lymphedema, varicose veins and different types of cancer. As a flavonoid, it also exhibits anti-inflammatory, antioxidant, and antimutagenic properties [7–12]. It has been shown to improve factors associated with diabetic complications. Blood parameters of glycation and oxidative stress were measured in type 1 diabetic patients before and after intervention with a diosmin-containing flavonoid mixture. A decrease in glycosylated hemoglobin was accompanied by an increase in glutathione peroxidase [13], demonstrating long-term decrease in blood glucose levels and increased antioxidant activity [6].

The eventual objective of the present study is to determine the antihyperglycemic property of diosmin by assessing activities of the key metabolic enzymes concerned with carbohydrate metabolism in streptozotocin-nicotinamide-induced diabetic rats.

2. Materials and methods

2.1. Animals

Male albino Wistar strain rats weight about 180 to 220 g were procured from Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and they were maintained in an air-conditioned room

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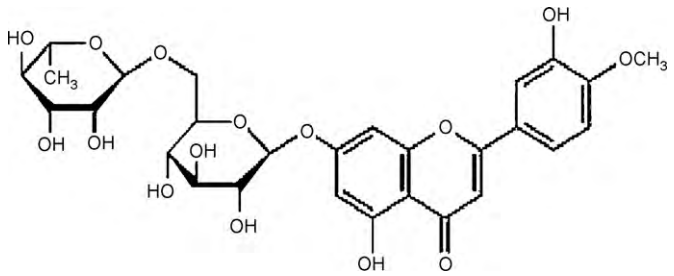

Fig. 1. Structure of diosmin.

(25 ± 2 °C) with a 12-h light: 12-h dark cycle, and were allowed access to food (standard pellet diet) and water *ad libitum*. Studies were carried out in accordance with Indian National Law on Animal Care and Use. Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg No. 566/2008), Annamalai University, Annamalainagar, provided ethical clearance.

2.2. Chemicals

Diosmin and streptozotocin were purchased from Sigma Chemical Company, St Louis, MO, USA, and nicotinamide was obtained from Ranbaxy Chemicals Ltd., Mumbai, India. All the other chemicals and reagents were used of analytical grade.

2.3. Induction of diabetes in rats

Streptozotocin was freshly dissolved in (0.1 M, pH 4.5) citrate buffer and nicotinamide was dissolved in normal physiological saline and maintained on ice prior to use. Non-insulin-dependent diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal injection of streptozotocin (45 mg/kg b.w), 15 min after the intraperitoneal administration of nicotinamide (110 mg/kg b.w) [14]. The elevated plasma glucose was determined and those rats with fasting glucose levels greater than 250 mg/dL were served as diabetic rats and used in the study. Treatment with diosmin was started on the third day after streptozotocin induction.

2.4. Experimental procedure

The animals were separated into six groups ($n = 6$), a total of 36 rats (24 diabetic surviving rats, 12 normal control rats) were used. Diosmin was dissolved in vehicle solution of 0.6% dimethylsulfoxide (DMSO) and different doses of diosmin were administered orally using an intragastric tube for a period of 45 days.

- Group I: normal control (vehicle treated; DMSO: 1 ml/kg b.w)
- Group II: normal + diosmin (100 mg/kg b.w)
- Group III: diabetic control
- Group IV: diabetic + diosmin (25 mg/kg b.w)
- Group V: diabetic + diosmin (50 mg/kg b.w)
- Group VI: diabetic + diosmin (100 mg/kg b.w).

The initial and final body weight of the various groups were recorded. At the end of the experimental period, the animals were fasted overnight, anesthetized using ketamine hydrochloride (24 mg/kg b.w, intramuscular injection), and sacrificed by cervical decapitation. Blood samples were collected in tubes containing potassium oxalate and sodium fluoride (3:1) mixture for the estimation of plasma glucose and insulin. Hemoglobin and glycosylated hemoglobin levels were estimated in whole blood samples. Liver was immediately dissected, washed in ice-cold saline to remove the blood. The liver was selected because it is one of the tissues showing a high rate of free radical generation.

2.5. Analytical procedure

Plasma glucose levels were estimated using a commercial kit (Sigma Diagnostics Pvt. Ltd., Baroda, India) by the method of Trinder [15]. Plasma insulin was assayed by ELISA kit (Boeheringer-Manneheim Kit, Manneheim, Germany). Hemoglobin and glycosylated hemoglobin were estimated by diagnostic kit (Agappe Diagnostic Pvt. Ltd., India) [16].

Hepatic hexokinase was assayed by the method of Brandstrup et al. [17]. The reaction mixture in a total volume of 5.3 ml contained the following: 1 ml of glucose (0.005 M) solution, 0.5 ml of adinosine triphosphate (0.072 M) solution, 0.1 ml of magnesium chloride (0.05 M) solution, 0.4 ml of potassium dihydrogen phosphate (0.0125 M), 0.4 ml of potassium chloride (0.1 M), 0.4 ml of sodium fluoride (0.5 M) and 2.5 ml of Tris-HCl buffer (0.01 M, pH 8.0). The mixture was pre-incubated at 37 °C for 5 min. The reaction was initiated by the addition of 2 ml of tissue homogenate. One millilitre of the reaction mixture was immediately transferred to the tubes containing 1 ml of 10% trichloroethanoic acid that was considered as zero time. A second aliquot was removed and deproteinised after 30 min incubation at 37 °C. The protein precipitate was removed by centrifugation and the residual glucose in the supernatant was estimated by the method of Trinder [15].

Glucose-6-phosphatase was measured by the method of Koide and Oda [18]. Incubation mixture contained 0.7 ml of citrate buffer (0.1 M, pH 6.5), 0.3 ml of substrate (0.01 M) and 0.3 ml of tissue homogenate. The reaction mixture was incubated at 37 °C for 1 h. Addition of 1 ml of 10% trichloroethanoic acid to the reaction tubes terminated the reaction of the enzyme. The suspension was centrifuged and the phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow [19]. The supernatant was made up to known volume. To this, 1 ml of ammonium molybdate was added followed by 0.4 ml of amino naphthol sulphonic acid. The blue colour developed after 20 min was read at 680 nm.

Fructose-1,6-bisphosphatase activity was measured by Gancedo and Gancedo [20]. The assay mixture in a final volume of 2 ml contained 1.2 ml of Tris-HCl buffer (0.1 M, pH 7.0), 0.1 ml of substrate (0.05 M), 0.25 ml of magnesium chloride (0.1 M), 0.1 ml of potassium chloride solution (0.1 M), 0.25 ml of ethylenediaminetetraacetic acid (0.001 M) solution and 0.1 ml of enzyme homogenate. The incubation was carried out at 37 °C for 5 min. The reaction was terminated by the addition of 10% trichloroethanoic acid. The suspension was centrifuged and the supernatant was used for phosphorus estimation by the method of Fiske and Subbarow [19]. The supernatant was made up to known volume. To this, 1 ml of ammonium molybdate was added followed by 0.4 ml of amino naphthol sulphonic acid. The blue colour developed after 20 min was read at 680 nm.

Protein content in the tissue homogenate was determined by the method of Lowry et al. [21]; 0.5 ml of the tissue homogenate was precipitated with 0.5 ml of 10% trichloroethanoic acid and centrifuged for 10 min and the precipitate was dissolved in 1.0 ml of 0.1 N sodium hydroxide. From this, 0.1 ml of aliquot was taken and made up to 1.0 ml with distilled water. Then, 4.5 ml of alkaline copper reagent was added and allowed to stand at room temperature for 10 min. After incubation, 0.5 ml of Folin's-Ciocalteau reagent was added and the blue colour developed was read at 620 nm after 20 min.

2.6. Statistical analysis

All the data were expressed as mean ± SD of number of experiments ($n = 6$). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS Version 15 (SPSS,

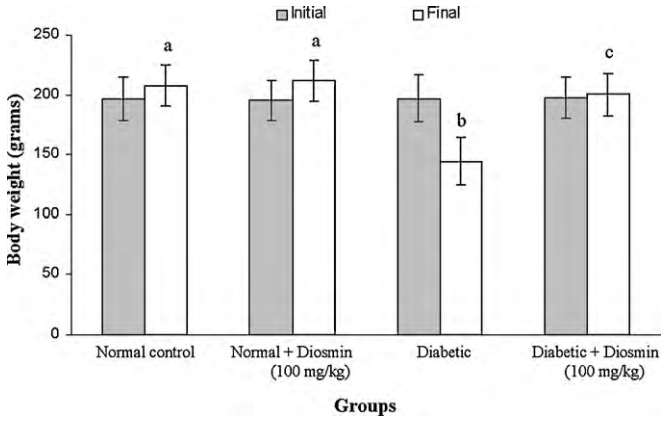

Fig. 2. Body weight in streptozotocin-induced diabetic rats before and after oral treatment of diosmin. Values are given as mean ± SD from six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p < 0.05$ (DMRT).

Cary, NC, USA) and the individual comparisons were obtained by Duncan's multiple range test (DMRT). Values are considered statistically significant when $P < 0.05$ [22].

3. Results

The changes in the body weight, food and water intake in control and experimental rats were deleted instead of are represented in Figs. 2 and 3. Food and water intake were elevated whereas the body weight significantly decreased in diabetic rats compared with normal control rats. In diabetic rats treated with diosmin 100 mg/kg b.w significantly decreased the food and water intake and also increased body weight in diabetic rats when compared to diabetic control rats.

Fig. 4 showed the levels of plasma glucose and insulin in normal control and experimental rats. The levels of plasma glucose were significantly increased whereas plasma insulin levels were significantly decreased in diabetic control rats. In diosmin treated (all doses), a significant decrease in plasma glucose levels and significant increase in insulin levels were observed at the end of the experimental period. Diosmin at a dose of 100 mg/kg b.w showed a highly significant effect than other two doses (25 and 50 mg/kg b.w). Based on these data, the effective dose was fixed at 100 mg/kg b.w and used for further analysis.

The levels of total hemoglobin and glycosylated hemoglobin in normal control and experimental animals are depicted in Table 1. The diabetic rats showed significant decrease in the levels of total hemoglobin and significant increase in the levels of glycosylated hemoglobin when compared with normal control rats. The levels of total hemoglobin and glycosylated hemoglobin were extensively reversed by the administration of diosmin in diabetic rats. Normal animals treated with diosmin at a dosage 100 mg/kg b.w did not show any significant changes in plasma glucose, insulin, hemoglobin and glycosylated hemoglobin.

Table 2 showed the changes in the activities of hexokinase and glucose-6-phosphate dehydrogenase in the liver of normal control and experimental rats. The activities of hexokinase and glucose-6-phosphate dehydrogenase were significantly decreased in diabetic rats when compared with normal control rats. Oral administration of diosmin to diabetic rats extensively increased the activities of these hepatic enzymes.

Table 3 depicted the activities of gluconeogenic enzymes (glucose-6-phosphatase and fructose-1,6-bisphosphatase) in normal control and experimental rats. The actions of glucose-6-phosphatase and fructose-1,6-bisphosphatase were significantly

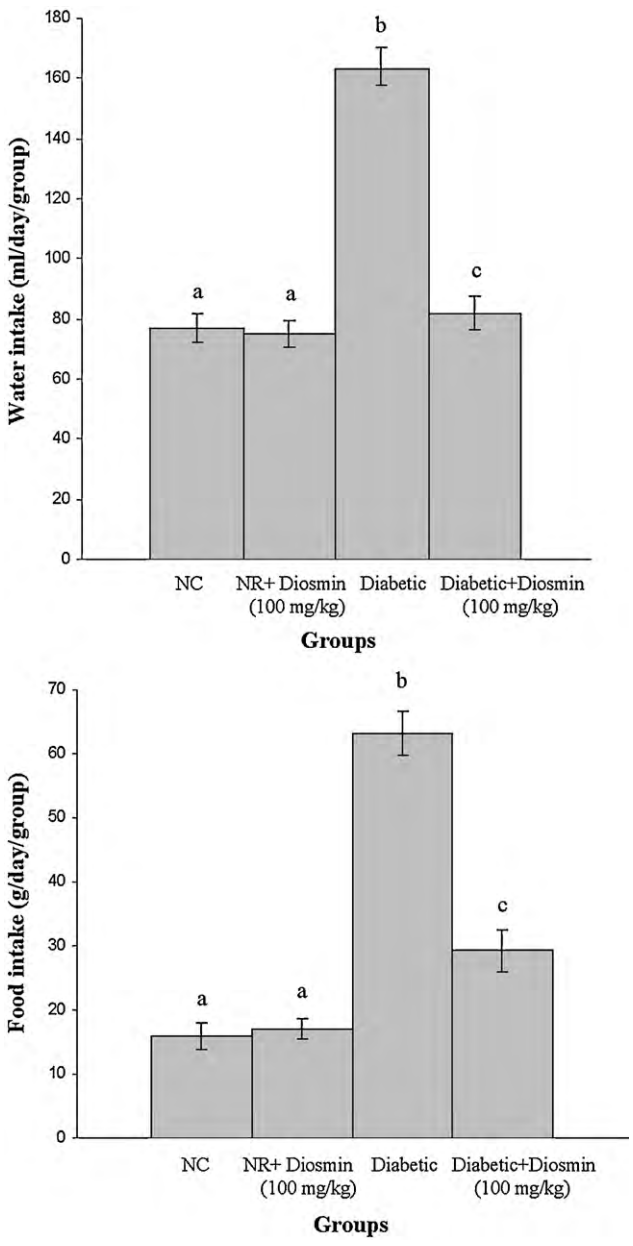

Fig. 3. Water and food intake in streptozotocin diabetic rats before and after oral treatment of diosmin. Values are given as mean ± SD from six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p < 0.05$ (DMRT); NC: normal control; NR: normal.

increased in diabetic rats. Oral administration of diosmin to diabetic rats inverted the changes in the activities of these hepatic enzymes.

4. Discussion

Diabetes mellitus is a serious illness with multiple complications and premature mortality, accounting for at least 10% of total health care expenditure in many countries [23]. Global postulates that three fourth of the world population cannot afford the products of allopathic medicine and thus, have to rely upon the use of traditional medicines, which are largely derived from plants [24]. Some substances have shown antidiabetic effect by influencing β-cell to stimulate insulin secretion and restore insulin sensitivity [25]. Diosmin found in citrus fruits, an active ingredient

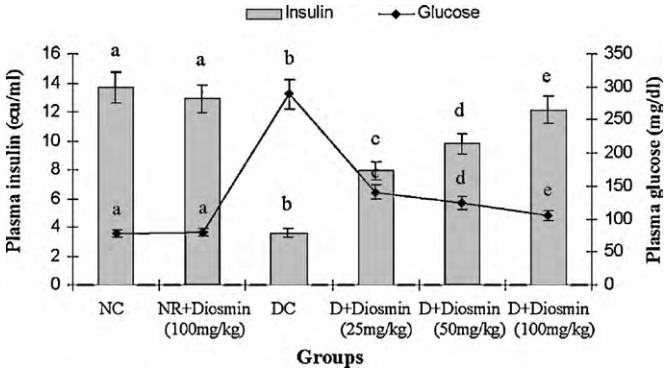

Fig. 4. Changes in the levels of plasma glucose and insulin in normal control and experimental rats. Values are given as mean ± SD from six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p < 0.05$ (DMRT); NC: normal control; NR: normal; DC: diabetic control.

Table 1
Effect of diosmin on the levels of hemoglobin and glycosylated hemoglobin in normal control and experimental rats.

Groups	Total hemoglobin (g/dL)	Glycosylated hemoglobin (mg/gHb)
Normal control	13.05 ± 0.91^{a}	0.29 ± 0.04^{a}
Normal + diosmin (100 mg/kg)	12.83 ± 0.96^{a}	0.27 ± 0.03^{a}
Diabetic control	7.96 ± 0.44^{b}	0.84 ± 0.07^{b}
Diabetic + diosmin (100 mg/kg)	11.82 ± 0.83^{c}	0.42 ± 0.03^{c}

Values are given as mean ± SD from six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p < 0.05$ (DMRT).

of detralex (a purified flavonoid fraction composed of 90% diosmin and 10% hesperidin) used for the treatment of chronic venous insufficiency [26–28].

This study evaluates the effect of diosmin by measuring the activities of key enzymes involved in carbohydrate metabolism in the liver of non-diabetic and streptozotocin-induced diabetic rats. Streptozotocin is a selective β-cell genotoxicant and when administered in a single high dose, it induces a rapid onset of diabetes by generating sufficient levels of DNA adducts to cause over activation of poly adenosine diphosphate ribose synthetase in the base excision repair pathway [29].

Table 2
Effect of diosmin on changes in the activities of hexokinase and glucose-6-phosphate dehydrogenase in normal control and experimental rat liver.

Groups	Hexokinase (units/g protein)[1]	Glucose-6-phosphate dehydrogenase ($\times 10^{-4}$ ml U/mg protein)
Normal control	152.63 ± 11.62^{a}	4.78 ± 0.36^{a}
Normal + diosmin (100 mg/kg)	155.08 ± 11.87^{a}	5.01 ± 0.38^{a}
Diabetic control	102.65 ± 7.86^{b}	2.23 ± 0.17^{b}
Diabetic + diosmin (100 mg/kg)	141.27 ± 10.81^{ca}	3.54 ± 0.27^{c}

Values are given as mean ± SD from six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p < 0.05$ (DMRT); [1]: μmoles of glucose phosphorylated/min

Table 3
Effect of diosmin on changes in the activities of glucose-6-phosphatase and fructose-1,6 bisphosphatase in normal control and experimental rat liver.

Groups	Glucose-6-phosphatase (U/mg protein)[1]	Fructose-1, 6-bisphosphatase (U/mg protein)[2]
Normal control	0.19 ± 0.01^{a}	0.34 ± 0.03^{a}
Normal + diosmin (100 mg/kg)	0.18 ± 0.01^{a}	0.32 ± 0.02^{a}
Diabetic control	0.27 ± 0.02^{b}	0.61 ± 0.05^{b}
Diabetic + diosmin (100 mg/kg)	0.22 ± 0.02^{c}	0.38 ± 0.03^{ca}

Values are given as mean ± SD from six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p < 0.05$ (DMRT); [1]: μmoles of Pi liberated/min; [2]: μmoles of Pi liberated/hour.

Liver is mainly responsible for maintaining normal concentrations of blood glucose by its ability to store glucose as glycogen and to produce glucose from glycogen breakdown or from gluconeogenic precursors [30]. Selective destruction of pancreatic β-cells by streptozotocin during experimental diabetes results in the decreased plasma insulin levels. This in turn leads to the defective glucose oxidation and causes hyperglycemia. The fundamental mechanism underlying hyperglycemia in diabetes mellitus involves over-production (excessive hepatic glycogenolysis and gluconeogenesis) and decreased utilisation of glucose by the tissues [31].

The daily administration of diosmin to diabetic rats for 45 days caused a statistically significant reduction in food and fluid intakes and an increase in the body weight. This could be the result of improved glycemic control produced by diosmin. Insulin generally has an anabolic effect on protein metabolism in that it stimulates protein synthesis and retards protein degradation [32]. In uncontrolled or poorly controlled diabetes, there is an increased glycosylation of a number of proteins, including hemoglobin [33]. Glycosylated hemoglobin was 3.4–5.8% of total hemoglobin in normal human red blood cells [34] and it was found to increase in diabetic patients up to 16% [35]. The level of glycosylated hemoglobin is monitored as a reliable index of glycemic control in diabetes [36], and useful tool to assess the management of diabetes mellitus. The glycosylated hemoglobin level reflects the average blood glucose concentration over the preceding 6–8 weeks [32]. In our study also, hemoglobin decreased and glycosylated hemoglobin increased in diabetic rats and administration of diosmin controls the glycation of hemoglobin by an increase in glutathione peroxidase and thus decreases the level of glycosylated hemoglobin in experimental rats [13].

Liver is an important organ that plays a pivotal role in glycolysis and gluconeogenesis. A partial or total deficiency of insulin causes derangement in carbohydrate metabolism that decreases the activities of hexokinase, glucokinase and phosphofructokinase [37], causing impaired peripheral glucose utilization and augmented hepatic glucose production. The main objective of the study is to determine whether diosmin is implicated as an antihyperglycemic agent, which modulates key enzymes involved in glucose metabolism. In our study, diosmin administration to diabetic rats modulated key glucose metabolizing enzymes in liver, which result in normal blood glucose homeostasis.

One of the key enzymes in the catabolism of glucose is hexokinase, the first enzyme of glycolysis [38,39], which phosphorylates glucose by transferring a phosphoryl group from adinosine triphosphate to form glucose-6-phosphate [40]. Hexokinase is both an insulin-dependent and an insulin-sensitive enzyme and is almost completely inhibited or inactivated in the diabetic rat liver in the absence of insulin [41]. Hexokinase insufficiency in diabetic rats can cause decreased utilization of glucose for energy production [42]. Similar results were obtained in the present study. Administration of diosmin to diabetic rats resulted in a significant reversal in the activity of hexokinase. The increased plasma insulin and decreased glucose in diabetic rats noticed in diosmin administration may also be as a result of increased hepatic hexokinase activity, thereby increased glycolysis.

A decrease in the activity of glucose-6-phosphate dehydrogenase slows down the pentose phosphate pathway in diabetic conditions [43]. Diabetic rats treated with diosmin showed significantly increased liver glucose-6-phosphate dehydrogenase activity, via enhanced secretion of insulin, which might increase the influx of glucose into the pentoses monophosphate shunt and

this resulted in an increased production of the reducing agent, nicotinamide adinine diphosphate, with concominant decrease in oxidative stress [44].

Glucose-6-phosphatase plays a vital role in the homeostasis of blood glucose [45]. However, the activity of glucose-6-phosphatase was known to be inhibiting under hyperglycaemic conditions [46]. Fructose-1,6-bisphosphatase is one of the key enzyme of the gluconeogenic pathway. The activities of the gluconeogenic enzymes such as glucose-6-phosphatase and fructose-1,6-bisphosphatase, increased significantly in the liver and kidney of diabetic rats [47], which may be due to insulin deficiency. In diabetic rats treated with diosmin, the activities of these enzymes were significantly lowered, which might be due to increased secretion of insulin.

5. Conclusion

In conclusion, the administration of diosmin resulted in a significant restoration of the plasma glucose, insulin, glycosylated hemoglobin, and the activities of carbohydrate metabolic enzymes. The present study suggests that diosmin enhances the glycolytic enzymes and controls glucose metabolism in streptozotocin-nicotinamide-induced diabetic rats. Therefore, diosmin possesses antihyperglycemic activity by stimulating the insulin production from the existing β-cells of pancreas.

Conflict of interest statement

None.

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