Antihyperglycemic Effect of an Important Phytocompound - Phloretin on Streptozotocin Induced Diabetes: An Experimental Study

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Authors' contributions

This work was carried out in collaboration between both authors. The corresponding author RU designed the research problem and wrote the protocol. The first author TN performed the research work and wrote the initial draft of manuscript. The corresponding author RU corrected the final format of manuscript. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aim: To investigate the antihyperglycemic effect of phloretin on streptozotocin induced diabetic rats.
Materials and Methods: Diabetes mellitus was induced in adult male albino rats of Wistar strain weight ranges between 180-200 g by intraperitoneal administration of streptozotocin (STZ) (60 mg/kg b.w). Phloretin was administered orally to diabetic rats at two different doses like 25 and 50mg/kg b.w. The antidiabetic potential of phloretin was evaluated by analyzing the changes in body weight, blood glucose, total hemoglobin, glycosylated hemoglobin, insulin, liver glycogen and carbohydrate metabolizing enzymes like hexokinase, glucose-6-phosphate dehydrogenase, glucose-6-phosphatase and fructose-1, 6-bisphosphatase in the experimental rats.
Results: Diabetic rats showed increased level of glucose and glycosylated hemoglobin and

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decreased level of body weight, serum insulin, hemoglobin and liver glycogen. Increased activities of gluconeogenic enzymes such as glucose-6-phosphatase and fructose-1, 6-bisphosphatase and decreased activities of hexokinase and glucose-6-phosphate dehydrogenase were observed in diabetic rats. The phloretin treated diabetic rats showed significantly reduced level of blood glucose and glycosylated hemoglobin and significantly increased level of body weight, serum insulin and liver glycogen. The altered activities of key enzymes of carbohydrate metabolism were significantly reverted to near normal in phloretin administered diabetic rats.

Conclusion: The present findings suggest that phloretin may be useful in the treatment of diabetes mellitus and confirmed its antihyperglycemic effect mediated through the regulation of carbohydrate metabolic enzyme activities.

Keywords: Phloretin; streptozotocin; insulin; diabetes mellitus; antihyperglycemic effect.

1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disease with the highest rate of prevalence and mortality worldwide that is caused by an absolute or relative lack of insulin and/or reduced insulin activity, which results in hyperglycemia 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 [4]. Due to the side effects of antihyperglycemic agents for the treatment of diabetes, currently there is growing interest on plants and plant derived products [5]. The World Health Organization expert committee is also considering complementary and alternative approaches, including the use of herbal medicine with antidiabetic properties due to their natural origin and less side effects [6].

Globally prevalence of diabetes mellitus is an upward trend. The International Diabetes Foundation estimates that 366 million adults aged 20-79 were affected by diabetes worldwide in 2011 and this figure is expected to rise 552 million by the year 2030, with most of the increase coming from developing countries [7]. Liver is one of the chief storage organs for glucose reserve in the body and plays a crucial role in blood glucose homeostasis. It has been reported that the diabetic condition decrease the activities of enzymes in the glycolytic and pentose phosphate pathways, while increasing the activities of gluconeogenic and glycogenolytic pathways [8]. Liver cells have been used as an *in vitro* model for evaluating or screening the antihyperglycemic effect of plant or its constituents [9].

Medicinal plant based drug discovery continues to provide new and important leads against various pharmacological targets including diabetes [10]. A number of medicinal plants traditionally used in various herbal preparations for the management of diabetes and only a few of them have been proven scientifically [11,12]. A few pharmaceutical trials are used to develop new biological agents derived from plants proved to be effective in the treatment of hyperglycemic threat [13]. Due to defective insulin secretion during diabetic condition, liver gluconeogenesis contributes to the elevation of blood glucose [14]. Numerous medicinal plants and their products have been proved to modulate the carbohydrate metabolic enzymes thereby help in maintaining the glucose homeostasis [15,16,17].

Flavonoids are currently considered as promising natural substances to develop the modern therapy options against diabetes [18]. These groups of plant secondary metabolite nutraceuticals are well known for broad spectrum biological activities including antihyperglycemic effect. A few experimental evidences have demonstrated that the hypoglycemic effect of flavonoids are either through regulation of enzymes involved in carbohydrate metabolism or by stimulation of peripheral tissues for glucose uptake [19,20].

Among the natural products, phloretin compounds are attracting much interest because of their beneficial effects. Phloretin, a natural active compound, belongs to flavonoids, exists in sap of apple, pear and other fruits and vegetables. Phloretin has been studied as a possible penetration enhancer for skin-based drug delivery [21], attenuates inflammation by

antagonizing prostaglandins [22] and protects the skin from UV light-induced photodamage [23]. It can serve the purposes of antioxidation, antitumor, antibiosis and parahormone under physiological context [24,25,26]. Phloretin is one of the antimutagenic factors that can be used for hepatoma treatment and other tumors, and thus it is a potential chemotherapeutic agent. There are 4 hydroxyl groups (-OH) in its chemical structure, which can inhibit the trans-membrane transport of saccharides leading to apoptosis of malignant cells [27].

There are many pharmacological studies using phloretin, but there is no study on the antidiabetic activity of phloretin. So, the present study was designed to evaluate the antihyperglycemic activity of phloretin by the analysis of biochemical parameters and the activities of carbohydrate metabolic enzymes in streptozotocin (STZ) induced diabetic rats.

2. MATERIALS AND METHODS

2.1 Chemicals

Phloretin (2',4',6'-trihydroxy-3-(p-hydroxyphenyl)-propiophenone; purity>98%) and Streptozotocin were purchased from Sigma Chemicals Company, St. Louis, Mo. USA. All other chemicals and reagents were of analytical grade and purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India.

2.2 Animals

Male albino rats (*Rattusnorvegicus*) weight ranges between 180-200g were used in the present investigation. The animals were housed in standard polypropylene cages and maintained under controlled room temperature and humidity with 12-h light and 12-h dark cycle. During the experimental period, the animals were fed with a diet of commercially available standard laboratory pellets purchased from Hindustan Lever Ltd, Bangalore and provided free access of water *ad libitum*.

2.3 Induction of Diabetes Mellitus

Diabetes mellitus was induced in overnight fasted experimental rats by a single intraperitoneal injection of STZ (60 mg/kg b.w) dissolved in freshly prepared citrate buffer (0.1 M, pH 4.5). After five days, the blood glucose was analyzed and determined the rats with fasting blood glucose greater than 250 mg/dl were used in the present study.

2.4 Experimental Design

The total numbers of 30 rats were randomly divided into five groups of six rats in each group (24 diabetic surviving and 6 normal).

Group I: Normal control

Group II: Diabetic control

Group III: Diabetic rats treated with phloretin (25 mg/kg b.w.)

Group IV: Diabetic rats treated with phloretin (50 mg/kg b.w.)

Group V: Diabetic rats treated with glibenclamide (600 µg/kg b.w.)

Two different doses of phloretin such as 25 mg and 50 mg/kg b.w were fixed for this study based on the previous report on chemopreventive role of phloretin against oral cancer [28]. Phloretin and glibenclamide were given orally to diabetic rats for 45 days daily at 10.00 a.m using intragastric tube. No death of the diabetic rats was observed till the end of study. The initial and final body weight of rats in each group was recorded. At the end of experimental period, the rats were deprived of food overnight and sacrificed by cervical decapitation. Blood was collected and the serum was separated for the estimation of glucose and insulin. Liver was dissected out, washed in ice-cold saline patted dry and weighed. The liver homogenate was used for biochemical investigations.

2.5 Biochemical Analysis

Serum glucose was estimated using a commercial kit (Sigma Diagnostics Pvt. Ltd., Baroda, India) by the method of Trinder [29]. Serum insulin was assayed by ELISA kit (Boeheringer-Manneheim Kit, Manneheim, Germany) [30]. Hemoglobin and glycated hemoglobin were estimated by diagnostic kit (AgappeDiagnostic Pvt. Ltd., India) [31]. The estimation of protein was carried out by the method of Lowry et al. [32]. Glycogen content was assayed by the method of Ong and Khoo [33]. For this experiment, the liver samples were homogenised with 10 volumes of ice-cold 30% KOH while frozen and boiled at 100℃ for 30 min. Glycogen was precipitated with ethanol, washed and resolubilized in water and then the glycogen content was determined by treated with

anthrone reagent. The green colour developed was read at 625 nm.

A portion of the liver tissue was dissected out, washed with ice-cold saline and homogenized with 0.1 M Tris-HCl buffer (pH 7.4) for the assay of key enzymes of carbohydrate metabolism. The homogenate was centrifuged at 2000 g for 5 min to remove the debris and the supernatant was used for the evaluation of enzymes of carbohydrate metabolism.

2.5.1 Assay of hexokinase

Hexokinase was assayed by the method of Brandstrup et al. [34]. In this method, the incubation mixture was prepared and it contained 1.0 ml of glucose, 0.5 ml of ATP solution, 0.1 ml of $MgCl_2$, 0.4 ml of K_2HPO_4, 0.4 ml of KCl, 0.1 ml of NaF and 2.5 ml of buffer. This was preincubated at 37°C for 5 min and then the reaction was initiated by the addition of 0.5 ml of tissue homogenate. 1.0 ml of the reaction mixture was immediately transferred to a tube containing 1.0 ml of TCA (control). A second aliquot was removed and deproteinised after 30 min of incubation at 37°C. The protein precipitate was removed by centrifugation and the residual glucose in supernatant was estimated in all the tubes by the method of Trinder [29]. The enzyme activity was expressed as µmol of glucose phosphorylated/hour/mg protein.

2.5.2 Assay of glucose-6-phosphate dehydrogenase

Glucose- 6-phosphate dehydrogenase was measured by the method of Ells and Kirkman [35]. In this method, the incubation mixture was prepared and it contained 1ml of Tris-HCl buffer (0.05 M, pH 7.5), 0.1 ml of magnesium chloride, 0.1 ml of $NADP^+$, 0.5 ml of phenazinemethosulphate, 0.4 ml of 2,6-dichlorophenol indo phenol dye solution and 0.5 ml of liver homogenate. The contents were incubated at 37°C for 10 min. The reaction was initiated by the addition of 0.5 ml of glucose- 6-phosphate. The absorbance was read at 640 nm against water blank at 1 min intervals for 3-5 min using spectrophotometer.

2.5.3 Assay of glucose-6-phosphatase

Glucose-6-phosphatase was assayed by the method of Koide and Oda [36]. In this method, the reaction 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 and it was incubated at 37°C for 1 h. The reaction of the enzyme was terminated by the addition of 1 ml of 10% trichloroethanoicacid. The suspension was centrifuged and the phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow [37]. The supernatant was made up to known volume. To this, 1 ml of ammonium molybdate followed by 0.4 ml of amino naphtholsulphonic acid was added. The blue color developed after 20 min was read at 680 nm.

2.5.4 Assay of fructose-1, 6-bisphosphatase

Fructose-1, 6-bisphosphatase activity was measured by Gancedo and Gancedo [38]. 2 ml of assay mixture 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 (0.1 M), 0.25 ml of ethylene diaminetetraacetic acid (0.001 M) solution and 0.1 ml of enzyme homogenate. The assay mixture was incubated at 37°C for 5 min. The reaction was terminated by the addition of 1 ml of 10% trichloroethanoicacid. The suspension was centrifuged and then the supernatant was used for phosphorus estimation by the method of Fiske and Subbarow [37]. The supernatant was made up to known volume. To this, 1 ml of ammonium molybdate was added followed by 0.4 ml of aminonaphtholsulphonic acid. The blue color developed after 20 min was read at 680 nm.

2.6 Statistical Analysis

The results were expressed as mean ± S.D of six samples from each group (n=6). The statistical significance was evaluated by one way Analysis of Variance (ANOVA) and the individual comparisons were obtained by Duncan's Multiple Range Test (DMRT).

3. RESULTS

During the experimental period, the changes in body weight of normal and experimental rats were measured and the results are represented in Table 1. The body weight was significantly decreased ($P<0.05$) in diabetic rats when compared with normal control rats. But, the body weight was significantly increased ($P<0.05$) in diabetic rats treated with phloretin (25 mg and 50 mg/kg b.w) and glibenclamide (600 µg/kg b.w.) when compared with diabetic rats.

The levels of blood glucose, total hemoglobin (Hb), glycated hemoglobin (HbA1c), insulin and liver glycogen in normal and experimental animals were determined and the values are depicted in Table 2. The diabetic rats showed significant increase (*P*<0.05) in the levels of glucose and glycated hemoglobin and significant decrease (*P*<0.05) in the levels of total hemoglobin, insulin and liver glycogen when compared with normal control rats. The levels of blood glucose, total hemoglobin, glycated hemoglobin, insulin and liver glycogen were extensively reversed by the administration of phloretin at the dose of 50mg/kg b.w as like as the standard drug glibenclamide(600µg/kg b.w) treated diabetic rats.

Table 3 showed that the changes in the activities of hexokinase and glucose-6-phosphate dehydrogenase in the liver of normal control and experimental rats. The hexokinase and glucose-6-phosphate dehydrogenase activities were significantly decreased (*P*<0.05) in diabetic rats when compared with normal control rats. Oral administration of phloretin at two different doses like 25 and 50 mg/kg b.w to diabetic rats were significantly increased (*P*<0.05) the activities of hexokinase and glucose-6-phosphate dehydrogenaseas like as glibenclamide treated diabetic rats.

Table 4 represented the activities of gluconeogenic enzymes like glucose-6-phosphatase and fructose-1, 6-bisphosphatase in the liver of normal control and experimental rats. In this study, the levels of glucose-6-phosphatase and fructose-1,6-bisphosphatase were significantly increased (*P*<0.05) in diabetic rats when compared with normal control rats. Oral administration of phloretin and the standard drug glibenclamide were significantly decreased (*P*<0.05)the activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase in liver of STZ-induced diabetic rats when compared with diabetic control rats.

Table 1. Effect of phloretin on body weight

Groups	Initial weight (g)	Final weight (g)	Change in body Weight (g)	Change in body weight (%)
Normal control	180±12.50	206±13.44[c]	26±1.12[c]	+14.44
Diabetic control	185±12.95	164±9.10[a]	21±2.45[a]	-11.35
Diabetic + Phloretin (25mg/kg b.w)	185±12.95	200±14.00[c]	15±0.35[b]	+8.10
Diabetic+Phloretin (50mg/kg b.w)	180±12.50	205±14.35[c]	25±1.05[c]	+13.88
Diabetic+Glibenclamide (600µg/kg b.w)	190±13.30	205±14.35[c]	15±1.05[b]	+7.89

Values are expressed as mean ± SD of six rats from each group. Values are not sharing a common superscript letter differ significantly at 5% level (P<0.05) using Duncan's Multiple Range Test (DMRT)

Table 2. Effect of phloretin on blood glucose, total hemoglobin, glycated hemoglobin, insulin and liver glycogen

Groups	Glucose (mg/dl)	Total hemoglobin (g/dl)	Glycated hemoglobin (mg/g Hb)	Insulin (µU/ml)	Glycogen (mg/g)
Normal control	85.23±5.96[b]	14.54±1.15[c]	3.24±0.19[c]	53.21±1.98[c]	56.46±3.94[c]
Diabetic control	284±22.68[a]	7.87±0.69[a]	6.75±0.32[a]	32.25±1.24[a]	28.63±2.00[a]
Diabetic+Phloretin (25 mg/kg b.w)	98±20.06[b]	13.80±1.10[b]	3.85±0.21[b]	45.57±1.35[b]	42.76±2.98[b]
Diabetic+Phloretin (50 mg/kg b.w)	90±7.28[b]	15.14±1.12[c]	3.20±0.21[c]	52.65±1.85[c]	54.87±3.78[c]
Diabetic+Glibenclamide (600 µg/kg b.w)	97±20.77[b]	14.81±1.03[bc]	3.37±0.19[c]	51.74±1.62[c]	45.32±3.17[d]

Values are expressed as mean ± SD of six rats from each group. Values are not sharing a common superscript letter differ significantlyat 5% level (P< 0.05) using Duncan's Multiple Range Test (DMRT)

Table 3. Effect of phloretin on the activities of hexokinase and glucose-6-phosphate dehydrogenase in the liver

Groups	Hexokinase (µmoles of glucose phosphorylated/h/mg protein)	Glucose-6-phosphate dehydrogenase (µmoles of NADPH formed/min/mg protein)
Normal control	0.30±0.04[c]	5.28±0.06[c]
Diabetic control	0.14±0.01[a]	3.05±0.08[a]
Diabetic + Phloretin (25 mg/kg b.w)	0.20±0.02[b]	3.75±0.05[b]
Diabetic + Phloretin (50 mg/kg b.w)	0.28±0.04[c]	5.18±0.09[c]
Diabetic+Glibenclamide (600 µg/kg b.w)	0.27±0.04[c]	4.94±0.07[d]

Values are expressed as mean ± SD of six rats from each group. Values are not sharing a common superscript letter differ significantly at 5% level (P<0.05) using Duncan's Multiple Range Test (DMRT)

Table 4. Effect of phloretin on the activities of glucose-6-phosphatase and fructose-1, 6-bisphosphatase in the liver

Groups	Glucose-6-phosphatase (µmoles of inorganic phosphate liberated/min/mg protein)	Fructose-1,6-bisphosphatase (µmoles of inorganic phosphate liberated/h/mg protein
Normal control	10.38±0.05[c]	4.84±0.03[c]
Diabetic control	17.38±0.08[a]	7.85±0.09[a]
Diabetic+Phloretin (25 mg/kg b.w)	14.71±0.02[b]	5.82±0.15[c]
Diabetic+Phloretin (50 mg/kg b.w)	10.99±0.05[c]	4.88±0.04[c]
Diabetic+Glibenclamide (600 µg/kg b.w)	11.10±0.06[c]	4.95±0.04[d]

Values are expressed as mean ± SD of six rats from each group. Values are not sharing a common superscript letter differ significantly at 5% level (P<0.05) using Duncan's Multiple Range Test (DMRT)

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 [39]. Global postulates that three fourth of the world population cannot afford the products of allopathic medicine and thus they have to rely upon the use of traditional medicines are largely derived from plants [40]. Some substances have shown antidiabetic effect by influencing β - cell to stimulate insulin secretion and restore insulin sensitivity [41].

In the present study, we observed that the loss of body weight and excess of food and water intake by STZ-induced diabetic rats than normal rats. These results coincide with earlier report [42]. The characteristic loss of body weight is mainly due to increased water intake, polyuria, dehydration, muscle wasting, excessive hair loss and increased food intake [43]. Hence, a notable gradual decrease in body weight might be the result of protein wasting due to the unavailability of carbohydrates for energy metabolism and the loss or degradation of structural proteins [44].

The non-enzymatic, irreversible covalent bonding of glucose with Hb in the circulation leads to the formation of HbA1c, which is used to predict the diabetic risk of individuals [45]. About 16% increase of HbA1c in patients with diabetes mellitus and the amount of increase is directly proportional to the fasting blood glucose level. In diabetes, the excess glucose present in blood reacts with Hb and thus Hb level was decreased in diabetic rats. In the present study, the level of HbA1c in diabetic rats was significantly increased when compared to normal control rats. It indicates their poor glycemic control and this result is consistent with other studies [46,47]. Administration of phloretin prevents the formation of HbA1c and increasing the level of Hb in

diabetic rats. This may be due to the restoration of blood glucose and reducing the intensity of Hb glycation.

Liver is mainly responsible for maintaining the normal level of blood glucose by its ability to store glucose as glycogen and to produce glucose from glycogen breakdown or from gluconeogenic precursors [48]. During the experimental period, the selective destruction of pancreatic β-cells by streptozotocin leads to the decreased level of plasma insulin and it cause the defective glucose oxidation and 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 [49]. In the present study, the administration of phloretin to diabetic rats induced the insulin secretion significantly and thus decreases the blood glucose level. Similarly, the oral administration of phloretin to C57BL BKS-DB mice significantly decreased the blood glucose and improved glucose tolerance was reported [50].

Glycogen deposition in peripheral tissues and liver occurs after meal is a physiological response in mammals to maintain the blood glucose concentration [51]. Diabetes mellitus impairs the normal capacity of the liver to synthesize glycogen and low content may be attributed to lack of insulin. In this study, oral administration of phloretin significantly increased the level of liver glycogen which indicates that the better insulin activity in diabetic rats. These results are in agreement with the previous study, in that lowering of blood glucose level was accompanied by increased level of liver glycogen after S-allylcysteine supplementation to STZ induced diabetic rats [52].

Liver is an important organ that plays a vital role in glycolysis and gluconeogenesis. Hexokinase is an insulin-dependent and an insulin-sensitive enzyme and it is almost completely inhibited or inactivated in the liver of diabetic rats due to the absence of insulin [53]. Hexokinase insufficiency in diabetic rats can cause decreased utilization of glucose for energy production [54]. Similar results were obtained in the present study and thus the administration of phloretin to diabetic rats showed significant reversal in the activity of hexokinase. In this study, the increased level of serum insulin and decreased level of glucose were observed in diabetic rats treated with phloretin. The diabetic rats treated with phloretin showed increased hepatic hexokinase activity and thereby increased glycolysis.These results are in agreement with the previous study, in that they reported that the flavonoid compound esculetin improved hexokinase activity in STZ-induced diabetic rats [55]. A decrease in the activity of glucose-6-phosphate dehydrogenase slows down the pentose phosphate pathway in diabetic condition [56]. In this study, diabetic rats treated with phloretin showed significantly increased liver glucose-6-phosphate dehydrogenase activity through enhanced secretion of insulin.

Glucose-6-phosphatase plays a vital role in the homeostasis of blood glucose [57]. Fructose-1, 6-bisphosphatase is one of the key enzyme of the gluconeogenic pathway. In this study, the activities of gluconeogenic enzymes such as glucose-6-phosphatase and fructose-1, 6-bisphosphatase were increased significantly in the liver of diabetic rats. The activities of glucose-6-phosphatase and fructose-1, 6-bisphosphatase were significantly lowered in diabetic rats treated with phloretin which might be due to increased secretion of insulin. Similarly, the researchers reported that the flavonoid umbeliferone improves glucose metabolism in diabetic rats by the inhibition of gluconeogenesis [58].

5. CONCLUSION

The administration of phloretin to diabetic rats showed that the significant restoration of body weight, blood glucose, insulin, haemoglobin, glyc ated hemoglobin, liver glycogen and key enzymes of carbohydrate metabolism. So, the present study confirmed that the phloretin enhances the glycolytic enzymes and control the glucose metabolism in streptozotocin induced diabetic rats. Therefore from this study, we concluded that the phloretin might be an effective plant based antihyperglycemic agent in future.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Authors have hereby declared that the principles of laboratory animal care were followed and all experiments have been examined and approved by the Institutional Animal Ethical Committee of Bharathidasan University, Tiruchirappalli-612

024, Tamilnadu, India as per the guidelines of CPCSEA, India.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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