The Effect of Cherry Sticks Extract on the Levels of Glycoproteins in Alloxan-Induced Experimental Diabetic Mice

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Abstract. This study was designed to evaluate the effect of ethanolic cherry sticks extract on the levels of glycoproteins in alloxan-induced diabetic mice. Forty-five adult male albino mice were divided equally into three groups: Group 1: control, Group 2: diabetic mice, Group 3: diabetic mice treated with cherry sticks extract as well as to eighteen mice treated with cherry sticks extract only for toxicity test. All treatments were administered via an intragastric tube. Diabetes was induced in the mice of Group 3 by an intraperitoneal injection with 100 mg/kg body weight of alloxan. Oral administration of cherry sticks extract at a concentration of 250 mg/kg body weight for 15 days significantly reduced the levels of blood glucose, glycosylated hemoglobin, urea, and creatinine as well as those of hexose, hexosamine, fucose, and sialic acid in the diabetic mice treated with the cherry sticks extract as compared to untreated diabetic mice, with no adverse effects in mice treated only with cherry sticks extract. In conclusion, cherry sticks extract proved to have a beneficial effect on the diabetic mice in this study. In light of these advantageous results, it is advisable to broaden the scale of use of cherry sticks extract in a trial to alleviate the adverse effects of diabetes.

Key words: blood glucose level, sialic acid, diabetic and cherry sticks.

Introduction

Diabetes mellitus, a leading metabolic disorder worldwide, is characterized by hyperglycemia associated with impairments in insulin secretion and/or insulin action, as well as alterations in the intermediary metabolism of carbohydrates, proteins, and lipids. The number of people with type I and type II diabetes are dramatically increasing worldwide. Diabetes mellitus is one of the most prevalent metabolic disorders in both Western and developing countries. According to the World Health Organization, diabetes currently affects 170 million people, and this figure is expected to double by 2025 [1, 2].

Glycoproteins perform multiple complex functions in the cell surface, including serving as receptors for many hormones and viruses, cell-cell recognition, cellular adhesion, and cell differentiation [3]. Measuring glycoproteins provides useful information about membrane structural integrity and function [4]. Protein-bound hexose in the cell membrane creates a hydrophobic nature, whereas protein-bound hexosamine provides cationic charges on the cell surface membrane and makes the membrane more polar [4, 5]. Sialic acid is widely distributed in human tissues and in circulation; it is chiefly present as the terminal sugar of oligosaccharide chains of glycoproteins. Profound changes in the metabolism of glycoproteins and alterations in membrane glycosylation pattern have been well documented in diabetes mellitus [6, 7, 8]. In recent times, many traditionally important medicinal plants have been tested for efficacy against impaired glycoprotein levels in diabetes [9, 10].

Cherries are consumed as fresh fruit and have unique health benefits for many inflammatory diseases [11]. The sour cherry (Prunus cerasus L.), also known as tart cherry, is a species of cherry native to Europe and western Asia and belongs to the family Rosaceae. Sour cherry may garner new interest, due mainly to the fact that it can be considered a “functional food” because of its high content of antioxidant compounds [12, 13]. Therefore, it has been suggested that the activity of cherries could be
mainly related to anthocyanins [12]. Recent studies using anthocyanins on in vitro experimental systems have confirmed the potential potency of these compounds.

Demonstrable benefits of anthocyanins include protection against liver injuries, significant reduction of blood pressure, strong anti-inflammatory activity, and the suppression human cancer cell proliferation[14, 15]. The consumption of anthocyanins may play a significant role in preventing lifestyle-related diseases such as cancer, diabetes, and neurological and cardiovascular diseases [16]. The finding that sour cherries contain high levels of anthocyanins that possess strong antioxidant and anti-inflammatory properties has attracted much attention to this species [12].

Therefore, the present study was designed to evaluate the pharmacological effect of ethanolic extract of cherry sticks on the levels of blood glucose, glycosylated hemoglobin, urea, and creatinine as well as on glycoproteins (protein bound hexose, protein bound hexosamine, fucose and sialic acid) in both normal and alloxan-induced diabetic mice.

Materials and Methods
Reagents. All chemicals (except alloxan-monohydrate from Sigma chemicals) used were of reagent grade (supplied by Either Merck or Fluka) and were used as supplied.

Extraction procedure. The cherry sticks were collected from the cherry fruit. Cherry sticks (50 g) were dried and ground with a grinder (Moulinex, France) into a powder prepared for extraction. The powder was extracted by 500 ml of hydro-ethanol mixture (80:20 v/v) for 6 hours. This step was repeated three times. The filtrate was pooled and concentrated under vacuum at a temperature not exceeding 50°C and dissolved in normal saline.

Procedure of Thin layer chromatography (TLC). The TLC plates were pre-washed with methanol and activated by being kept at 115°C for 30 minutes. Aliquots were applied on pre-coated silica gel aluminum plates 60 F254 (20 × 10 cm, 200 μm thickness; E. Merck, Germany). Samples were spotted on the TLC plate in the form of bands with 8 mm using a Hamilton microliter syringe. The TLC plate was saturated in a chromatographic tank containing chloroform: benzene: methanol (60: 25: 15 v/v/v) used as mobile phase. After running the sample, the TLC plate was stained with iodine vapor.

Animals. Sixty-three albino mice weighing 20 - 23 g, aged 6 - 8 weeks (obtained from Biotechnology Division at University of Technology, Baghdad, Iraq) were kept in clean and dry cages in the laboratory where the research was done (Prof. Abbas A. Mohammed’s Lab). They were maintained at a temperature range of 25 ± 5°C, 60-70% humidity, 12 hours of artificial lighting (8:00 to 20:00) and supplied with water and food ad libitum. The animals were procedured, maintained, and used in accordance with the ‘Guide for the Care and Use of Laboratory Animals in Biotechnology Division, and approved by the University of Technology, Animal Ethical Committee’. The mice were divided into groups of 18 mice for the toxicity test and 45 mice for diabetic experiments.

Toxicity test. The toxicity of the ethanolic extract of cherry sticks was tested against 18 albino mice divided into 6 groups of 3 mice, each group receiving a different dose: 50, 100, 150, 250, 350 and 500 mg/kg body weight. The toxicity of extract of cherry sticks was evaluated on the basis of body weight loss, clinical observation, and mortality.

Treatment with Alloxan. Diabetes was induced in the mice by intra-peritoneal administration of Alloxan, in a dose of 100 mg/kg body weight, dissolved in normal saline. Since this drug is capable of producing initial fatal hypoglycemia as a result of the massive pancreatic insulin release, the animals were allowed to drink 5% glucose solution overnight to overcome drug-induced hypoglycemia. After 3 days, successful induction of diabetes was confirmed by the presence of glucose in urine (indicated by Benedict’s test) and elevated blood glucose levels in the range above 300 mg/dL, and used for the cherry sticks extract treatment.

Experimental design of diabetic animals. Forty-five adult male albino mice were used in the current study. They were equally divided into three groups, fifteen animals in each. All treatments were given orally using a modified plastic syringe (intra-gastric tube) at a single daily dose (0.25 ml/kg body weight) for 15 consecutive days as follows: Group I: Control mice. Group II: Diabetic control mice. Group III: Diabetic mice administered orally with cherry sticks extract (250 mg kg⁻¹ body weight) in aqueous solution for 15 consecutive days.

Biochemical assays. At the end of day 15, the animals of all groups were subjected to overnight fasting and were then anesthetized with chloroform. Blood was collected using EDTA as an anticoagulant in order to biochemical assays (Prof. Abbas A. Mohammed’s Lab).
Glycosylated hemoglobin was estimated by the method of Sudhakar Nayak and Pattabiraman [17], modified by Bannon [18]. Briefly, saline-washed red blood cells were treated with water for lysis and incubated at 37°C for 15 minutes before oxalate: HCl solution was added and mixed. The filtrate was heated in a boiling water bath for 4 hours, cooled with ice-cold water, treated with 40% TCA, and again centrifuged at 1000 g for 10 minutes. The supernatant obtained was then heated with 80% phenol and H$_2$SO$_4$, and the color developed was read at 480 nm after 30 minutes.

The level of glucose was estimated by the method of Sasaki et al [19], that of urea by the method of Natelson et al [20], and those of plasma protein according to the method of biuret according to the manufacturer’s instructions in the Croma test kit (Cat No. 1153005; linear chemicals, S.I., Spain). Plasma creatinine levels were estimated by the method of Brod and Sirota [21].

**Extraction and determination of glycoproteins.**

Protein-bound hexoses were estimated by the method of Dubois and Gilles [22]. To 0.1 mL of plasma, 5.0 mL of 95% ethanol was added, mixed and then centrifuged (1500xg for 10 minutes). The precipitate was dissolved in 1.0 mL of 0.1 N NaOH. Subsequently, 1.0 mL of distilled water and 1.0 mL of standards (20-100 μg) were set up along with the test. 8.5 mL of orcinol sulfuric acid reagent was added to all the tubes, which were kept in a water bath for exactly 15 minutes at 90°C. The tubes were cooled in tap water and the color developed was read at 540 nm against a blank.

Protein-bound fucose was estimated by the method of Dische and Shettle [23]. Two tubes, each containing 0.1 mL of sample (labeled as control and test), were taken. 5 mL of 95% ethanol was added, mixed well, and then centrifuged (1500xg for 10 min.). The precipitate was dissolved in 1.0 mL of 0.1 N NaOH; 1 mL of distilled water served as blank. A series of standards in 1.0 mL volume were also set up along with the test. All the tubes were kept in ice-cold conditions, and 4.5 mL of H$_2$SO$_4$-water mixture was added. The tubes were kept in a boiling water bath for 3 min. and cooled. The 0.1 mL of cysteine reagent was added to all the tubes except the control and kept for 60 minutes at room temperature. The color developed was read at 396 nm and 430 nm against the blank. The fucose content was calculated by finding the differences in the readings obtained at 396 nm and 430 nm and then subtracting the values obtained without cysteine.

Protein-bound hexosamine was estimated by the method of Wagner [24]. To 1.0 mL of plasma, 2.5 mL of 3 N HCl was added, and the mixture was kept for 6 hours in a boiling water bath before being neutralized with 6 N NaOH. To 0.8 mL of the neutralized sample, 0.6 mL of acetyl acetone reagent was added. The tubes were heated in a boiling water bath for 30 minutes. After cooling, 2.0 mL of Ehrlich’s reagent was added and mixed well. The blank contained 0.8 mL of distilled water. Blank and standards were processed similarly. The color developed was read at 540 nm.

Protein-bound sialic acid was estimated by the resorcinol method of Svennerholm [25]. 20 μL of each concentration of calibration samples or plasma samples was put in clean and sterile test tubes. 980 μL of distilled water was added to each tube. The resulting solution was then mixed and put in an ice bath. One mL of resorcinol reagent was added to each tube. The tubes were put in a water bath at 100°C for 15 minutes and then transferred to an ice bath for 10 minutes. 2 mL of (Butyl acetate : Methanol v/v) solution was added and the resulting solution was well mixed. The samples were centrifuged at 1500xg for 10 minutes and readings were obtained at 580 nm.

**Statistical analysis.** All the grouped data were statistically evaluated with SPSS/10.5 software. Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test; P values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as the Mean ± S.D.

**Results**

**Thin Layer Chromatography.** In the present study, different solvent systems were tried to resolve the components of ethanolic cherry sticks extract. However, the Rf value of the tested extract was 0.85, 0.71, and 0.45.

**Determination of toxicity.** To evaluate the deleterious effects of cherry sticks extract treatments on mice throughout the investigated period (15 days), the body weight was considered a parameter for this regard (Figure 1). Six days later, the mice were left without administration of cherries sticks extract. The fact that no animals died or lost body weight during 21 days of post-administration with cherry sticks extract indicated that the treatment was well-tolerated.

**Effect of cherry sticks extract on blood glucose and glycosylated hemoglobin.** The diabetic mice exhibited a significant increase (P<0.05) in blood glucose and glycosylated hemoglobin levels when compared with the control group. Upon oral administration of cherry sticks extract, hypoglycemic
activity by reducing blood glucose and glycosylated hemoglobin was recorded; the levels were adjusted to those of the control mice (Table 1).

Table 1 shows the levels of total proteins, urea, and creatinine of the control and experimental groups of mice. These biochemical variables were significantly altered in diabetic mice when compared to control mice. Upon oral administration of cherry sticks extract to diabetic mice, the levels improved to those of the control mice. No statistical significance was observed between control mice and mice treated with cherries sticks extract.

**Effect of cherry sticks extract on glycoproteins.**

The levels of plasma glycoprotein of control and experimental mice were shown in Table 2. The levels of glycoproteins, hexose, hexosamine, fucose and sialic acid were significantly increased ($P<0.05$) in diabetic mice when compared with control mice. Upon oral administration of cherry sticks extract, the levels were significantly reversed to those of control mice. Also, no statistical significance was observed between control mice and mice treated with cherries sticks extract.

**Discussion**

The management of diabetes with agents devoid of any side effects still represents a challenge to the medical system. This concern has led to an increased demand for natural products with anti-hyperglycemic activity that have fewer side effects. The present study demonstrated that oral administration of cherry sticks extract did not show cytotoxic effects (considered death or loss of body weight). However, there are no published data examining the effect of cherry sticks extract on the toxicity of mice.

Alloxan causes diabetes through its ability to destroy the insulin-producing beta cells of the pancreas [26], [27]. In vitro studies have shown that alloxan is selectively toxic to pancreatic beta cells, leading to the induction of cell necrosis [28], [29].
The cytotoxic action of alloxan is mediated by reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration, leading to the rapid destruction of beta cells [30]. According to previous studies, plant extracts have anti-hyperglycemic effects by promoting the regeneration of \( \beta \)-cells or by protecting these cells from destruction, by restricting the glucose load, and by promoting unrestricted endogenous insulin action. Anti-hyperglycemic effects may also be caused by the action of plant extracts on \( \beta \)-cells, causing \( \beta \)-cells to release insulin or activate the insulin receptors to absorb the blood sugar and stimulate peripheral glucose consumption [31], [32]. Thus, the cherry sticks extract may bring about its hypoglycemic action through stimulating the surviving or remnant \( \beta \)-cells of Langerhans islets to release more insulin.

Although the action mechanism of the cherry sticks extract is unknown, the presence of biologically active ingredients such as cyanidin 3- glucoside, cyanidin 3-rutinoside, cyanidin 3-sophoroside, pelargonidin 3-glucoside, pelargonidin 3- rutinoside, 3-glucoside, and peonidin 3-rutinoside has been confirmed in sour cherries and might be responsible for the fruit’s medicinal properties [11]. In addition, anthocyanins have been anecdotally reported to have anti-diabetic activity. Previous results indicated that pelargonidin-3-galactoside and its aglycone, pelargonidin, caused a 1.4-fold increase in insulin secretion [33]. It was recently found that consuming 40 g of sour cherry juice per day for six weeks resulted in significantly reduced glycosylated hemoglobin levels, body weight, and blood pressure as well as improved blood lipids profiles in 19 diabetic patients with hyperlipidemia [34].

In the present study, the diabetic mice had shown higher levels of glycosylated hemoglobin compared to those of the control mice, indicating their poor glycemic control; after 15 days of cherry sticks treatment, the glycosylated hemoglobin significantly decreased compared to the Alloxan-induced diabetic mice only, indicating a decrease in the status of glycation. Diabetes occurs due to the excess glucose present in the blood reacting with hemoglobin to form glycosylated hemoglobin. The rate of glycation is proportional to the concentration of blood glucose [35]. Glycosylated hemoglobin has been found to increase over a long period of time in diabetes [36]. There is an evidence that glycation itself may induce the formation of oxygen-derived free radicals in diabetic condition [37]. Thus, it has been suggested that oxidative stress can play an important role in the tissue damage associated with diabetes and complications. Therefore,

### Table 1: Level of blood glucose, glycosylated hemoglobin, total proteins, urea and creatinine in control and experimental groups of mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic + cherries sticks extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Glucose (mg dL(^{-1}))</td>
<td>79.00 ± 3.25</td>
<td>370.16 ± 21.20*</td>
<td>90.53 ± 7.14**</td>
</tr>
<tr>
<td>Glycosylated Hemoglobin (g dL(^{-1}))</td>
<td>2.84 ± 0.12</td>
<td>6.2 ± 0.28*</td>
<td>2.90 ± 0.18**</td>
</tr>
<tr>
<td>Total Protein (g dL(^{-1}))</td>
<td>7.85 ± 0.96</td>
<td>4.26 ± 0.69*</td>
<td>7.00 ± 1.32**</td>
</tr>
<tr>
<td>Urea (mg dL(^{-1}))</td>
<td>33.00 ± 3.21</td>
<td>46.34 ± 3.44*</td>
<td>29.57 ± 1.42**</td>
</tr>
<tr>
<td>Creatinine (mg dL(^{-1}))</td>
<td>0.86 ± 0.50</td>
<td>3.12 ± 0.99*</td>
<td>0.90 ± 0.73**</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD fifteen animals each; * Statistically significant as compared with control; ** Statistically significant as compared with diabetic; \( ^b \) No significant differences as compared with control.
The effect of cherry sticks extract on the levels of glycoproteins

The measurement of glycosylated hemoglobin is supposed to be very sensitive index for glycemic control [38].

Administration of cherry sticks extract to diabetic mice significantly inhibits proteolysis caused by Alloxan and thus maintained the levels of total proteins to the control levels. The increased protein catabolism may feed gluconeogenesis in the liver during diabetes [39]. Dighe et al. [40] have reported that accelerated proteolysis of uncontrolled diabetes occurs as a result of the deranged glucagon-mediated regulation of cAMP formation in insulin deficiency. This accounts for the observed decrease in the total protein content in the Alloxan-induced diabetic mice.

Diabetic mice manifest a negative nitrogen balance related to enhanced proteolysis in skeletal muscle and other tissues. Impaired nitrogen balance coupled with lowered protein synthesis leads to increased concentrations of urea and creatinine in the blood. This ultimately results in an impaired renal function in diabetic animals [41]. The administration of cherry sticks extract to diabetic mice significantly decreased the level of blood urea and creatinine.

In this study, the levels of glycoproteins (hexose, hexosamine, fucose and sialic acid) in the plasma of Alloxan-diabetic mice were significantly higher than those of both the control mice and the cherry sticks extract-treated mice. Abnormalities in the metabolism of glycoproteins are observed in both naturally occurring and experimental diabetes [42]. The increases in plasma glycoprotein components have been reported to be associated with the severity and duration of diabetes. Decreased incorporation of the carbohydrate structure and composition to these in circulation. The vascular complications that involve complexes of protein-carbohydrate molecules could contribute to an increase in plasma glycoproteins [10].

Fucose is member of a group of essential sugars that the body requires for functional cell-to-cell communication, and its metabolism appear to be altered under various disease conditions, such as diabetes mellitus [43]. Due to increased glycosylation in the diabetic state, fucose levels could be increased. The serum proteins haptoglobin, α-1 acid glycoprotein, and α1-antitrypsin are synthesized in liver. The metabolism and synthesis of these proteins may be altered in diabetes, leading to changes in serum in the hyperglycemia stage and accelerating the synthesis of basement membrane components i.e., glycoproteins [44]. The utilization of glucose was depressed by insulin-dependent pathways [32], thereby enhancing the formation of hexose, hexosamine, and fucose for the accumulation of glycoproteins [45]. Experiments conducted by Pari and Murugan showed elevated levels of fucose in diabetic animals [46]. Our results suggest that the increased fucosylated proteins in diabetic

### Table 2: Levels of glycoproteins (mg dL⁻¹) in plasma of control and experimental groups of mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hexose</th>
<th>Hexosamine</th>
<th>Fucose</th>
<th>Sialic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.41 ± 5.96</td>
<td>74.15 ± 4.53</td>
<td>35.26 ± 2.51</td>
<td>60.32 ± 3.25</td>
</tr>
<tr>
<td>Diabetic</td>
<td>126.14 ± 8.44*</td>
<td>87.54 ± 5.25*</td>
<td>41.12 ± 2.21*</td>
<td>80.35 ± 5.24*</td>
</tr>
<tr>
<td>Diabetic + cherries sticks extract</td>
<td>92.43 ± 6.73** b</td>
<td>70.41 ± 5.77** b</td>
<td>38.27 ± 2.70** b</td>
<td>64.24 ± 4.67** b</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD fifteen animals each. * Statistically significant as compared with control; ** Statistically significant as compared with diabetic; b No significant differences as compared with control.
mice could be due to an increase in the synthesis and/or decrease in the degradation of these proteins.

Sialic acid is a terminal component of the non-reducing end of the carbohydrate chains of glycoproteins and glycolipids, which are essential constituents of many hormones and enzymes present in serum and tissues. Sialic acid is an important constituent for the characteristic changes of transformed cells; the liver is the major site involved in the synthesis of sialic acid and other glycoproteins [47]. The synthesized glycoproteins are made to circulate in blood. There is a pronounced increase in serum rather than in other organs. The decrease in the content of sialic acid in tissues may be due to its utilization in the synthesis of fibronectin, which contains sialic acid residues in the core structure [48]. The biosynthesis of the carbohydrate moieties of glycoprotein forms the insulin independent pathways for the use of glucose 6-phosphate. But the deficiency of insulin during diabetes produces derangement of glycoprotein metabolism, resulting in the thickening of the basal membrane of pancreatic beta cells. Agents with antioxidant or free radical scavenging properties may inhibit oxidative reactions associated with glycation. In this context, previous studies have shown that decreases in hyperglycemia could lead to a decrease in glycoprotein levels [49]. Administration of cherry sticks extract to diabetic mice resulted in a significant reversal of these changes to near normal values.

Conclusion

In conclusion, oral administration of cherry sticks extract exhibits a beneficial effect on glycoproteins, as well as a protective effect against Alloxan-induced diabetes in mice and thus provides a rationale for the use of cherries sticks in Ayurvedic medicinal treatment.

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References