Effect of *Citrullus colocynthis* Extract on Glycated Hemoglobin Formation *(In Vitro)*

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ABSTRACT

Objective: Diabetes mellitus (DM) is typically a disorder of carbohydrate, fat, and protein metabolism. It develops due to a lack of or loss associated with insulin and/or resistance to insulin. Regarding complications of chemical substance use, drugs with few complications and high-reliability tannins are needed. This study aimed to determine the effect and mechanism of action of *Citrullus colocynthis* extract on the formation of glycated hemoglobin (HbA1c).

Materials and Methods: A solution containing hemoglobin and glucose was incubated for 1, 2, 3, 4, 30, and 60 days by adding *Citrullus colocynthis* extract or glutathione. Quantitative measurement of HbA1c was performed using ion-exchange chromatography. Data were analyzed using ANOVA and two-way repeated measures test. A $p<0.05$ was considered statistically significant.

Results: The *Citrullus colocynthis* extract in hyperglycemic conditions and with increasing time reduced the formation of HbA1c and thus inhibited the production of glycated proteins. By increasing the time and after initiation of reaction of extract concentrations (0, 0.1, 0.3, 0.5, and 1 g/dL), presently, there was a significant decrease in the formation of HbA1C compared to those in the control group ($p<0.05$). The decrease in glycation has been dose dependent.

Conclusion: Therefore, *Citrullus colocynthis* could directly reduce the formation of HbA1c.

Keywords: Diabetes mellitus, hemoglobin, *Citrullus colocynthis*, glycation

Introduction

Diabetes is a common endocrine disorder worldwide with increasing incidence and one of the five main causes of death among countries [1]. Diabetes is a disorder of carbohydrate, fat, and protein metabolism that is caused by lack or decrease of insulin and/or resistance to insulin [2]. Researchers showed that environmental factors, as well as genetic and autoimmunity factors, also play a role in the development of diabetes [3]. In type I diabetes, beta-pancreatic cells are destroyed and unable to generate insulin. In type II diabetes, the most important cause of the disease is resistance to insulin, which results in a decrease in response to insulin in tissues and increase in hepatic glucose production that leads to hyperglycemia. This type of diabetes is the most common form in human societies and frequently observed in individuals aged >40 years and those with obesity [4]. The incidence of diabetes increases with insulin secretion disorder, insulin resistance, and overproduction of hepatic glucose (fasting hyperglycemia) [5]. Diabetes usually leads to retinopathy, cataracts, neuropathy, atherosclerosis, and delayed wound healing [6]. One consequence of type II diabetes is the progression of atherosclerosis. Indeed, it has been specified that type II diabetes is common with several risk factors for atherosclerosis, and atherosclerosis develops in many cases due to resistance to insulin and hyperglycemia [7]. Protein glycation increases, and formation of advanced glycated end products (AGEPs) occurs whenever proteins are subjected to recovering glucose. Its level depends on the intensity of hyperglycemia and its presence and duration in the body [8]. Glycation of proteins leads to change in their structures and performances, in turn developing complications [9]. The formation of glycated proteins and AGEPs leads to the production of free radicals through auto-oxidation of glucose and results in glycation of proteins [10]. Free radicals can damage lipids, proteins, and nucleotides and probably tissues in individuals with diabetes [11]. It is possible that Maillard reaction occurs between proteins and

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glucose during hyperglycemia (Maillard reaction has a role in food corruption and change in taste and loss of nutrients). Indeed, nonenzymatic reactions in which protein chains and lipids and/or nuclides are connected are called glycation. The formation of glycated proteins and AGEPs play a role in diabetes, kidney failure, and Alzheimer's disease [12]. Given these descriptions, special attention has been paid to compounds that can control glycation. These compounds can control formation of glycated protein and AGEPs by blocking carbonyl groups in reducing sugars [13]. Regarding the abovementioned disadvantages of protein glycation, it is necessary to control these reactions to improve diabetes complications. Some drugs that can break cross-links in glycated protein and have a role in treating diabetes are administered [14]. However, concerning the complications of these chemical drugs, there is a need for drugs with few complications and high reliability that can be used in the long term, and traditional medicine is gaining great attention in this area. Medicinal plants are among the natural materials with few possible side effects. Recent studies have confirmed plants' antioxidant, antidiabetes, and hyperglycemic properties [15]. Antioxidants have protective effect against free radicals from glycation [16]. Epidemiologic studies showed that consuming fruits and vegetables decreases the complications of chronic diseases, such as cardiovascular disease, cancer, and diabetes [17]. Phytochemicals, such as flavonoids, phenols, and organo-sulfur compounds, are one of the most important and effective compounds in fruits and vegetables that have antioxidant effects [18]. Traditionally, pharmaceutical plants have had a special position in medical science for treatment of common human diseases due to ease of access and fewer side effects, especially metabolic diseases, such as diabetes.

Moreover, investigations Kaewnarin and et al. [19] on the effectiveness of medicinal plants with hypoglycemic properties has increased recently to reduce the effects of diabetes [19]. Most herbs contain significant amounts of antioxidants including tocopherols (vitamin E), carotenoids, ascorbic acid (vitamin C), flavonoids, and tannins. One of these plants whose antidiabetes and hypoglycemic effects have been shown in previous studies is *Citrullus colocynthis*. This plant grows naturally in deserts of many tropical countries, such as Iran and western Iraq, and is called hanzal and alqam in ancient books. In study Mahmoodi and et al. [20] Its fruit is traditionally used in Kerman province (Iran) to decrease blood sugar level (20). Furthermore, its fruit is used for treating digestive disorders and diabetes; however, in study Lakshmi and et al. [21] there has been some acute toxicities reported after consuming this plant [21]. Shi and et al. [22] showed that extracts of this plant contain glycosides and saponin that, according to studies, leads to control of lipid peroxidation and cessation of reactive oxygen species (ROS) production [22]. This study aimed to investigate one possible mechanism for the beneficial hypoglycemic effect of this plant. Recent studies have proven antioxidant and antiglycosylation effects of medicinal plants.

Incubation with glucose

We use human hemoglobin that was purchased from Sigma Company *(Sigmax; America),* D-glucose, and sodium phosphate buffer, 4.0 mM, for in vitro tests. All solutions were prepared in phosphate buffer. Different D-glucose concentrations (5, 10, 20, and 40 mM) were used for incubation with hemoglobin to provide normoglycemic and hyperglycemic conditions, and hemoglobin in phosphate buffer without glucose was considered as control.

Hemoglobin treated with *Citrullus colocynthis* extract

To determine the protective effect of *Citrullus colocynthis* on hemoglobin glycation, the hemoglobin solution was preincubated at different doses of *Citrullus colocynthis* extract (0.1, 0.3, 0.5, and 1 g/dL) [23] at 37°C for 1 h. Then, different glucose concentrations (5, 10, 20, and 40 mM) were added. To measure the level of hemoglobin glycation, the amount of glycated hemoglobin (HbA1c) was determined by *ion-exchange chromatography.* With this, using the columns and its protocol, HbA1C was obtained (Biosystem; *America*). Biosystem is a kit containing chromatographic columns accompanied with chemical reagent, which should be used at room temperature. It functions based on spectrophotometer ion exchange. According to the kit instructions, we used chemical reagents with a separate column for each sample and, finally, collected the rinsed liquid from the column (HbA1C). We mixed the hemolysate and a chemical reagent to attain total HbA1C. Finally, the spectrophotometer was accessed by a device with a wavelength of 415 nm. HbA1C level was calculated using the following formula: each test was repeated three times.

$$\%HbA1c = 100 \times HbA1c/\text{total hemoglobin}$$

This is an extremely time-consuming (approximately 1 h) and temperature-sensitive method and should be performed very carefully.

Statistical analysis

In the statistical data analysis, the software The Statistical Package for the Social Sciences (SPSS) version 18 (IBM Corp.; Armonk, NY, USA) was used. All experiments were conducted thrice for every separate sample, and all results achieved were reported as mean values of the ANOVA (to compare the different concentrations of aqueous extract of watermelon), and two-way repeated measures test (to determine the effect of Abuja watermelon extract on different days) was also utilized for data analysis. A $p<0.05$ indicated statistical significance.

Results

The effect of *Citrullus colocynthis* extract on HbA1c formation after days 1, 2, 3, 4, 30, and 60 [24] is shown in Figures 1-4. Our findings demonstrated that *Citrullus colocynthis* extract in hyperglycemic conditions and with increasing time reduced the formation of HbA1C and therefore inhibited the production of glycated protein. By increasing the time and after the initiation of the reaction with extract concentrations (0, 0.1, 0.3, 0.5, and 1 g/dL), there was a significant decrease in the formation of HbA1C compared to the control group, which has a dose dependent decrease ($p<0.05$).

Figure 1 shows the amount of glycated hemoglobin (HbA1c) treated by 5 mM glucose and various concentrations of *Citrullus colocynthis* extract on days 1, 2, 3, and 4. None of the concentrations were significant compared to those in the control group, but on days 30 and 60, all concentrations were significant compared to those in the control group (Figure 1).

Figure 2 shows the amount of HbA1c incubated with 10 mM glucose and different concentrations of *Citrullus colocynthis* extract on days 1, 2, and 3. None of the concentrations were significant compared to those in the control group, and the amount of HbA1c incubated with 10 mM glucose and various concentrations of *Citrullus colocynthis* extract on days 4, 30, and 60. All concentrations have been significant compared to those in the control group (Figure 2).

Figure 3 shows the amount of HbA1c incubated with 20 mM glucose and various concentrations of *Citrullus colocynthis* extract on day 1. None of the concentrations were significant compared to those in the control group, and day 2 concentrations of 0.5 and 1 were significant compared to those in the control group. On days 3, 4, 30, and 60, all concentrations have been significant compared to those in the control group (Figure 3).

Figure 3 shows the amount of HbA1c incubated with 40 mM glucose and various concentrations of *Citrullus colocynthis* extract on days 1, 2, and 3. None of the concentrations were significant

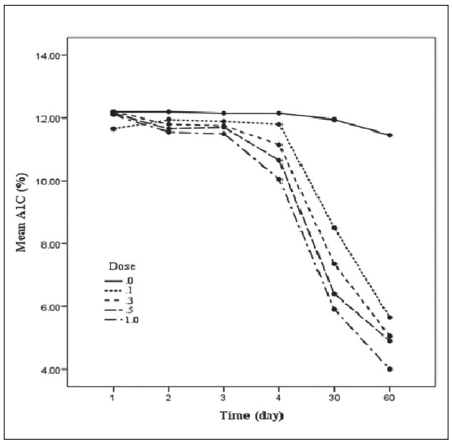

Figure 1. HbA1c incubated with 5 mM glucose and different doses of *Citrullus colocynthis* extract (0, 0.1, 0.3, 0.5, and 1 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 0 or positive control: HbA1c incubated with 5 mM glucose in days 1, 2, 3, 4, 30, and 60
Dose 0.1: HbA1c incubated with 5 mM glucose + *Citrullus colocynthis* extract 0.1 g/dL in days 1, 2, 3, 4, 30, and 60
Dose 0.3: HbA1c incubated with 5 mM glucose + *Citrullus colocynthis* extract 0.3 g/dL in days 1, 2, 3, 4, 30, and 60
Dose 0.5: HbA1c incubated with 5 mM glucose + *Citrullus colocynthis* extract 0.5 g/dL in days 1, 2, 3, 4, 30, and 60
Dose 1: HbA1c incubated with 5 mM glucose + *Citrullus colocynthis* extract 1 g/dL in days 1, 2, 3, 4, 30, and 60
(p<0.05, mean±SD)

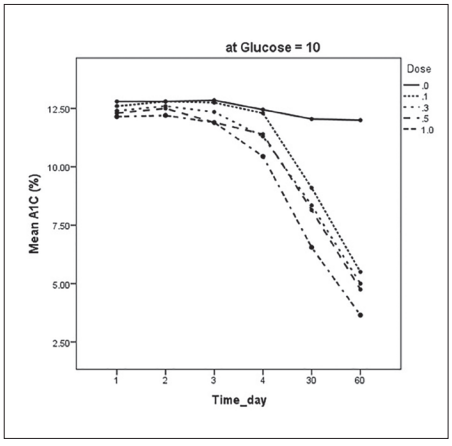

Figure 2. HbA1c incubated with 10 mM glucose and different doses of *Citrullus colocynthis* extract (0, 0.1, 0.3, 0.5, and 1 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 0 or positive control: HbA1c incubated with 10 mM glucose in days 1, 2, 3, 4, 30, and 60
Dose 0.1: HbA1c incubated with 10 mM glucose + *Citrullus colocynthis* extract (0.1 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 0.3: HbA1c incubated with 10 mM glucose + *Citrullus colocynthis* extract (0.3g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 0.5: HbA1c incubated with 10 mM glucose + *Citrullus colocynthis* extract (0.5 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 1: HbA1c incubated with 10 mM glucose + *Citrullus colocynthis* extract (1 g/dL) in days 1, 2, 3, 4, 30, and 60
(p<0.05, mean±SD)

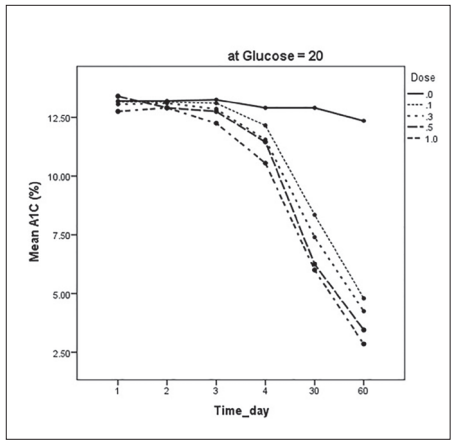

Figure 3. HbA1c incubated with 20 mM glucose and different doses of *Citrullus colocynthis* extract (0, 0.1, 0.3, 0.5, and 1 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 0 or positive control: HbA1c incubated with 20 mM glucose in days 1, 2, 3, 4, 30, and 60
Dose 0.1: HbA1c incubated with 20 mM glucose + *Citrullus colocynthis* extract (0.1 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 0.3: HbA1c incubated with 20 mM glucose + *Citrullus colocynthis* extract (0.3 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 0.5: HbA1c incubated with 20 mM glucose + *Citrullus colocynthis* extract (0.5 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 1: HbA1c incubated with 20 mM glucose + *Citrullus colocynthis* extract (1 g/dL) in days 1, 2, 3, 4, 30, and 60
(p<0.05, mean±SD)

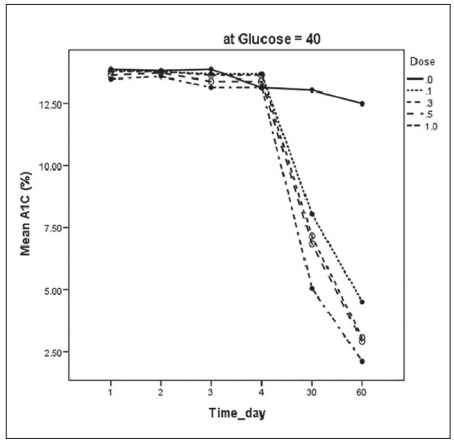

Figure 4. HbA1c incubated by 40 mM glucose and different doses of *Citrullus colocynthis* extract (0, 0.1, 0.3, 0.5, and 1 g/dL) in days 1, 2, 3, 4, 30, and 60 days
Dose 0 or positive control: HbA1c incubated with 40 mM glucose in days 1, 2, 3, 4, 30, and 60
Dose 0.1: HbA1c incubated with 40 mM glucose + *Citrullus colocynthis* extract (0.1 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 0.3: HbA1c incubated with 40 mM glucose + *Citrullus colocynthis* extract (0.3 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 0.5: HbA1c incubated with 40 mM glucose + *Citrullus colocynthis* extract (0.5 g/dL) in days 1, 2, 3, 4, 30, and 60
Dose 1: HbA1c incubated with 40 mM glucose + *Citrullus colocynthis* extract (1 g/dL) in days 1, 2, 3, 4, 30, and 60
(p<0.05, mean±SD)

compared to those in the control group. In day 4, concentrations of 0.5 and 1 were significant compared to those in the control group. In days 30 and 60, all concentrations have been significant compared to those in the control group (Figure 4).

Discussion

Diabetes is one of the most common diseases worldwide. This serious complication may result from increased glycation of healthy proteins as a consequence of associated chronic hyperglycemia. Most proteins (including hemoglobin) react with glucose and form covalent combinations without the requirement of enzymes. This chemical reaction is called nonenzymatic glycosylation. Accumulation of AGEPs leads to diabetes complications because enzymatic glycation causes reverting of nonenzymatic glycation process and creating free amino groups. The enzymatic glycation of the system contradicts the glycation process in mammal cells. This system phosphorylates the fructose, which is connected to the lysine of the glycated proteins by use of furosemide 3 kinase (FN3K), and therefore causes instability of these products. Finally, the glycated proteins are dialyzed. In individuals with diabetes, the process of enzymatic glycation is a condition resulting from the abundant hyperglycemic periods, and the none of the glycation frequently continues. HbA1C is an inductor combination, which is reversible, but after the inner reformation of this combination, stable HbA1C is formed. When hemoglobin is glycated, its efficiency is reduced, which leads to a pathologic condition [25]. In the case of other glycated proteins, there is a pathological state including serum glycated albumin (GA), which is involved in many pathologic processes. GA is the major form of circulating Amadori-type glycated proteins and an early precursor of AGEPs), which bind to receptor for AGE (RAGEs), opening the floodgate of deleterious downstream signals, including increased ROS production, inflammatory cell activation, inappropriate increase of angiotensin II (Ang II), and release of growth factors [26]. AGE increases the progression of cardiovascular diseases directly and indirectly. Aggregation in different bodies and reaction with RAGE induce oxidative stress and increase inflammation and extracellular matrix transformation. It causes a disorder in endothelial function and leads to an increase in plaque production, thus resulting in atherosclerosis in diabetes. Regardless of the use of drugs for diabetes, several patients still develop chronic complications. Although enhanced control of the blood sugar level is an important measure to retard the development of serious complications, it may be useful to administer drugs that directly inhibit protein gly-

cation. Acetylating agents [27] and antioxidants [28-30] are known to inhibit particular protein glycation. Subsequently, the development of diabetic cataract was retarded by the administration of aspirin and tocopherol [31, 32]. Nagai and et al. [14] showed to improve diabetes problems, drugs that may break cross-links inside glycated protein are administered and improved diabetes [14]. Despite the results of these chemical medications, there is a need for drugs by minimum complications and high reliability that may be used in the long term, and traditional remedies receive great attention throughout this area. Medicinal crops are among the organic materials with few side effects. Matsuura and et al. [15] have confirmed plant's antioxidant, antidiabetes, and antihyperglycemic components [15]. Traditionally, pharmaceutical plants' life had special position within medical sciences in the treatment of common human diseases, with easy access and fewer unwanted side effects, especially in metabolic diseases, such as diabetes. Moreover, Kaewnarin and et al. [19] showed that investigating the effectiveness of medicinal plants' life has increased recently, decreasing the textural outcomes in diabetes [19]. In this study, the effect of the *Citrullus colocynthis* extract on the formation of HbA1C and production of glycated proteins were investigated days 1, 2, 3, 4, 30, and 60. Our findings show that the *Citrullus colocynthis* extract in hyperglycemic conditions with increasing time decreased the formation of HbA1C and thus inhibited the production of glycated proteins. By increasing time and after the initiation of the reaction of extract concentrations (0, 0.1, 0.3, 0.5, and 1 g/dL), there was a significant decrease in the formation of HbA1C compared to those in the control group ($p<0.05$), which was less dose dependent. *Citrullus colocynthis* as an important herbal medicine is known to have antioxidant properties [33-35]. Additionally, it is found helpful in asthma, rheumatism, sciatica, gout, paralysis, leprosy, epilepsy, and expulsion associated with intestinal parasites. It can also be used as purgative for chronic constipation and a widely popular abortifacient [36]. In Mediterranean nations, infusion of *Citrullus colocynthis* is traditionally used as antidiabetic medication. One clinical trial in Iran involving 50 patients concluded that the fruit pulp of the plant was effective in improving the glycemic effect of patients with type II diabetes without severe adverse effects. This also inhibits protein glycation [20-22]. The amount involved in glycation usually depends on the blood glucose level. Even though the particular blood glucose level has not been significantly different, typically, the amount of glycated hemoglobin was significantly different following treatment by *Citrullus colocynthis* extract in previous animal studies [20-22]. This suggests that *Citrullus colocynthis* extract may also directly prevent glycation. Since glutathione is an antioxidant, the reduced formation of glycated hemoglobin from the treatment associated with glutathione in this study was considered to obtain its antioxidant effect. *Citrullus colocynthis* (5 g/dL) was more effective in preventing lipid oxidation compared with quercetin (1 mM); besides, this same mechanism might have caused *Citrullus colocynthis* (5 g/dL) to prevent the formation of HbA1C.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Rafsanjan University of Medical Sciences: (grant ID: 9.3591) 2015.12.4

Informed Consent: The study was an in vitro and we use only the human hemoglobin that was purchased from Sigma Company.

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Author Contribution: Concept – M.M.; Design - M.M.; Supervision - M.M., M.H.; Resources - M.N.K.; Materials - M.M., S.N., M.B.G., S.F.P.; Data Collection and/or Processing - S.N., M.B.G., S.F.P., M.N.K.; Analysis and/or Interpretation - M.N.K., M.R.H.; Literature Search - M.N.K.; Writing Manuscript - M.M., M.N.K.; Critical Review - M.M.

Conflict of Interest: The authors of have no conflict of interest to declare.

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