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...
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 nucleic acids 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 hypoglycemic 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 tocochromers (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 anticyglycosylation effects of medicinal plants.

### Incubation with glucose

We use human hemoglobin that was purchased from Sigma Company (Sigma; 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.

\[
\%\text{HbA1C} = \frac{100 \times \text{HbA1C}}{\text{total hemoglobin}}
\]

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, 3, and 4. 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
minister drugs that directly inhibit protein gly.

doing in atherosclerosis in diabetes. Regardless of

the use of drugs for diabetes, several patients

ing serious complications, it may be useful to ad.

Discussion

Diabetes is one of the most common diseases

Figure 1. HbA1c incubated with 5 mM glucose

Figure 3. HbA1c incubated with 20 mM glucose

Figure 2. HbA1c incubated with 10 mM glucose

Figure 4. HbA1c incubated by 40 mM glucose

Figure 49
Acetylation 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, anti-diabetes, and anti-hyperglycemic 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, Kaewinarin and et al. [19] showed that investigating the effectiveness of medicinal plants’ life has increased recently, decreasing the textual 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 an anti-diabetic 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.

Peer-review: Externally peer-reviewed.

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

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

Financial Disclosure: This project was financially supported by Vice Chancellor for Research of Rafsanjan University of Medical Sciences, Rafsanjan, Iran.

References
1. Zinman B, Gerich J, Nathan DM. The diabetes control and complications trial/epidemiology of diabetes interventions and complications study: 30th anniversary presentations. Diabetes Care 2014; 37: B [CrossRef]
2. Beagley J, Guariguata L, Weil C, Motala AA. Global estimates of undiagnosed diabetes in adults. Diabetes Res Clin Pract 2014; 103: 150-60. [CrossRef]
3. Butalia S, Kaplan GG, Khokhar B, Rabi DM. The effect of an advanced glycation end-product crosslink breaker and exercise training on vascular function in older individuals: a randomized factorial design trial. Exp Gerontol 2013; 48: 1509-17. [CrossRef]
4. Nagai R, Murray DB, Metz TO, Baynes JW. Cation: a fundamental mechanism of action of AGE inhibitors, AGE breakers, and other inhibitors of diabetes complications. Diabetes 2012; 61: 549-59. [CrossRef]
5. Matsuura N, Aradate T, Kurosaka C, et al. Potent protein glycation inhibition of plantagoiside in Plantago major seeds. Biomed Res Int 2014; 2014: 208539. [CrossRef]
6. Al-Rawi NH. Diabetes, Oxidative Stress, Antioxidants and Saliva: A Review. INTECH Open Access Publisher; 2012.
7. Cazzola R, Cestaro B. Antioxidant Spices and Herbs Used in Diabetes-Chapter 9. 2014. [CrossRef]
8. Agati G, Azzarello E, Pollastri S, Tattini M. Flavonoids as antioxidants in plants: location and functional significance. Plant Sci 2012; 196: 67-76. [CrossRef]
9. Kaewinarin K, Niamsup H, Shank L, Rakicrathram N. Antioxidant and anti-glycation activities of some edible and medicinal plants. Chiang Mai J Sci 2014; 41: 105-16.
10. Mahmoudi M, Hossein J, Hossein-zijood S, Poodlavand V, Asadpour M, Eghbali H. The Effect of Citrullus Colocynthis Hydroalcoholic Extract on in vitro Albumin Glycation. JRMUS 2013; 12: 3-14. [CrossRef]
11. Lakshmi B, Sendrayanurmal V, Subramanian S. Beneficial Effects of Citrullus Colocynthis Seeds Extract Studied in Alloxan-induced Diabetic Rats. Int J Pharm Sci 2013; 19: 47-55.
12. Shi C, Karim S, Wang C, Zhao M, Murtaza G. A review on anti-diabetic activity of Citrus sinensis Schrad. Acta Pol Pharm 2014; 71: 363-7.
13. Choafook and productwantsa A, Farzami B, Mohammadi-Barborti A. The inhibitory effect of garlic extract on formation of glycated hemoglobin and AGEPs. J Med Sci 2007; 7: 1039-43. [CrossRef]
14. Turk Z, Mesic R, Benko B. Comparison of advanced glycation endproducts on haemoglobin (Hb-AGE) and hemoglobin A1c for the assessment of diabetic control. Clinica Chimica Acta 1998; 277: 159-70. [CrossRef]
25. Jing Z, Kuang L, Wang Y, et al. ADMA: a specific biomarker for pathologic progress in diabetic microvascular complications? Biomark Med 2016; 10: 385-95. [CrossRef]

26. Gan T, Liao B, Xu G. The clinical usefulness of glycated albumin in patients with diabetes and chronic kidney disease: Progress and challenges. J Diabetes Complications 2018; 32: 876-84. [CrossRef]

27. Rendell M, Nierenberg J, Brannan C, et al. Inhibition of glycation of albumin and hemoglobin by acetylation in vitro and in vivo. J Lab Clin Med 1986; 108: 286-93.

28. Chevion S, Hofmann M, Ziegler R, Chevion M, Nawroth PP. The antioxidant properties of thioctic acid: characterization by cyclic voltammetry. Biochem Mol Biol Int 1997; 41: 317-27. [CrossRef]

29. Lal S, Chithra P, Chandrakasan G. The possible relevance of antioxidative glycosylation in glucose mediated alterations of proteins: an in vitro study on myofibrillar proteins. Mol Cell Biochem 1996; 154: 95-100. [CrossRef]

30. Slight SH, Feather MS, Ortwenth BJ. Glycation of lens proteins by the oxidation products of ascorbic acid. Biochimica et Biophysica Acta 1990; 1038: 367-74. [CrossRef]

31. Ceriello A, Giugliano D, Quatraro A, Dello Russo P, Torella R. A preliminary note on inhibiting effect of alpha-tocopherol (vit. E) on protein glycation. Diabetes Metab 1988; 14: 40-2.

32. Jain SK, McVie R, Jaramillo JJ, Palmer M, Smith T. Effect of modest vitamin E supplementation on blood glycated hemoglobin and triglyceride levels and red cell indices in type I diabetic patients. J Am Coll Nutr 1996; 15: 458-61. [CrossRef]

33. Shaikh J, Shaikh D, Rahman AB, Shafi S. Antimicrobial and toxicological studies on fruit pulp of Citrullus colocynthis L. Pak J Pharm Sci 2016; 29: 9-15.

34. Rizvi M, Saeed A, Zubairy N. Medicinal plants history, cultivation and uses. Karachi: Hamdard Institut Adv Studies Res 2007: 85-7.

35. Kumar S, Kumar D, SAROHA K, Singh N, Vashishta B. Antioxidant and free radical scavenging potential of Citrullus colocynthis (L.) Schrad. methanolic fruit extract. Acta Pharmaceutica 2008; 58: 215-20. [CrossRef]

36. Usmanghani K, Saeed A, Alam MT. Indusyunic medicine: traditional medicine of herbal, animal, and mineral origin in Pakistan: Dept. of Pharmacognosy, Faculty of Pharmacy, University of Karachi; 1997.