Introduction

Proteins represent the most prevalent biological macromolecules present in all cells and cellular compartments (1). The non-enzymatic glycosylation reaction of proteins, which predominantly takes place in various proteins within the body, including hemoglobin (Hb), albumin (Alb), and collagen, has been identified as the primary pathogenic factor in chronic diabetic disorders (2). Scientists have confirmed that diabetes mellitus is caused by the phenomenon of glycosylation (3). According to previous research, Alb serves as a primary carrier for fatty acids, significantly influencing the efficacy of numerous drugs and leading to metabolic alterations in specific ligands (4). Furthermore, previous studies have indicated that the binding of certain drugs to Alb could impact that of other drugs (5). Serum Alb is one of the most crucial proteins in the body that undergoes non-enzymatic glycosylation (6). Studies have demonstrated that the concentration of glycosylated Alb and advanced glycation end-products increases in the serum of diabetic individuals (7). Previous studies have indicated that the glycosylation of Alb leads to a significantly higher increase in electrophoretic mobility when compared to natural serum Alb (8). Moreover, according to prior research, the formation of glycosylated Hb is directly dependent on the glucose concentration (9). Non-enzymatic glycosylation increases with an increase in plasma glucose levels (10). According to evidence, measuring glycosylated Hb is a standard method for long-term blood glucose control and provides a reliable diagnosis for diabetes (11). Given that glycosylation of proteins is an oxidation reaction, it seems that the presence of antioxidant compounds such as vitamins E and C, as well as some natural compounds such as flavonoids and volatile essences, known as potent antioxidant compounds and widely distributed in nature, can inhibit or halt the occurrence of this reaction (12). In this case, many secondary complications of diabetes can be reduced or prevented.

Effects of Ethanolic Extract of Walnut Leaf on Albumin and Hemoglobin Glycosylation In Vitro

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Abstract

Background: One of the complications of diabetes mellitus is the glycosylation of various proteins in the body. The purpose of this study was to explore the glycosylation reaction of albumin (Alb) and hemoglobin (Hb) in the presence of varying concentrations of the hydroalcoholic extract derived from dried walnut leaves.

Materials and Methods: The plants were gathered and then underwent extraction by employing the maceration technique. Next, 1 mL of the Alb solution, with a concentration of 50 mg/mL, and 1 mL of the Hb solution, with a concentration of 50 mg/mL, were separately exposed to various concentrations of the walnut leaf hydroalcoholic extract (0.1, 0.25, 0.5, 1, and 0 mg/mL). Additionally, 1 mL of the glucose solution (30 mg/mL for Alb and 20 mg/mL for Hb) was added to each, along with 0.5 mL of phosphate buffer (pH = 7.4, concentration of 0.01 M). This mixture was left for 72 hours, and the absorbance of the final solution was measured at 443 nm.

Results: According to the results, the most significant inhibition of Alb glycosylation was observed in the presence of the hydroalcoholic extract from walnut leaves at a concentration of 25% µg/mL. Moreover, the highest percentage of inhibition of glycosylated Hb was found in the presence of 5% µg/mL of the hydroalcoholic extract from dried walnut leaves.

Conclusion: The hydroalcoholic extract from dried walnut leaves was observed to moderately reduce the glycosylation of both Alb and Hb.

Keywords: Diabetes, Glycosylation, Albumin, Hemoglobin, Walnut leaves

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insulin secretion or insulin resistance in the body’s cells (14). Walnut, belonging to the Juglandaceae family, is cultivated in Iran (15). Its leaves are utilized for the treatment of rheumatic pain, fever, skin diseases, and diabetes (16). According to previous research, inhibiting the non-enzymatic binding of glucose to proteins could potentially reduce the complications associated with diabetes mellitus (17). Therefore, this study aimed to examine the glycosylation reaction of Alb and Hb in the presence of various concentrations of hydroalcoholic extract from dried walnut leaves.

Materials and Methods

Extraction
Walnut leaves were collected from the Bahadoran garden in Isfahan in early June. The leaves were then separated from their stems and washed at room temperature until completely dry. After obtaining and drying the desired plant material, extraction was performed using the soaking method. Specifically, 100 g of the plant powder was soaked in 70% ethanol for 72 hours on a shaker at laboratory temperature, allowing for complete extraction of the plant compounds. The resulting solution was separated by filtering through filter paper and then placed in a rotary evaporator at 70 °C to remove the ethanol solvent, resulting in concentrated extracts. These extracts were then spread on plates to dry completely, and finally, the samples were covered with foil and stored in the refrigerator.

Glycosylation of Hemoglobin and Albumin
Substances were purchased from Pars Azmoon Company, Isfahan. Then, 1 mL of the Alb solution (Sigma-A9418) with a concentration of 50 mg/mL and 1 mL of the Hb solution (H2500 Sigma) with a concentration of 50 mg/mL, each with 0.5 mL of phosphate buffer (pH = 7.4, concentration of 0.01 M), were separately incubated for 72 hours in the presence of different concentrations of the walnut leaf hydroalcoholic extract (0.1, 0.25, 0.5, 1, and 0 mg/mL) and 1 mL of the glucose solution (30 mg/mL for Alb and 20 mg/mL for Hb), along with gentamycin. The samples of Hb and Alb proteins with a concentration of 50 mg/mL were prepared in glucose solutions of various concentrations and incubated for 72, 48, and 24 hours. Additionally, a control group containing all components, except for the extract, was included for comparison.

Measurement of Protein Glycosylation
After the completion of the incubation period, each test tube was washed twice with 1 mL of 21% trichloroacetic acid and centrifuged at 3000 rpm for 10 minutes. The resulting precipitates were separated again by centrifugation at 3000 rpm for 10 minutes using 0.5 mL of 40% trichloroacetic acid. The supernatant was separated, and then, at 40 °C, it was mixed with 0.5 mL of 0.05 M thiobarbituric acid for 30 minutes. Finally, the absorbance of the final solution was measured at 443 nm.

Statistical Analysis
The analyses were conducted using SPSS software, version 21. A one-way analysis of variance was employed if the assumptions were met (normality and homogeneity of variance). Otherwise, the non-parametric Kruskal-Wallis test was utilized, and a significance level of 0.05 was considered for all tests. A repeated measures analysis of variance was used to compare the percentage of Alb glycosylation over time and at different glucose concentrations.

Results
As shown in Figure 1, the mean percentage of Alb glycosylation over 72 hours was significantly higher than the other two time points. At a glucose concentration of 30 mg/mL, the percentage of glycosylation was the highest at all three time points and significantly different from the other concentrations, except for 40 mg/mL. Thus, the exposure time has led to a significant increase in the level of Alb protein glycosylation (P<0.05). The percentage of Alb glycosylation inhibition at different glucose concentrations investigated over the 72-hour period was approximately the same and demonstrated no statistically significant difference (P=0.86, Figure 2). Similarly, the percentage of Hb glycosylation inhibition at different glucose concentrations over the 72-hour period showed no statistically significant difference (P=0.85, Figure 3). Therefore, the mean percentage of Alb glycosylation inhibition was significantly higher than that of Hb at all concentrations (P<0.05, Figure 4).

Discussion
Glycosylation is a type of oxidation reaction that can be inhibited or significantly reduced by antioxidant compounds (18). It is worth mentioning that the increase
The effect of walnut leaf on albumin and hemoglobin glycosylation

Diabetes mellitus is a metabolic disorder characterized by chronic elevation of blood glucose levels, which results in disruptions in the metabolism of carbohydrates, lipids, and proteins (20). Chronic hyperglycemia is the primary cause of secondary complications in diabetes (21). Despite numerous reports, there is evidence indicating the involvement of non-enzymatic glycosylation of proteins in the pathophysiology of diabetes (22). A significant proportion of deaths in diabetic patients arise from complications, collectively referred to as micro and macrovascular complications. These complications can lead to kidney failure, eye problems, cardiovascular issues, and central nervous system disorders. The key clinical symptom indicating these complications is an elevation in blood sugar levels (23). Due to protein glycosylation reactions, their nature and spatial structure change, and their biochemical activity is altered. This alteration leads to the onset of various diseases such as atherosclerosis, retinopathy, and neuropathy (24). According to previous research, insulin is essential for the utilization of glucose in various body tissues, including muscle tissue (25). In diabetes mellitus, the decrease in insulin and reduced tissue sensitivity to insulin lead to diminished glucose absorption by these tissues, increasing blood glucose concentration (26). Consequently, there is a higher likelihood of protein glycosylation reactions, such as reactions with Alb (27). In this study, incubation time and glucose concentration were identified as the primary factors influencing protein glycosylation. The findings of the present study revealed that the glycosylation of Alb protein increases only up to a concentration of 30 mg/mL with an increase in time, indicating a correlation with the rise in glucose concentration. Based on these results, both the duration of elevated blood glucose and the level of blood glucose are involved in the glycosylation process. Non-enzymatic glycosylation poses a threat to Alb. The outcomes of this study align with those of previous research (28). Plants, due to the abundance of active ingredients, prevent the production of glycated products and reduce the products of this reaction (29).

Figure 2. The Mean Percentage Inhibition of Albumin Glycosylation at Different Extract Concentrations

Figure 3. The Mean Percentage Inhibition of Hemoglobin Glycosylation at Various Extract Concentrations

Figure 4. Average Percentage of Glycated Albumin and Hemoglobin Inhibition at Different Glucose Concentrations
lower compared to Alb. Nevertheless, Hb was still less glycated in the presence of various concentrations of the extract compared to the control group.

**Conclusion**

Based on the obtained results, the hydroalcoholic extract of dried walnut leaves demonstrated inhibitory effects on non-enzymatic glycosylation of both Alb and Hb, with a greater inhibitory effect on Alb compared to Hb. This difference in the inhibitory effect may be attributed to structural differences in the molecular composition of Alb. Furthermore, the current study results revealed that the hydroalcoholic extract of walnut leaves reduces the formation of glycated products of Alb. Therefore, it is plausible to suggest that further clinical research evaluate the effects of the walnut leaf extract on reducing the glycation of Alb and Hb. Considering the hypoglycemic and antiglycemic effects of walnut leaves and their role in inhibiting non-enzymatic glycation of sugar-protein bonds, the medicinal use of this plant with a specific dosage could partially prevent the complications of diabetes.

**Authors’ Contribution**

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**Competing Interests**

The authors declare that there is no conflict of interests.

**Ethical Approval**

Not applicable.

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**References**


