Inhibitory potential of pure isoflavonoids, red clover, and alfalfa extracts on hemoglobin glycosylation

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Original Article

Abstract

BACKGROUND: Non-enzymatic glycosylation of hemoglobin is complications of diabetes. Antioxidant system imbalance can result in the emergence of free radicals' destructive effects in the long-term. Red clover (Trifolium pratense L.) and alfalfa (Medicago sativa L.) contain isoflavonoids and have antioxidant activity. This experimental study evaluated the inhibitory activity of pure isoflavonoids (daidzein and genistein), red clover and alfalfa extracts on hemoglobin glycosylation.

METHODS: This study was performed in Iran. Stock solution of hydroalcoholic extracts of red clover and alfalfa in concentrations of 1 and 10 g/100 ml and stock solution of daidzein and genistein in concentrations of 250 ng, 500 ng, 25 µg and 250 µg/100 ml were prepared as case groups. Control group was without hydroalcoholic extracts of plants and pure isoflavonoids. All experiments were performed in triplicate. Hemoglobin was prepared and antioxidant activities were investigated to estimate degree of nonenzymatic hemoglobin glycosylation.

RESULTS: There was no significantly difference between used extracts (extract of red clover and alfalfa) and control of the hemoglobin glycosylation but using daidzein ($P = 0.046$, 0.029 and 0.021, respectively) and genistein ($P = 0.034$, 0.036 and 0.028) significantly inhibited ($P < 0.050$) this reaction in 25 µg/100 ml, 250 and 500 ng/100 ml concentrations when compared to control. in 25 µg/100 ml, 250 ng and 500 ng/100 ml concentrations percentage of inhibition were 32, 80 and 74.5% respectively with used of daidzein and were 21, 83 and 76% respectively with consumption of genistein.

CONCLUSION: According to decrease of glycation of hemoglobin with isoflavonoids, two used plant in this study containing isoflavonoid may be useful on diabetes.

Keywords: Glycosylation, Genistein, Medicago sativa, Trifolium

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Introduction

The non-enzymatic glycation of hemoglobin having been established and shown to be significantly increased in diabetes.[1] Measurement of glycated hemoglobin has proven to be particularly useful in monitoring the effectiveness of therapy in diabetes.[1,2] The major factor responsible for the elevated basal glucose level in the diabetic group was a decreased efficiency in the tissue uptake of glucose.[3]

Control of plasma glucose could prevent the progression of most of the complications of diabetes and hemoglobinA1c is the most important criterion controlling these long-term complications.[4] Dramatically increase of the worldwide prevalence of type 2 diabetes is a true challenge for modern medicine. Thus, dietary supplements that can modulate glucose homeostasis would be desirable.[5] Use of medicinal plants for amelioration of various metabolic disorders is finding favor with researches owing to their lesser side-effects.[6] Despite considerable progress in the treatment of diabetes by oral hypoglycemic agents, search for newer drugs continues because the existing synthetic drugs have several limitations.[7] The herbal drugs with antidiabetic activity are yet to be commercially formulated as modern medicines, even though they have been acclaimed for their therapeutic properties in the traditional systems of medicine.[7] The plants provide a potential source of hypoglycemic drugs because many plants and plant-derived compounds have been used in the treatment of diabetes.[7] Several investigators have implicated the role of free radical mediated pathology in diabetes mellitus.[8,9]

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Since the glycosylation of protein is an oxidative reaction[10] Therefore, antioxidants should be able to prevent this reaction. Antioxidants have attracted attention in recent years in scavenging and combating the effects of free radicals.[11,12] Recently, a great deal of interest has been directed toward the bioactivity of flavonoids as dietary sources of antioxidants.[13]

Red clover (Trifolium pratense L.) and alfalfa (Medicago sativa L.) (belongs to the leguminosae family) have been used traditionally as a medicine.[14,15] These plants contain high concentrations of isoflavonoids[16] such as genistein and daidzein.[17,18] Isoflavonoids are secondary metabolites that can be divided into isoflavones and pterocarpans.[19] Certain isoflavones found in red clover leaves include daidzein, genistein, pratensein and prunetin.[20,21] Several in vitro, animals and human studies have shown isoflavones to have antidiabetic properties.[21-24]

In the studies, effects of red clover and alfalfa were investigated on diabetes, for example, the results of Gray and Flatt[14] demonstrated the presence of anti-hyperglycemic, insulin-releasing and insulin-like activity in alfalfa.

According to the effect of isoflavonoids on diabetes treatment, this experimental study aimed to assessment the inhibitory activity of pure isoflavonoids (daidzein and genistein), red clover and alfalfa and on hemoglobin glycosylation.

Materials and Methods

This study was performed in Iran. Red Clover and alfalfa collected before sprouting from Semirum District, Isfahan, Iran. The plant specimen was authenticated and deposited at the Herbarium of the College of Sciences, Isfahan University. The aerial parts of the plants were dried in the shade, and whole parts of the plants were crushed in a miller.[25] Pure isoflavonoids (daidzein and genistein) were purchased from Sigma (Sigma Chemical Co., USA).

A total of 50 g of dried plants material was soaked in 85% aqueous-methanol (1/10, w/v) for 12 h. The extract was filtered through a Buchner funnel. The plant residue was re-extracted then with 50% methanol for additional 6 h. The resulting extracts were evaporated in vacuum to one-third of the original volume. Chlorophyll, oil and carbon were extracted using decanter and chloroform.[26]

1 ml prepared plant extract was evaporated in Benmary 40 °C[27] then was dissolved in Dimethyl sulfoxide (Sigma Chemical Co., USA) to obtain a stock solution.[28] Plants stock solution was prepared in concentrations of 1 and 10 g/100 ml.[29]

Daidzein and genistein stock solution were prepared in concentrations of 250 ng, 500 ng, 25 µg and 250 µg/100 ml in methanol and were used to investigate antioxidant effect on hemoglobin glycosylation.

These stocks were used as case group. Control group was without hydroalcoholic extracts of plants and pure isoflavonoids.

Freshly blood was taken from healthy volunteers and was separated by centrifugation at 3000 rpm for 10 min. Erythrocytes were washed with 5 vol of the phosphate buffered saline three times. The buffy coat was carefully removed with each wash. At the last washing, the cells were centrifuged at 2800 rpm for 5 min to obtain packed cells with a constant volume. The upper layers (containing hemoglobin) were taken with a dropper.[30] The hemoglobin concentrations were estimated by Drabkin and Austin[31] method.

The antioxidant activities of two hydroalcoholic extracts and pure flavonoids were investigated by estimating the degree of nonenzymatic hemoglobin glycosylation.

The assay was performed with adding 60 mg/100 ml of hemoglobin solution, and 1 ml of gentamycin (20 mg/100 ml), in 0.01 M phosphate buffer (pH 7.4) in absence and presence of 2 g/100 ml concentration of glucose for 72 h. The mixture was incubated in dark at room temperature for 72 h. Degree of glycosylation of hemoglobin was measured colorimetrically at 443 nm. Rate of absorption with control was considered as 100% glycosylation.[32,33] The experiment was performed in triplicate.[34,35]

The glycosylation of hemoglobin percentage was calculated according to the following equation:

Percentage of hemoglobin glycosylation = $(A-B)/C\times100$

Where A was the absorbance in the presence of the extracts or flavonoids without glucose, B was the absorbance of the extracts or flavonoids in the presence of the glucose and C was the absorbance of the control.[36]

Statistical evaluation was conducted with SPSS for Windows (version 14, SPSS Inc., Chicago, IL, USA) and values were expressed as mean ± standard deviation. Independent samples t test was applied to compare groups means. Difference between concentrations in the case group was carried out using the analysis of variance (one-way ANOVA) and Duncan's post-hoc. $P < 0.050$ were considered statistically significant.

Results

As shown in table 1, inhibitory activity of both hydroalcoholic extracts in 1 and 10 g/100 ml concentrations had no statistically significant difference with control group although percentage of inhibition with alfalfa extract was more than red clover extract in both of used concentrations.

Table 2 illustrates the effect of daidzein and genistein on the inhibition percent of hemoglobin glycosylation. Daidzein in 25 µg/100 ml, 250 and 500 ng/100 ml concentrations can significantly inhibit (P = 0.046, 0.029 and 0.021, respectively) glycosylation of hemoglobin in comparison with control group.

Hemoglobin glycosylation had a significant difference in 25 and 250 µg/100 ml concentrations of daidzein and genistein as compared to 250 ng and 500 ng/100 ml concentrations.

The highest inhibitory activity of hemoglobin glycosylation was 80% in 250 ng/100 ml concentration. Genistein significantly inhibited (P = 0.034, 0.036 and 0.028 respectively) hemoglobin glycosylation in 25 µg/100 ml, 250 ng and 500 ng/100 ml concentrations as compared to control group. Percentage of hemoglobin glycosylation inhibition with using of genistein in 250 ng/100 ml concentration was highest (83%).

Discussion

Despite insulin therapy, diabetic patients suffer from some chronic clinical complications due to high blood glucose which induces non-enzymatic glycosylation of natural proteins such as hemoglobin, lens proteins, biomembrane proteins, albumin, collagen and myelin.[37]

Glycated hemoglobin has attained significant prominence in the modern world of the medicinal biology due to its use as a scale in the long-term control of diabetes mellitus.[38,39] There are several herbs, roots, fruits and other plant materials that are used to treat diabetes throughout the world.[40]

Table 1. Effect of red clover and alfalfa extract on inhibition percentage of haemoglobin glycosylation

Group		Concentration (g/100 ml)	Absorption (means ± SD)	Inhibition percentage of haemoglobin glycosylation	P
Case	Red clover	1	0.319 ± 0.06	3	0.265
		10	0.309 ± 0.30	6	0.153
	Alfalfa	1	0.301 ± 0.02	8	0.092
		10	0.291 ± 0.11	11	0.075
Control		-	0.326 ± 0.07	0	

Independent samples t test were applied to compare groups means; SD: Standard deviation

Table 2. Effect of daidzein and genistein on inhibition percent of haemoglobin glycosylation

		Concentration (ng or µg/100 ml)	Means ± SD of absorption (confidence interval)	Inhibition percent of haemoglobin glycosylation	P
Daidzein	Case	250 ng	0.066 ± 0.04*	80.0	0.021
		500 ng	0.083 ± 0.2*	74.5	0.029
		25 µg	0.272 ± 0.4*,**,§	32.0	0.046
		250 µg	0.310 ± 0.02**,§	5.0	0.122
	Control	0	0.316 ± 0.1	0.0	
Genistein	Case	250 ng	0.056 ± 0.06*	83.0	0.028
		500 ng	0.078 ± 0.09*	76.0	0.036
		25 µg	0.260 ± 0.2*,**,§	21.0	0.034
		250 µg	0.322 ± 0.6**,§	2.0	0.201
	Control	0	0.316 ± 0.1	0.0	

* Significant difference between used concentration of daidzein in comparison with control; ** Each significant difference with 250 ng; § Each significant difference with 500 ng; P values are significant P < 0.050; Independent samples t-test were applied to compare groups means; Difference between concentrations in case group were applied using the analysis of variance and Duncan's post-hoc; SD: Standard deviation

According to our results, extracts of plant (red clover and alfalfa) in used concentrations can't inhibit glycosylation of hemoglobin while daidzein and genistein in 25 µg/100 ml, 250 and 500 ng/100 ml concentrations significantly reduced its. In our study, increasing of plant extract amount containing most of isoflavonoid may considerably inhibit hemoglobin glycosylation.

Alfalfa has an antihyperglycemic property and insulin-releasing action[41] that is known in both of animal and human studies.[14,42] These activates of alfalfa extracts may be useful for type 2 diabetes and especially important for patients with "pre-diabetic" state for diabetes prevention. These patients already manifest abnormalities of glucose metabolism and could benefit from a low-risk, inexpensive, food-based intervention.[5]

According to Daisy and Rajathi orally administered aqueous extracts (400 mg/kg body weight) of Clitoria ternatea leaves (leguminosae family) and flowers significantly reduced glycosylated hemoglobin in rats.[43] In the other study by Amer et al.,[44] daily intake of Trifolium alexandrinum extract (leguminosae family) in drinking water for 4 weeks immediately caused significant decreases in glycated hemoglobin levels in diabetic rats. They expressed these effects may be due to the presence of a high content of flavonoids, which acts synergistically as antioxidants.

In the study carried out by James et al.,[25] the subsequent administration of Hibiscus cannabinus methanolic leaf (containing antioxidant) extract inhibit hemoglobin glycosylation, where a concentration of 20 mg/ml of the extract gave a significant inhibition by yielding hemoglobin concentration of 1.877 ± 0.40 µg/ml. This observed effect might be attributed by the presence of bioactive compounds in the plant extract such as flavonoids, alkaloids, phenols and sterols. This needs further investigation specific bio active compound responsible for such activities.[25] The main isoflavones in red clover are biochanin A and formononetin, which are both abundantly found in leaves.[17]

Winiarska et al.[5] and Asgary et al.[45] and Asgary et al.[46] carried out different studies on the effect of antioxidants on hemoglobin glycosylation. In one of these researches,[46] they measured the inhibition percentage of haemoglobin glycosylation in the presence of three different concentrations (0.5, 5, 10 microg/ml) of several flavonoids. The results demonstrated that biochanin A (isoflavonoid) inhibited haemoglobin glycosylation 100%. They expressed antioxidants able to prevent haemoglobin glycosylation reaction. According to their studies, plants containing flavonoids can be utilized to inhibit or treat complication of diabetes.

In the study by Adisa et al.,[47] inhibitory effect of flavonoid-rich methanolic extract of cnestis ferruginea on glycosilation was investigated in concentrations of 10, 20, 30 µg/ml and hemoglobin glycosylation reduced in these concentrations.

In the animal study by Kamalakkannan and Prince,[48] flavonoids effect was investigated in diabetic rats. In their study, Rutin (a polyphenolic flavonoid) was orally administered to rats for a period of 45 days, and this flavonoid significantly decreased glycosylated hemoglobin in them.

Selvaraj et al.[49] investigated the effect of lipoic acid and taurine antioxidants on glycosylation of hemoglobin. They revealed glycated hemoglobin levels were higher in erythrocytes incubated with 50 mmol/l glucose concentrations than in erythrocytes incubated with 5 mmol/l glucose and the increase in glycated hemoglobin levels was blocked significantly when erythrocytes were pretreated with either lipoic acid or taurine (25, 50, 100, 150 µmol/l). They mentioned antioxidants can partially inhibit the formation of glycated hemoglobin by lowering the levels of lipid peroxides. Based on the favorable efficiency of these herbal medicines containing isoflavonoid on diabetes, additional studies are needed to investigate effect of the other extraction assays and the other concentrations of these plants on glycosylation of hemoglobin.

Further studies are needed to identify the effect of other concentrations of red clover and alfalfa extracts on inhibition of hemoglobin glycosylation.

Conclusion

This suggests that the isoflavonoids inhibit hemoglobin glycosylation. Isoflavonoids could be improved diabetes by inhibition this reaction. These components exert beneficial effects on glycosylation of hemoglobin through their antioxidative actions, therefore, two used plant in this study containing isoflavonoid may be useful in minimizing glycation of hemoglobin.

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Conflict of Interests

Authors have no conflict of interests.

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