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DIFFERENT FRACTIONS OF ACANTHUS (*GUNDELIA TOURNEFORTII* L) SUPPRESS AGE FORMATION AND PROTEIN OXIDATION ON IN VITRO GLUCOSE MEDIATED PROTEIN OXIDATION

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Abstract

Non-enzymatic glycation of proteins and formation of AGE are the important factors of pathogenesis of chronic complications of diabetes. Since these events lead to the destruction of proper function of proteins, using antioxidant compounds originated from plants can be considered as a therapy to prevent such chronic effects.
In this study, the effect of different fractions of artichoke (*Gundelia Tournefortii*) of Lorestan province on the inhibition of the non-enzymatic glycation process of bovine serum albumin (BSA) was investigated in a model of glucose-mediated protein oxidation using total advanced glycation end products (AGEs) and pentosidine fluorescence measurement as well as protein oxidation markers including protein carbonyl (PCO) formation and loss of protein thiol groups. In addition, the phenol and flavonoid contents and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the different fractions were determined.
Results indicated that the different fractions of the *G. Tournefortii*, especially ethyl acetate fraction in a manner of dose-dependent (100, 250 and 500 µg/ml), inhibited formation of AGE and pentosidine. In addition, the ethyl acetate fraction possesses the highest antioxidant activity and total phenolic and flavonoid contents.

Keywords: *advance glycation end products; antiglycation activity; diabetes; G. Tournefortii.*

Introduction

Diabetes is one of the most important human metabolic diseases which more than 150 million people in the world suffer from it and a significant number of them remains unknown. Diabetes is a costly disease and in many countries, it leads to blindness as well as the amputation or even the chronic renal failure in the people between the ages of 20-70. Since its treatment is still unknown in many cases, it is possible to reduce its prevalence and consequences only by timely understanding and proper care [1,2]. As a major factor in oxidative stress, hyperglycemia is a contributing factor in diabetes type 1 and 2. Through direct production of ROS, or by changing the balance of restoration in oxidative stress, hyperglycemia plays its role and it has some considered mechanism including increase in polyol pathway flux, increase in the formation of advanced glycation end products (AGEs) within the cell, activation of protein kinase C and overproduction of superoxide by the mitochondrial electron transport chain [2-4]. Non-enzymatic glycation of proteins and AGE formation are important factors of pathogenesis of chronic diabetes. Non-enzymatic reaction between amino groups of proteins and carbonyl group of reducing sugars or other carbonyl products is known as Millard reaction [3, 5]. This reaction is divided into three basic stages: primary, intermediate, and final.

At the initial stage, glucose (or other reducing sugars such as fructose, pentose, galactose mannose, and xylulose)) reacts to the free amino group to form an unstable combination; known as the more stable product, Schiff base changes into the Amadori product [5].

In the intermediate stage, Amadori products decompose to a variety of active dicarbonyl compounds such as glyoxal, methyl glyoxal, and deoxyglucosones through dehydration, oxidation, and other chemical reactions. In the final stage of glycation, through oxidation reactions, dehydration, and circularization, irreversible compounds called AGEs are created. The intracellular formation of AGEs damages the target cells by several main mechanisms [5].

On the other hand, glucose auto oxidation can form H_2O_2 and keto aldehyde under the influence of metal ions. Keto aldehydes can react with the amino group of keto amines and these keto amines ultimately lead to the formation of AGEs [6]. Thus, free radicals have an important role in production of AGE components and in destruction of proteins.

One of the anti-AGE strategies is using antioxidants, especially with plants origin. Artichoke plant with the scientific name of *G. Tournefortii* is from the Asteraceae family. In traditional medicine of Turkey, the dried grains use for treatment of some diseases, while the fresh leaves of this plant is diuretic. Also, in Turkey, traditionally, the stem of this plant used for treatment of diarrhea, stomach pain, bronchitis, kidney stone, and neck's inflammation [7]. Also in Jordan, the local people use artichoke plant for treatment of diabetes [8]. The result of a study in Iran approves the traditional medicine theory of protective effects of artichoke in the treatment of liver diseases [9]. Another study in Turkey has demonstrated that the methanol extract of the aerial parts and its seeds have significant antioxidant potency compared to alpha-tocopherol [10]. In addition, this extract has inhibitory effect on the activity of Glutathione S-transferase enzyme. They showed that the polyphenol content of a plant seed is more than its aerial parts; and therefore, the antioxidant potency of seed is more than the other parts. In this study, at first, the antioxidant property of artichoke plant in Lorestan has been investigated. Regarding the high antioxidant activity of the plant and the link between glycation and oxidation, we decided to investigate the in vitro antiglycation activity of different organic fractions of *G. Tournefortii*.

Materials and methods

Materials

5,5´-dithiobisnitro benzoic acid (DTNB) and bovine serum albumin (BSA) were obtained from Merck (Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Fluka (Buchs, Switzerland). glucose, catechin, ascorbic acid, 2,4-dinitrophenylhydrazine (DNPH), Folin–Ciocalteu's reagent (FCR), sodium benzoate and trichloroacetic acid (TCA) were obtained from Sigma (St. Louis, MO,

USA). All other reagents were of analytical reagent (AR) grade.

Plant material

Artichoke's rootstock plant were collected in May and June of 2015 in Noor Abad city (Lorestan, Iran) and identified by Dr Khodayari (Department of Biology, Faculty of Sciences, University of Lorestan, Iran) and a voucher specimen (Lu 120) was deposited in Lorestan university herbarium. The collected materials were dried at room temperature away from sun light. The dried leaves were pulverized and kept at 8 °C for further use.

Preparation of different fractions

Three hundred grams of the plant powder was extracted three times (3×24 h) with ethanol (80%, v/v) at room temperature. The extracts were filtrated, concentrated using a rotary evaporator and then dried to a residue by lyophilization [11]. The average yield of the extracts was 20%. The residue re-dissolved in water and was subjected to fractionation processes. The extract was fractionized first with diethyl ether for four times at room temperature. The extracted liquid phase was then re-fractionized with ethyl acetate for four times. The resulting three fractions (diethyl ether, ethyl acetate and Aqueous) were evaporated under vacuum to dryness to give the diethyl ether, ethyl acetate, and Aqueous fractions, respectively. They were quantitatively re-dissolved in ethanol to a 10 mg/ml concentration. The stock solutions were kept at -20 °C in the dark for future analyses.

Determination of total phenolic content

Total phenolic content of the different fractions of the plant were determined with the Folin–Ciocalteu's reagent (FCR) according to a published method [12]. Each sample (0.5 ml) was mixed with 2.5 ml FCR (diluted 1:10, v/v) followed by 2 ml of Na_2CO_3 (7.5%, v/v) solution. The absorbance was then measured at 765 nm after incubation at 30 °C for 90 min. Results were expressed as gallic acid equivalents (mg gallic acid/g dried fraction).

Determination of total flavonoid content

The total flavonoid content of different fractions of the *G. Tournefortii* was evaluated by colorimetric methods described in the literature [13]. Each sample (0.5 ml) was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a $NaNO_2$ solution (15%). After 6 min, 0.15 ml of an $AlCl_3$ solution (10%) was added and allowed to stand for 6 min, then 2 ml of NaOH solution (4%) was added to the mixture. Immediately, water was added to bring the final volume to 5 ml and the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was then determined at 510 nm versus water blank. Results were expressed as catechin equivalents (mg catechin/g dried fraction).

DPPH radical scavenging activity

The DPPH test is widely used to evaluate the free-radical scavenging capacity of antioxidants. Radical scavenging activities of the different fractions were measured according to the method of Blois [14]. Briefly, 1 ml each of sample with variable concentrations (50-200 µg/ml in ethanol) was added to 1 ml of a DPPH solution (0.2 mM in ethanol) as the free radical source and kept for 30 min at room temperature. The decrease in the solution absorbance was measured at 517 nm. Vitamin C was used as the positive control. The DPPH radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%) = $[(A_o - A_1 / A_o) \times 100]$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of test or the standard sample.

Detection of hydroxyl radicals generated by sugar autoxidation

In vitro hydroxyl radical detection by benzoate hydroxylation was carried out by using method described by Hunt et al [15]. Briefly, reaction mixtures contained sodium benzoate (1 mM), potassium phosphate buffer (100 mM), pH 7.2, glucose (500 mM) and CuSO4 (0.1 mM) in the presence or absence of various concentrations (100, 250 and 500 µg/ml) of different fractions were incubated for 4 day at 37°C. The decrease in benzoate hydroxylation, measured by the fluorescence intensity (excitation and emission maxima of 380 and 410 nm, respectively) correlates with the hydroxyl radical scavenging activity of the fraction. The results were expressed in terms of percentage inhibition.

In vitro glycation of BSA

BSA (10 mg/ml) was modified in vitro at 37 °C by glucose (500 mM), as a reducing sugar. All incubations were carried out in 0.2 M phosphate buffer, pH 7.4, in the absence and

presence of various concentrations (100, 250 and 500 µg/ml) of different fractions and aminoguanidine (AG) (1 mM). Aliquots were taken 21 day and dialyzed extensively against phosphate buffer to remove extra-unbound sugar, and any other impurities [16].

Fructoseamine assay

Samples glycated with glucose was assayed for Amadori products by the method of Johnson et al [17]. Briefly, 1 ml of NBT reagent (0.5 mM NBT in 0.2M sodium carbonate buffer, pH 10.4) was added to the samples and the mixture was incubated at 37°C. Absorbance at 530 nm against a reagent blank was measured after 1 h incubation.

Total AGEs and pentosidine fluorescence measurement

The total AGEs formation can be generally followed by measuring their characteristic fluorescence using the excitation and emission maxima of 370 and 440 nm, respectively. Pentosidine is an amino acid adduct, ascending by reaction between lysine, arginine and sugars. Its presence is characterized by a typical fluorescence (Varian-spectrofluorometer, model Cary) with excitation and emission maxima at 335 and 385 nm, respectively [16].

Determination of protein carbonyl content

From the including markers of proteins oxidative damage is the formation of protein carbonyl, so the measurement of its amount is an appropriate way for recognizing the level of protein oxidative damage. The measurement of amount of protein carbonyl was done based on the existing methods of scientific articles [18]. Carbonyl content was calculated based on the molar extinction coefficient DNPH ($\varepsilon = 2.2 \times 10^4$ $cm^{-1} M^{-1}$) and results was expressed in terms of percentage inhibition.

Thiol group measurement

Another marker of oxidative damage in proteins is the loss of thiol groups. Thiol groups of native and the modified BSA were measured according to Ellman's assay [19] using 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). The content of thiol groups was obtained based on the L-cystein standard curve.

Statistics analysis

Statistical analyses were performed by t-test. All data were expressed as mean of ±SD. Data were statistically significant when the $P<0.05$.

Results

Total phenolic and flavonoid content

In this study, the Folin-Ciocalteu and the aluminum chloride ($AlC1_3$) reactants were used to estimate the phenolic and flavonoid content. Among different fractions, the ethyl acetate fraction with 335.85 mg gallic acid/ gram of dried fraction and 303.2 mg catechin/ gram of dried fraction had the highest amount of phenol and flavonoid contents, respectively (Table 1). The general order of phenol and flavonoid compounds, respectively, is as following:

Ethyl acetate fraction > Aqueous fraction > Diethyl ether fraction

Ethyl acetate fraction > Diethyl ether fraction > Aqueous fraction `

DPPH radical scavenging activity

In Table 2, the effective concentration of different fractions and the standard compound (vitamin C) for scavenging 50% of DPPH in a form of IC_{50} are given. As it is shown in Table 2, different fractions in a concentration-dependent manner are the scavenging activities of free radicals. Ethyl acetate fraction had the highest activity; and compared to vitamin C with IC_{50} equal to 3.5 µg/ml, it had much better reaction.

According to the IC50 value, the order of scavenging activities of DPPH radicals is as follow:

Ethyl acetate fraction > Diethyl ether fraction > Aqueous fraction

Inhibitory effect of different fractions on benzoate hydroxylation

In investigation of different fractions of *G. tournefortii* on benzoate hydroxylation, as it is clear in Figure 1, different fractions in the concentration-dependent process lead to the inhibition of benzoate hydroxylation. In the meantime, ethyl acetate fraction has the highest ability whether in inhibition of production or even in collecting hydroxyl radicals.

The effect of different fractions of G. tournefortii on Amadori production

Formation of Amadori products was studied by measuring fructosamine. Fructosamine test is a colorimetric measurement for determining the amount glycation of a protein based on the power and ability of Amadori products to reduce NBT to a tetrazinolyl radical. When BSA

was incubated with glucose, increase in the amount of Amadori products was gradually seen. While in the presence of different fractions (Fig. 2), there was not any significant effect on formation of Amadori products.

Inhibited production of AGE compounds by different fractions of the G. tournefortii

According to Fig 3, it has been concluded that in the presence of different concentrations of different fractions of *G. tournefortii,* the fluorescence intensity decreased significantly; in the meantime, ethyl acetate fraction had the highest inhibitory activity and consequently, it is comparable to amino guanidine as a typical inhibitor. In addition, the influence of different fractions on the formation process of pentosidine as an index component of AGE was measured by using the typical fluorescence (Ex 335, Em 385). According to Fig 4, pentosidine fluorescence intensity increased during the incubation and different fractions of *G. tournefortii* in various concentrations and with a dose-dependent trend leads to the reduction of the fluorescence related to pentosidine. Results in these two assays showed the significant inhibitory effect of ethyl acetate fractions on the process of AGE formation.

The effect of different fractions of G. tournefortii on proteins oxidation

The most molecular changes in proteins occurred in the process of glycation of the proteins are PCO formation and losing (oxidation) thiol groups that show the destruction of proteins oxidation. Based on our results, BSA incubation with glucose increases PCO formation and adding different concentrations in different fractions of *G. tournefortii* decreased PCO production in a dose-dependent manner (Table 3). In addition, protein incubation with glucose decreased thiol groups' content which shows thiol groups oxidize during AGE formation phenomenon; nevertheless, adding various concentrations of different fractions of *G. tournefortii* considerably increased thiol groups content (Table 4). As it was seen, among fractions, the ethyl acetate fraction has the highest ability in improving PCO formation and thiol group's oxidation.

Discussion

Oxidative stress is extremely related to diabetes and its side effects; so that during both types 1 and 2 diabetes, and even in the absence of diabetic side effects, the oxidative stress will increase in blood; and treatment with antioxidants like vitamin E and melatonin decreases diabetic side effects [20]. Many factors are responsible for producing free radicals under the condition of hyperglycemia. Among these factors, we can refer to protein glycation (AGE formation), autoxidation of glucose, polyol pathway, and activation of protein kinas C. In addition to the above cases, the decrease in efficiency of anti-oxidants systems in diabetes can be regarded as other main factor in producing free radicals and oxidative stress. Generally, anti-oxidants with different mechanisms affect oxidative processes. These mechanisms usually include prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides and scavenging of free radicals [21]. Many studies have shown that the huge part of antioxidant property in plants is due to their phenol content, especially flavonoids [22].

In our study, DPPH test was used for studying the anti-oxidant ability in *G. tournefortii.* In DPPH test, the antioxidant's ability in scavenging DPPH radicals directly is related to their hydrogen-donating ability. In the case of *G. tournefortii*, the ethyl acetate fraction has the highest potential in scavenging DPPH radicals. On the other hand, this fraction has the highest amount of phenol and flavonoid compositions. Given the ability of phenol compounds in transferring hydrogen, it is logical that ethyl acetate fraction has the highest potential in scavenging these free radicals.

One of the destructive effects of hyperglycemia is AGE formation. A lot of studies showed the effect of these compounds on destructive and long lasting side effects of diabetes [1, 4, 5].

In fact, this point that the glycation along with oxidation reactions destruct the correct function of proteins is a strong reason for the effect of AGE on diabetes pathology. On the other hand, these reactions are regarded as the main source of ROS formation and carbonyl active compounds. Recent studies show the interference of free radicals like H_2O_2, super oxide, and hydroxyl radicals in AGE formation reactions [23-25]. In addition, autoxidation of sugars like glucose lead to formation of H_2O_2 and keto-aldehydes. The ketoaldehydes can in

turn react with amino groups of proteins forming ketoimines. These ketoimines may finally lead to AGEs formations, and this is regarded as another pathway in the production of free radicals and carbonyl active compounds under the condition of hyperglycemia [15].

At least, these points theoretically show the value of anti-oxidants in preventing from AGE formation [26-28]. Generally, AGE formation inhibitors are divided into three categories [27-29]: quenchers of dicarbonyl intermediates (hydrazines) like aminoguanidine, chelating compounds of transition metal ions (Phytate and penicillamine), and anti-oxidants (super oxide dismutase, catalase, vitamins C and E). Consumption of aminoguanidine has been stopped due to some deleterious side effects such as drug resistance and hepatotoxicity [29]. On the other hand, it does not appear practical or desirable to inhibit glycoxidation by chelating all transition metal ions in vivo. Thus, using antioxidant compounds may be an appropriate strategy in preventing from AGE formation [26].

Generally, the glycation reactions are divided into two parts. In the first step, the accomplished reactions lead to the production of Amadori compounds; and in the second step the produced Amadori compounds create AGE compounds over various reactions [30]. It was suggested that free radicals and oxidative reactions did not play any role in the Amadori compounds production during the first step, while they played an important role in AGE compound production during the second stage [31-32]. In our study, the measurement of fructosamine indicates that different fractions of *G. tournefortii*, except its concentration, have no effect on the formation of Amadori products during the first step of glycation. In contrast, using the technical fluorescence as a confirmed method for determining the ultimate compound of AGE was applied in our study. Results showed the different fractions, especially ethyl acetate fraction in a manner of dose-dependent, inhibited the AGE formation and pentosidine. These results were in accordance with other studies that showed antioxidative potency of the extracts of *Allium cepa* [33], *Ilex paraguariensis* [34], *Teucrium polium* [1] and green tea [35] might account for their inhibitory effect on AGEs formation. In addition, the results obtained from these studies indicated that inhibitory capacity of flavonoids against protein glycation was remarkably related to their scavenging effect on free radicals derived from glycoxidation process. In our study, the presence of antioxidant phenolic compounds, mainly flavonoids, in different fractions, especially ethyl acetate fraction has been demonstrated. Due to the strong anti-oxidant property of ethyl acetate fraction as well as its ability to collect ROS, its feature for inhibition of glycation reaction and AGE formation can be related to the antioxidant feature of the plant that comes from its high phenolic and flavonoid contents.

The most molecular changes in proteins during process of protein glycation and AGE formation are PCO formation and protein thiol group oxidation that show the oxidative injury to proteins. Due to the formation of free radicals during glycation pathway and in the autoxidative glycation process, it is very important to investigate these oxidative parameters. As a result, for considering the reason of proteins oxidative damage during glycation reaction, the amounts of PCO and thiol groups were measured. Results strongly expressed that glucose (reducing sugar used in the test) can increase PCO and oxidation of thiol groups after 21 days; while the different fractions, especially ethyl acetate fraction in a concentration-dependent process, led to the reduction of PCO and an increase in thiol groups number. According to Wolff's hypothesis which expresses that hydroxyl radicals production in the phenomenon of "autoxidative glycation" takes the responsibility of oxidative protein damages and AGE's formation [6]; it was decided to noticeably examine the effect of different fractions on hydroxyl radicals production during glucose autoxidation. The results of benzoate hydroxylation indicate that the different fractions of *G. tournefortii* lead to the inhibition or collection of hydroxyl radicals, among which, the ethyl acetate fraction showed the highest ability. This inhibitory effect is likely due to the direct scavenging of hydroxyl radicals or chelating of transition metals and leading to less hydroxyl radicals production, or it may have both effects. Our findings not only indicated the involvement of oxidative reactions in AGE formation, but they

also showed that antioxidants with plant origin can be regarded as AGE inhibitors.

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Table 1. Total phenolic and flavonoid contents of different fractions of the *G. tournefortii*

Sample	Total Phenolic Content [a]	Total flavonoid Content [b]
Diethyl ether fraction	74.15 ± 0.83	22.0 ±1.59
Ethyl acetate Fraction	335.84 ± 3.47	303.2±4.7
Aqueous fraction	95.08 ± 0.71	11.7±0.53

Each value represents the mean ± SD (n=3).

Table 2: The DPPH radical scavenging activity of different fractions of the *G. tournefortii* at various concentrations (200-50 µg/ml).

	DPPH radical Scavenging (%)			IC50 (µg/ml)
Concentration	**50 µg/ml**	**100 µg/ml**	**200 µg/ml**	
Diethyl ether fraction	12.1 ± 1.9	23.7 ± 1.7	49.3 ± 2. 1	204 ±3.1
Ethyl acetate Fraction	91.7 ± 1.1	93.2 ± 6.2	94.8 ± 2.4	-
Aqueous fraction	4.6 ± 0.2	11.5 ± 0.5	26.4 ± 2.0	367±3.5
Ascorbic acid	-	-	-	3.5 ±1.8

Results of three means in independent experiment is ±SD.
[a] Total phenolic content was expressed as mg gallic acid equivalents/g dried fraction.
[b]Total flavonoid content was expressed as mg catechin equivalents/g dried fraction.

Figure 1. The inhibitory effect of different fractions of *G. tournefortii* on benzoate hydroxylation. Different fractions in a manner of dose-dependent lead to the inhibition of benzoate hydroxylation. Results of three means in independent experiment are ±SD.

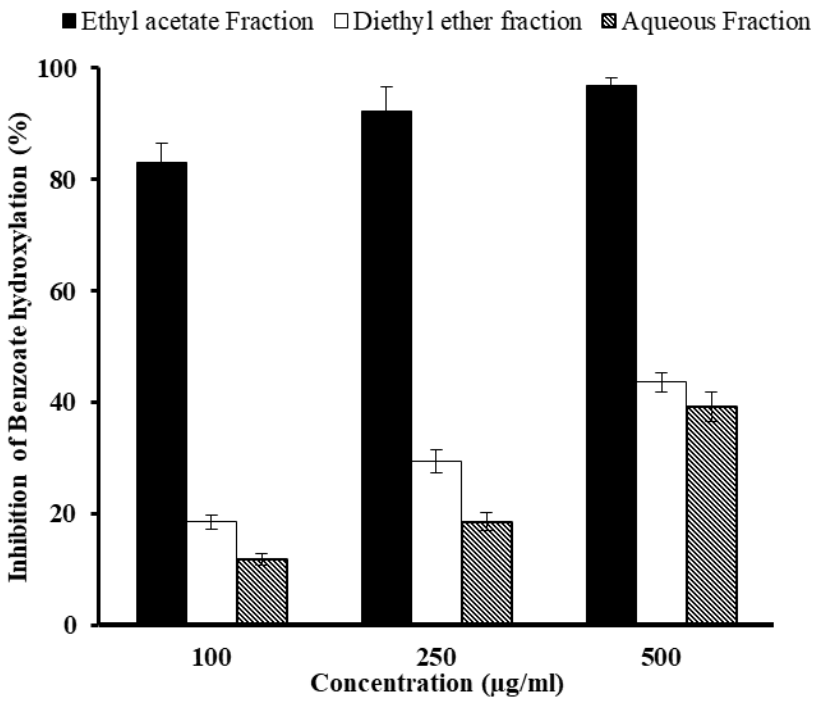

Figure 1. The inhibitory effect of different fractions of *G. tournefortii* on benzoate hydroxylation. Different fractions in a manner of dose-dependent lead to the inhibition of benzoate hydroxylation. Results of three means in independent experiment are ±SD.

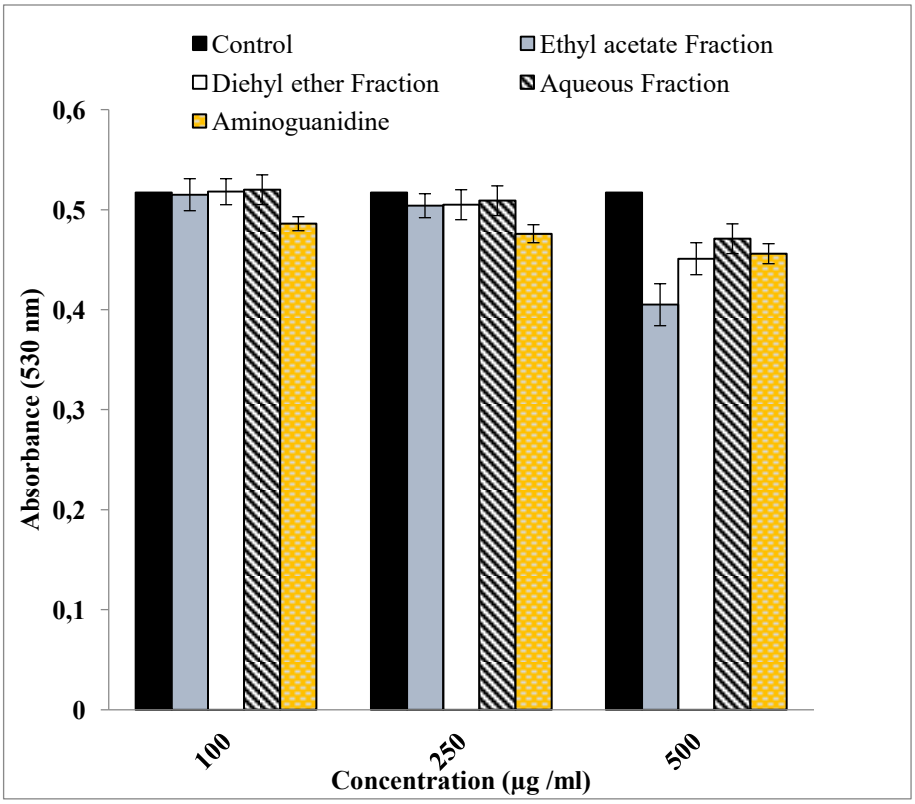

Figure 2. The effect of different fractions of *G. tournefortii* and aminoguanidine on formation process of Amadori products. The average result of 3 independent tests is ±SD.

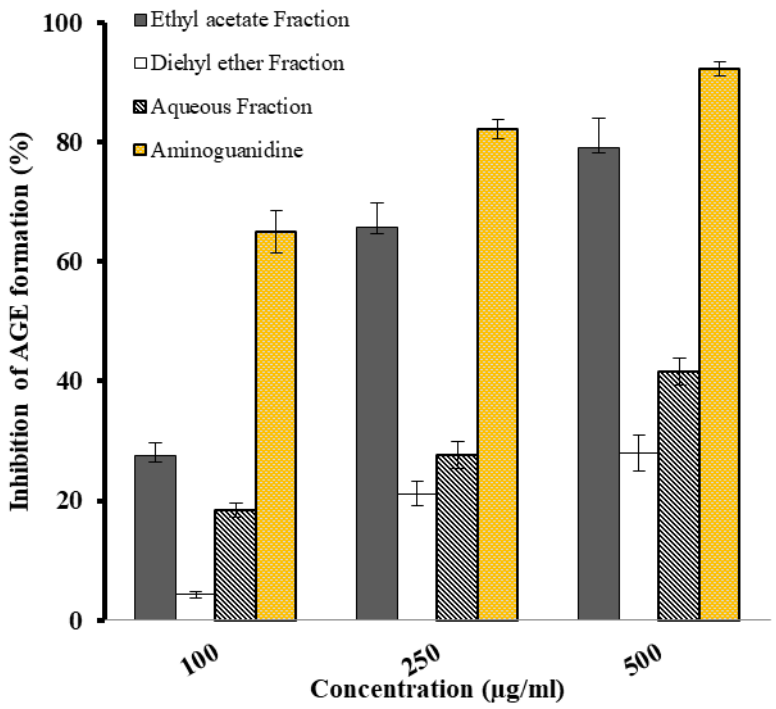

Figure 3. The inhibitory effects of different fractions of *G. tournefortii* on AGE formation in the presence of glucose. Results of three means in independent experiment are ±SD.

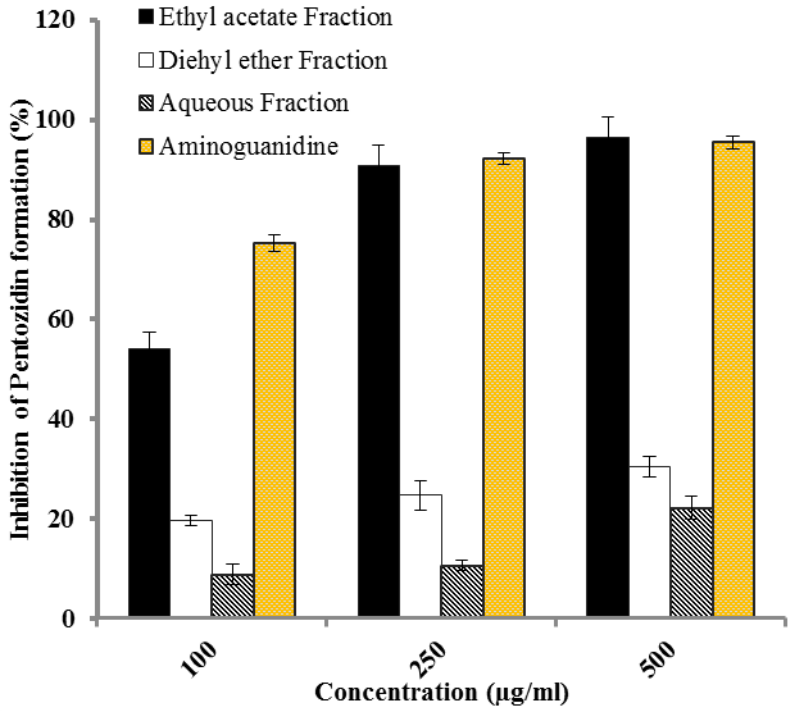

Figure 4. The inhibitory effect of *G. tournefortii* different fractions on pentosidine formation as one of the important component of AGE index. Results of three means in independent experiment are ±SD.

Table 3. The inhibitory effects of different fractions of *G. tournefortii* at various concentrations and aminoguandin on PCO formation in the presence of glucose.

Concentration	100 µg/ml	200 µg/ml	500 µg/ml
		Inhibition (%)	
Diethyl ether fraction	5.0 ± 0.7*	16.8 ± 0.7	30.2 ± 2. 1
Ethyl acetate fraction	9.50 ± 1.1	21.50 ± 0.2	40.0 ± 6.4
Aqueous fraction	3.0 ± 0.1	4.5 ± 0.5	18.1 ± 3.0
Aminoguanidine	35.0 ± 3.5	52.1 ± 3.9	67.23 ± 4.5

Results of three means in independent experiment are ±SD.

Table 4. The effects of different fractions of *G. tournefortii* and aminoguandin at various concentrations on thiol group content in the presence of glucose.

Concentration	100 µg/ml	200 µg/ml	500 µg/ml
Diethyl ether fraction	6.7 ± 0.9	16.7 ± 1.1	31.5 ± 3. 2
Ethyl acetate Fraction	8.7 ± 0.8	146.3 ± 7.2	228.7 ± 11.4
Aqueous fraction	5.3 ± 0.6	11.4 ± 1.5	13.4 ± 1.0
AG (1 mM)	24.1± 1.5	172.1 ± 7.4	242.7± 9.5

Results of three means in independent experiment are ±SD.