Iranian Journal of Pharmaceutical Sciences
Winter 2010: 6(1): 33-50
ijps.sums.ac.ir

Original Article

Salvia reuterana Extract Prevents Formation of Advanced Glycation End Products: An *In Vitro* Study

Mohammad Ali Esmaeili*, Mohammad Reza Kanani, Ali Sonboli

Department of Biology, Medicinal Plants and Drug Research Institute, Shahid Beheshti University, Tehran, Iran

Abstract

In this study, we examined the antioxidant activities of methanolic extract of three endemic species of *Salvia* from Iran (*S. lachnocalyx, S. reuterana* and *S. sahendica*) employing various established *in vitro* systems including ferric reducing antioxidant power, Trolox equivalent antioxidant capacity assay and scavenging of 1,1-diphenyl-2-picrylhydrazyl radical. The results revealed that *S. reuterana* extract possess the highest antioxidant activity, total phenolic and flavonoid contents among these three species. Regarding the link between glycation and oxidation, we proposed that the *S. reuterana* extract might possess significant *in vitro* antiglycation activities as well. The *S. reuterana* extract also showed strong inhibitory effects on the production of Amadori products and advanced glycation end products from bovine serum albumin in the presence of fructose in term of protein carbonyl formation and thiol oxidation. The structural changes of bovine serum albumin (BSA) with fructose, in the presence of *S. reuterana* extract were evaluated by circular dichroism and fluorescence techniques. Regarding enhancing the helicity of the protein and preventing helix decrement in the secondary structure of BSA in the presence of fructose, it can be concluded that *S. reuterana* extract and its constituents may act as an anti-glycation agent for bovine serum albumin.

Keywords: Advanced glycation end products; Antiglycation; Antioxidant; *S. reuterana*.
Received: February 14, 2009; *Accepted:* August 22, 2009

1. Introduction

Free radicals and other reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical and hydrogen peroxide are an entire class of highly reactive molecules derived from the normal metabolism of oxygen or from exogenous factors and agents [1]. ROS are not only strongly associated with lipid peroxidation resulting in deterioration of food materials, but also are involved in the development of pathology of numerous chronic diseases, including carcinogenesis, coronary heart disease, diabetes, neurodegeneration and many other health problems related to aging [2-5]. Diabetic patients are more susceptible to oxidative attack than normal subjects owing to their higher production of ROS [6] and lower content of antioxidants

*Corresponding author: Mohammad Ali Esmaeili, Department of Biology, Medicinal Plants and Drug Research Institute, Shahid Beheshti University, Evin, Tehran, Iran.
Tel: (+98)21-29903025; Fax: (+98)21-29903023
E-mail: m_esmaeili@sbu.ac.ir

(GSH, vitamins C and E) [7]. Several mechanisms appear to be involved in hyperglycemia-mediated oxidative stress, such as glucose autoxidation, protein glycation, and formation of advanced glycation end products (AGEs) [8].

Non-enzymatic protein glycation occurs primarily through the formation of a Schiff base between the aldehyde (or keto) groups of the sugars and amino groups of the protein chain [9]. The Schiff base thus generated subsequently goes through a number of reactions to yield fluorescent brown pigments called advanced glycation end products [10-13]. It is also known that a heterogeneous collection of AGEs are formed by sequential glycation and oxidation reactions termed glycoxidation. Some AGEs such as carboxymethyllysine (CML) and pentosidine have become highly useful biomarkers of glycoxidative damages [14]. The accumulation of the reaction products of protein glycation (non-enzymatic reaction of proteins with glucose and other reducing sugars) in living organisms leads to structural and functional modifications of tissue proteins [14]. Therefore, targeting the glycation pathways might have broad and beneficial effects on aging and age-related diseases. In consideration of the significance of glycoxidative stress to diabetic pathology, a supplement of antioxidants in response to the inhibition of protein modification should be a theoretical strategy for preventing diabetic complications [15]. This hypothesis has been supported by the clinical results which indicated that the development of Type 2 diabetes may be reduced by the intake of antioxidants in diets [16]. Fruits, vegetables, and beverages are important dietary sources of polyphenols [17]. Strong evidences from animal studies show that the glycation inhibitor, aminoguanidine, attenuates the development of a range of diabetic vascular complications. However, some toxicity problems have been encountered in clinical trials with aminoguanidine [18]. Therefore, there has been an increasing interest in the use of plant compounds as antidiabetic compounds [19]. In a continuing effort to search for antioxidants from plant materials as therapeutic agents, we are interested in the development of antioxidants that have potent antiglycation activity.

The genus *Salvia*, with about 700 species, is one of the most widespread members of the Lamiaceae family. An unusually large number of useful secondary metabolites, belonging to various chemical groups, such as essential oils, terpenoid compounds and phenolic derivatives, have been isolated from the genus, which features prominently in the pharmacopoeias of many countries throughout the world [20].

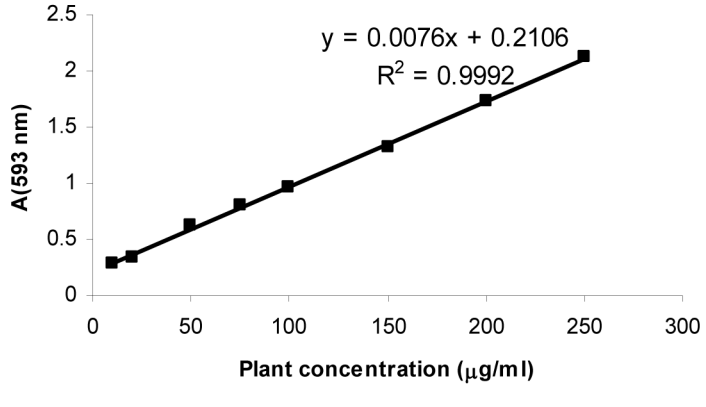

Figure 1. Reducing power of *Salvia reuterana* extract (25-250 µg/ml) in FRAP assay. Each value represents the mean±SD.

Our unpublished data concerning the biological activities of some endemic *Salvia* species in Iran confirms that this genus has a great potential, especially in the antioxidant systems, for the food and cosmetic industries. In this paper, at first we compare antioxidant activity of three endemic species of *Salvia* (*S. lachnocalyx, S. reuterana and S. sahendica*) and secondly, regarding the link between glycation and oxidation, we decided to examine the inhibitory effect of *S. reuterana* extract (high antioxidant activity) on glycation-and oxidation-dependent damages to albumin induced by fructose. In addition, the interaction of bovine serum albumin (BSA) with fructose, in the absence and presence of plant extract, was studied by circular dichroism and fluorescence techniques.

2. Materials and methods

2.1. Chemicals

Butylated hydroxytoluene (BHT), Nitroblue tetrazolium (NBT), 5,5'-dithiobisnitro benzoic acid (DTNB) and bovine serum albumin (BSA) were obtained from Merck (Germany). Fructose, catechin, L-ascorbic acid, 2,4-dinitrophenylhydrazine (DNPH), Folin-Ciocalteu's reagent (FCR) and sodium benzoate were obtained from Sigma (St. Louis, MO, USA). Thioflavin T (ThT),2,4, 6-trinitrobenzene sulfonic acid (TNBSA) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Fluka (Buchs, Switzerland). All other reagents used were of analytical grade.

2.2. Plant material

The aerial parts (leaves and flowers) of plants were collected from different area of Iran. The plants were identified by A. Sonboli and colleagues (Department of Biology, Medicinal Plants and Drug Research Institute, Shahid Beheshti University, Velenjak, Tehran, Iran) and a voucher specimen was deposited in herbarium of Medicinal Plants and Drug Research Institute, Shahid Beheshti University, Velenjak, Tehran, Iran. Herbarium information of the plant species listed below:

1. *Salvia reuterana* (Boiss.) Buhse, Tabriz, Iran, June 2008, voucher herbarium specimen (No. MPH-1321).
2. *S.* sahendica (Boiss.) Buhse, Tabriz, Iran, June 2004, voucher herbarium specimen (No. MPH-848).
3. *S. lachnocalyx* Hedge, Fars, Iran, June 2004, voucher herbarium specimen (No. MPH-674).

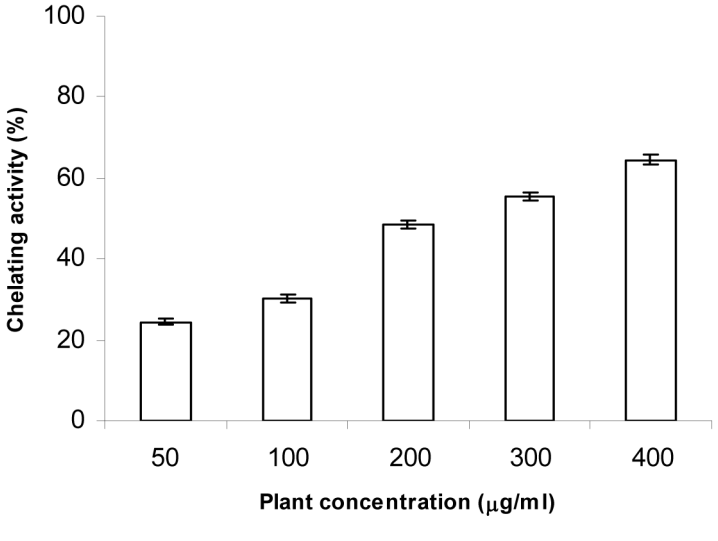

Figure 2. Chelating activity of different concentrations (50-400 μg/ml) of *S. reuterana*. Each value represents the mean±SD.

The plant aerial parts were air-dried, protected from direct sunlight, and then powdered. The powdered plant material (100 g) was extracted three times with methanol at room temperature overnight. The methanolic extracts were filtered and concentrated under reduced pressure on a rotary evaporator. All of the extracts obtained were used in antioxidant measurements. The methanolic extract of *S. reuterana* as polyphenolic-rich extract with powerful antioxidant activity was also used in *in vitro* glycation studies.

2.3. Determination of total phenols and flavonoids levels

The total phenolics content of the plant extract was determined according to the Folin & Ciocalteu (FC) procedure method [21]. The FC reagent oxidizes phenols in plant extract and changes in the dark blue color (λ=765 nm) are monitored by UV visible. 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. A calibration curve, i.e., absorbance versus gallic acid concentration, was taken. Results were expressed as gallic acid equivalents (mg gallic acid/g dried extract).

Determination of the flavonoid content was achieved using the method described by Maksimovic *et al.* [22] by addition of aluminum chloride reagent to the solution containing the extract. 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 aluminum chloride ($AlCl_3$) 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 prepared water blank and concentrations of flavonoids were deduced from a standard curve and calculated in mg catechin equivalent.

2.4. DPPH free radicals scavenging activity assay

Radical scavenging capacity was determined according to the technique reported by Blois [23]. An aliquot of 1.5 ml of 0.25 mM DPPH solution in ethanol and 1.5 ml of extract at various concentrations were mixed. The mixture was shaken vigorously

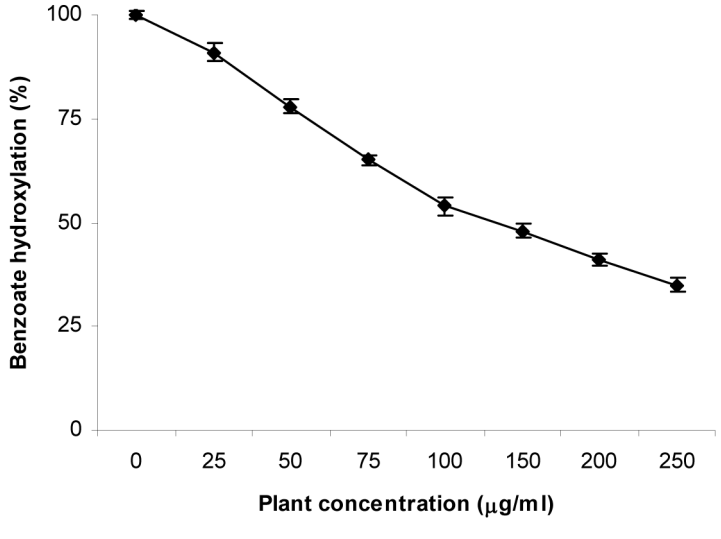

Figure 3. Inhibitory effect of *S. reuterana* extract on hydroxyl radical generation induced by fructose autoxidation in the presence of transition metals. Benzoate hydroxylation measured by the fluorescence intensity at excitation 308 nm and emission 410 nm. Each value represents the mean±SD.

Table 1. Total phenolic and flavonoid contents of three *Salvia* species extracts. Each value represents the mean±SD.

Extracts	Total phenolic[a]	Total flavonoid[b]
S. reuterana	85.10±2.80	46.21±2.10
S. sahendica	64.50±3.40	35.70±2.40
S. lachnocalyx	48.82±1.34	16.44±1.80

[a]Total phenolic content was expressed as mg gallic acid equivalents/g dried extract; [b]Total flavonoid content was expressed as mg catechin equivalents/g dried extract.

and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring the absorbance at 517 nm with a Varian spectrophotometer. The DPPH radicals scavenging activity was calculated according to the following equation:

Scavenging activity=$[(A_0-A_1/A_0)\times 100]$

Where A_0 is the absorbance of the control (blank, without extract) and A_1 is the absorbance in the presence of the extract or standard sample.

2.5. Fe^{2+} chelating activity assay

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis *et al.* [24]. To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}-Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as Chelating rate = $(A_0-A_1)/A_0\times 100$, where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

2.6. Ferric reducing ability power (FRAP)

The FRAP method measures the absorption change that appears when the TPTZ (2,4,6-tri-pyridyl-*s*-triazine)-Fe^{3+} complex is reduced to the TPTZ-Fe^{2+} form in the presence of antioxidants. An intense blue colour, with absorption maximum at 595 nm, develops [25]. Briefly, the FRAP reagent contained 2.5 ml of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl plus 2.5 ml

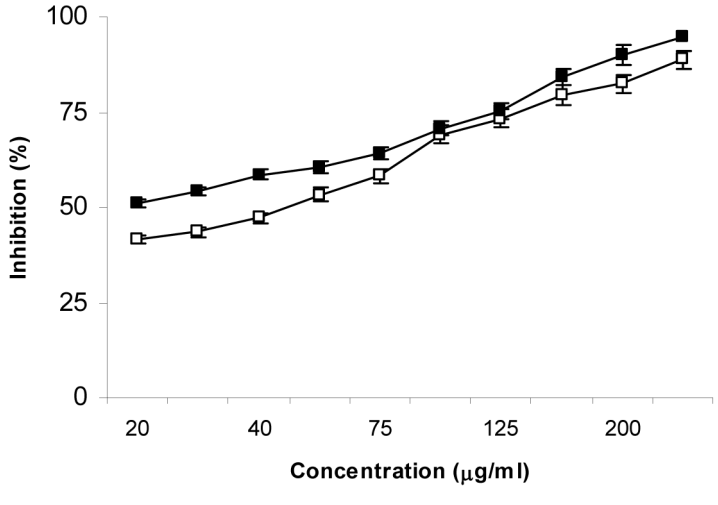

Figure 4. Inhibitory effect of different concentrations (20-250 µg/ml) of *Salvia reuterana* extract (□) and aminoguanidine (AG) (■) on AGEs formation in the BSA-fructose system. Each value represents the mean±SD. Aminoguanidine was used as positive control. IC_{50} (the concentration required for 50% inhibition of the intensity of fluorescence relative to negative control) were calculated from the dose inhibition curve. IC_{50} value for plant extract and AG are 43 and 20 (µg/ml), respectively.

of 20 mM $FeCl_3$ and 25 ml of 0.3 M acetate buffer, pH 3.6, was freshly prepared. The extracts were dissolved in ethanol at a concentration of 1 mg/ml. An aliquot of 0.2 ml of solution was mixed with 1.8 ml of FRAP reagent and the absorption of the reaction mixture was measured at 595 nm. Ethanolic solutions of known Fe (II) concentration, in the range of 50-500 µM ($FeSO_4$), were used for obtaining the calibration curve. The FRAP value represents the ratio between the slope of the linear plot for reducing Fe^{3+}-TPTZ reagent by plant extract compared to the slope of the plot for $FeSO_4$.

2.7. Antioxidant capacity determined by radical cation ($ABTS^+$)

ABTS assay was based on the method of Re *et al.* [26] with slight modifications. ABTS radical cation ($ABTS^+$) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm. After addition of 25 µl of sample or Trolox standard to 2 ml of diluted $ABTS^+$ solution, absorbance at 734 nm was measured at exactly 6 min. The decrease in absorption at 734 nm was used for calculating TEAC values. A standard curve was prepared by measuring the reduction in absorbance of $ABTSo^+$ solution at different concentrations of Trolox. Appropriate blank measurements were carried out and the values recorded. Results were expressed as Trolox equivalent antioxidant capacity (TEAC).

2.8. Determination of hydroxyl radicals in sugar autoxidation process

Hydroxyl radical detection by benzoate hydroxylation was carried out as described [27]. Briefly, reaction mixtures contained sodium benzoate (1 mM), potassium phosphate buffer (100 mM), pH 7.2, fructose (100 mM) and $CuSO_4$ (0.1 mM) in the presence or absence of different concentration of methanolic extract of S. *reuterana* (25-250 µg/ml) was incubated for 4 day at 37 °C. The decrease in benzoate hydroxylation, measured by the fluorescence intensity

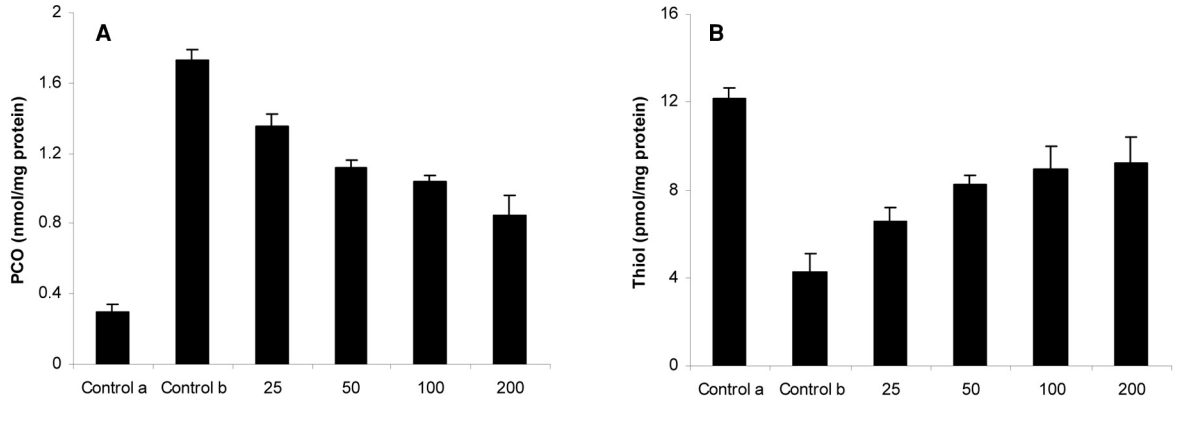

Figure 5. Effect of different concentrations (0-250 µg/ml) of *S. reuterana* extracts on protein carbonyl (PCO) formation (A) and thiol group oxidation (B) in the presence of BSA and fructose. BSA (10 mg/ml) was incubated with fructose (100 mM) in phosphate buffer (pH 7.4) in a total volume of 3 ml at 37 °C for 21 days in the absence and/or the presence of different concentrations of *S. reuterana* extract. PCO formation and thiol groups of samples were measured by DNPH and Ellman's methods, respectively. Control (a) represents the PCO value of BSA (A) and thiol group value (B) in absence of fructose and plant extract and control (b) represents the PCO value of BSA (A) and thiol group value (B) in BSA/fructose system without plant extract. Each value represents the mean±S.D. (n=3).

Table 2. Antioxidant activity of different *Salvia* species extracts in DPPH, TEAC and FRAP assays as compared to ascorbic acid and catechin.

Materials	DPPH (IC50)[a]	TEAC value	FRAP value
S. reuterana	15.01±1.00	0.74±0.04	0.34±0.01
S. sahendica	17.14±0.53	0.63±0.06	0.21±0.07
S. lachnocalyx	>100	0.35±0.01	0.15±0.01
Ascorbic acid	-	0.96±0.03	0.95±0.05
Catechin	-	0.95±0.04	0.97±0.02
BHT	18.21±0.47	-	-

a: IC_{50} values were represented as µg/ml.

(excitation and emission maxima of 308 and 410 nm, respectively) correlates with the hydroxyl radical scavenging activity of the extract. The results were expressed in terms of percentage inhibition, calculated from a control measurement of the reaction mixture consisted of reducing sugars. For prevention of effect of autofluorescence and coloring substances of plant extract in the reaction mixture, aiquots of samples were transferred to new tube and 10 µl of 100 % (w/v) Trichloroacetic acid (TCA) was added to each tube. The supernatant was removed after agitation and centrifugation (15000 rpm, 4 °C), then the precipitated compounds was dissolved with 400 µl buffer (PBS) to serve for screening [28].

2.9. In vitro glycation of bovine serum albumin

According to a slightly modified method of Yamaguchi *et al*. [29], bovine serum albumin (BSA, 10 mg/ml) was incubated in fructose (100 mM) and sodium azide (0.02 %) with or without $CuSO_4$ (100 µM) in 0.2 M phosphate buffer (pH 7.4). All dishes were autoclaved prior to use to inactivate proteases, and all solutions were filter-sterilized (0.2 µm membrane). The samples in capped vials were protected from light and incubated at 37 °C for 21 days. Samples were then dialyzed against sodium phosphate buffer at 4 °C for 48 h. This removed reversibly bound and unbound sugars from the BSA solutions. Following dialysis, the samples were stored at -20 °C in small aliquots prior to analysis . The dialysis experiments were repeated three times and the results were similar. After dialysis, the protein concentration of samples was determined in triplicate by BCA assay.

2.10. Determination of total advanced glycation endproducts (AGEs)

Aliquots of samples (100 µl) were

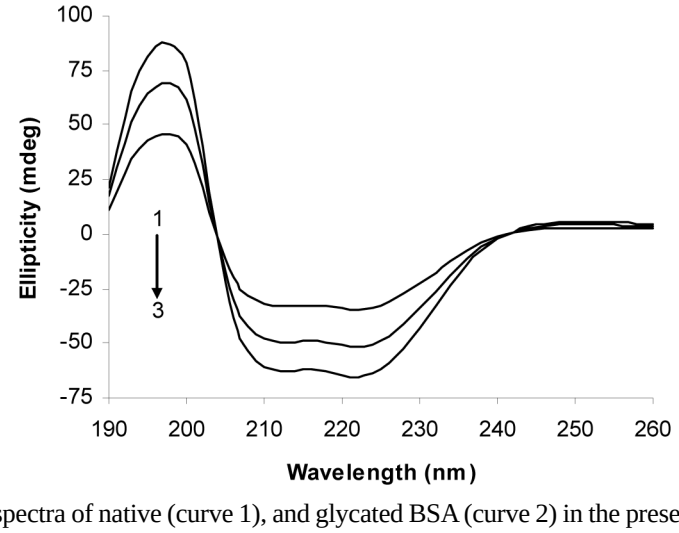

Figure 6. Far-UV CD spectra of native (curve 1), and glycated BSA (curve 2) in the presence of *S. reuterana* extract (curve 3). A 1-mm path length cell was used for CD analyses.

transferred to new tube and 10 µl of 100 % (w/v) TCA was added to each tube. The supernatant containing sugar, plant extract and the interfering substances was removed after agitation and centrifugation (15000 rpm, 4 °C), then the precipitate of AGEs-BSA was dissolved with 400 µl buffer (PBS) to serve for screening. The fluorescence intensity of glycated materials was measured at 370 nm excitation and 440 nm emission using Varian-spectrofluorometer, Cary Eclipse model . The IC_{50} (the concentration that resulted in 50% inhibition of the activity) was estimated for each test sample from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

2.11. Determination of protein carbonyl content

For determination of protein carbonyl content in the samples, 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added to the reaction mixture. Samples were incubated for 30 min. at room temperature. Then, 1 ml of cold TCA (10%, w/v) was added to the mixture and centrifuged at 3000 g for 10 min. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 ml of guanidine hydrocholoride (6 M, pH 2.3). The absorbance of the sample was read at 370 nm [30]. The data were expressed in terms of percentage of inhibition, calculated from a control measurement of the reaction mixture without the test sample.

2.12. Thiol groups measurement

Thiol groups of native and modified BSA were determined spectrophotometrically with the use of DTNB [31]. Four µl of samples were added to 46 µl of phosphate buffer (0.2 M, pH 6.8) and 50 µl of phosphate buffer containing 1 mM DTNB (5,5'-dithiobis-2-nitrobenzonic acid) in a 96-well plate. After 10 min. of incubation at room temperature, absorbance was measured at 412 nm. The free thiol concentration of samples was calculated based on the standard curve prepared by using various concentration of L-cysteine.

2.13. Circular dichroism experiments

The secondary structural changes of glycated samples were evaluated by far-UV CD spectroscopy. Measurements were recorded over wavelength range of 200-250 nm using an AVIV spectropolarimeter, model 215 (USA) with a 0.1 cm path length sample cell. All CD measurements were carried out at 25 °C with the help of a thermostatically controlled cell holder. Spectra were collected at 1nm intervals, with a scan speed of 20 nm/min. Each spectrum was the average of two scans and the noise in the data was smoothed using the AVIV 215 software.

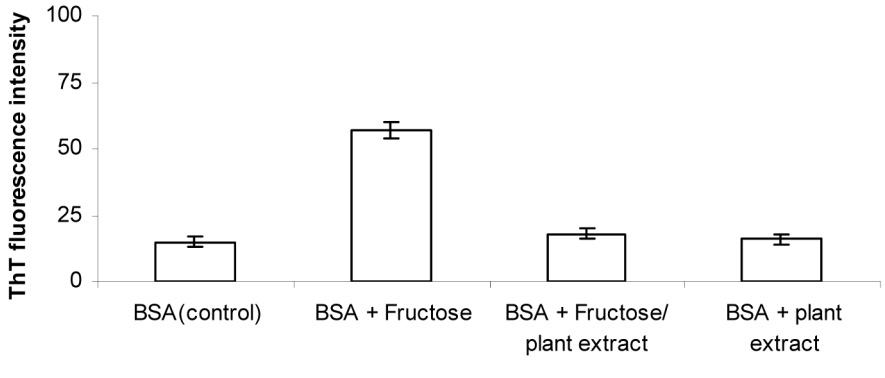

Figure 7. Thioflavin T fluorescence intensity of BSA incubated in the presence of fructose and *S. reuterana* extract. Thioflavin T fluorescence intensity was measured by the fluorescence intensity at excitation 308 nm and emission 410 nm.

2.14. Determination of fibrillar state with thioflavin T

The fibrillar state of the incubated BSA was determined via thioflavin T (Th T), a reagent used for detecting the β-sheet configuration in proteins [32]. The fluorescence of BSA (0.2 mg/ml) and 10 μM ThT reagent in 100 mM phosphate buffer, pH 7.4, was measured at 370 nm excitation and 440 nm emission using Varian-spectrofluorometer, model Cary Eclipse.

2.15. Lysine modification

The determination of the level of free lysine residues was carried out based on Sharma method [33]. The amount of free lysine in each sample was obtained from the mean of three independent experiments. Valine was used as a standard.

2.16. Statistical analysis

The results were presented as means±SD. Statistical analyses were performed using student's t-test. Differences at $p<0.05$ were considered statistically significant.

3. Results

3.1. Total phenolic and flavonoid content

Various methanolic *Salvia* species extracts were investigated regarding their composition by different colorimetric techniques, such as the content of total phenolic compounds by the Folin-Ciocalteu assay and flavonoids by $AlCl_3$ reagent. Total phenolic content of each *Salvia* species methanolic extract was reported as mg gallic acid equivalent per g dried extract. According to the Table 1, *S. reuterana* has the highest total phenolic level with a value of 85.1±2.8 μg/mgμ1. Total flavonoid contents of different *Salvia* species extracts also expressed as mg catechin equivalent per g dried extract. The results, as presented in Table 1, show that the flavonoid contents of the *Salvia* species extract have the following order:

S. *reuterana*>*S. sahendica*>*S. lachnocalyx*

Based on these data, the methanolic extract of *S. reuterana* possesses the highest content of phenolics and flavonoids.

3.2. Antioxidant activity of plants extracts using different in vitro anti-oxidant assay system DPPH scavenging

A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods [23]. It has also been used to quantify antioxidants in complex biological systems in recent years. The IC_{50} values (defined as the concentration of test compound required to produce 50% inhibition) for DPPH scavenging by different species of *Salvia* are shown in Table 2. According to the findings presented in Table 2, the highest scavenging activity was found for methanolic extract of *S. reuterana* (15.01±1.00 μg/ml), followed by *S. sahendica*

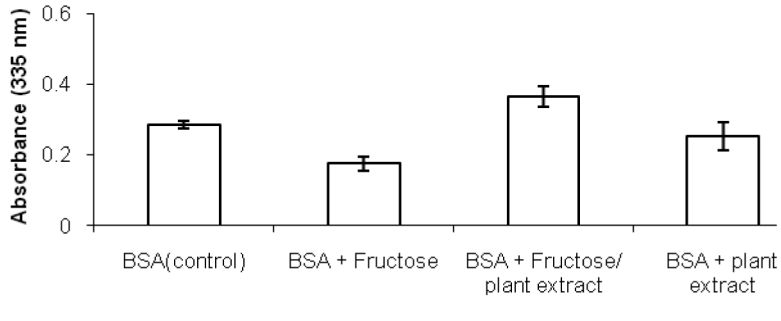

Figure 8. TNBSA absorbance of BSA incubated for 21 days in the presence of fructose and *S. reuterana* extract.

(17.14±0.53 µg/ml). *S. lachnocalyx* exhibited the weakest antioxidant activity in this test system of which IC_{50} value is >100 µg/ml.

3.3. FRAP and TEAC methods

FRAP assay measures the reducing ability of antioxidant that react with ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and produce a coloured ferrous tripyridyltriazine (Fe^{2+}-TPTZ) [25]. Using this assay, the FRAP value of *S. reuterana* extract was found to be 0.34 compared to ascorbic acid and catechin with FRAP value of 0.95 and 0.97, respectively (Table 2). The results represented in Figure 1, showed that *S. reuterana* extract has a potent reducing ability in a dose-dependent manner, pointing to its antioxidant activity. The reducing ability of the extracts was strongly correlated with the phenolic and flavonoid levels. These results were in agreement with Soong and Barlow [34] who found a strong correlation between total phenolic content and FRAP assay. Rice-Evans *et al*. [35] reported that phenolic compounds exhibited redox properties, (i.e. act as reducing agents, hydrogen donators and singlet oxygen quenchers). The redox potential of phenolic phytochemicals plays a crucial role in determining the antioxidant properties [35]. In addition, the antioxidant ability of *S. reuterana* extract to scavenge the blue-green colored $ABTS^{\cdot+}$ radical cation was measured relative to the radical scavenging ability of Trolox. The result as shown in Table 2 clearly indicates that the methanolic extract of *S. reuterana* has the highest reducing power with an EC value of 0.74 compared to other *Salvia* extracts.

In the metal chelating assay, ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with consequent decrease in the intensity of the red color of the complex. Our results (Figure 2) showed that the extract of *S. reuterana* was capable of chelating ferrous ions by 24.5, 30.2, 48.5, 55.3 and 64.6% at the extract concentrations of 50, 100, 200, 300 and 400 µg/ml, respectively. Based on these data, it is clear that the *S. reuterana* extract contained high antioxidant activity and chelating properties. According to our results obtained by antioxidant assays we used, *S. reuterana* extract has high antioxidant agent in glycation processing.

3.4. Inhibitory effect of S. reuterana on benzoate hydroxylation

Benzoate hydroxylation test which scavenges $OH^{\cdot}$ generated by Fenton reaction gives a weak fluorescent intensity, while after incubation of protein in the presence of fructose; products with strong fluorescent intensity are formed (36). Spectrofluorometric changes have been measured to detect the damage caused by hydroxyl radicals. Antioxidants present in plant compete with benzoate within the scavenging reaction and reduce the fluorescence. The effect of different concentrations of *S. reuterana* extract (25-250 µg/ml) on benzoate hydroxylation is shown in Figure 3. As shown, increasing the concentration of *S.reuterana* extract is associated with lower values of hydroxylated benzoate.

3.5. Evaluation of anti-glycation property

The inhibition study for the production of AGEs was carried out in different concentrations of *S. reuterana* extract. The extract was able to inhibit the production of AGEs by 53.3% in 50 µg/ml concentration as the same concentration of aminoguanidine (AG) which resulted in 60.4% inhibition (Figure 4). In addition, it is evident from Figure 4 that different concentrations of *S. reuterana* extract (20-250 µg/ml) have significantly decreased the formation of AGEs with an IC_{50} value (the concentration required for a 50% inhibition of the intensity of

fluorescence relative to negative control) of 43 µg/ml. In that respect, *S. reuterana* extract activity is comparable to the effect of AG with an IC_{50} value of 20 µg/ml as a known glycation inhibitor. Detection of the absorbance was very useful to make a rough estimation of the total degree of modification with regard to lysine and arginine modifications in the glycated protein. Plant extract acted as a glycation inhibitor because of its free radical scavenging property. The effectiveness of plant extract in reducing superoxide anions and AGEs formation can speak about its potential uses for diabetic patients [19].

3.6. Effect of S. reuterana extract on protein carbonyl production

Oxidative stress is known to introduce carbonyl groups into the amino acid side chains of proteins [37]. Oxidative modifications of BSA preparations were demonstrated using a combination of carbonyl assay and the thiol groups. To evaluate whether *S. reuterana* can reduce the protein glycation in glycation process, we evaluated the extent of protein carbonyl formation after three weeks by DNPH reagent. As shown in Figure 5A, glycation elicited a significant increase of carbonylation of BSA in the presence of fructose compared to the control sample without reducing sugar. However, as shown in Figure 5A, a significant effect on the inhibition of protein oxidation due to glycation was exerted in a dose-dependent manner; by methanolic *S. reuterana* extract that indicate significant anti-protein oxidation activities.

To further characterize the oxidative modifications in our preparations, we examined whether the content of free thiol groups in albumin was altered by the glycoxidation process. The determination of free thiol groups was performed according to Ellman's dosage [31] and results are shown in Figure 5B. Our results showed that addition of fructose to the BSA solution for 3 weeks decreased 64% of the free thiol groups, compared to the control sample. However, *S. reuterana* extract, in a dose-dependent manner and in the presence of fructose significantly inhibited oxidation of these thiol groups (Figure 5B).

3.7. Structural evaluation of the glycated samples

The structural changes of BSA occurring after incubation with fructose were evaluated in the presence of *S. reuterana* extract. The Far-UV CD spectrum of BSA is characterized by the presence of two strong negative bands at 208 and 222 nm, which represent the helical characteristics of BSA [38]. As shown in Figure 6, more negative ellipticity value of BSA was observed when BSA incubated in the presence of fructose and the extract relative to BSA control. ThioflavinT is a dye that interacts with amyloid fibril, probably by interacting with the specific quaternary structure of the β-pleated sheet, upon which interaction its fluorescence intensifies, while in its free form is only weakly fluorescent. It is indicated in Figure 7 that the fibrillar state of glycated BSA is enhanced compared to that of the BSA control. The results show that the intensity of ThT fluorescence for BSA in the presence of *S. reuterana* extract and BSA with fructose and *S. reuterana* extract are equal to that of the BSA control. Free lysine residues of BAS in glycation processes were also measured by TNBSA. As shown in Figure 8, the free lysine residues in glycated protein decreased by approximately 45% compared to the control sample, whereas in the presence of *S. reuterana* extract, the percent of free lysine residues is nearly similar to that of the control sample.

4. Disscussion

Oxidative stress is defined as a situation of serious imbalance between the production of free radicals (ROS) and antioxidant defense mechanisms, leading to potential tissue dysfunction and damage [1]. These reactive

oxygen species are generated in the early and advanced glycation processes. The glycation phenomenon corresponds to the non-enzymatic and non-oxidative covalent attachment of glucose molecule to protein. This process classically proceeds through early and advanced stages. In the early stage, reducing sugars such as glucose react with the amino groups of lysine side chains and the terminal amino group of proteins to form unstable Schiff bases and, through rearrangement, Amadori products. Then, this product undergoes slow and complex series of chemical reactions to form advanced glycation endproducts (AGEs) [39]. Extensive modification to lysine side chains and minor modification to arginine side chains have been found to occur during the AGE-formation process with glucose [40]. The accumulation of the reaction products of protein glycation in living organisms leads also to functional modifications of tissue proteins. From cross linking of proteins and interaction of AGE with their receptors and/or binding proteins, which leads to enhanced formation of reactive oxygen species with subsequent activation of nuclear factor-kB and release of pro-inflammatory cytokines, growth factors, and adhesion molecules [41]. AGE accumulation in collagen, a long-lived structural protein in the extracellular matrix region of the kidney, is thought to affect changes in elasticity, ionic charge, thickness, and turnover of basement membrane components [42]. Immunohistochemical studies using anti-AGE antibodies have revealed the presence of AGE-modified proteins in several tissues under pathological conditions, including the kidneys of patients with diabetic nephropathy [43], chronic renal failure [44], atherosclerotic lesions of arterial walls [45], and amyloid fibroids in hemodialysis-related amyloidosis [46] suggesting the potential involvement of AGE-modification in the pathogenesis of age-related disorders.

Oxidative stress in diabetes could originate from various processes, such as excessive production of oxygen radicals from the autoxidation of glucose, glycoxidized proteins, and glycoxidation of antioxidative proteins [47]. Although an elevated level of glucose had been thought to play a primary role in the Maillard reaction, the formation of AGEs is now known to result also from the action of various metabolites other than glucose such as galactose, sialic acid, mannose, glucose 6-phosphate, glyceraldehydes and fructose [48, 49]. In recent years, fructose consumption has largely elevated because of an increased consumption of soft drinks and other beverages and foods such as processed food, sauces, and ready-to-serve foods sweetened with sucrose and high-fructose corn syrup. As a reducing sugar, fructose has 300 times as many highly reactive chain structures as glucose [50]. In addition, fructose differs from glucose in the pathway for the formation of dicarbonyl compounds such as highly reactive 3-deoxyglucosone (3DG), which are intermediates in the advanced glycation stage. The primary pathway for 3DG formation from glucose is a reaction between glucose and amino acid residues, resulting in the formation of Amadori compounds, from which 3DG is produced. Fructose is converted to fructose-3-phosphate by phosphorylation, from which 3DG is formed [52]. In addition, Shin *et al*. showed another pathway that proceeds even in the absence of protein, i.e., the direct production of 3DG by the autoxidation of fructose without the formation of Amadori compounds [53]. It has also been suggested that dicarbonyl compounds such as 3DG and methylglyoxal are directly produced from fructose by cleavage from fructose to triose or catabolism of ketone bodies [53]. These may be associated with high reactivity of fructose in glycation. Also, researches have shown that fructose decreases glucose tolerance, increases insulin resistance and speeds up the process of glycation which

contributes to the development of normal aging as well as to some complications of diabetes. Thus, the discovery and design of inhibitors of the glycation reactions offer a promising therapeutic approach for the prevention of diabetic or other pathologic complications [14]. Although, the synthetic glycation inhibitor, aminoguanidine, a hydrazine-like small molecule, is a nucleophilic compound that traps reactive carbonyl intermediates such as MGO, GO, and 3-DG [17]. It is known to inhibit CML and CEL formation, cross-linking, and fluorescence in skin collagen of diabetic rats, and significantly retard the development of diabetic nephropathy. AG also caused correction of hyper-cholesterolemia and hypertriglyceridemia in the diabetic rats, consistent with effects of AG on dyslipidemia in humans [47]. Recently, the effects of metformin, pioglitazone, and pentoxyfylline on AGE formation were evaluated and demonstrated that all of these three drugs are potent inhibitors of glycation. Later studies by several investigators demonstrated that aminoguanidine retarded the development of diabetic complications including nephropathy, neuropathy, and vasculopathy [47]. Unfortunately, the clinical phases in the aminoguanidine treatment of patients with type-1 diabetes were ended due to serious complications in those patients.

There are reports of some natural substances isolated from plants with AGE-inhibitory effects. One such compound is resveratrol (3,4,5-trihydroxystilbene), a natural phytoesterogen found in grapes [54]. Resveratrol has been shown to inhibit AGE-induced proliferation and collagen synthesis activity in vascular smooth muscle. Another natural compound, curcumin, an active principle isolated from turmeric (*Curcuma longa*), has been known for its anti-oxidant and anti-inflammatory properties. Other studies have revealed curcumin to be a potent inhibitor of AGE formation and cross-linking of collagen in diabetic rats [55]. On the other hand, some herbal extracts and natural products have proven to be somewhat effective for inhibiting AGE formations [56].

As mentioned, cell and tissue damage by AGE comes from cross-linking and remodeling of structural proteins and affecting their physiological functions. Thus, there are many therapeutic strategies to suppress the protein glycation processes. In that respect, more attention should be considered is that the glycoxidized proteins generate reactive oxygen species (ROS) and also, ROS is generated by other reactions in the cascade of AGE formation such as MGO and Schiff's base pathways leading to lipoxidation and oxidative damage to cells. Therefore, strategies such as suppression of receptor signaling pathways (e.g. RAGE antagonists), and the use of antioxidants and α-oxoaldehyde scavengers have been tested. Antioxidant compounds such as vitamin C, vitamin E (α-tocopherol and the carotenoids including α-carotene, β-carotene, β-cryptoxantin, lutein, lycopene, and zeaxanthine) have been shown to have vascular-related effects that prevent or reverse nerve conduction velocity (NCV) deficits in experimental models. They have also shown to reduce *in vitro* and *in vivo* protein glycation [57]. Regarding this knowledge, more attention has been focused on preventing protein glycation by antioxidant from plant sources [18].

In this study, the antioxidant and antiglycation effect of three species of *Salvia* were studied. For evaluation of the antioxidant activity of plants extract, assays such as DPPH, FRAP and TEAC were performed. Based on our data, the methanolic extract of *S. reuterana* with high phenolic compounds levels possess potent antioxidant properties. Based on several studies reported recently [58], metal-catalyzed glucose and fructose autooxidation and oxidation of glycated residues can lead to ketoaldehyde and H_2O_2 formation. The ketoaldehydes react with

amino groups of proteins and form ketoimines. These ketoimines may finally lead to AGEs formations. The latter may be potent sources of free radicals and may be the important culprits in tissue damage [6]. Therefore, both the Amadori formation and the glucose autoxidation may contribute to the development of diabetic complications via free radical-induced protein oxidation and promotion of AGEs formations [59]. Thus, therapeutic approaches based on chelation of metal ions such as copper or iron may have dual inhibitory function on redox activity and suppressing ROS production. Based on these data, to study whether *S. reuterana* extract could affect fructose autoxidation in the presence of transition metal (Cu^{2+}), we use benzoate hydroxylation study. A significant decrease in benzoate hydroxylation was observed in the presence of *S. reuterana* extract. This probably indicates the *S. reuterana* extract can either scavenge hydroxyl radicals or chelate transition metals leading to less hydroxyl radical production, or it may have both effects.

Protein carbonyl formation and loss of protein thiol groups are indexes of protein oxidation [31, 37]. Protein carbonyl formation can occur via a variety of mechanisms, including oxidation by hypochloric acid (HOCl) [60], aldehydes, lipid peroxidation products [61], and metal-catalyzed oxidation (MCO) of proteins [37], such that many amino acids are susceptible to carbonyl oxidation. Increases in protein carbonyl levels have been reported in the synovial fluid of patients with rheumatoid arthritis as compared with patients with osteoarthritis [62] and experimental acute pancreatitis [63]. *S. reuterana* extract in a dose-dependent manner suppresses high reducing sugar-induced oxidative damages to protein by decreasing protein carbonyl formation and preserving protein thiols from oxidation. This indicates that *S. reuterana* extract by decreasing oxidative stress may be effective in preventing oxidative protein damages which are believed to occur under the glycoxidation processes.

There are two major questions regarding the protein conformational changes that may be associated with disease. First, it is important to know the possible effects of the change of conformation so that better structural insights into the pathogenesis of the disease are obtained. For example, the high loss of heme from glycated hemoglobin known to affect diabetes patients may imply that the structural integrity of the assembly of α- and β-chains in hemoglobin is impaired, as heme helps to maintain such integrity [38]. For non-heme proteins, glycation-induced overall changes in conformation have already been reported [64]. For serum albumin, glycation results in a higher propensity for formation of β-sheet. Based on literatures β-sheet generation is accompanied by loss of alpha helix in a compensatory way [38].

Based on data obtained from the far-UV CD spectrum, the fructation of BSA following three weeks incubation induced a loss of helical structure in BSA. However, the presence of plant extract alone induces an increase in the helicity of BSA (more negative ellipticity values). Therefore, plant extract may stabilize the native protein structure. The presence of plant extract in the protein/sugar system shows more helicity relative to the control. Thus, the extract prevents fructose from reducing the degree of secondary structure in BSA. To confirm this, ThT fluorescence, which is employed in the detection of amyloid fibril structures in proteins was performed. The results showed that the glycation of BSA induces the formation of the fibrillar state in BSA. However, there is no difference between the intensity of ThT fluorescence for BSA incubated with plant extract alone or with fructose compared to the ThT fluorescence intensity of BSA control. These data confirms the data obtained from CD-spectroscopy and show that *S. reuterana*t extract may stabilize

the native structure of BSA. Furthermore, the measurement of free lysine residues indicates that in the fructated BSA sample, there were approximately 40% less free lysine residues than in the control. However, in the presence of plant extract fructose cannot react with the lysine residues of BSA, thereby preventing AGE formation. Our studies on structural changes of glycated BAS, reveals that *S. reuterana* extract has potent compounds that inhibit the pathway of AGE formation by inhibiting the fructation of protein. BSA has a pI of approximately 5.9, thus, at pH 7.4 is negatively charged and cannot interact with polyanionic compounds in *S. reuterana* extract. Also, the results obtained from the measurement of free lysine residues confirm that plant extract does not react with amino groups in the lysine residues of BSA. Therefore, the free BSA lysine residues are masked by plant extract not allowing them to react with fructose.

Phenolic compounds occur as secondary metabolites in all plants. They embrace a considerable range of substances possessing an aromatic ring bearing one or more hydroxyl substituents, although a more precise definition is based on the metabolic origin, as these substances are derived through the shikimate pathway and phenylpropanoid metabolism. Interest in plant phenolics is also reported due to their physiological activity, which is dependent on their antioxidant activity, their ability to scavenge active oxygen species and electrophiles, their ability to inhibit nitrosation and to chelate metal ions, their potential for autooxidation and their ability to modulate certain cellular enzyme activities [65]. In addition, a strong capacity for the scavenging of the free radicals has been reported [66]. Most of the glycation inhibitory phytochemical constituents of plants have been reported to possess polyphenolic nature [56]. The active glycation inhibitory constituents of green tea have been regarded as polyphenols [67].

Chemical compositions and/or phytochemical compositions of other species of *Salvia* revealed the presence of phenolic compounds such as rosmarinic acid, salvianolic acids K and I. For instance, salvianolic acid, a rosmarinic acid dimer isolated from *S. officinalis*, had a very strong free radical-scavenging activity for DPPH and superoxide anion radicals [68]. Other researchers investigated the antioxidant capacity of rosmarinic acid, salvianolic acids K and I, sagecoumarin and sagerinic acid isolated from the same plant as well as a number of flavone glycosides such as luteolin 7-glucoside, 7-glucuronide, 30-glucuronide, 6-hydroxyluetolin 7-glucoside, and apigenin 6,8-di-C-glucoside. Consequently, the flavonoid glycosides were found to possess weaker DPPH-scavenging activity. Interestingly, bsitosterol isolated from *S. plebeia* was also found to be a strong antioxidant by the oxidative stability instrument (OSI) [69]. Thus, it was proposed that antioxidative effect of *S. reuterana* extract and its polyphenolic contents are, at least in part, involved in the AGEs inhibitory mechanisms.

5. Conclusion

It is concluded that, using different *in vitro* glycation models, the methanolic extract of the aerial parts of *S. reuterana*, as a poly phenolic-rich extract, showed a high inhibitory effect on formation of AGEs. In addition, methanolic extract of this plant exhibited significant decrease in protein carbonyl formation and increase in protein thiol levels and protect structural changes of BSA from glycation processes. Based on our results, *S. reuterana* extract has a high antiglycation potency which might be attributed to its antioxidative activity. Additional studies are needed to identification the antiglycation constituents of the plant.

Acknowledgments

The authors appreciate the financial

support of this investigation by the research council of Shahid Beheshti University.

References

[1] Halliwell B, Gutteridge JMC. Role of free radicals and catalitic metal ions in human disease: An overview. *Methods Enzymol* 1990; 186: 1-85.

[2] Kokkini S, Karousou R, Vokou D. Pattern of geographic variation of *Origanum vulgare* trichomes and essential oil content in Greece. *Biochem Sys Ecol* 1994; 22: 517-28.

[3] Pryor WA. The antioxidant nutrients and disease prevention-what do we know and what do we need to find out? *Am J Clin Nutr* 1991; 53: 391-3.

[4] Terao J, Piskula MK. Flavonoids as inhibitors of lipid peroxidation in membranes. In: Rice-Evans CA, Packer L, (editors). *Flavonoids in health and disease*. New York: Marcel Dekker, 1997; pp. 277-95.

[5] Duthie G. Lipid peroxidation. *Eur J Clin Nutr* 1993; 47: 759-64.

[6] Wolff SP, Dean RT. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. *Biochem J* 1987; 245: 243-50.

[7] Wen Y, Skidmore JC, Porter-Turner MM, Rea CA, Khokher MA, Singh BM. Relationship of glycation, antioxidant status and oxidative stress to vascular endothelial damage in diabetes. *Diabetes Obes Metab* 2002; 4: 305-8.

[8] Bonnefont-Rousselot D. Glucose and reactive oxygen species. *Curr Opin Clin Nutr Metab Care* 2002; 5: 561-8.

[9] Baynes JW, Watkins NG, Fisher CI, Hull CJ, Patrick JS, Ahmed MU, Dunn JA, Thorpe SR. In: Baynes JW, Monnier VM, (editors). *The maillard reaction in aging, diabetes, and nutrition*. New York: Alan R. Liss, Inc, 1989; pp. 4347-62.

[10] McPherson JD, Shilton B, Walton DJ. Role of fructose in glycation and cross-linking of proteins. *Biochemistry* 1988; 27: 1901-7.

[11] Suárez G, Rajaram R, Oronsky AL, Gawinowicz MA. Nonenzymatic glycation of bovine serum albumin by fructose (Fructation). *J Biol Chem* 1989; 264: 3674-9.

[12] Khalifah RG, Baynes JW, Hudson BG. Amadorins: Novel post-amadori inhibitors of advanced glycation reactions. *Biochem Biophys Res Commun* 1999; 257: 251-8.

[13] Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: A review. *Diabetologia* 2001; 44: 129-46.

[14] Rahbar S, Figarola JL. Novel inhibitors of advanced glycation endproducts. *Arch Biochem Biophys* 2003; 419: 63-79.

[15] Montonen J, Knekt P, Jarvinen R, Reunanen A. Dietary antioxidant intake and risk of type II diabetes. *Diabetes Care* 2004; 27: 362-6.

[16] Lampe JW. Spicing up a vegetarian diet: Chemo-preventive effects of hytochemicals. *Am J Clin Nutr* 2003; 78: 579S-83S.

[17] Thornalley PJ. Use of aminoguanidine (pimagedine) to prevent the formation of advanced glycation endproducts. *Arch Biochem Biophys* 2003; 419: 31-40.

[18] Rudnicki M, Oliveira MR, Pereira TV, Reginatto FH, Dal-Pizzol F, Moreira JCF. Antioxidant and antiglycation properties of Passiflora alata and *Passiflora edulis* extracts. *Food Chem* 2007; 100: 719-24.

[19] Gibbs RD. *Chemotaxonomy of flowering plants*. Vol. III, Montreal: McGill-Queen's University Press, 1974; pp. 1405-6.

[20] Banthorpe DV, Bilyard HD, Brown GD. Enol esters of caffeic acid in several genera of the Lamiaceae. *Phytochemistry* 1989; 28: 2109-13.

[21] Slinkard J, Singleton VL. Total phenol analysis: Automation and comparison with manual methods. *Am J Enol Viti Cult* 1977; 28: 49-55.

[22] Maksimovic Z, Malencié ND, Kovacevié N. Polyphenol contents and antioxidant activity of Maydis stigma extracts. *Bioresource Technol* 2005; 96: 873-7.

[23] Blois MS. Antioxidant determination by the use of a stable free radical. *Nature* 1958; 181: 1199-200.

[24] Dinis CP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys* 1994; 315: 161-9.

[25] Benzie IF, Strains JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal Biochem* 1996; 239: 70-6.

[26] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol Med* 1996; 26: 1231-7.

[27] Hunt JV, Dean RT, Wolff SP. Hydroxyl radical production and autoxidative glycosylation. Glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. *Biochem J* 1988; 256: 205-12.

[28] Matsuua N, T.aradate C, Sasaki H, Kojima M, Ohara J, Hasegawa M, Ubukata M. Screening

system for Maillard reaction inhibitor from natural product extracts. *J Health Sci* 2002; 48: 520-6.
[29] Yamaguchi F, Ariga T, Yoshimura Y, Nakazawa Y. Antioxidative and anti-glycation activity of garcinol from *Garcinia indica* fruit rind. *J Agr Food Chem* 2000; 48: 180-5.
[30] Ardestani A, Yazdanparast R. Antioxidant and free radical scavenging potential of *Achillea santolina* extracts. *Food Chem* 2007; 104: 21-9.
[31] Ellman L. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; 82: 70-7.
[32] Schmitt A, Schmitt J, Münch G, Gasic-Milencovic J. Characterization of advanced glycation end products for biochemical studies: Side chain modifications and fluorescence characteristics. *Anal Biochem* 2005; 338: 201-15.
[33] Sharma SD, Pandey BN, Mishra KP, Sivakami S. Amadori product and age formation during nonenzymatic glycosylation of bovine serum albumin *in vitro*. *J Biochem Mol Biol Biophys* 2002; 6: 233-42.
[34] Soong YY, Barlow PJ. Antioxidant activity and phenolic content of selected fruit seeds. *Food Chem* 2004; 88: 411-7.
[35] Rice-Evans CA, Miller NT, Paganga G. Antioxidant properties of phenolic compounds. *Trends Plant Sci* 1997; 4: 304-9.
[36] Gutteridge MG. Ferrous salt promoted damage to deoxyribose and benzoate. *Biochem J* 1987; 243: 709-14.
[37] Levine RL. Oxidative modification of glutamine synthetase. I. Inactivation is due to loss of one histidine residue. *J Biol Chem* 1983; 258: 11823-7.
[38] Moulick RG, Shibsekhar JBB, Basak RS, Anjan KD. Compensatory secondary structure alterations in protein glycation. *Biochim Biophys Acta* 2007; 1774: 233-42.
[39] Pennathur S, Heinecke JW. Mechanisms for oxidative stress in diabetic cardiovascular disease. *Antioxid Redox Signal* 2007; 9: 955-69.
[40] Cohen MP, Shea E, Chen S, Shearman CW. Glycated albumin increases oxidative stress, activates NF-kappa B and extracellular signal-regulated kinase (ERK), and stimulates ERK-dependent transforming growth factor-beta 1 production in macrophage RAW cells. *J Lab Clin Med* 2003; 141: 242-9.
[41] Lehmann R, Schleicher ED. Molecular mechanism of diabetic nephropathy. *Clin Chim Acta* 2000; 297: 135-44.
[42] Heidland K, Sebekova K, Schinzel R. Advanced glycation end products and the progressive course of renal disease. *Am J Kidney Dis* 2001; 38: S100-6.
[43] Vlassara H, Cai W, Crandal J, Goldberg T, Oberstein R, Dardaine V, Peppa M, Rayfield EJ. Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc Natl Acad Sci USA* 2003; 99: 15596-601.
[44] Bailey AJ, Paul RG, Knott L. Mechanisms of maturation and ageing of collagen. *Mech Ageing Dev* 1998; 106: 1-56.
[45] Yamada K, Miyahara Y, Hamaguchi K, Nakayama M, Nakano H, Nozaki O, Miura Y, Suzuki S, Tuchida H, Mirura N, Araki N, Horiuchi S. Immunohistochemical study of human advanced glycosylation end-products (AGE) in chronic renal failure. *Clin Nephrol* 1994; 42: 354-61.
[46] Kume S, Takeya M, Mori T, Araki N, Suzuki H, Horiuchi S, Kodama T, Miyauchi Y, Takabashi K. Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. *Am J Pathol* 1995; 147: 654-67.
[47] Brownlee M. Glycation and diabetic complications. *Diabetes* 1994; 43: 836-41.
[48] Urbanowski JC, Cohenford MA, Dain JA. Nonenzymatic galactosylation of human serum albumin *in vitro* preparation. *J Biol Chem* 1982; 257: 111-5.
[49] Zaman Z, Verwilghen RL. Non-enzymic glycosylation of horse spleen and rat liver ferritins. *Biochim Biophys Acta* 1981; 669: 120-4.
[50] Bunn HF, Higgins PJ. Reaction of monosaccharides with proteins: Possible evolutionary significance. *Science* 1981; 213: 222-4.
[52] Szwergold BS, Kappler S, Brown TR. Identification of fructose 3-phosphate in the lens of diabetic rats. *Science* 1990; 247: 451-4.
[53] Shin DB, Hayase F, Kato H. Polymerization of proteins caused by reaction with sugars and the formation of 3-deoxyglucosone under physiological conditions. *Agric Biol Chem* 1988; 52: 1451-8.
[54] Mizutari K, Ono T, Ikeda K, Kayashima K, Horiuchi S. Photo-enhanced modification of human skin elastin in actinic elastosis by N(ε)-(carboxymethyl)lysine, one of the glycoxidation products of the Maillard reaction. *J Invest Dermatol* 1997; 108: 797-802.
[55] Sajithlal GB, Chithra P, Chandrakasan G. Effect of curcumin on the advanced glycation and cross-linking of collagen in diabetic rats. *Biochem Pharmacol* 1998; 56: 1607-14.
[56] Kim Y, Kim Y. Protein glycation inhibitory and antioxidative activities of some plant extracts *in vitro*. *J Agric Food Chem* 2003; 51: 1586-91.
[57] Cameron NE, Cotter MA. Potential therapeutic

approaches to the treatment or prevention of diabetic neuropathy: Evidence from experimental studies. *Diabet Med* 1993; 10: 593-605.

[58] Yim MB, Yim HS, Lee C, Kang SO, Chock PO. Protein glycation: Creation of catalytic sites for free radical generation. *Ann NY Acad Sci* 2001; 928: 48-53.

[59] Takagi Y, Kashiwagi A, Tanaka Y, Asahina T, Kikkawa R, Shigeta Y. Significance of fructose-induced protein oxidation and formation of advanced glycation end product. *J Diabetes Complicat* 1995; 9: 87-91.

[60] Pereira WE, Hoyano Y, Summons RE, Bacon VE, Duffield AM. Chlorination studies. II. the reaction of aqueous hypochlorous acid with α-amino acids and dipeptides. *Biochim Biophys Acta* 1973; 313: 170-80.

[61] Blakeman DP, Ryan TP, Jolly RA, Petry TW. Diquat-dependent protein carbonyl formation. Identification of lipid-dependent and lipid-independent pathways. *Biochem Pharmacol* 1995; 50: 929-35.

[62] Chapman ML, Rubin BR, Gracy RW. Increased carbonyl content of proteins in synovial fluid from patients with rheumatoid arthritis. *J Rheumatol* 1989; 16: 15-8.

[63] Reinheckel T, Nedelev B, Prause J, Augustin W, Schulz HU, Lippert H, Halangk W. Occurrence of oxidatively modified proteins: An early event in experimental acute pancreatitis. *Free Radical Biol Med* 1998; 24: 393-400.

[64] Bouma B, Kroon-Batenburg LMJ, Wu Y, Brünjes B, Posthuma G, Kranenburg O, Groot PG, Voest EE, Gebbin MFBG. Glycation induces formation of amyloid cross-β structure in albumin. *J Biol Chem* 2003; 278: 41810-9.

[65] Antolovich M, Prenzler P, Robards K, Ryan D. Sample preparation in the determination of phenolic compounds in fruits. *Analyst* 2000; 125: 989-1009.

[66] Robards K, Antolovich M. Analytical chemistry of fruit bioflavonoids. *Analyst* 1997; 122: 11-34.

[67] Nakagawa T, Yokozawa T, Terasawa K, Shu S, Juneja LR. Protective activity of green tea against free radical- and glucose-mediated protein damage. *J Agric Food Chem* 2002; 50: 2418-22.

[68] Lu Y, Foo LY. Antioxidant activities of polyphenols from sage (*Salvia officinalis*). *Food Chem* 2001; 75: 197-202.

[69] Weng XC, Wang W. Antioxidant activity of compounds isolated from *Salvia plebeia. Food Chem* 2000; 71: 489-93.