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The ethanolic extract of *Eryngium billardierei* F. Delaroche restrains protein glycation in human serum albumin: An *in vitro* study

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Abstract: Protein glycation is directly associated with many pathological conditions. This study investigated the potential of *Eryngium billardierei* extract to inhibit the glycation process in human serum albumin (HSA). After preparation of the ethanolic extract of *E. billardierei*, the structural changes of glycated HSA in the absence and presence of different concentrations of *E. billardierei* extract were investigated using circular dichroism (CD), fluorescence spectroscopy and UV–Vis spectroscopy. The results confirmed that *E. billardierei* extract could reduce the formation of advanced glycation end products (AGEs) and Amadori products under *in vitro* glycation conditions and also improve HSA helical structure. In addition, a reduction in the HSA-cross amyloid formation was seen in the thioflavin T assay. The phytochemical analysis disclosed that *E. billardieri* extract is high in flavonoid and phenolic compounds. Accordingly, it could be concluded that the phenolics in *E. billardieri* extract could prevent glucose-induced HSA glycation. This study provides the rationale that *E. billardieri* extract could be implicated in controlling diabetes.

Keywords: AGEs; glycation; *Eryngium billardierei* extract; human serum albumin; spectroscopy.

INTRODUCTION

Fructose, glucose and other reducing sugars are necessary nutrients for the sustenance of human life. However, in the spontaneous non-enzymatic glycation process reducing carbohydrates, especially monosaccharides, bind covalently to the free amino groups of proteins and eventually cause the formation of toxic, heterogeneous, and irreversible advanced glycation end products (AGEs). The glycation of proteins is a main contributing issue to complications of diabetes

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mellitus.[1,2] Furthermore, AGE accumulation contributes to the progression of osteoporosis, and lifestyle-related diseases for example arteriosclerosis.[3] The two main sources of AGEs in the human body are the exogenous AGEs found in highly processed foods and the endogenous AGEs that are generated under oxidative stress and hyperglycemic conditions.[4] Various research have reported glycation of circulatory proteins, such as serum albumins.[5,6] Human serum albumin (HSA), a helical triple-domain structure protein, is a major contributor to oncotic blood pressure that includes 585 amino acids in its single polypeptide chain.[7–9] It has been shown that HSA could be involved in the formation of AGEs potentially.[10]

Recently, numerous compounds have been verified for glycation inhibitory potential *in vitro* and some success has been achieved with a few compounds being effective *in vivo* also. Herbs are known to contain functional components which can hinder glycation stress.[11] Furthermore, in comparison with synthetic substances, natural products have been found to be cheap, relatively non-toxic, and usable in an ingestible form.[12] The *Eryngium* is a plant of the Apiaceae family with 274 species, of which nine are native to Iran. *Eryngium billardierei* is applied considerably as a medicinal plant worldwide for the treatment of diverse ailments. The Persian name of the dominant *E. Billardieri* species is the Boqanq. In folk medicine, several parts of *E. Billardieri* are utilized for a wide range of diseases, such as scorpion bites, urinary infections, sinusitis, rheumatism, inflammatory disorders and wound healing. Previous studies showed that extracts obtained from the aerial and root parts of *E. billardieri* have anti-inflammatory, anti-oxidant, anti-bacterial, and anti-nociceptive effects.[13,14] Recent investigations also indicated the anti-diabetic effect of *E. Billardieri* extracts by improving the insulin resistance index and lipid profile, reducing G6Pase and PEPCK levels,[15] as well as reducing glucose levels and liver enzymes, and increasing the level of HDL to near normal.[16]

Accordingly, although previous studies highlighted the anti-diabetic effect of *E. billardieri in vivo*, research concerning the molecular mechanism of anti-glycation effects of *E. billardierei* on proteins is extremely limited. Hence, this study aims to analyze the anti-glycation potential of *E. billardierei* against glycated HAS, for the first time by employing a multi-spectroscopic approach. The results of this study can offer a platform to utilize *E. billardierei* in the treatment of aggregation-oriented disorders.

EXPERIMENTAL

Materials

Human serum albumin (> 96 %, lyophilized powder, fatty acid-free), D-glucose, nitro-blue tetrazolium (NBT), thioflavin T (ThT), acrylamide, *bis*-acrylamide, glycine, glycerol, *N′,N′*-tetramethylethylenediamine (TEMED), coomassie brilliant blue, and ethyleneamide-tetraacetic acid (EDTA) were acquired from Sigma Co. (USA). The dialysis tubing (cut off

10,000 MW) and membrane filters (25 mm in diameter, 0.2 μm pore size) were from Whatman (UK). Ethanol, di-sodium hydrogen phosphate (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4) and sodium azide (NaN_3) were obtained from Merck Co. (Germany). Plant materials were purchased from a local market in Tehran province (Iran). The voucher specimen (No. IAUH-12161) was confirmed by Avicenna Herbarium, Science and Research Branch, Islamic Azad University (Iran).

Preparation of ethanolic extract

The extraction procedure was adapted from previous research.[17] Briefly, the sepal and petal parts of *E. billardieri* were dried in an oven and ground to a fine powder with a mechanical grinder (Retsch, Germany). Afterward, 50 g of plant powder was macerated in 500 mL of ethanol (70 vol. %), covered with aluminum foil and kept in a shaker at room temperature. After 72 h, the extract was filtered through Whatman filter papers. Subsequently centrifuged at 3500 rpm for 20 min. Condensation was conducted by a rotary evaporator (Heidolph, Germany). The supernatant was dried at 37 °C and the obtained semisolid mass was kept at 4 °C for further analyses.

In-vitro *glycation of HSA*

Samples of HSA (1.5×10^{-5} M) were diluted to 10 mg mL^{-1} in sodium phosphate buffer (50 mM NaH_2PO_4/Na_2HPO_4 and 1 mM EDTA at pH 7.4 containing 0.02 % NaN_3 (to avoid microbial contamination) in capped vials under sterile conditions, which contained glucose (40 mM), glucose with different concentrations of ethanolic extract of *E. billardierei* (45, 105, 135, 175, 210 and 240 μg mL^{-1}), ethanol (70 vol. %) or no additive as a control (N-HSA). Protein concentration was determined spectrophotometrically with an extinction coefficient (*E1%*) of 5.30 at 280 nm[18] using a double-beam spectrophotometer (PG Instruments $T90^+$, UK). All dishes were autoclaved and all solutions were filter-sterilized (0.2 μm). The capped vials were incubated at 37 °C for 21 days and protected from light. This incubation time was selected to provide appropriate time for the completion of the intermediate stage of HSA glycation and production of Amadori products.[10] On completion of incubation, samples were dialyzed against sodium phosphate buffer for 48 h at 4 °C to remove excess amount of glucose.[19,20] After dialysis, all samples were aliquotted and stored at –20 °C for further analyses. The concentration of G-HSA (HSA incubated with glucose) was verified in triplicates by bicinchoninic acid protein (BCA) assay.

*Determination of total phenolic content (*TPC*)*

The *TPC* of *E. billardierei* at different concentrations was estimated utilizing the Folin–Ciocalteu (FC) method, as described earlier.[14] Briefly, 0.5 mL of the extract samples and standard gallic acid (GA) (10, 20, 40, 60, 80, 100 μg mL^{-1}) were positioned into the test tubes and mixed for 5 min at room temperature. Afterward, 2.5 mL of FC's reagent was mixed and shaken. After 5 min, 2 mL of sodium carbonate (2 %) was added and allowed to stand at room template for 2 h. The UV–Vis absorption was recorded by spectrophotometer at 760 nm. The blank was performed utilizing a reagent blank with solvent. The experiments were performed in triplicates. The calibration curve was plotted utilizing standard GA. The results were expressed as mg GAE/g dry extract.

*Determination of total flavonoid content (*TFC*)*

The test sample's *TFC* was determined utilizing the aluminum chloride method according to the previous method.[14] Quercetin (20–100 μg mL^{-1}) was utilized to make the calibration curve. Briefly, 1.5 mL of the extract with a defined concentration was mixed with 1.5 mL of a

solution of aluminum chloride, 2 %) and afterward 3 mL of potassium acetate (5 %) was added to the mixture. After 40 min incubation, the absorbance was measured at 415 nm. The experiments were performed in triplicates and average values were used. The TFC was expressed as mg QE/g dry weight of extract.

UV–Vis spectroscopy

The UV–Vis spectra of ethanolic extract (240 μg mL^{-1}), N-HSA (5 μM), G-HSA (5 μM), G-HSA (5 μM) in ethanol solution, and G-HSA with different concentrations of ethanolic extract of *E. billardierei* (45, 105, 135, 175, 210 and 240 μg mL^{-1}) were recorded by a double beam spectrophotometer in a wavelength range of 200–700 nm at 37 °C.

The intrinsic fluorescence measurements

The intrinsic fluorescence of tryptophan residue of N-HSA (5 μM), G-HSA (5 μM), G--HSA (5 μM) in ethanol solution and G-HSA with different concentrations of ethanolic extract of *E. billardierei* (45, 105, 135, 175, 210 and 240 μg mL^{-1}) was monitored *via* a BSFL-102 fluorescence spectrophotometer (BioLAB, Canada) utilizing a 1 cm quartz cell at 37 °C. The excitation wavelength was 285 nm. In all cases, 10 nm emission and excitation slits were utilized. For inner filter influence correction affected by the emission and excitation signals attenuation caused by the quencher absorption, Eq. (1) was applied:[21]

$$F_{corr} = F_{obs} \times 10^{(Ab_{ex} + Ab_{em})/2} \quad (1)$$

where Ab_{em}, Ab_{ex}, F_{obs} and F_{corr} are the mixture absorption at emission as well as excitation wavelengths, the observed fluorescence intensity, besides the corrected fluorescence intensity, respectively.

AGE Formation analysis

The emission spectra of N-HSA, G-HSA, G-HSA in ethanol solution and G-HSA in the presence of 45, 105, 135, 175, 210 and 240 μg mL^{-1} of *E. billardierei* extract at 370 nm excitation were acquired using BSFL-102 fluorescence spectrophotometer for the detection of AGE formation. Each point signifies the mean of three independent experiments.

Circular dichroism analysis

The secondary structural changes of N-HSA (5 μM), G-HSA (5 μM), G-HSA (5 μM) in ethanol solution and G-HSA in the presence of 175 and 240 μg mL^{-1} *E. billardierei* extract were monitored by model 215 circular dichroism (CD) spectrometer (AVIV, USA) with a scan speed of 20 nm min^{-1}. Measurements were recorded at 190–260 nm employing a quartz cell and a path length of 1 mm at 37 °C. Each spectrum was the average of three scans. Using subtracting the proper baseline, each CD spectrum was corrected. The spectrum of the CD buffer alone was subtracted from the spectrum of the sample containing N-HSA. However, since D-glucose exhibited a CD band around 190 nm, the CD curve of G-HSA was corrected by subtracting the spectral contribution of D-glucose-free HSA. The CD curves of G-HSA in the presence of *E. billardierei* extract were also corrected by subtracting the CD spectra of the samples containing glucose and *E. billardierei* extract mixtures. The CD curve of G-HSA in the presence of ethanol was corrected by subtracting the spectral contribution of ethanol and glucose mixtures. The CD spectra deconvolution software (CDNN, version 2.1) was applied to investigate the CD-spectra.

Determination of Amadori products

Samples were evaluated for Amadori products based on the NBT reaction with ketoamines. In carbonate buffer (pH 10.35) 0.18 μg mL^{-1} sample of protein was mixed with 0.03

mM NBT reagent and incubated for 1 h at 37 °C. Afterward, absorbance was monitored at 530 nm. Each point signifies the mean of three independent experiments.

ThT fluorescence analysis

A stock solution of ThT at 1 mM concentration was prepared in 0.01 M PBS at pH 7.4 and diluted to 0.1 μM. Then 10 μL of ThT was added to the samples (N-HSA (5 μM), G-HSA (5 μM), G-HSA (5 μM) in ethanol solution and G-HSA in the presence of 175 and 240 μg mL^{-1} *E. billardierei* extract) and then incubated for 30 min at 37 °C. Samples were scanned by BSFL-102 fluorescence spectrophotometer using an excitation/emission wavelength of 450/ /490 nm. In all cases, 10 nm emission and excitation slits were utilized. Spectra were corrected concerning the corresponding blank spectra.

Native-polyacrylamide gel electrophoresis (Native-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To test the effect of *E. billardierei extract* on glycation-induced protein aggregation, Native-PAGE and SDS-PAGE were performed *via* the standard protocol described by Schägger and von Jagow using 10 % acrylamide gel.[22] In native-PAGE electrophoresis, one aliquot of N-HSA, G-HSA, G-HSA in ethanol solution and G-HSA in the presence of 240 μg mL^{-1} extract solution were mixed with an equal volume of sample buffer (glycerol (30 %), 0.25 M Tris–HCl buffer, pH 6.8). Each lane was loaded with 20 mg of samples. Electrophoresis was carried out at 150 V for 110 min. The native proteins were visualized by incubating the gel in acetic acid (10 %) and methanol (40 %) for 25 min, Coomassie blue solution (0.02 % in methanol (30 %) and acetic acid (10 %)) for 25 min, and acetic acid (8 %) on an orbital shaker at room temperature overnight.

In SDS-PAGE electrophoresis, samples were dissolved in sample buffer (glycerol (30 %), 0.25 M Tris-HCl buffer, SDS (3.6 %), pH 6.8), and heated at 85 °C for 10 min. For reducing conditions, 50 mM dithiothreitol (DTT) was added to the sample buffer. Electrophoresis was performed at 80 V for 180 min, in running buffer (50 mM methysulfonic acid, 50 mM tris base, SDS (0.1 %), 1 mM ethylenediaminetetraacetic acid, pH 7.3). The polypeptide bands were visualized by incubating the gel in coomassie blue solution for 25 min and de-stained (ethanol (10 %) and acetic acid (7.5 %)) on an orbital shaker at room temperature overnight.

Statistical analysis

The data were recorded as mean ± standard deviation of the mean (n = 3). GraphPad Prism version 8.0 for Windows (GraphPad App, USA) was utilized for the analysis. The data were evaluated for significance utilizing a two-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Determination of TPC *and* TFC *of extract*

Phenolic compounds have a wide range of biochemical activities for instance anti-carcinogenic, anti-oxidant and anti-mutagenic.[14] Furthermore, it has been confirmed that phenolic-rich plant extracts inhibited sugar-induced protein glycation.[23] Fig. 1a displays the *TPC* of *E. billardierei* extract (from sepal and petal parts) with different concentrations and with the values derived from a calibration curve (y = 0.0106x + 0.0544, R^2 = 0.99) of GA (10–100 μg mL^{-1}). As displayed, total phenolics in the *E. billardierei* extract with the different concentrations varied from 5.01 to 22.15 mg GAE/g dry extract. Additionally, the ana-

lysis of variance confirmed that the content was increased ($P < 0.05$) with the increasing concentration of extract.

Flavonoids are secondary, anti-oxidant, metabolites whose strength is verified by the position and number of free –OH groups. Fig. 1b exhibits the *TFC* of *E. billardierei* extract with various concentrations and quercetin (20–100 μg mL^{-1}) was utilized to make the calibration curve. Values were derived from a calibration curve ($y = 0.0070x + 0.0195$, $R^2 = 0.98$). As displayed, *TFC* in *E. billardierei* extract varied from 4.01 to 20.05 mg QE/g DW of the extract. Consequently, the *E. billardierei* extract proved to have significant total flavonoids and phenolics content. Our data were in agreement with those of Daneshzadeh *et al*. who determined the *TPC* and *TFC* of *E. billardierei* extract.[14] However, it should be mentioned that Daneshzadeh *et al*. prepared their extract from both leaves and flowers of *E. billardierei*.

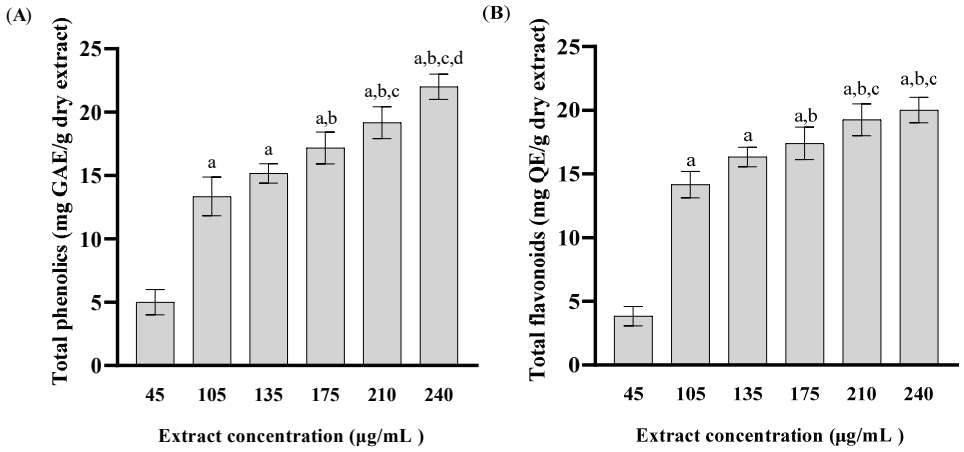

Fig. 1. a) *TPC* and b) *TFC* of *Eryngium billardieri* extract at different concentrations. $P < 0.05$ compared with the [a]45, [b]105, [c]135 and [d]175 μg mL^{-1} of extract.

UV–Vis spectroscopy results

The UV–Vis spectroscopy of the ethanolic extraction of *E. billardierei* demonstrated a maximum absorption band (λ_{max}) at about 266 nm (Fig. 2A). According to the literature, the spectra of phenolic acids and flavonoids have obvious λ_{max} at *ca*. 270 or 340 nm.[24] Furthermore, phenolic acids with the benzoic acid carbon framework have their λ_{max} in the 200 to 290 nm range.[25] Generally, flavonols in the form of glycosides, exhibit λ_{max} in the wavelength range of 260–355 nm.[24] Consequently, the *E. billardierei* extract was found to have remarkable total flavonoids and phenolics content. Additionally, there are no signals in the saponins spectrum (380 to 700 nm),[26] which confirmed the absence of sap-

onins in the *E. billardierei* extract. This observation aligns with *TPC* and *TFC* determination as mentioned earlier.

To supply information on the structural impact of the ethanolic *E. billardieri* extract on G-HSA, the UV–Vis absorption spectra of G-HSA in the absence and presence of different concentrations of *E. billardieri* extract were monitored by applying the mixture of glucose and *E. billardieri* extract at the same concentration as the reference solution. As shown in the inset of Fig. 2B, HSA has two

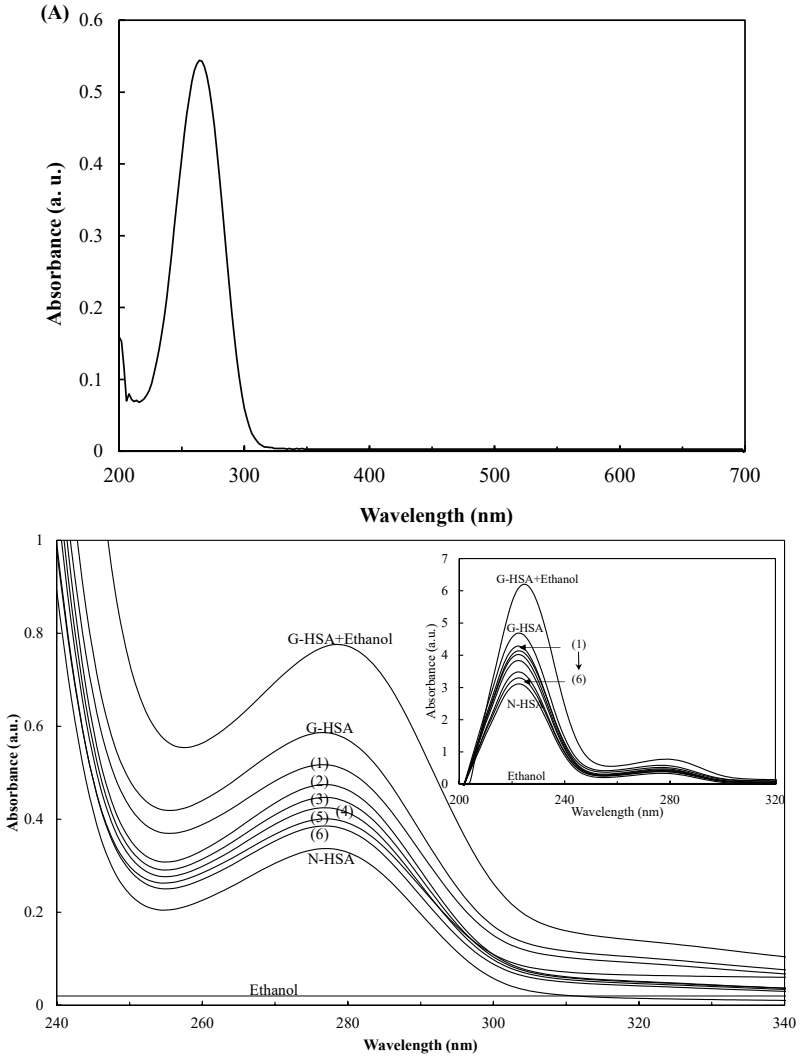

Fig. 2. The UV–Vis spectra of: a) the *E. billardierei* extract and b) N-HSA, G-HSA, G-HSA in ethanolic solution, ethanol and G-HSA in the presence of 45 (1), 105 (2), 135 (3), 175 (4), 210 (5) and 240 (6) μg mL^{-1} of *E. billardierei* extract.

λ_{max}: *ca*. 230 and 380 nm. The λ_{max} at 230 nm belongs to the n→π transition of C=O in the backbone of HSA and it additionally displays the α-helix content in the protein.[27] The λ_{max} at *ca*. 280 nm belongs to tryptophan, tyrosine and phenylalanine residues absorption.[28] According to the literature, the absorbance values in the range of 0.2–0.8 are known to offer the highest precision.[29] However, as could be found in Fig. 2B, the UV absorbance values for peptide bonds (*ca*. 230 nm) of HSA are above this range, therefore, the alterations in λ_{max} at 280 nm were investigated. The increase in λ_{max} (hyperchromicity) at 280 nm for G-HSA was observed (more than two times) with no emergence of a new peak, which could be attributed to alterations in the microenvironment of aromatic amino acids or variation of aromatic amino acids.[19] Similar observations have been reported upon incubation of HSA with glucose.[19,20] This result implies that the unfolding and cross-linking of protein helices during nonenzymatic glycation changes the conformation of HSA.[30] It is well-known that any structural perturbations hamper the normal carrying capacity of HSA.[7]

The solvent employed for the extraction of plants is crucial. The choice of solvent depends on the solvent availability, the nature of the bioactive compounds, the part of a plant to be extracted, and the type of plant. Usually, polar solvents, for instance ethanol and water, are employed in the extraction of polar compounds. Ethanol is nontoxic at low concentrations and is self-preservative at a concentration above 20 %.[31] Additionally, some results suggested that the ethanolic extract of some plants potentially have hypoglycemic effect.[32,33] Consequently, in this investigation we employed the ethanolic extract of *E. billardieri*. Thus, the effects of ethanol on the glycation of HSA were also investigated. The λ_{max} at 280 nm for G-HSA incubated in ethanol solution was even higher than G-HSA, which could be attributed to more structural alterations in G-HSA. However, As can be found in this figure the presence of different concentrations of *E. billardieri* extract in G-HSA solutions resulted in a reduction of λ_{max} in a dose-dependent manner. It should be noted that *E. billardieri* extract was added to both reference and sample cuvettes, thus, reduction in λ_{max} could be a result of the prevention of AGEs formation in HSA *in vitro*.

Tryptophan (Trp)-fluorescence measurements

One of the valuable techniques to discover protein–ligand interaction is fluorescence spectroscopy. Generally, the intrinsic fluorescence of HSA is achieved by the lone Trp residue of HSA which is located at position 214 (the Trp_{214} residue) in the hydrophobic cavity of subdomain IIA (Sudlow I).[34] Any variations in polarity around the Trp_{214} residue (fluorophore) could be evaluated by the shift in the fluorescence emission peak ($\lambda_{max,em}$). Thus, the variations in the tertiary structure of G-HSA in the presence of *E. billardieri* extract were determined by fluorescence spectroscopy (Fig. 3). The fluorescence intensity of G-HSA at excit-

ation of 285 nm decreased (about 46 %) with no obvious shift at the $\lambda_{max,em}$, thus it could be assumed that the binding of glucose to HSA is accompanied by fluctuations in the dielectric environment of the indole ring of Trp_{214} residue. Various studies have reported a decrease in the fluorescence intensity of HSA upon glycation.[2,10,35] The fluorescence intensity of G-HSA incubated in ethanol soluteion decreased noticeably, *i.e*., ethanol could induce more fluctuations in the microenvironment of the Trp_{214} residue. However, the presence of *E. billardieri* extracts restored the fluorescence of G-HSA towards native-like in a concentration-dependent manner, suggesting the fact that hydrophobic residues that get exposed upon glycation remain buried in the presence of *E. billardieri* extract.

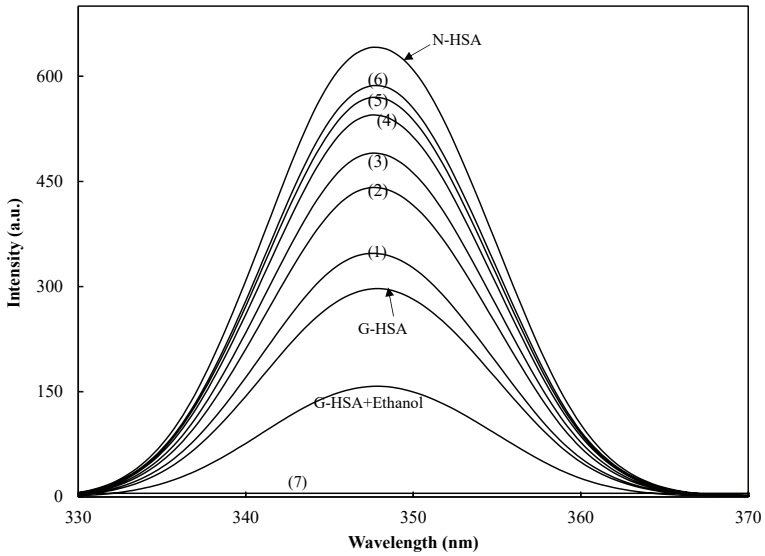

Fig. 3. Fluorescence emission spectra of f N-HSA, G-HSA, G-HSA in ethanolic solution and G-HSA in the presence of 45 (1), 105 (2), 135 (3), 175 (4), 210 (5), and 240 (6) μg mL^{-1} of *E. billardierei* extract and *E. billardierei* extract alone (7).

AGEs specific fluorescence

Regularly the specific fluorescence of AGEs is retorted to distinguish AGE formation *in vitro*. The fluorescent AGEs are the heterocyclic aromatic amines having fluorescence at a specific wavelength and giving a characteristic peak. As shown in Fig. 4, N-HSA disclosed an insignificant and weak AGE fluorescence suggesting absence of AGE adduct formation in the native protein. However, there was an obvious enhancement in the AGE-specific fluorescence in G-HSA compared to N-HSA signifying AGE formation in HSA.[19] Surprisingly, a noticeable enhancement in the AGE-specific fluorescence in G-HSA incubated in ethanol compared to G-HSA was observed. Thus, our result revealed that ethanol

could accelerate the formation of AGE products. This observation is in agreement with those of Wu *et al.* who observed the relationship between ethanol concentration and AGE production.[19] Conversely, in the presence of *E. billardieri* extract, there was a reduction (in a concentration-dependent manner) in the fluorescence intensity compared to G-HSA, indicating that *E. billardieri* extract (especially at higher concentrations) could inhibit AGE formation potentially.[6] This observation is in line with other assays suggesting *E. billardieri* extract as a potent anti-glycating agent.

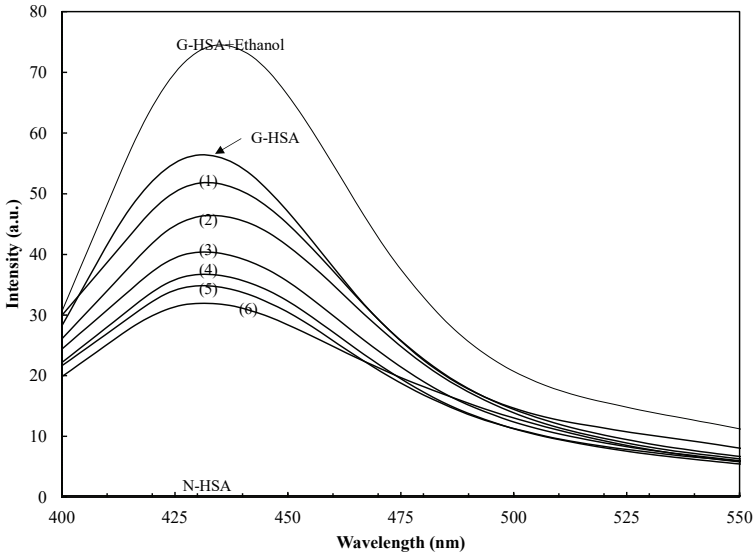

Fig. 4. The fluorescence intensity after excitation at 370 nm of N-HSA, G-HSA, G-HSA in ethanolic solution and G-HSA in the presence of 45 (1), 105 (2), 135 (3), 175 (4), 210 (5) and 240 (6) μg mL^{-1} of extract.

Determination of Amadori products consuming NBT-reducing activity

Glycation is a spontaneous reaction between the free amino groups of proteins and aldehyde or ketone groups of reduced sugars upon covalent bond formation.[20] Glycation occurs in three stages: 1) Schiff base production, 2) Amadori product formation and 3) AGE formation. Amadori product is a ketoamine that is made during the glycation process as an important and stable intermediate in the AGEs formation. The formation of Amadori products and AGEs plays a significant role in the pathogenesis of various diseases including diabetes.[10] Amadori products can reduce NBT reagent and subsequently make colored formazan dye with λ_{max} at 530 nm. Fig. 5 compares the formation of this dye in G-HSA in the absence and presence of *E. billardieri* extract. According to this figure, the content of Amadori product formation was increased in the G-HSA sample which is

in agreement with the previous observations.[6,10] In the G-HSA incubated in ethanolic solution sample a remarkable increase in Amadori product formation was observed which is in agreement with our AGEs specific fluorescence results. Conversely, the presence of *E. billardieri* extract (175 and 240 μg mL^{-1}, the two high concentrations of the extract in which the most changes in G-HSA were observed) caused a significant reduction in the content of Amadori product formation implying that the presence of *E. billardieri* extract resists non-enzymatic glycation of HSA.

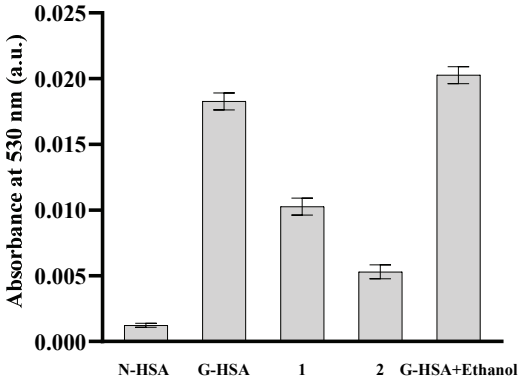

Fig. 5. NBT absorbance at 530 nm of N-HSA, G-HSA, G-HSA in ethanolic solution and G-HSA in the presence of 175 (1) and 240 (2) μg mL^{-1} of *E. billardierei* extract.

Secondary structure analysis

Protein glycation could produce protein aggregation directly. Insoluble aggregates can create an amyloid cross-structure, initiating protein stability and structure to be modified.[23] Thus, the modifications in the secondary structure of HSA were monitored by far-UV CD spectroscopy. The far-UV CD spectra of N-HSA demonstrated two double minimums at *ca*. 222 and 208 nm (Fig. 6), which demonstrated the $\pi \rightarrow \pi^*$ transition and the $n \rightarrow \pi^*$ transition of the α-helical structure of HSA, respectively.[7] Fig. 6 indicates loss of helical structure as determined by a reduction in the negative ellipticity at 208 and 222 nm in G-HSA compared to the control (N-HSA). As shown in Table I, the ratio of α-helix for N-HSA was found to be 57.9 %; however, for G-HSA it was found to be 40.1 %. This result is in agreement with previous studies.[10,19,20] G-HSA incubated in ethanolic solution exhibited a meaningful fall in the θ values at 208 and 222 nm, and a significant increase in β-sheet content was also observed. Conversely, in the presence of 175 or 240 μg mL^{-1} *E. billardieri* extract the α-helix ratio was found to be 48.4 and 50.3 %, respectively. Generally, protein glycation influences the production of protein aggregation directly. Insoluble aggregates can produce an amyloid cross-structure, making protein stability and structure to be modified. Accordingly, the presence of *E. billardieri* extract resisted secondary

structural alterations and restored the far-UV CD spectra towards native-like in a dose-dependent manner indicating a potent anti-glycation agent.

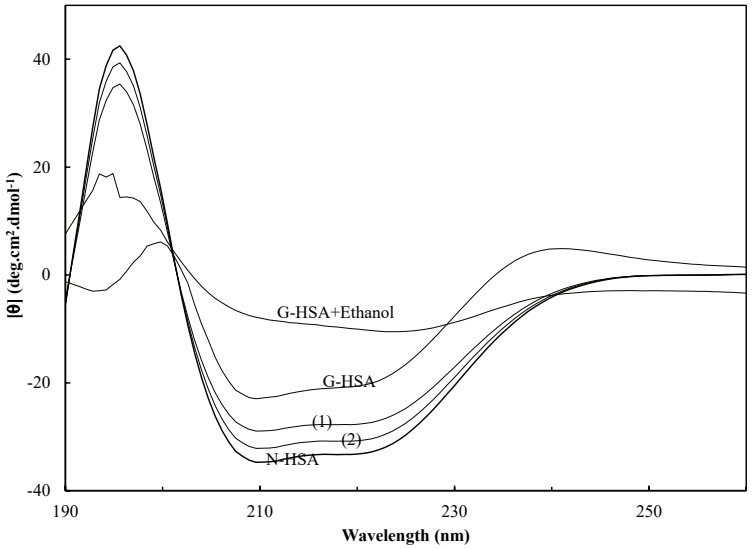

Fig. 6. The CD spectra of N-HSA, G-HSA, G-HSA in ethanolic solution and G-HSA in the presence of 175 (1) and 240 (2) μg mL^{-1} of *E. billardierei* extract.

TABLE I. Content of the secondary structure of HSA and glycated HSA in the absence and presence of the *E. billardierei* extract and ethanol at 37 °C

Sample	α-Helix, %	β-Sheet, %	Random coil, %
N-HSA	57.9	23.3	18.8
G-HSA	40.1	30.1	29.8
G-HSA+extract (175 μg mL^{-1})	48.4	24.1	27.5
G-HSA+extract (240 μg mL^{-1})	50.3	25.7	24.0
G-HSA in ethanolic solution	21.6	43.3	35.1

Determination of fibrillar state with ThT

ThT is a biomarker that interacts with the amyloid fibril structure of proteins making the enhancement of fluorescence intensity in the range of 480–490 nm upon excitation at 450 nm.[6] Accordingly, to determine if *E. billardieri* extract acts as an inhibitor of the amyloid-like aggregates, the fibrillar state with ThT was studied. According to Fig. 7, N-HSA showed minimal fluorescence indicating HSA to be in its native conformation. However, the fluorescence intensity of G-HSA was enhanced compared to N-HSA suggesting that aggregate formation has taken place.[6] The fluorescence intensity of G-HSA incubated in ethanol compared to G-HSA was also enhanced considerably exhibiting an increase in amyloid cross-conformation,[23] *i.e.*, ethanol could induce amyloid-like aggregates

in G-HSA, significantly. This observation is in agreement with our secondary structure analysis. However, G-HSA in the presence of *E. billardieri* extract (175 and 240 μg mL^{-1}) displayed decreased ThT fluorescence. Thus, the presence of *E. billardieri* extract could inhibit fibril formation. Hence, it can be expressed that *E. billardieri* extract is a potent mixture that can prevent fibril formation, help protein maintain its native conformation, and prevent several pathological conditions associated with protein aggregation.

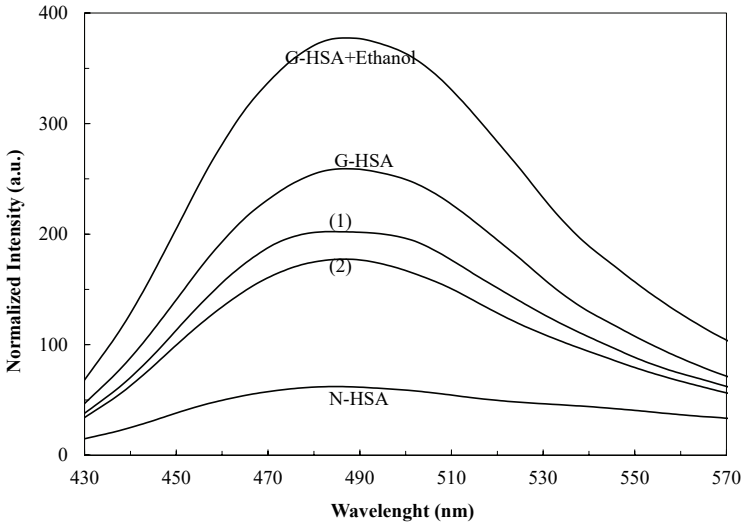

Fig. 7. ThT fluorescence emission spectra of N-HSA, G-HSA, G-HSA in ethanolic solution and G-HSA in the presence of 175 (1) and 240 (2) μg mL^{-1} of *E. billardierei* extract.

Native-PAGE and SDS-PAGE Electrophoresis

HSA is a 66 kDa protein with an isoelectric point (p*I*) of 4.7, *i.e.*, the surfaces of HSA carry negative charges at pH > 4.7 and positive charges in solutions at pH < 4.7.[36] Thus, at neutral pH (7.4), HSA has regions that are strongly negatively charged even though it also has positively charged pockets.[7] The attachment of glucose affects the surface charge of HSA which is attributed to the surface amino acids modification.[36] It has been reported that the pI of the G-HSA has a higher acidic value than N-HSA.[37] Fig. 8a exposed that G-HSA revealed a faster anodic migration than N-HSA, indicating that significant glycation had occurred. As observed in Fig. 8a, G-HSA in ethanolic solution revealed a faster anodic migration than G-HSA. However, treatment of G-HSA with 240 μg mL^{-1} of *E. billardierei* extract restored the electrophoretic migration toward N-HSA demonstrating the protective role of *E. billardierei* extract against glucose-mediated glycation.

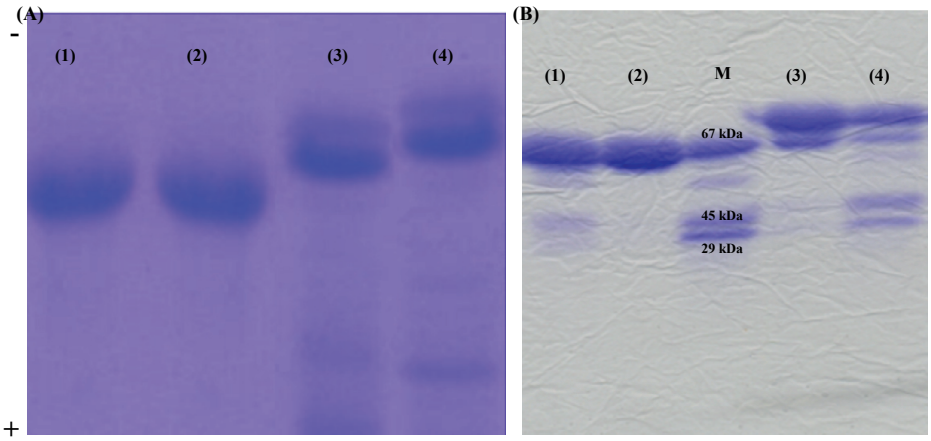

Fig. 8. a) Native-PAGE and b) SDS-PAGE of the effects of *E. billardierei* extract on protein aggregation. Lane 1: N-HSA, lane 2: G-HSA in the presence of 240 μg mL^{-1} of extract, lane 3: G-HSA and lane 4: G-HSA in ethanolic solution. M: molecular mass marker.

It has been accepted that after prolonged glycation, protein could form micelle-like aggregates.[23] The glycation of albumin causes structural changes and an increment in the total molecular weight (*MW*) of the protein. It has been shown that glycation could promote strong conformational changes in protein that affect both tertiary and secondary structures. Hence, glycation could promote amyloid aggregation in protein both reducing the helical content and supporting *β*-cross structure formation that rapidly evolves to the formation of amyloid aggregates.[38] The electrophoretic mobility of N-HSA, G-HSA and *E. billardierei* extract-treated HSA samples was also evaluated on SDS-PAGE. As shown in Fig. 8b, N-HSA displayed a single parental band, while G-HSA presented multiple bands. The other band with lower MW may include the fragments of HSA.[19] Furthermore, the relative mobility of G-HSA and N-HSA was different. G-HSA sample demonstrated reduced mobility compared to N-HSA indicating the attachment of glucose molecules. This observation is in agreement with previous studies in which glycation of HSA by glucose resulted in reduced electrophoretic mobility.[39] G-HSA in ethanolic solution revealed the same relative mobility as the G-HSA sample. Lambrecht *et al.* stated that aqueous ethanol with a concentration of 50 vol. % could result in the formation of disulfide bonds between BSA, notably.[40] Siddique *et al.* exposed that the formation of disulfide bonds played a critical function in the formation of protein aggregates.[41] Feng *et al.* also noted that ethanol could induce disulfide cross-linking and perform a fundamental role in BSA and whey protein isolates aggregate formation.[42] Accordingly, it could be concluded that ethanol-induced disulfide cross-linking plays a crucial role in G-HSA aggregate formation. Treatment of G-HSA with *E. billardierei* extract

restored the electrophoretic migration toward N-HSA demonstrating the protective role of *E. billardierei* extract against glucose-mediated glycation.

Protein glycation directly influences protein aggregation production. Insoluble aggregates can create an amyloid cross-structure, generating protein stability and structure to be changed. In summary, our findings prove that the presence of *E. billardieri* extract decreased Amadori product formation and structural alterations of HSA incubated with glucose. Previous studies verified that phenolic-rich plant extracts prevented sugar-induced protein glycation.[6,43] The phytochemical analysis disclosed that *E. billardieri* extract is high in flavonoid and phenolic compounds. It has been proved that through the glycation process, bioactive constituents such as kaempferol, salicylic acid, carvacrol, gallic acid, rutin and ferulic acid could employ their glycation inhibition impacts by chelating redox-inducing transition metal ions, scavenging free radicals, and neutralizing reactive carbonyl intermediates. Thus, it could be concluded that the phenolics in *E. billardieri* extract could prevent glucose-induced HSA glycation. This favorable effect could aid in lowering the risk of diabetes complications. However, further research is required to verify the actual mechanism of *E. billardieri* extract inhibition and the active chemicals involved in the process.

CONCLUSION

AGEs are correlated with pathophysiological conditions accordingly making studies, related to protein glycation, of clinical importance. To the best of our knowledge, this research is the first attempt to clarify the effects of *E. billardieri* extract on the structure of HSA from a molecular point of view. The findings of this paper verify that the ethanolic extract of *E. billardieri* could inhibit the pathway of AGE formation by interfering with the glycation of HSA. This study provides insight into the ability of *E. billardieri* extract to prevent the glycation and unfolding of HSA. Hence, although further investigations are warranted to draw firm conclusions, the presence of *E. billardieri* extract in foods could inhibit glycation reactions under hyperglycemia providing protection against pathogenic impacts of AGEs during diabetes.

ИЗВОД

ЕТАНОЛНИ ЕКСТРАКТ *Eryngium billardierei* F. DELAROCHE СПРЕЧАВА ГЛИКОВАЊЕ ХУМАНОГ СЕРУМСКОГ АЛБУМИНА: СТУДИЈА *IN VITRO*

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Гликовање протеина је директно повезано са многим патолошким стањима. У овој студији је испитан потенцијал екстракта *Eryngium billardierei* да инхибира процес гликовања хуманог серумског албумина (HSA). Структурне промене глико-

ваног HSA су испитане у одсуству и присуству различитих концентрација етанолног екстракта *E. Billardierei* користећи методе циркуларног дихроизма, флуоресцентне и UV–Vis спектроскопије. Резултати су показали да екстракт *E. billardierei* може смањити стварање крајњих производа узнапредованог гликовања (AGE) и Амадори производа у *in vitro* условима, као и да повећа удео хеликса у структури HSA. Такође је, применом тиофлавин T теста, утврђено смањење стварања унакрсних амилоидних влакана HSA. Фитохемијска анализа је указала на висок садржај флавоноидних и фенолних једињења у екстракту *E. billardieri*, што упућује на закључак да ова фенолна једињења могу спречити гликовање HSA индуковано глукозом. Резултати ове студије потврђују да се екстракт *E. billardieri* може укључити у контролу дијабетеса.

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