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ORIGINAL ARTICLE

Inhibition of Glycation-induced Cytotoxicity, Protein Glycation, and Activity of Proteolytic Enzymes by Extract from *Perovskia atriplicifolia* Roots

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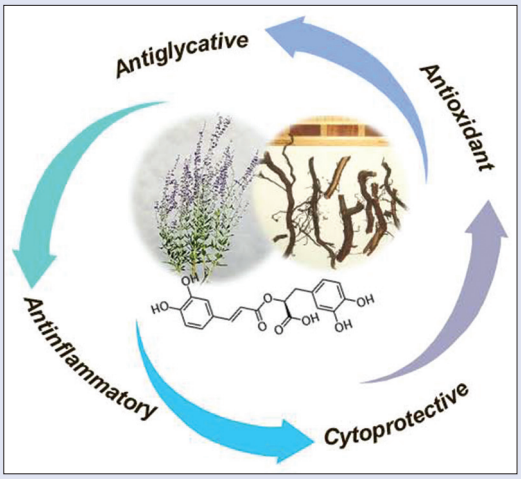
ABSTRACT

Background: Protein glycation and glycotoxicity belong to the main oxidative-stress related complications in diabetes. *Perovskia* species are used in Asian folk medicine as antidiabetic herbs. **Objective:** The aim of this study was to verify the ability of the methanolic extract from *Perovskia atriplicifolia* Benth. roots to diminish glycation of albumin and to prevent cell damage *in vitro*. Furthermore, we tested the extract for *in vitro* antioxidant activity and inhibition of elastase and collagenase. **Material and Methods:** The aqueous methanol extract was analyzed by UHPLC-MS for the content of polyphenols and terpenoids. The prevention of glycated albumin-induced cell damage was tested in four mammalian cell lines (peripheral blood mononuclear cells, human embryonic kidney cells – HEK293, normal human fibroblasts, and Chinese hamster ovary cells) with the 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium assay. **Results:** Glycated albumin is significantly more toxic than native human serum albumin (LC_{50} from 35.00 to 48.34 μg/mL vs. 5.47–9.10 μg/mL, respectively). The extract, rich in rosmarinic acid (344.27 mg/g dry mass), mitigated the glycated albumin toxicity, and increased glycated albumin-treated cell survival by more than 50%. The inhibition of advanced glycation endproduct formation was confirmed by monitoring conformational changes. The free radical scavenging activity was higher than Trolox and metal reducing power was one-third to half that of ascorbic acid. The activity of elastase and collagenase was inhibited by 54.75% ± 6.87% and 60.03% ± 7.22%, respectively. **Conclusions:** The results confirm antiglycative and antiglycotoxic potential of *Perovskia* root and its traditional antidiabetic use. The high activity can be attributed to rosmarinic acid abundance.

Key words: Advanced glycation end-products, antioxidant, cytoprotective, rosmarinic acid

SUMMARY

- Perovskia is a small genus of aromatic shrubby plants growing in arid regions of Central and South Asia. Different parts are used in folk medicine as antiparasitic, anti-infectious and antidiabetic remedy. Here, we have studied the extract from roots for inhibition of: glycation-induced cytotoxicity, human serum albumin glycation, inflammation-related enzymes, as well as for antioxidant activity. Result: the extract from P. atriplicifolia roots inhibited protein glycation and AGE-induced toxicity in cell cultures. The mechanism is likely to rely on the antioxidant activity of high content of rosmarinic acid.



Abbreviations used: AGE: advanced glycation end-products; DPPH: 2,2-diphenyl-1-picrylhydrazyl; HSA: human serum albumin.

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INTRODUCTION

Several species of *Perovskia* are distributed in arid and semi-arid regions of Central and South Asia, but only three of them have been studied for phytochemical and pharmacological properties. *Perovskia atriplicifolia* Benth. (Lamiaceae) is a popular and undemanding ornamental shrub, cultivated worldwide for its spectacular blooming and pleasant scent. Its natural distribution ranges from East of Iran, through Pakistan and Afghanistan, to Tibet and South-West China. It is a folk medicinal herb in the areas of natural occurrence. Most of the published data refer to the composition and activities of essential oils from aerial parts. Roots of *Perovskia* contain considerable amounts of red-colored quinoid norabietanoids called tanshinones and are considered an alternative source of these valuable pharmacologically active compounds in addition to the

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renowned Traditional Chinese Medicine drug – *Salvia miltiorrhiza rhizoma* and *radix*. Typically for Nepetoideae subfamily of Lamiaceae, *Perovskia* is also abundant in rosmarinic acid, another plant natural product known for its numerous health promoting properties.[1-3] Other compounds present in different parts of this plant include other phenolic acids, lignans, flavonoids, and di- and triterpenoids.[2,4-9] *P. atriplicifolia* is used as folk medicine to treat a variety of ailments, including diabetes.[10-12] However, the anecdotic information on the antidiabetic use of *Perovskia* sp. in the regions of their natural distribution has not been confirmed by any experimental data.

Nonenzymatic reaction between reducing sugar and free amino group of proteins, also known as Maillard reaction, leads to the formation of glycated protein termed Amadori product. Further rearrangement, oxidation, and reduction of the Amadori product result in the formation of several advanced glycation end products (AGEs) such as pentosidine, carboxymethyllysine, crossline, and pyralline. Several studies have reported that AGEs are generated in the diabetic milieu as a result of chronic hyperglycemia and enhanced oxidative stress. AGEs through direct and receptor-dependent pathways, promote the development, and progression of diabetic complications, including neuropathy, nephropathy, and cardiovascular disease. The oxidation process is believed to play an important role in AGEs formation. Further oxidation of Amadori product leads to the formation of intermediate carbonyl compounds that can react with the nearby lysine or arginine residues to form protein crosslink and AGEs. The reactive carbonyl compounds may also be generated from the metal ion-catalyzed autoxidation of glucose. Therefore, agents with antioxidative or metal-chelating property may retard the process of AGEs formation by preventing further oxidation of Amadori product and metal-catalyzed glucose oxidation. In this regard, several natural compounds known to possess antioxidative property, such as curcumin, rutin, garcinol, and flavonoid-rich extracts, have been shown to prevent AGEs formation *in vitro* and *in vivo*.[13]

Human serum albumin (HSA) is the most abundant plasma protein in human serum. It is known to function as a multipurpose transport protein and may also perform a protective role *in vivo* as a circulating antioxidant. The *in vitro* exposure of proteins to glucose in phosphate buffer results in the nonenzymatic covalent attachment of glucose to lysine side chains in a manner that resembles that observed *in vivo*. Circulating AGEs can elicit cellular damage by binding to several membrane receptors. For example, receptor for AGE (RAGE) expression is upregulated in diabetes and inflammation, causing intracellular oxidative stress.[13] Therefore, inhibiting AGE formation and moderating their detrimental effects on cells is essential for preventing diabetes- and inflammation-related complications.[14]

In this study, we have tested the potential of methanolic extract from *P. atriplicifolia* roots to prevent *in vitro* formation of AGE, cytoprotective activity against AGE-induced toxicity in several human cell lines. Further, we proved the ability to inhibit two proteolytic enzymes involved in inflammatory processes - elastase and collagenase. In addition, we verified *in vitro* antioxidant power of this extract using several screening assays based on complementary mechanisms.

MATERIAL AND METHODS

Reagents

The chemicals, enzymes, and other reagents were from Sigma-Aldrich Chemical Company (St. Louis, USA) unless stated otherwise.

Plant material

Roots of *P. atriplicifolia* were obtained from plants cultivated in the certified collection of the Botanical Garden of Medicinal Plants at the Medical University of Wroclaw, Poland (identity was confirmed using Flora of Pakistan, URL: http://www.tropicos.org/Project/Pakistan - last Accessed for the purpose of source plant identification on 1.10.2016; the sample specimens are stored in the Botanical Garden herbarium under "Lamiaceae-*P. atriplicifolia* 2013/1-4"). The material was collected from 8-year-old plant in October 2013; the roots were cleaned from soil, cut, and dried in the shade at ambient temperature (between 21°C and 25°C). Immediately, before extraction, the roost was pulverized in an herbal mill (Vorwerk, Germany).

Preparation of extracts

A volume of 100 g of accurately weighed ground roots were soaked in 500 mL of 80% (v/v) aqueous methanol and sonicated at 35°C for 6 h. After the extraction, the solvent was removed through a filter paper and the extraction procedure repeated two times. The solvent was evaporated under reduced pressure in the rotary evaporator (Laborota, Heidolph, Germany). The extraction yield was 10.71 g. The portions of the dried extract were dissolved in appropriate solvents at various concentrations for further experiments.

LC-MS analysis of the extract composition

The extract was dissolved in 80% aqueous MeOH (LC-MS grade from JT Baker, UK; LCMS grade ultrapure water from Merck, Germany), filtered through the 0.22 μm syringe filter (Carl Roth, Germany) at the concentration of 1 mg/mL and analyzed for tanshinones and rosmarinic acid content as described in our previous paper.[9]

Preparation of glycated albumin

The preparation of glycated HSA was based on the modified previously described[15] method with bovine serum albumin. HSA was dissolved in 100 mM phosphate buffer pH 7.4 to give a stock solution of 4 mg/mL. This was subsequently diluted with glucose stock solutions dissolved in the same buffer, to form incubation mixtures that contained a final concentration of 2 mg/mL HSA and 50 mM glucose with or without addition of the *Perovskia* extract at 100 μg/mL. Solutions were filter sterilized (0.22 μm filters) and incubated in capped vials at 37°C for up to 8 weeks. Reversibly, bound and nonbound glucose was removed from HSA by exhaustive dialysis against 50 mM phosphate buffer pH 7.4 and the HSA samples stored until used in the further experiments at 4°C.

5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium cytotoxicity assay

For this assay, peripheral blood mononuclear cells, human embryonic kidney cells (HEK293), human normal fibroblast, and Chinese hamster ovary (CHO) cell lines were used and cytotoxicity of extracts, albumin, and glycated albumin with and without extracts on these cells was assessed. Cell lines were purchased from Sigma-Aldrich (USA) and stored frozen until used for the experiments. For each experiment, cultures were seeded from frozen stocks. Cells were maintained in Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 10% fetal bovine serum. All cell lines were incubated at 37°C in a 5% CO_2 atmosphere and were in the logarithmic phase of growth at the time of the 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium (MTS) assays. Cells were harvested and seeded into 96-well tissue culture plates at a density of 2×10^4 cells per well with 200 μl aliquots of medium treated with glycated (HSA-G) or nonglycated albumin (HSA) as negative and positive controls and glycated albumin with the tested extract at the concentration of 100 μg/mL. The same extract concentration was also uses as control with nonglycated albumin. The cells were allowed to adhere to the wells for 24 h at 37°C in a humidified atmosphere optimized with 5% CO_2 in air. The next day, 40 μl of MTS reagent: 2 mg/mL MTS, and

0.21 mg/mL phenazine ethosulfate in phosphate-buffered saline (PBS), was added to the wells and incubated for another 4 h. All experiments were performed at least three times. Blank was prepared by replacing the MTS reagent with the same volume of PBS buffer. After the 4 h exposure, the endpoint absorbance was determined at 492 nm. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells that served as negative control. In a preliminary experiment, the extract was also tested for potential effect on cell viability at the concentration of 100 μg/mL.

In vitro antiglycative activity

Congo red assay

Congo red binding to amyloid cross β-structure was estimated by measuring absorbance at 530 nm as mentioned by Klunk *et al.*[16] Briefly, glycated samples (500 μL) were incubated with 100 μL of 100 μM (in PBS with 10% [v/v] ethanol) Congo red (Merck, Germany) for 20 min at room temperature. Absorbance was recorded for the Congo red-incubated samples as well as for Congo red background.

Intrinsic fluorescence measurements

Tryptophan specific fluorescence of native HSA and HSA glycated with 50 mM glucose was measured by a Jasco FP-770 fluorimeter (Tokyo, Japan). Both native as well as glycated-HSA were excited at 280 nm. Native HSA showed a strong emission peak at 340 nm because of its single tryptophan residue (Trp 214). Glycation-induced structural change in glycated-HSA was evident from 43.2% loss in intensity.

Circular dichroism spectropolarimetry

Circular dichroism (CD) spectra of HSA–G mixtures treated with extracts and control samples were recorded in the far-ultraviolet (UV) region (190–250 nm) on a Jasco J-720 spectropolarimeter (Tokyo, Japan) using a quartz cuvette of 1 mm path length. Spectra obtained are averages of 10 consecutive scans performed using a bandwidth of 1 nm, response time of 2 s and a scan speed of 20 nm/min and baseline corrected by subtracting corresponding blanks.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli[17] with 5% stacking and 10% separating gels. One aliquot of glycated BSA solution was mixed with an equal volume of SDS sample buffer (20 mg/mL SDS, 30% glycerol, 0.25 M Tris–HCl buffer, pH 6.8) and boiled for 3 min. After electrophoresis, the gel was visualized by Coomassie brilliant blue R-250 staining.

In vitro antioxidant activity

Determination of total phenol content

Total phenol content was estimated as gallic acid equivalents (mg gallic acid/g extract) according to Singleton and Rossi.[18] In brief, 100 μl aliquot of extract was transferred to a 10 mL volumetric flask, containing calcium 6.0 mL H_2O, to which 500 μl Folin–Ciocalteu's reagent (Avantor, Poland) was subsequently added. After 1 min, 1.5 mL of 20% (w/v) Na_2CO_3 was added and the volume was made up to 10 mL with H_2O. After 2 h of incubation at 25°C, the absorbance was measured at 765 nm and compared to a gallic acid calibration curve.

DPPH• free radical scavenging

The ability of extracts to scavenge DPPH• radicals was determined using a modified method described in our previous paper.[19] In brief, a 50 μl aliquot of extracts was mixed with 450 μl Tris–HCl buffer (50 mmol/l, pH 7.4) and 500 μl (0.2 mmol/l) DPPH• dissolved in methanol. The final concentrations of extracts were 1, 2, 5, 10, 25, 50, and 100 μg/mL. After a 30-min reaction period, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated using following equation:

$$\text{Percentage inhibition} = ([\text{Abscontrol} - \text{Abs sample}]/\text{Abscontrol}) \times 100 \quad (1)$$

The results were also expressed as EC_{50} - the concentration needed to cause half of maximum response calculated from nonlinear regression curve using GraphPad Prism 5.0 (La Jolla, USA). Rosmarinic acid (Fluka, Switzerland) and Trolox (Sigma-Aldrich, USA) were used as positive controls.

$ABTS^{•+}$ free radical scavenging

The ability of extracts to scavenge $ABTS^{•+}$ radical scavenging was determined by the method of Re *et al.*[20] The $ABTS^{•+}$ radical was generated by reacting an (7 mmol/L) ABTS (2,2-Azinobis (3-ethylbenzothiazoline-6-sulphonate) diammonium salt) (Sigma-Aldrich, St. Louis, USA) aqueous solution with $K_2S_2O_8$ (2.45 mmol/L, final concentration) in the dark for 12–16 h, at ambient temperature, and adjusting the Abs 734 nm to 0.700 (7 0.020) with ethanol. Next, 1.485 mL $ABTS^{•+}$ solution was added to 15 μl sample, the absorbance at 734 nm was recorded 1 min after initial mixing and subsequently at 5 min intervals (40 min, in to). The results are expressed as the Trolox equivalent antioxidant capacity (TEAC, mmol Trolox per g of extract) at different time intervals.

Reducing power

Iron(III) to iron(II) reduction

The iron(III) reductive capacity of the extracts was assessed spectrophotometrically according to the method of Oyaizu.[21] In brief, 1 mL of dissolved compound was mixed with 2.5 mL phosphate buffer (0.2 mol/l, pH 6.6) and 2.5 mL of a 10 g/L potassium hexacyanoferrate (III) ($K_3(Fe(CN)_6)$) solution. After 30 min at 50°C, 2.5 mL of a 100 g/L aqueous trichloroacetic acid solution was added and the mixture was centrifuged for 10 min (1800 rpm). Finally, a 2.5 mL aliquot was mixed with 2.5 mL ultrapure water and 0.5 mL of a 1 g/L $FeCl_3$ solution and the absorbance was recorded at 700 nm. The data are presented as ascorbic acid equivalents (AAEs; mmol ascorbic acid/g sample) calculated using ascorbic acid calibration curve.

Phosphomolybdenum test

The modified method of Prieto *et al.*[22] was used as described in our previous paper.[19] The extract at the concentrations range of 10–500 μg/mL was mixed with the reagent solution containing ammonium molybdate tetrahydrate (4 mM), tribasic sodium phosphate dihydrate (28 mM), sulfuric acid (600 mM) (all from Avantor, Poland, analytical grade). The samples were incubated at 90°C for 60 min. After cooling down to room temperature, the absorbance of the green Mo(V) complex (max. absorption at 695 nm) was measured as a result of the reduction of Mo(VI) to Mo(V). For reference, appropriate solutions of ascorbic acid were used, and the reducing capacity of the analyzed fractions was expressed as the AAEs calculated from K1/K2 ratio where K1 was the slope coefficient of linear dose response function of the samples, and K2 – the slope coefficient of ascorbic acid.

Inhibition of elastase and collagenase activity

Elastase

The reaction mixture contained: 250 μl of 700 μM substrate N-Succinyl-ALA-ALA-ALA p-Nitroanilide (SucALA3PNA, Sigma-Aldrich, USA) dissolved in 100 mM TRIS-HCl (pH 8.0) buffer, 100 μl of *Perovskia* extract solution and 250 μl of porcine pancreatic elastase (Sigma-Aldrich, USA) with activity adjusted to 0.5 U/mL in the same buffer. The mixtures prepared in the 48-well polystyrene plate were incubated for 1 h at 37°C in a microplate shaker (DTS-4, ELMI, Latvia). Thereafter, the reaction was stopped by adding 500 μl of 2 mg/mL

soybean trypsin inhibitor (Sigma-Aldrich, USA) and absorbance was read using microplate spectrophotomater (μQuant, Biotek, USA) at λ = 405 nm against a blank. Results are expressed as inhibition percent compared to the sample without extract. Oleanolic acid (Carl Roth, Germany) was used as a reference inhibitor of enzyme activity.

Collagenase

The inhibition of collagenase activity was tested using chromogenic substrate FALGPA (N-(3-(2-furyl) acryl)-Leu-Gly-Pro-Ala) (Sigma-Aldrich USA). The substrate was dissolved in TRICINE buffer (50 mM of N-(Tri (hydroxymethyl) methyl) glycine, containing $CaCl_2$-10 mM and 0.4 M NaCl at pH = 7.5). The reagent mix contained 280 μl of substrate solution and 20 μl of enzyme solution (1.6 U/mL in TRICINE buffer) with or without tested extract. The absorbance change at λ = 345 nm was monitored at 25°C every 1 min for 30 min against a blank. Oleanolic acid was used as a reference inhibitor. Negative control contained enzyme solution without an inhibitor. The activity of extract was expressed as percent of inhibition of enzymatic activity.

Statistical analysis

DPPH, phosphomolybdenum, and enzyme inhibition assays were performed twice in eight repetitions. All other experiments were performed twice, each time in triplicates. Means, standard errors, standard deviation, one-way ANOVA, and paired *t*-test were calculated from replicates within the experiments and analyses were done using SPSS version 22 (IBM, Armonk, USA). Statistical significance was accepted at a level of $P < 0.05$. Dose response (EC_{50}, IC_{50}, LC_{50}) was calculated from nonlinear regression using GraphPad Prism version 5.0.

RESULTS

5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium cytotoxicity assay

To address the question whether the cell-damaging effect of AGE mitigates in the presence of extract, we tested its effect on viability of four mammalian (three human and one of hamster) cell lines. As shown in Figure 1a, the glycated albumin exerts the toxic effect in much lower doses than native HSA (LC_{50} from 35.00 to 48.34 μg/mL vs. 5.47–9.10 μg/mL, respectively). The percentage of cell viability also dramatically dropped down to 37.13-41.35% depending on the cell line [Figure 1b]. The co-incubation with *Perovskia* extract mitigated G-HSA toxicity that was demonstrated both by increased cell survival and higher LC_{50} of G-HSA (24.34–31.67 μg/mL) in the presence of extract. The differences in the treatment response between the four cell lines were not statistically significant (at $P < 0.01$) in case of cell survival rate. However, when LC_{50} was considered, the HEK and CHO cells were clearly more sensitive to the presence of native HSA and less than two other lines sensitive to G-HSA, whereas the beneficial effect of the extract was only slightly lower for those lines.

In the used concentration (30 μg/mL), the toxicity of G-HSA was not completely abolished (77.94%–89.26% viability rate compared to the control). The extract was not toxic (no decrease in cell survival) to any of the tested cell lines (data not shown).

Influence on human serum albumin glycation

Congo red binding assay

Congo red binding assay can be used to estimate the degree of modification occurred within secondary structure of protein. Congo red has a specific binding affinity to the β-sheet structure of proteins and exhibits specific absorption at 530 nm after binding. The dye binds to hydrophobic clefts between antiparallel β-strands. Therefore, to assess whether our compounds may bring about any protection in

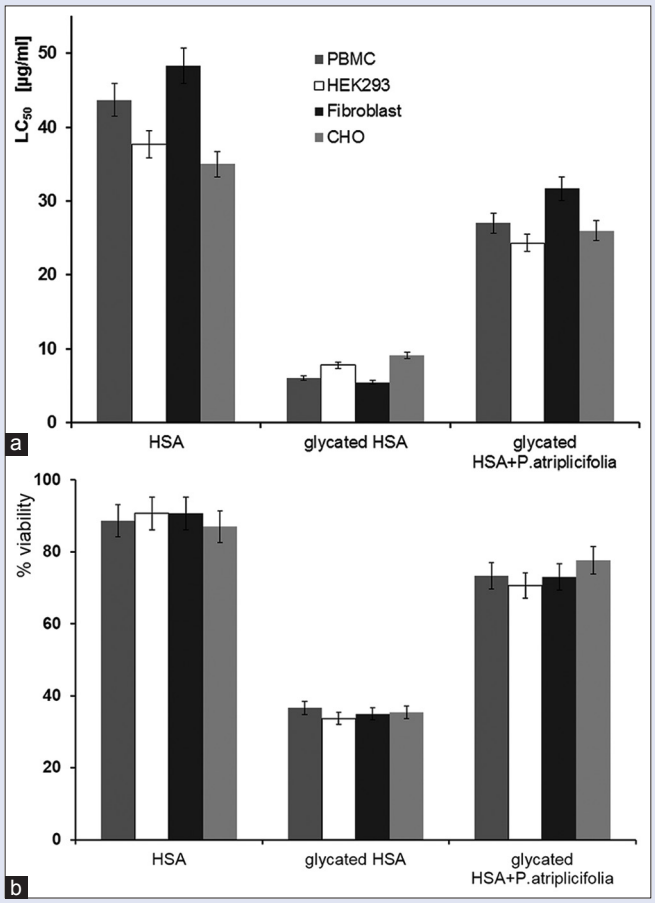

Figure 1: Effect of *Perovskia atriplicifolia* extract on *in vitro* viability of cell lines treated with glycated human serum albumin: (a) expressed as LC_{50} of added albumin, showing significant increase tolerance of cells to advanced glycation end products-induced toxicity; (b) expressed as cell survival percentage, showing partial restoring of viability of cells challenged with glycated HAS in the presence of extract

secondary structure of HSA, the Congo red staining was performed. The time dependence plot of spectral intensities of Congo red-HSA-AGE solutions [Figure 2] shows the different extent of glycosylation process. While the affinity of Congo red for glycated HSA is raised noticeably over the measure period (12 weeks), a decrease in the signal was observed in the presence of the extract ($P < 0.05$). This observation makes it evident that the extract protected the structural transition from α-helix to cross-β structure. One possible explanation is that the extracts bearing potentials to suppress alterations in the alpha conformers by concealing the glycation sites and lowering the extent of solvent-accessible surface area thereby producing barriers for cross β-structure formation.

Intrinsic fluorescence measurements

Native HSA showed a strong emission peak at 340 nm because of its single tryptophan residue (Trp 214). To investigate if AGE formation proceeds in the HSA–glucose system, autofluorescence measurements were performed. Glycation-induced structural change in glycated-HSA was evident from ~50% loss in intensity. Fluorescence intensity of glycated HSA was quenched with an obvious red shift in the presence of extract, suggesting its ability to prevent AGE formation [Figure 3].

Circular dichroism spectropolarimetry

To analyze HSA-G structure in greater detail, CD spectropolarimetry was performed. Far-UV-CD spectra of albumin, between 190 and

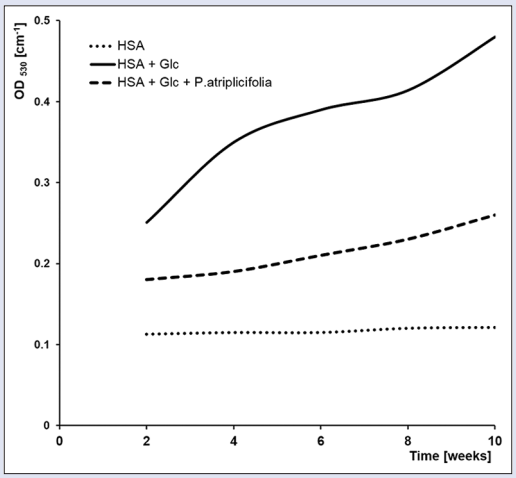

Figure 2: Time evolution of secondary structure alteration in human serum albumin monitored by Congo Red binding assay at 530 nm

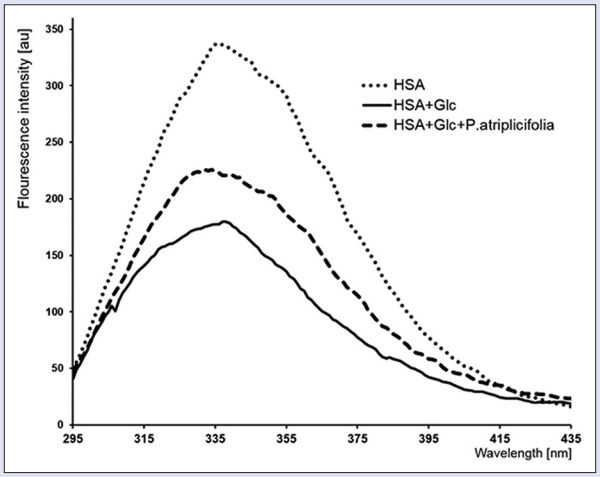

Figure 3: Fluorescence spectra of glycated samples of human serum albumin that were obtained in the wavelength range of 295–445 nm after excitation at 270 nm in the presence and absence of 30 μg/mL *Perovskia atriplicifolia* extract

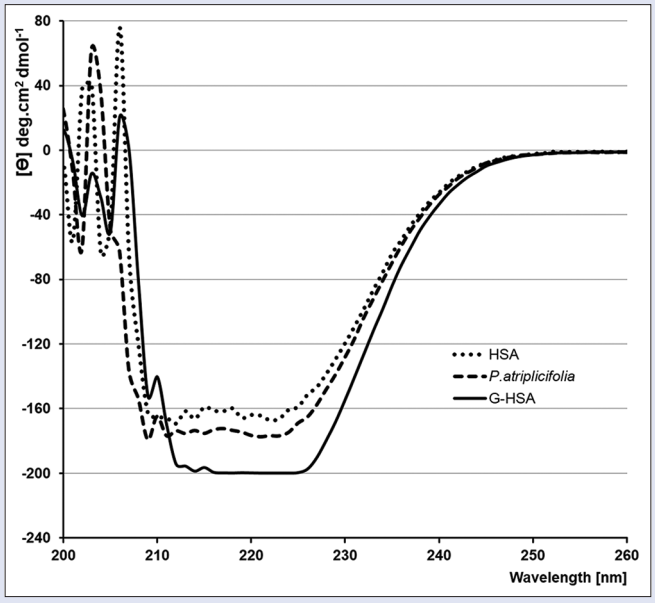

Figure 4: Far-ultraviolet circular dichroism spectra of native and glycated human serum albumin in the presence and absence of *Perovskia atriplicifolia* root extract. The circular dichroism data were expressed as molar ellipticity (deg cm^2/dmol)

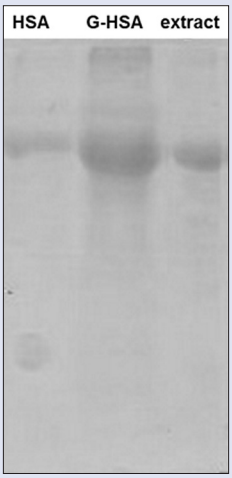

Figure 5: Electrophoretic pattern of native human serum albumin (lane on the left) and human serum albumin incubated for 8 week with 50 mM glucose without (middle lane) or with *Perovskia atriplicifolia* extract (lane on the right). The mobility retardation of G-human serum albumin is decreased in the presence of the extract

250 nm, after 14 weeks incubation with glucose was recorded [Figure 4]. Glycation leads to large alteration in the secondary structure of HSA, which appreciably protected by the presence of *Perovskia* extract. CD spectra of control solution indicate a significant alteration in the content of β-conformer even after this period, whereas the most considerable beta structure formation occurs on glycation within the same period. Hence, the higher level of α-helix obtained in the experiment could be attributed to antiglycative effects of the extract.

Electrophoretic pattern of native and glycated human serum albumin

Figure 5 shows the migration pattern of native and glycated samples in 10% SDS-PAGE. As compared to native HSA, glycated sample showed appreciable change in electrophoretic mobility with concomitant increase in band intensity. Moreover, HSA incubated with 50 mM glucose showed maximum change in electrophoretic mobility accompanied with an increase in band intensity. Increased hyperchromicity and change in electrophoretic migration pattern of G-HSA were also significantly alleviated in the presence of the tested extract. The preliminary experiment with a higher dose of extract did not suggest a significant increase in the antiglycative effect, though.

Antioxidant activity

Free radical scavenging

DPPH• free radical scavenging

This was estimated using nitrogen-centered free radical (●DPPH), which serves as the reaction indicator molecule. The EC_{50} of the extract in scavenging 100 μM DPPH was 14.2 μg/mL, whereas EC_{50} of pure rosmarinic acid was 6.24 μg/mL whereas that of Trolox was above 250 μg/mL (calcium 1 mmol/L). The maximum 100% of DPPH was reduced by the extract in a concentration of 25 μg/mL after 5 min of

incubation. Hence, the *Perovskia* extract is a more efficient DPPH radical scavenger than Trolox, and most of its activity is attributable to high rosmarinic acid content.

ABTS$^{\bullet+}$ free radical scavenging

Another antioxidant activity screening method, applicable for both lipophilic and hydrophilic antioxidants is ABTS radical cation decolorization assay. The antioxidant activity is presented in the form of TEAC values. The TEAC value (mmol) of tested extract increased from 0.64 ± 0.08 after 1 min up to 5.31 ± 0.30 in 10 min ($P < 0.05$), after which it decreased again down to 1.00 ± 0.09 and then did not change significantly ($P > 0.05$). Hence, free radical scavengers contained in *Perovskia* extract are acting quickly in comparison to Trolox and their sum activity is also long lasting.

Reducing power

To assess the electron-donating properties of the extract, an ability to reduce iron (III) and molybdenum (VI) were investigated. The results are presented as AAE values (w/w), the higher the AAE value the greater the electron-donating power of the sample [Figure 2]. AAE for Fe (III) was 0.527 ± 0.018 whereas for Mo (VI) it was 0.308 ± 0.014.

The obtained AAE values show that the extract can donate electrons to reduce both transition metals with a strength of 1/3 (Mo) to more than half (Fe) of that of ascorbic acid ($P < 0.05$).

Inhibition of proteolytic enzymes

Both elastase and collagenase were significantly inhibited *in vitro*. Maximum inhibition reached about 60% at 30 μg/mL and was not statistically different ($P < 0.05$) between the enzymes (collagenase – 60.03% ± 7.22% and elastase - 54.75% ± 6.87%). Elastase was inhibited in a dose-dependent way with IC_{50} – 21.39 μg/mL ± 3.44. In case of collagenase, the dose response was not proportional, so we were not able to calculate a reliable IC_{50} value. This complex response is likely to result from enzyme interacting with several extract components thus obscuring the mechanisms of activity inhibition by individual substances.

Phytochemical composition

Total phenol content

Total phenol content of *P. atriplicifolia* was estimated as 126 ± 8 mg gallic acid/g (dry wt.) extract.

LC-MS analysis

The major component in the dried extract was rosmarinic acid that constitutes over 30% of dry mass (344.27 mg/g) and it is the only polyphenol present in detectable amount. This value exceeds the so called total phenol content, but this discrepancy arises from the differences in reactivity of gallic and rosmarinic acids with Folin-Ciocalteu reagent. Other constituents detected using LC-MS belong to nor-abietanoid quinoid diterpenoids (tanshinones). Sum of three major tanshinones (cryptotanshinone, 1β-hydroxycryptotanshinone, 1-oxocryptotanshinone) reached only about 15 mg/g of dry extract.[9]

DISCUSSION

All observed effects are most likely caused by the high content of rosmarinic acid in the extract. This compound is widely recognized as antioxidant and anti-inflammatory agent and occurs in several taxa, such as Lamiaceae and Boraginaceae. Its antiglycative properties were confirmed previously in several reports on such medicinal herbs as *Salvia* sp., *Melissa officinalis, Mentha spicata*, and other Lamiaceae.[23-25] The numerous other bioactivities have been also reviewed.[1,26] However, the ability of a rosmarinic acid-rich extract to protect mammalian cells against damage induced by glycated albumin has not been reported, yet. Of course, the presence of other, minor compounds such as flavone glycosides and tanshinones could also influence the overall activity of the *Perovskia* root extract.

AGEs arise from the reaction of sugars with side chains and the N-terminus of proteins and are thought to be involved in the pathogenesis of several diseases by inducing oxidative stress, inflammation and cell death presumably mediated through activation of the RAGE. To address the question whether the cell damaging effect of AGE mitigates in presence of extract, we tested its effect on viability of four mammalian (three human and one of hamster) cell lines.

Alleviation of glycotoxicity in various cells, including kidney-derived cells suggests potential for further research on the prevention of hyperglycemia-induced injuries to which the kidneys are one of the most vulnerable targets. One has to bear in mind that the used cell lines, including HEK293 are not a fully relevant model system for *in vivo* conditions. On the other hand, we obtained similar results using four different mammalian lines to prove the more universal than cell-line specific activity of tested extract. In the study of Tupe *et al*.,[24] using HEK293 and red blood cells, some other plant extracts have been also proved to be efficient protective agents. One of the extracts in the above cited report was from *Mentha arvensis*, but these authors did not mention rosmarinic acid as one of the active components. The extracts were prepared by maceration with water but their composition was not characterized. In our study, we only tested the survival rate of cells, but from the results obtained by Tupe *et al*.[24] we can presume that the other parameters are likely to correspond to the previous study.

On the other hand, using reduction-based cell viability assays were shown to affect the assay results,[26] so we checked the influence of the extracts alone on the cell survival rate and on the reduction of the reagent mix. No detectable increase of absorbance that might result from the interference was observed.

The further mechanism that can protect from hyperglycemic injury is inhibition of protein glycation. Using HSA as a model system offers a higher relevance to phytotherapy than the most commonly used (and much more affordable) bovine albumin. *Perovskia* extract shows versatile mechanisms of action reducing different markers of AGE formation.

The effect of protecting HSA from conformational changes was similar to that observed in our previous study on rosmarinic acid-rich extract of lemon balm using BSA as model albumin.[23] However, the lemon balm extract was used in 20 time higher concentration, so the activity of *Perovskia* proved to be remarkably superior.

High antioxidant activity is a typical property of plants from Lamiaceae family and rosmarinic acid is usually mentioned as one of the prominent antioxidants.[3,27] Its high content in *Perovskia* root extract explains the efficiency in both tested mechanisms of antioxidant activity – free radical scavenging and reducing transition metal ions.

The ability to inhibit pro-inflammatory enzyme activity is essential for preventing tissue injury resulting from advanced phase of inflammation, which is also frequent complication of diabetic conditions. Many polyphenols or polyphenol rich herbs are known to inhibit proinflammatory proteases.[28,29]

Anti-inflammatory properties were also studied on several lignans isolated from chloroform extract of *P. atriplicifolia* leaves.[4] Taxiresinol was the most potent among six compounds and inhibited leukotriene C4 release from rat basophiles with IC_{50} of 3.49 μM. However, in our study, we did not detect these lignans in the hydromethanolic extract, probably because chloroform is a better solvent for obtaining nonpolar compounds. Ultrasonic-assisted extraction is an efficient way to obtaining rosmarinic acid enriched alcohol extracts from plant material.[30]

Rosmarinic acid is responsible for preventing complications of diabetes related to inflammatory[31] and neuropathic conditions.[32] Mostly, high antioxidant potential of rosmarinic acid and other highly hydroxylated phenolics is regarded as a major drive of glycation inhibition.[23] However, in *P. atriplicifolia* extract, other compounds such as diterpenoids despite having moderate or weak antioxidant properties, can also contribute to the total effect. Such compounds could for example exert their effect through transition metal chelation.[33,34]

Plant polyphenols are also able to counteract diabetic complications by a variety of other mechanisms, for example, by stimulating glucose metabolism,[35] aldose reductase,[36] and α-glucosidase,[37] or interacting with peroxisome proliferator-activated receptor γ.[38]

Rosmarinic acid as an antioxidant constituent of plant extracts can be also generally considered as nontoxic to cells, even those prone to oxidative stress such as neurons or nephrons.[39]

CONCLUSIONS

Our results demonstrate the high potential of this little known but emerging, folk medicinal plant in inhibition of several steps of protein glycation and protecting viability of cells harmed by glycated albumin. As a consequence, *Perovskia* roots offer a valuable combination of antiglycative, antioxidant and proteolytic enzyme inhibition and should be explored as a potential herbal drug for prevention of oxidative stress and inflammation-based complications of diabetes. Further studies are necessary, in particular an *in vivo* verification of pharmacological activities, but the contribution and mechanism of action of each individual compound from the extract should be also investigated. *P. atriplicifolia*, an easy to grow and widespread ornamental plant is in this respect one of the very promising sources of bioactive principle.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Petersen M, Simmonds MS. Rosmarinic acid. Phytochemistry 2003;62:121-5.
2. Senol FS, Slusarczyk S, Matkowski A, Pérez-Garrido A, Girón-Rodríguez F, Cerón-Carrasco JP, *et al.* Selective *in vitro* and in silico butyrylcholinesterase inhibitory activity of diterpenes and rosmarinic acid isolated from *Perovskia atriplicifolia* Benth. and *Salvia glutinosa* L. Phytochemistry 2017;133:33-44.
3. Ślusarczyk S, Hajnos M, Skalicka-Woźniak K, Matkowski A. Antioxidant activity of polyphenols from *Lycopus lucidus* Turcz. Food Chem 2009;113:134-8.
4. Ahmad I, Waheed A, Tahir NB, Rais AK. Anti-inflammatory constituents from *Perovskia atriplicifolia*. Pharm Biol 2015;53:1628-31.
5. Jiang ZY, Yu YJ, Huang CG, Huang XZ, Hu QF, Yang GY, *et al.* Icetexane diterpenoids from *Perovskia atriplicifolia*. Planta Med 2015;81:241-6.
6. Perveen S, Khan SB, Malik A, Tareen RB, Nawaz SA, Choudhary MI. Phenolic constituents from *Perovskia atriplicifolia*. Nat Prod Res 2006;20:347-53.
7. Perveen S, Malik A, Noor AT, Tareen RB. Pervosides A and B, new isoferulyl glucosides from *Perovskia atriplicifolia*. J Asian Nat Prod Res 2008;10:1105-8.
8. Perveen S, Malik A, Tareen RB. Structural determination of atricins A and B, new triterpenes from *Perovskia atriplicifolia*, by 1D and 2D NMR spectroscopy. Magn Reson Chem 2009;47:266-9.
9. Slusarczyk S, Topolski J, Domaradzki K, Adams M, Hamburger M, Matkowski A. Isolation and fast selective determination of nor-abietanoid diterpenoids from *Perovskia atriplicifolia* roots using LC-ESI-MS/MS with multiple reaction monitoring. Nat Prod Commun 2015;10:1149-52.
10. Shah AJ, Rasheed M, Jabeen Q, Ahmed A, Tareen RB, Gilani AH, *et al.* Chemical analysis and calcium channel blocking activity of the essential oil of *Perovskia abrotanoides*. Nat Prod Commun 2013;8:1633-6.
11. Tarawneh A, León F, Pettaway S, Elokely KM, Klein ML, Lambert J, *et al.* Flavonoids from *Perovskia atriplicifolia* and their *in vitro* displacement of the respective radioligands for human opioid and cannabinoid receptors. J Nat Prod 2015;78:1461-5.
12. Tareen RB, Bibi T, Khan MA, Ahmad M, Zafar M. Indigenous knowledge of folk medicine by the women of Kalat and Khuzdar regions of Balochistan, Pakistan. Pak J Bot 2010;42:1465-85.
13. Peyroux J, Sternberg M. Advanced glycation endproducts (AGEs): Pharmacological inhibition in diabetes. Pathol Biol (Paris) 2006;54:405-19.
14. Elosta A, Ghous T, Ahmed N. Natural products as anti-glycation agents: Possible therapeutic potential for diabetic complications. Curr Diabetes Rev 2012;8:92-108.
15. Miroliaei M, Khazaei S, Moshkelgosha S, Shirvani M. Inhibitory effects of Lemon balm (*Melissa officinalis* L.) extract on the formation of advanced glycation end products. Food Chem 2011;129:267-71.
16. Klunk WE, Jacob RF, Mason RP. Quantifying amyloid beta-peptide (Abeta) aggregation using the Congo red-Abeta (CR-abeta) spectrophotometric assay. Anal Biochem 1999;266:66-76.
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
18. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 1965;16:144-58.
19. Wozniak D, Drys A, Matkowski A. Antiradical and antioxidant activity of flavones from *Scutellariae baicalensis* radix. Nat Prod Res 2015;29:1567-70.
20. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26:1231-7.
21. Oyaizu M. Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr Diet 1986;44:307-15.
22. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of Vitamin E. Anal Biochem 1999;269:337-41.
23. Ma HY, Gao HY, Sun L, Huang J, Xu XM, Wu LJ. Constituents with α-glucosidase and advanced glycation end-product formation inhibitory activities from *Salvia miltiorrhiza* Bge. J Nat Med 2011;65:37-42.
24. Tupe RS, Sankhe NM, Shaikh SA, Kemse NG, Khaire AA, Phatak DV, *et al.* Nutraceutical properties of dietary plants extracts: Prevention of diabetic nephropathy through inhibition of glycation and toxicity to erythrocytes and HEK293 cells. Pharm Biol 2015;53:40-50.
25. Bulgakov VP, Inyushkina YV, Fedoreyev SA. Rosmarinic acid and its derivatives: Biotechnology and applications. Crit Rev Biotechnol 2012;32:203-17.
26. Wang P, Henning SM, Heber D. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. PLoS One 2010;5:e10202.
27. Matkowski A. Antioxidant activity of extracts and different solvent fractions of *Glechoma hederacea* L. and *Orthosiphon stamineus* (Benth.) Kudo. Adv Clin Exp Med 2008;17:615-24.
28. Thring TS, Hili P, Naughton DP. Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. BMC Complement Altern Med 2009;9:27.
29. Wittenauer J, Mäckle S, Sussmann D, Schweiggert-Weisz U, Carle R. Inhibitory effects of polyphenols from grape pomace extract on collagenase and elastase activity. Fitoterapia 2015;101:179-87.
30. Martins FS, da Conceição EC. Evaluation of extraction method on the chemical composition in *Apeiba tibourbou* Aubl's extracts. Pharmacogn Mag 2015;11:368-73.
31. Lucarini R, Bernardes WA, Ferreira DS, Tozatti MG, Furtado R, Bastos JK, *et al. In vivo* analgesic and anti-inflammatory activities of *Rosmarinus officinalis* aqueous extracts, rosmarinic acid and its acetyl ester derivative. Pharm Biol 2013;51:1087-90.
32. Hasanein P, Mohammad Zaheri L. Effects of rosmarinic acid on an experimental model of painful diabetic neuropathy in rats. Pharm Biol 2014;52:1398-402.
33. Upadhyay A, Tuenter E, Ahmad R, Amin A, Exarchou V, Apers S, *et al.* Kavalactones, a novel class of protein glycation and lipid peroxidation inhibitors. Planta Med 2014;80:1001-8.
34. Upadhyay A, Tuenter E, Amin A, Exarchou V, Hermans N, Apers S, *et al.* 5-O-demethylnobiletin, a polymethoxylated flavonoid, from *Citrus depressa* Hayata peel prevents protein glycation. J Funct Foods 2014;11:243-9.

35. Eid HM, Nachar A, Thong F, Sweeney G, Haddad PS. The molecular basis of the antidiabetic action of quercetin in cultured skeletal muscle cells and hepatocytes. Pharmacogn Mag 2015;11:74-81.
36. Somani G, Sathaye S. Bioactive fraction of *Saraca indica* prevents diabetes induced cataractogenesis: An aldose reductase inhibitory activity. Pharmacogn Mag 2015;11:102-10.
37. Chiabchalard A, Nooron N. Antihyperglycemic effects of *Pandanus amaryllifolius* Roxb. leaf extract. Pharmacogn Mag 2015;11:117-22.
38. Lee M, Sung SH. Platyphylloside isolated from *Betula platyphylla* inhibit adipocyte differentiation and induce lipolysis via regulating adipokines including PPARγ in 3T3-L1 Cells. Pharmacogn Mag 2016;12:276-281.
39. Andrade JM, Biegelmeyer R, Dresch RR, Maurmann N, Pranke P, Henriques AT. *In vitro* antioxidant and enzymatic approaches to evaluate neuroprotector potential of blechnum extracts without cytotoxicity to human stem cells. Pharmacogn Mag 2016;12:171-7.