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).

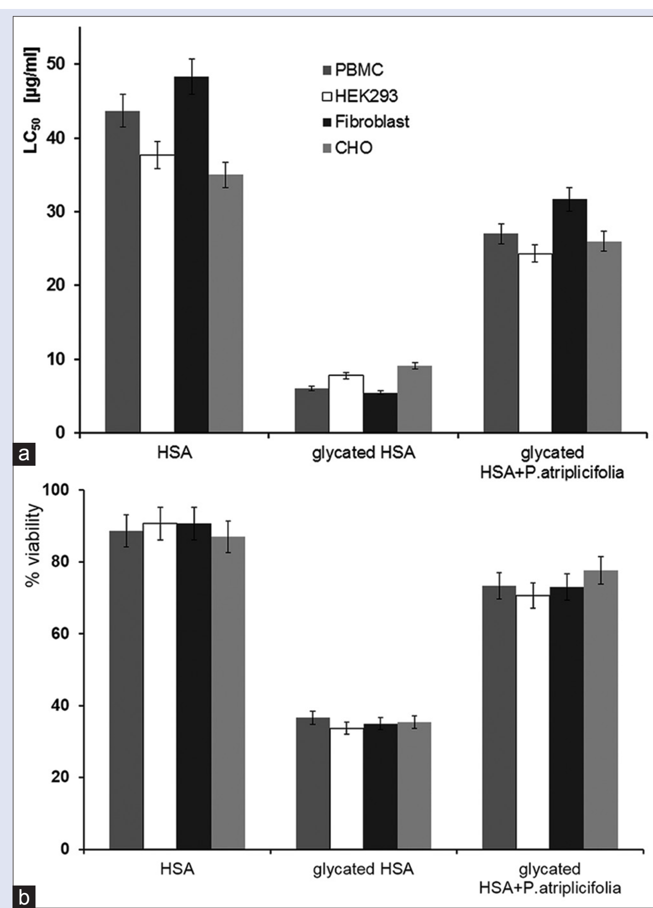

Figure 1: Effect of *Perovskia atriplicifolia* extract on *in vitro* viability of cell lines treated with glycated human serum albumin: (a) expressed as LC_{50} of added albumin, showing significant increase tolerance of cells to advanced glycation end products-induced toxicity; (b) expressed as cell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

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 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

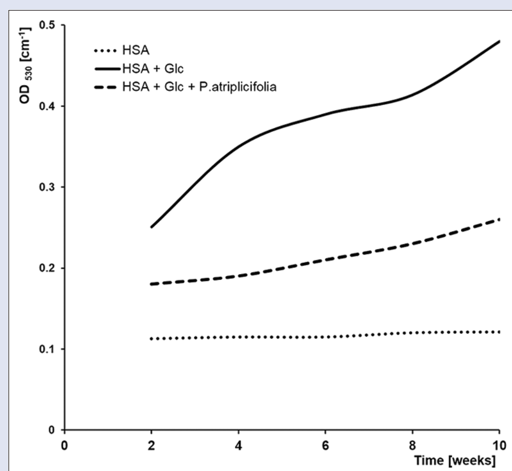

Figure 2: Time evolution of secondary structure alteration in human serum albumin monitored by Congo Red binding assay at 530 nm

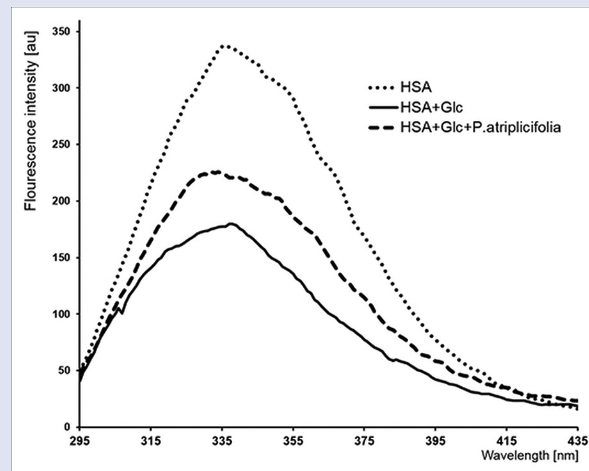

Figure 3: Fluorescence spectra of glycated samples of human serum albumin that were obtained in the wavelength range of 295–445 nm after excitation at 270 nm in the presence and absence of 30 μg/mL *Perovskia atriplicifolia* extract

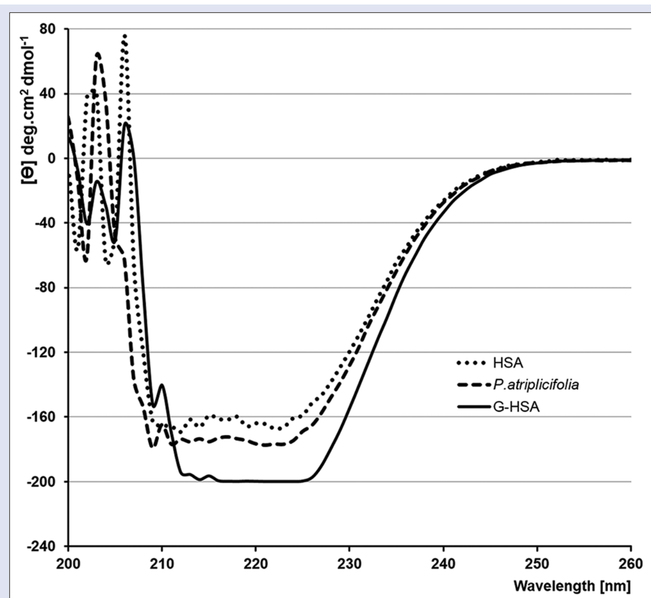

Figure 4: Far-ultraviolet circular dichroism spectra of native and glycated human serum albumin in the presence and absence of *Perovskia atriplicifolia* root extract. The circular dichroism data were expressed as molar ellipticity (deg cm²/dmol)

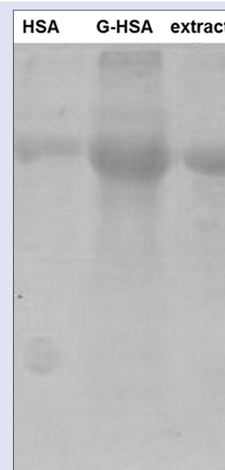

Figure 5: Electrophoretic pattern of native human serum albumin (lane on the left) and human serum albumin incubated for 8 week with 50 mM glucose without (middle lane) or with *Perovskia atriplicifolia* extract (lane on the right). The mobility retardation of G-human serum albumin is decreased in the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

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