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Inhibitory effects of Lemon balm (*Melissa officinalis*, L.) extract on the formation of advanced glycation end products

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ABSTRACT

Lemon balm (*Melissa officinalis*) is a medicinal herb possessing functional compounds with unexplored anti-glycative action. The anti-glycative activity of Lemon balm extract was evaluated in the bovine serum albumin (BSA)/glucose system. The level of glycation, conformational alterations and protein binding to RAGE receptors were assessed by specific fluorescence, Congo red binding assay, circular dichroism, ligand and Western blotting. Ethanol fractions of *Melissa* leaf exhibited the highest inhibitory effects on the formation of advanced glycation end products (AGEs) and the late stage of glycation process. Significant alteration in the secondary structure of albumin was observed upon glycation, which was mitigated by applying the herb extract. Moreover, upon treatment with balm extract, glycated albumin adopts a secondary structure impeding its detection by RAGE receptors of microglial cells. Our results represent the anti-glycative properties of *Melissa* extract and its application for possible treatment of AGE-associated diseases.

1. Introduction

Protein glycation is a haphazard process resulting from the binding of sugars to the free amine residues of proteins. The newly generated modified molecules are known as 'advanced glycation end-products' (AGEs). Considering the multifactorial pathways and complexity of reactions involved in AGE formation, those compounds possessing inhibitory effects in the mechanism-based-AGE-formation are ideal candidates against biomolecular damage in AGE-associated diseases. In these pathways, glycoxidised proteins produce common toxic species like reactive carbonyl compounds and reactive oxygen species (ROS), leading to promotion of degenerative events in diabetic complications, atherosclerosis and Alzheimer's disease. (Ahmed, 2005; Baynes, 1991; Rahbar & Figarola, 2003; Ravelojaona, Molinari, Gesztesi, & Robert, 2007). A number of studies have revealed that cell and tissue damage by AGE comes from protein modifications, conformational conversion and functional impairments. Protein remodelling has been evidenced for the glycated bovine serum albumin (BSA) whereby cross-β structure appears in the protein molecule (Bouma et al., 2003). Therefore, it is of therapeutic concern to discover medicines for targeting each committed step and harmful molecules of glycation cascade through intervention within, thereby controlling the overall conformational changes of glycated proteins. In this respect, natural medicines with a spectrum of functional molecules seem to be promising tools with the least side effects and as superior alternatives to synthetic drugs.

Lemon balm (*Melissa officinalis*, L.) is a medicinal herb of the family Lamiaceae (Zargari, 1990). Medicinal preparation of the herb has a long tradition in Iran, particularly for treatment of indigestion, anaemia and cardiac failure (Anon, 2002; Zargari, 1990). Furthermore, antioxidant properties, metal-chelation and free radical scavenging activity of *Melissa* extract have been extensively identified (Dastmalchi et al., 2008). Such different modes of action are of considerable therapeutic concern in the treatment of hyperglycaemia. Despite established beneficial capacities in treatment of Alzheimer's diseases (Perry, Pickering, Wang, Houghton, & Perry, 1998), it has not been identified how balm may help in preventing the negative effects of AGE-induced toxicity. To validate whether balm extract possess *in vitro* anti-glycative action, BSA as a model protein, was subjected to glucose treatment and the induced alterations were assessed with and without the extract.

2. Materials and methods

All chemicals used in this study were obtained from Sigma–Aldrich or Merck. All other chemicals were of reagent grade.

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2.1. Plant extracts preparation

The fresh Lemon balm leaves obtained from the Institute of Medicinal Plants, Halejerd, Iran, and were subjected to air drying. The samples (1 g) were then powdered with a mill and shaked gently with 5 ml dichloromethane (Merck, Cat. No. 106051). After filtration, samples were extracted with 45% (V/V) aqueous ethanol (Merck, Cat. No. 818760), the ratio of material to the extractant (phenolic acids) was 1:5.4 (w/v). The extracts were filtered, the filtrates were freeze-dried and stored at 4 °C. The dried extract was weighed and diluted appropriately with water (pH ≈ 7.3) and immediately used in assays. The concentration of the extract was 2 mg/ml in the test solutions.

2.2. Preparation of glycated albumin

Glycated BSA was prepared and characterised as described previously (Bourdon, Loreau, & Blache, 1999; Valencia et al., 2004; Westwood & Thornalley, 1995). In brief, BSA (Sigma–Aldrich, Cat. No. A7906) (0.75 mM) was incubated at 37 °C in a solution of D-glucose (Aldrich, Cat. No. 158968) (50 mM) in 0.1 M phosphate buffer (pH 7.4) in the presence or absence of *M. officinalis* extract. To prevent bacterial contamination, 0.02% (w/v) NaN_3 (Sigma–Aldrich, Cat. No. S8032) was added to the solution and it was filtered through a low protein binding filter (Millex.®-GV 0.22 μm filter unit, Millipore). Aliquots were taken from BSA–glucose solution after each period of incubation and were extensively dialysed against autoclaved phosphate buffer saline (PBS) at 4 °C to remove free glucose molecules. Pure BSA was incubated under the same conditions as a control sample.

2.3. Determining protein glycation and AGEs formation

The amount of glycation in BSA was determined using brown staining method. Optical density of 1 mg/ml protein of each sample (pH 7.4) was recorded by measuring the absorbance at 340 nm in a Shimadzu UV60A spectrophotometer. Glycation was also confirmed by AGE-related auto-fluorescence assay. Fluorescence of relevant samples (0.15 mg/ml) was measured after exciting at 370 nm, and monitoring the emission at 400–450 nm using a Hitachi F-2500 spectrofluorometer. Correction for spectra was done with the appropriate protein and buffer blanks.

2.4. Congo red assay

Congo red (Merck, Cat. No. 101340) binding assay was performed by measuring the absorbance for AGE–BSA and BSA (control) separately, as well as for Congo red background, based on the well documented method (Klunk, Jacob, & Mason, 1999). For this purpose, 800 μl of protein solution (100 μM) was incubated with 200 μl of Congo red solution (100 μM Congo red in phosphate buffer saline–ethanol 10% (v/v)). Absorbance of respected samples was recorded at 530 nm.

2.5. Circular dichroism (CD) spectropolarimetry

All far-UV CD spectra were obtained at room temperature and recorded on a JASCO J-715 spectropolarimeter using solutions with a protein concentration of about 0.15–0.2 mg/ml. All spectra resulted from averageing four scans and were corrected for the respective blanks. Results are expressed as molar ellipticity, $[\Theta]$ (deg cm^2 $dmol^{-1}$), based on a mean amino acid residue weight (MRW). The molar ellipticity was determined as $[\Theta]\lambda = (\Theta \times 100\ MRW)/(cl)$, where l, is the light path length in centimetres, c, is the protein concentration in mg/ml, and Θ is the measured ellipticity in degrees at the relevant wavelength. The relative percentages of the secondary structure elements were estimated using SELCON3 software.

2.6. Microglial cell culture

Primary cultures of microglia from neocortex of newborn rats (Wistar strain) were prepared from mixed glial cultures according to the procedure of Giulian and Baker with some modifications (Giulian & Baker, 1986). Briefly, the brain cortex tissue was minced in nutrient medium after removing the meninges. To obtain a cell suspension, the cells were dissociated by triturating with firepolished Pasteur pipettes. The cell suspension was plated at a density of 5×10^4 cells/cm^2 into 25 cm^2 tissue culture flasks (Nunc) in Dulbecco's Modified Eagle's Medium, DMEM and 10% FCS at 37 °C with 5% CO_2. Cells were fed every 4 days with a half change of the medium. After 2 weeks, cultures contained glial cells including rounded microglial cells mostly localised on the top of the astrocyte monolayer. The loosely adherent microglial cells were recovered by vigorous agitation for 30 min in an orbital shaker at 150 rpm and 37 °C (Eugenín et al., 2001). After centrifugation at 1000 rpm for 5 min, cells were cultured on 24 multiwell plates in DMEM supplemented with 10% foetal calf serum. Non-adherent cells were discarded after 15 min and attached cells, mostly microglial, were harvested and plated at a final density of 1×10^6 cells/ml on 24 multiwell plates as with the previous step.

2.7. Western and ligand blot analysis

Pooled microglial cell extract were separated on SDS–PAGE and transferred to nitrocellulose, and incubated with various AGE–BSA which were glycated in the presence or absence of *M. officinalis* extract. Bound BSA was detected on membranes using anti-BSA antibodies.

3. Results and discussion

The sequence of events triggering glucose-induced protein damage, might be categorised into three overlapping stages viz. early (Amadori), intermediate (protein cross-link and carbonyl group formation) and late stage (post-Amadori). The first stage initiated with the advancement of the Millard reaction (Verzil et al., 2000). Brownish colour measurement is among the approaches to determination of protein–AGE adducts. It was performed to monitor the extent of BSA glycation. Glycation brought about colour alteration in albumin solution with a significant degree of modification during 16 weeks of incubation (Fig. 1). The lower

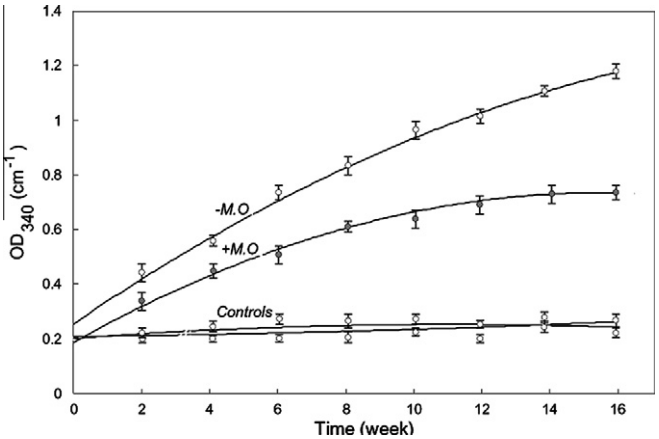

Fig. 1. Brownish colour absorbance of albumin at 340 nm. Samples were incubated with glucose for 16 weeks with and without MO extract. In control samples, BSA was incubated with and without the herb extract in the absence of glucose.

absorbance at relevant wavelength for the treated samples with *Melissa* extract indicates its efficacy on alleviating the glycation reaction tends to be more pronounced after 8 weeks incubation. A remarkable decrease of glycation (to a degree of ~40%) reveals the extract function in attenuation of Millard reaction during the initial stage of glucose-induced protein damage. Since no oxidation reaction has been proposed to occur during the formation of Amadori products (Fu et al., 1994), the inhibitory effects of *balm* extract may result from properties rather than its recognised antioxidant activity (Dastmalchi et al., 2008). Similarly, BSA glycation has not been suppressed by the extract of *Teucrium polium* in spite of known antioxidant action (Ardestani & Yazdanparast, 2007). Moreover, the metal-chelating property of *guava* leaf extract has been proven to be responsible for alleviation of the Amadori reaction in BSA glycation system (Wu, Hsieh, Wang, & Chen, 2009). Based on the verified efficacy of *Melissa* extract in chelating transition metals (Dastmalchi et al., 2008), a similar suggestion may be made for the present observations. The rationale behind this speculation includes the previous report that, diethylenetriaminepentaacetic acid, an inhibitor of glucose autoxidation with known metal-chelating activity, reduced albumin glycation (Wolff & Dean, 1987). Chelating activity of AGE inhibitors has been suggested in contributing to the inhibitory effects on AGE formation and protection against development of diabetic complications (Price, Rhett, Thorpe, & Baynes, 2001). Accordingly, a tight association between metal-chelation and AGEs inhibitory action of *Melissa* leaf extract is suggestive. Furthermore, the strong relationship between glucose associated oxidative modifications carried out by transition metals, like copper (Cu) and iron (Fe), and oxidative damage in neurodegenerative disorders, along with the beneficial effects of balm extract in treatment of Alzheimer's disease (Akhondzadeh et al., 2003; Akhondzadeh, Noroozian, Mohammadi, Ohadinia, & Moin, 2003), hold good evidences for the above suggestion.

Auto-fluorescence signals are believed to originate from protein–AGE adducts (Fu et al., 1994). To investigate if AGE formation proceeds in the BSA–glucose system, autofluorescence measurements were performed. The glycophore fluorescence (λ_{ex} = 370 nm) spectra of albumin–AGE preparations were recorded and compared with those in the presence of *Melissa* extract. As reported in Fig. 2, a significant decrease (nearly 50%) of fluorescence intensity at about 430–460 nm was observed in albumin incubated with the balm extract with an obvious red shift from 435 nm to 447 nm. This spectral contribution is attributed to the fact that glycated BSA turns into a less polar molecule owing to exposing its hydrophobic sites in respect to the native molecules (Rondeau et al., 2010). Treatment with *Melissa* extract resulted in a profound prevention of such structural changes, keeping the protein molecule closer to its native polar conformation. The behaviour of the extract in this respect resembles that of molecular chaperones which blocks the exposed hydrophobic surfaces of their substrate proteins. These observations specify the anti-AGEs properties of *Melissa* extract assigning to its rosmarinic acid content: 96.45 ± 0.13 mg/g (Dastmalchi et al., 2008), one of the most abundant caffeic acid esters in the extract, possessing post-Amadori suppressive effects (Kim et al., 2010).

It is well known that glycation brings about definitive modifications in the secondary structure of native BSA (Rondeau et al., 2010). To assess whether the extract holds any protective effect on the secondary structure alteration, a Congo red binding assay was performed. Congo red has a specific binding affinity to the β-sheet structure of proteins and exhibits a specific absorption at 530 nm after binding. The dye binds to hydrophobic clefts between anti-parallel β strands (Klunk et al., 1999). Time dependent plot of spectral intensities of dye–protein–AGE solutions revealed a noticeable difference in intensity of Congo red absorbance at 530 nm between samples glycated in the presence or absence of *Melissa* extract indicating that the extract inhibits the transition of α to β conformer during glycation (Fig. 3). This prompted us to analyze the albumin–AGE structure in greater detail with CD spectropolarimetry. Far-UV CD spectra of glycated solutions indicated a large alteration in the secondary structure of untreated BSA which was protected appreciably by applying the herb extract (Fig. 4). Quantitative analysis of the spectra established the obtained results of structural alterations in treated, modified and control samples. A considerable content of β-structure was evident in the glycated albumin comparable with the extract treated and control samples (Table 1). Higher amount of α-helix in the presence of *Melissa* extract could be ascribe to the anti-glycative effects. The fact that glycation induces refolding of initially globular albumin into amyloid fibrils, comprising of a cross-β structure, has already been reported (Bouma et al., 2003). One possible explanation is that the extract has potential to arrest changes 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. Accordingly, we conclude that the extract has a potential to block the refolding of albumin into

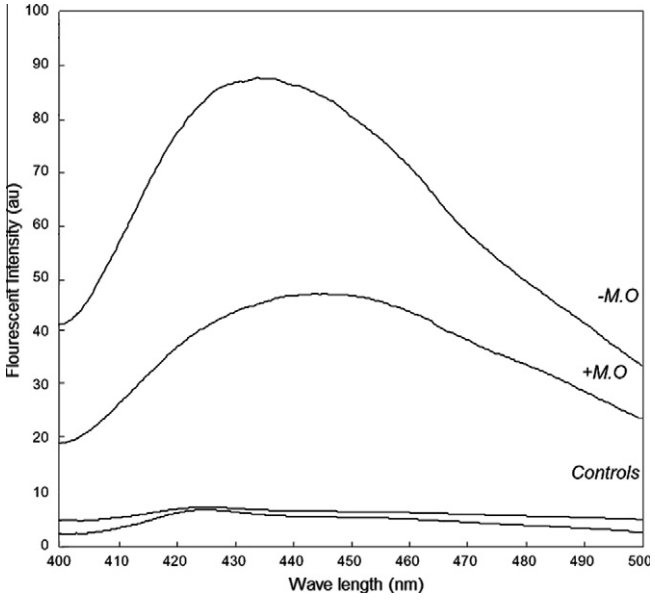

Fig. 2. Fluorescence spectra of glycated samples of albumin were obtained in the wavelength range of 400–500 nm after excitation at 370 nm in the presence and absence of *Melissa* extract.

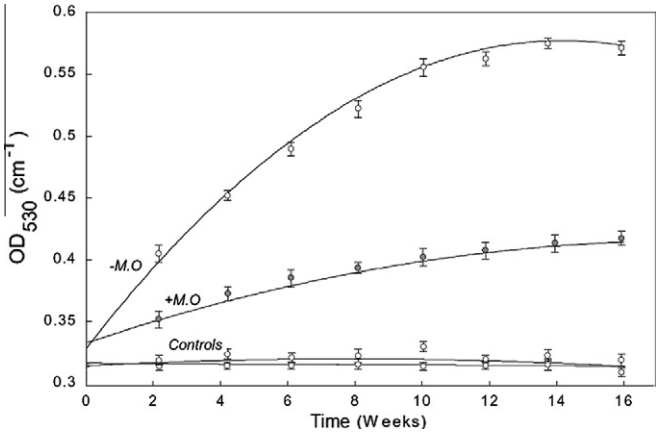

Fig. 3. Time evolution of secondary structure alteration in BSA monitored by Congo red binding assay at 530 nm.

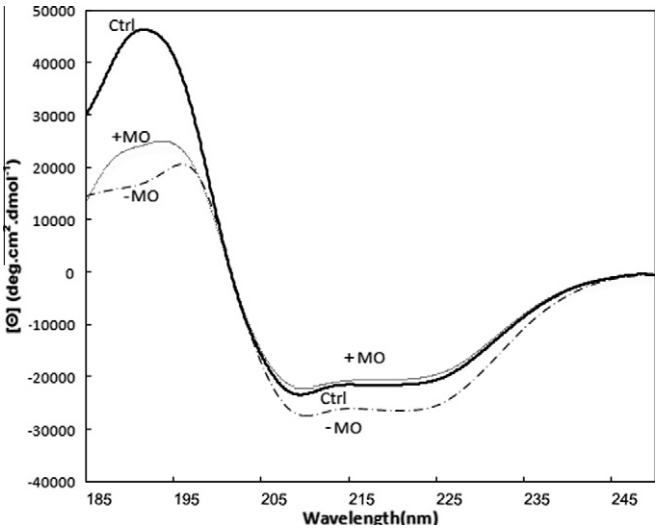

Fig. 4. Far-UV CD spectra of native albumin (Ctrls.), glycation in the presence (+MO) and absence (−MO) of the extract. The CD data were expressed as molar ellipticity (deg cm^2 $dmol^{-1}$).

Table 1
Effect of the Lemon balm extract on secondary structural contents[a] of BSA. SELCON3 software was used for estimating the relative percentages of the secondary structural elements.

Sample	Unglycated	Glycated	Extract treated
α-Helix	65.8 ± 2.21	37.1 ± 1.6	53.2 ± 1.89
β-Sheet	3.13 ± 0.11	15.34 ± 1.03	4.23 ± 0.74

[a] The values refer to percentage of total structure, and the rest is random coil.

cross-β structure as a consequence of its anti-amyloid property. This structural protection is in accordance with the aforementioned chaperone–like properties by which the extract reserved the protein's native-like conformation during glycation.

One of the mechanisms by which AGEs exert toxicity is binding to their receptors (RAGE) (Rahbar & Figarola, 2003). To investigate whether similar complexes can occur in the presence of *Melissa* extract, a ligand blotting experiment was performed. Our data showed that normally glycated albumin has a high affinity to the immobilised RAGE, un-glycated samples did not show such attraction (Fig. 5). Surprisingly, when albumin molecules were subjected to glucose solution in the presence of the extract, lower affinity to

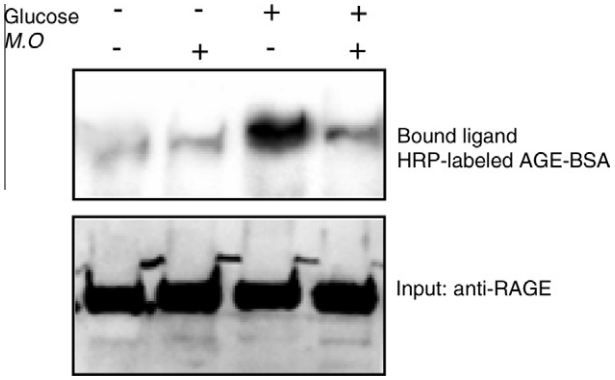

Fig. 5. Microglial cell extract was separated by SDS–PAGE under non-reducing conditions, transferred to a nitrocellulose membrane, and incubated with un-glycated BSA or BSA which was glycated in the presence or absence of MO extract. After washing, BSA was detected using a polyclonal antiserum.

RAGE receptors was observed even less than that of untreated samples. A very week signal was obtained for binding of un-glycated BSA to RAGE receptors. Weak immunoreactivity is also apparent for BSA exclusively incubated with *Melissa* extract in the absence of glucose (Fig. 5). It has been reported that duration of incubation with glucose has a direct correlation with cytotoxicity of AGEs-modified BSA (Loske et al., 1998). At the molecular level, this is owing to the contribution of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) to AGE mediated induced damage. At the cellular level, numerous studies support the view that interaction of AGEs with cell surface receptors such as RAGE elicits ROS generation and vascular inflammation (Yan, Ramasamy, & Schmidt, 2008). Accordingly, cytotoxic effects of AGEs should be overcome by *Melissa* extract with known either ROS or RNS scavenging activities (Dastmalchi et al., 2008), properties similar to those reported for aminoguanidine, a powerful inhibitor of AGE formation (Corbett et al., 1992), or as evident in Fig. 5, through the intervention with multiligand receptors on microglial cells. Again, the herb extract would afford a protective effect against AGE-induced toxicity, since fewer AGEs would be available to recognise and bind to their RAGE receptors. AGE-inhibitors could also modify the structure of AGEs in such a way that it inhibits their binding to and recognition by AGE receptors including RAGE. Of major interest in this context is the suggestion of Bouma and coworkers who have proposed the appearance of a unique recognition signal within globular protein structures upon glycation, termed as cross-β structure and substantiated for glycated BSA (Bouma et al., 2003). The observed protective effects of the extract from Lemon balm, on transition of secondary structure from α-helix to cross-β structure in albumin–AGE, strongly support this view. Suppression of receptor signalling pathways (e.g. RAGE antagonists) evidenced in this report, could be explained by considering the fact that MO extract has the capability of interacting with the cross-β structure pathway during glycation of BSA.

4. Conclusion

M. officinalis extracts hold potential interventions with multifactorial pathways and mechanisms underlying the AGEs formation. Our results revealed the anti-AGEs property of Lemon balm extract and its association with metal-chelating activity. A relationship is also hypothesised between AGEs inhibitory action and the chaperone-like activity of the herb extract. The efficacy of *Melissa* extract in protecting the structural integrity of BSA during glycation is verified and ascribed to the extract capacity in evading the cross-β structure pathway of the glycation process. All these features introduce an outstanding natural resource with dual antioxidant and anti-AGE functions, considerable protection against glucose-induced cellular damage, remarkable prospect for treatment of complications such as diabetes, ageing and a broad range of other conformational disorders.

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