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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.

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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

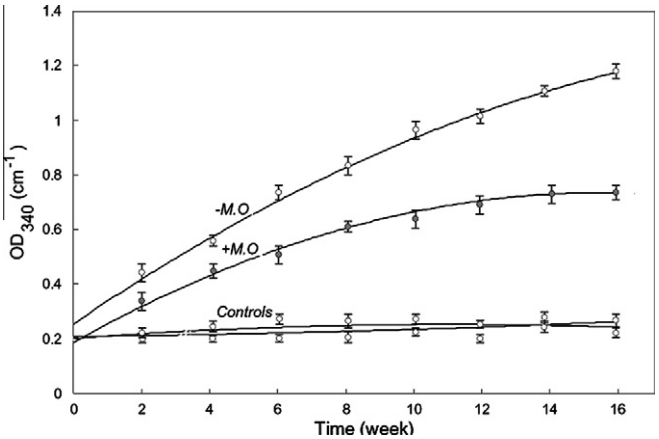

Fig. 1. Brownish colour absorbance of albumin at 340 nm. Samples were incubated with glucose for 16 weeks with and without MO extract. In control samples, BSA was incubated with and without the herb extract in the absence of glucose.

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