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Protection against Advanced Glycation End Products and the Mode of Action of Lemon Balm on Hemoglobin Fructose-Mediated Glycation

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Abstract

The present communication reports on changes in the secondary and tertiary structures of Hemoglobin (Hb) upon fructation. The presence of Lemon balm (LB) provided significant protection toward the formation of early (HbA1c) and advanced glycation end products (Hb-AGEs), as evident from circular dichroism (CD) and fluorescence studies. The degree of AGE modification and its inhibition were characterized with intrinsic fluorescence, the fibrillar state, and the exposure of hydrophobic clusters. An increased signal of intrinsic fluorescence was observed upon glycation, particularly in the later stage of fructation. Glycation mediated fibril formation and its inhibition were assessed by Thioflavin T (ThT) assay. These sequence of events were parallel with an increase in β-sheet content from ~2 to 13% for Hb-AGE, as evidenced by Congo red binding and CD. Furthermore, the late stage of glycation was also marked by an increase loss of heme moiety, confirming the relatively high affinity of ANS toward glycated-globin which was mitigated by applying the balm extract. However, the activity of balm can be attributed to inhibition of oxidative stress and free radicals derived from Hb-AGE, or chelation of metal ions producing in Fenton reaction. Overall, the presence of LB provided significant protection against AGE-induced deleterious processes, can qualify the herb as an effective AGE-inhibitor with potential prevention toward diabetic complications arising from Hb glycation.

Keywords: Human hemoglobin; Protein glycation; Hb-AGE; Folk medicine; Fructation

Introduction

Formation of advanced glycation end products (AGEs) is a hallmark of long-standing hyperglycemia. Glycation (sometimes called non-enzymatic glycosylation) is the result of the covalent bonding of a sugar molecule, such as glucose or fructose, to a protein or lipid molecule, without the controlling action of an enzyme. Glycation may occur either inside the body (endogenous glycation) or outside the body (exogenous glycation). It is a haphazard process that impairs the functioning of biomolecules. *In vivo*, advanced glycation of proteins has major contribution to the biomolecular damage in chronic diseases, such as diabetes, neural and microvascular abnormalities, and Alzheimer's disease [1]. It seems that intracellular AGE can be derived in multiple pathways, the Maillard reaction of asparagine and lysine representing the principal one. Via direct and receptor-dependent manner, AGEs exert their pathogenetic role in the development of multiple diabetic complications, such as atherosclerosis, retinopathy, neuropathy and nephropathy [2,3]. Therefore, the occurrence of extensive protein glycation in targeted tissues and/or organ sites causes interruption of normal protein structure and biological function, prompting the search for suitable medicines that could control the process. However, long-standing hyperglycemia has revealed to promote spontaneous glycation of Hb and causes severe progression of Hb-AGE formation [4]. Hb-AGE has substantial contribution to intracellular generation of reactive species of oxygen (ROS), nitrogen (RNS), and free radicals in RBC [5,6]. In this respect, several alterations are assumed to influence the biological function with the formation of glycated-Hb including: substantial remodeling of native conformation, intrinsic secondary structural changes, heme dissociation, decreased amino and free thiol group content, etc. The heterogeneous glycation products derived from Hb are often used as glycation models for *in vivo* and *in vitro* studies to quality the involvement of AGEs in cellular pathophysiology. Thus, based on these crucial aspects, many efforts have been devoted to finding effective anti-glycative agents.

A key aim of therapy in diabetic patients is to reduce hyperglycaemia by modification of the diet. However, dietary compliance is often difficult, and an alternative is to use pharmacological compounds that can reduce AGEs. Numerous compounds have been investigated for anti-glycation activity but their use in humans is still debatable. Natural medicines abundant in polyphenols might have therapeutic modalities for intervention in multifactor and complex reactions of AGE formation in different oxidative-based diseases. Melissa officinalis L., commonly known as lemon balm (LB), is a folk medicine characterized by unique high polyphenolic content [7,8] with medicinal properties such as, anti-inflammatory, antiviral, memory enhancer, cardiac tonic, anti-depressant, sleeping aid, and antioxidant activity [9-14]. Phenolic acids, the main active ingredients present in the extract of LB are reported for anti-oxidative [15], anti-proliferative [16], anti-inflammatory [17], anti-angiogenic [18] and anti-cancer properties [19,20]. The traditional use of LB extract has been proven in lowering blood glucose levels [21,22]. Accordingly, controlled studies were conducted to determine if balm could interfere with the chemical processes and mechanisms that ultimately lead to Hb-AGE. To identify the effect of LB in limiting the progression of AGE-induced toxicity, Hb was exposed to fructose and the pattern of structural alterations, heme degradation, fibril formation, lysine content, and the related oxidation fashion were monitored in the presence of lemon balm.

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Materials and Methods

Materials

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

Plant extraction

The extract of LB was prepared as previously described [23]. The LB leaves were air-dried and made into powder. 1 g of the powder was shaken gently with 5 mL dichloromethane. After filtration, samples were extracted with 45% aqueous ethanol, filtered, freeze-dried and stored at 4°C. The dried extract was weighed, diluted appropriately with water, and immediately used in assays. The concentration of the extract was 2 mg/mL in the test solutions.

High Performance Liquid Chromatography (HPLC)

The contents of RA in extracts from LB were determined by HPLC performed on a Hitachi liquid chromatograph (Hitachi, Ltd., Tokyo, Japan), which consisted of a model L-2130 pump and a model L-2455 photo diode array detector set at 280 nm. A reversed phase LiChrosphere RP-18 column was used for HPLC analysis. Elution was carried out at room temperature and utilized 2% (v/v) acetic acid in water as solvent A and 0.5% acetic acid in water and acetonitrile (50:50, v/v) as solvent B. Phenolic compounds were identified by comparison of their retention times (Rt) and UV-Vis spectra [24].

Glycation analysis

Preparation of glycated hemoglobin: Glycated Hb was prepared and characterized as described previously [25,26]. Hb (0.75 mM) was incubated at 37°C in a solution of D-fructose (50 mM) in 0.1 M phosphate buffer saline (PBS, pH 7.4) in the presence or absence of Melissa extract (0.2 mg/mL). 0.02% (w/v) NaN_3 was added to the solution to prevent bacterial contamination and filtered through a low protein binding filter (Millex-GV 0.22 µm filter unit, Millipore). Aliquots were taken from the Hb-fructose solution after each period of incubation and extensively dialyzed against autoclaved PBS at 4°C to remove unreacted fructose. Pure Hb was incubated under the same conditions as the control sample.

Determination of glycation: The amount of glycation in Hb was determined using the brown staining method [27]. Optical density of 1 mg/mL protein of each sample (pH 7.4) was recorded by measuring the absorbance at 340 nm with a UV60A spectrophotometer (Shimadzu).

Determination of free amino groups using fluorescamine: 1 µl protein solution (10 mg/ml), 100 µl Na_2HPO_4 (100 mM), 45 µl distilled water, and 50 µl fluorescamine reagent solution (1 mM fluorescamine in acetonitrile) were mixed and incubated for 10-15 min in dark in a 96-well plate. The fluorescence intensity of fluorescamine was measured at excitation and emission wavelengths, 390/490 nm in a Carry spectrofluorometer. The percentage of free amino groups was calculated according the relation:

$$\text{Percentage of free amino group} = \frac{\text{Fluorescene emission of HB in desired condition}}{\text{Fluorescence emission of Hb}} \times 100\%$$

AGEs analysis: The amount of glycation was evaluated by an AGE-related auto-fluorescence assay. Fluorescence was measured for relevant samples (0.15 mg/mL) recorded at wavelengths of 370/440 nm [28]. Correction for spectra was done with appropriate protein and buffer blanks.

ThT Binding assay: ThT fluorescent assay was performed as an indicator of amyloid fibrillation [29]. 10 µL of Hb samples were added to 590 µL of 25 µM ThT solution (2.5 mM ThT stock solution in 25 mM sodium phosphate buffer, pH 6, passed through a 0.45 µm filter paper). Fluorescent emission spectra were recorded at 490 nm with an excitation wavelength of 445 nm. The excitation and emission slit widths were set as 5 and 10 nm, respectively [30].

Heme release measurements: Heme loss associated with fructation was monitored by measuring the absorbance spectrum of fructated Hb. Aliquots were pulled on a daily basis for 6 days. The average of three independent measurements of the absorbance at the Soret peak (415 nm) was recorded as a function of incubation time. Absorption spectra were recorded for Hb-fr samples after 30 days incubation in varying concentrations of sugars to follow the status of heme moiety.

Congo red assay: Congo red (CR) binding assay was performed as described previously [23]. Binding of CR was detected by measuring the absorbance at 530 nm. 800 µL of protein solution (100 µM) was incubated with 200 µL of CR solution (100 µM Congo red in PBS containing ethanol 10% (v/v)). Absorbance at 530 nm was recorded for CR-incubated samples, as well as for CR and protein for background correction.

Circular dichroism spectropolarimetry: Far-UV CD spectra of the samples were recorded at ambient condition on a J-715 spectropolarimeter (Jasco) using solutions with a protein concentration of about 0.15 to 0.2 mg/mL. Each spectrum after correction for the respective blanks is the average of four scans. Results are expressed as molar ellipticity, [Θ] (deg cm^2 $dmol^{-1}$) based on a mean amino acid residue weight (MRW). The molar ellipticity was determined as $[\Theta]_\lambda = (\Theta \times 100MRW)/(cl)$, where c is the protein concentration in mg/mL, l is the light path length in centimeters, and Θ is the measured ellipticity in degrees at a wavelength λ. The relative percentages of the secondary structural elements were estimated using SELCON3 software.

Cell culture and MTS assay

Three mammalian cell lines, human embryonic kidney cells (HEK293), normal human fibroblasts, and Chinese hamster ovary cells (CHO), were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% antibiotic solution at 37°C, and 5% CO_2 atmosphere. They were in the logarithmic phase of growth at the time of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Cells were harvested for the viability test and seeded into a 96-well tissue culture plate at a density of 1×10^4 cells per well. The cells were allowed to adhere to the wells for 24 h at 37°C in a humidified atmosphere optimized with 5% CO_2. After replacement of the medium, Hb, Hb-fr, and Hb-fr-LB were added to the cells seeded on wells. Cells were grown for an additional 24 h before being subjected to viability test. MTS was added to wells the next day and incubated for 4 h. All experiments were performed at least three times. Phosphate-buffered saline (PBS) was used as a negative. The toxic endpoints were determined at 492 nm after 4 h exposure. Viability of cells defined as the inhibitory concentration (IC_{50}):

The estimated IC_{50} values of samples (concentration causing death of 50% of cells)

$$\%viability = \left(\frac{Test\ abs}{Control\ abs}\right) \times 100\%$$

Statistical analysis

All experiments were performed in triplicates. Mean, standard error, standard deviation, one-way ANOVA, and paired t-test were calculated from replicates within the experiments and analyses were done using SPSS Version 22. Statistical significance was accepted at a

level of $P<0.05$.

Results and Discussion

To address the question if the early product of Hb-fructose (HbA_1c) mitigates in the presence of extract, we tested its effect on browning reactions (usually Maillard type reactions). There was a significant difference between OD_{340} of fructated-Hb and balm treated samples (Figure 1). The lower absorbance at relevant wavelength for the treated samples represents the attenuation of the Maillard reaction during the initial stage of fructation. From chemical viewpoint, AGEs are formed by a three-step mechanism in which intense brown pigments appear at the early stage [27]. Since the generation of superoxide radicals is favored by transition metals at the glycoxidation phase of the Maillard reaction [31-33], established superoxide radical scavenging with metal chelating activity of balm extract are suggested in contributing to the observed inhibitory [12,23]. Similar metal chelating activity has been reported for guava leaf extract [34]. Figure 2 shows the extent of reacted amino groups of glycated samples using fluorescamine assay [35]. The amount of free lysines in HB was decreased significantly (70%) in the presence of fructose, while LB appreciably protected them from glycation (Figure 2).

It has been shown that fructose modifies the secondary structure of Hb by exposing the solvent-accessible surface area of the sites prone to glycation [36]. The CR method is a well-known assay for cross-β structure in the glycated protein and displays specific absorption at 530 nm after binding [37]. An amyloid-specific increase in intensity at 530 nm was observed when the absorbance was recorded for solutions of CR-Hb samples (Figure 3). Fructose-induced alteration in the canonical secondary structure of native Hb was reduced with the addition of LB extract. The late stage of Hb glycation has been marked by an increase of β-structure at the expense of α-helical structure [38]. The reduction in OD_{530} for LB extract treated samples (Figure 3) concluded that LB extract is able to prevent the conversion of native α-helical structure to β- conformer.

A CD spectropolarimetry was employed in the far-UV region (200 to 250 nm) to independently confirm the results obtained with CR. It was observed that the extent of alterations was much higher for the samples incubated with fructose than with the control. The addition of LB significantly reduced those structural alterations and shielded the secondary structure of Hb from harmful effects of fructose (Figure 4). The α-helix content of the HbA_0 and Fr-Hb samples estimated by the adsorption at 222 nm was approximately 62% and 41%, respectively, comparable with the samples treated with LB extract (Table 1). These observations were consistent with the proposed anti-amyloid properties of LB extract [23] in retarding the changes in α-conformers by shielding the glycation sites, lowering the extent of solvent accessible surface area, and forming a barrier for cross β-structure formation. It has been reported that glycation induces refolding of the globular proteins into amyloid fibrils comprising of cross-β structure [39]. The behavior of LB extract in this respect resembles that of molecular chaperones that block the hydrophobic surfaces of proteins.

At the early stage of glycation, fructose controls the Hb structure such that the heme moiety is no longer engaged in Hb structure and released from the protein [26]. This heme liberation from Hb is responsible for the majority of the pathology associated with diabetic complications [38]. The extent of fructose-induced damage was significantly higher for native Hb samples than those treated with LB extract (Figure 5). Fructation affects the structural integrity of the assembly of α- and β-chains in Hb as heme helps to maintain the overall integrity. Glycation gives rise to major conformational changes in the heme pocket. The process attenuates the forces holding the heme moiety to Hb, which leads to its release from the hydrophobic pocket. This is consistent with the hypothesis that ROS production follows heme liberation upon Hb fructation [25]. Glycation of ceruloplasmin by methylglyoxal also leads to liberation of copper ions [39]. Release of trace metals, such as iron and copper, from metalloproteins is attributed to ROS production, which leads to changes in protein structure [25,40]. It was proposed that the inhibitory effects of LB extract result from its recognized ROS scavenging properties [12] because the addition of LB extract impeded ROS production at the early stage of autoxidative fructation (Figure 1). The reverse effect of LB extract on heme degradation and conformational alteration of Hb originates from its controlling action on the β-amyloid structure formation. A feature of glycated proteins is the propensity to condense into amyloid fibrils, which are characterized by a specific quaternary structure element known as cross-β structure [41]. In contrast to the holo structure, removal of the prosthetic group from many proteins results in apo forms which commonly leads to the formation of the amyloid structure [29]. A synergistic mechanism is proposed for LB action in that antioxidant and ROS scavenging activities are engaged

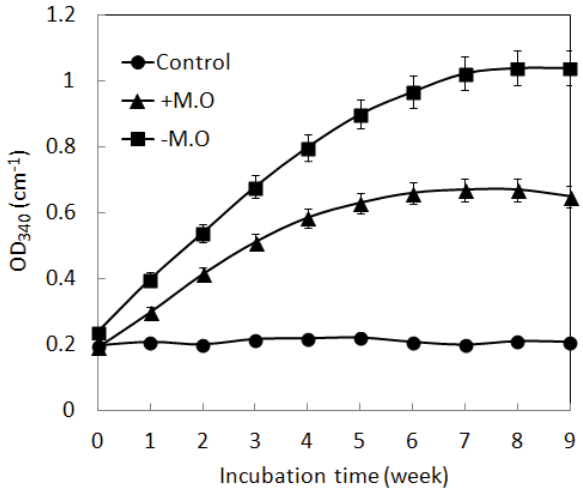

Figure 1: Brownish color absorbance of Hb at 340 nm. Samples were incubated with fructose for 9 weeks in the presence and absence of LB extract. Control samples for Hb were incubated with and without LB extract in the absence of fructose.

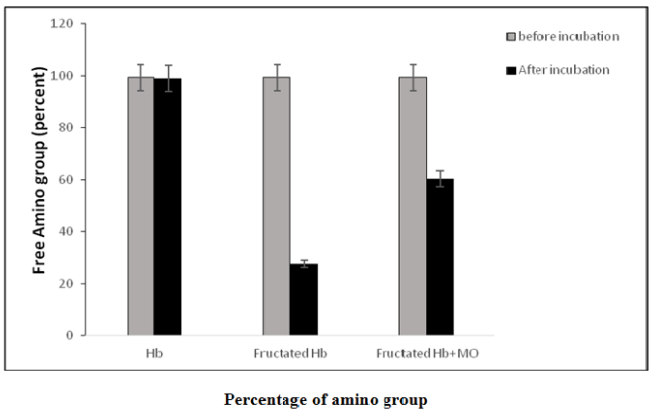

Figure 2: Percentage of free amino groups after incubation of hemoglobin samples for 5 weeks in 50 mM phosphate buffer, pH 7.4 at 37°C with 1) no additives (control), 2) 50 mM fructose, and 3) 50 mM fructose and 10 µg/ml MO extract.

Sample	Control	-M.O	+M.O
α-Helix	62.52 ± 1.1	41.21 ± 1.31	59.16 ± 3.1
β-Sheet	2.11 ± 0.8	13.07 ± 1.2	7.1 ± 1.5

Table 1: Effect of lemon balm extract on secondary structural content of Hb. SELCON3 software was used for estimating the relative percentages of the secondary structural elements.

in carbonyl generation during Hb-fructation. It can be concluded that fructose is capable of inducing amyloid cross-β formation in the Hb structure based on CD spectral and CR staining results.

A binding assay specific to the amyloid marker ThT was used to further determine if such structural changes occur in the fructated Hb. ThT fluorescent emission was enhanced in Hb-AGE and significantly reduced in the LB-treated samples (Figure 6). It can be concluded based on the ThT fluorescent and CR staining data that high levels of fructose promote fibrillation in Hb structure and high levels of LB extract diminish fibrillation in Hb structure. The results are similar to the observed effect of acetylsalicylic acid on Hb structure, in which the agent suppresses β-sheet formation as a precursor to amyloid formation [42]. LB suppresses the ability of fructose to trigger the switch in Hb from the native globular fold into the amyloid cross-β structure upon covalent binding.

The most common way of measuring glycation-induced alterations in proteins is a tryptophan fluorescent assay. Auto-fluorescent phenomena associated with the formation of a structurally distinct fluorophore ($\lambda_{ex}/\lambda_{em}$=370/440 nm) is the hallmark of Hb-AGE production at the late stage of Hb glycation [43]. The auto-fluorescent spectra of the samples were recorded at an emission wavelength of 440 nm with and without the LB extract (Figure 7). Fructose increased the signals from AGE adducts mostly because of the appearance of species that correspond to high levels of protein modification. A conformational switchover in the protein molecules during the process of fructation gave rise to fluorescent Hb–AGE adducts, whereas HbA1c failed to exhibit such fluorescent signals. Variations in the glycated adduct cannot be ruled out because glycation leads to the formation of both HbA1c and Hb-AGE with different auto-fluorescent signals. The emission spectra at 450 nm continued to increase monotonically with a slight blue shift. This is further evidence for Hb–AGE formation during the 9 weeks of incubation (Figure 8). A significant decrease in the fluorescent intensity of AGEs was observed with LB addition as shown in the inset of Figure 8. The fluorescent intensity was quenched by 54% with the addition of 1.0 mg/mL of LB extract compared to the control sample.

An ANS fluorescence assay was performed to further evaluate the effect of LB against AGE formation and to quantify the accessible surface-located hydrophobic regions of the protein [44]. Analyses of ANS emission peaks indicated that fructose dramatically increases fluorescent intensity and shifts the emission spectrum by 20 nm (hypsochromic effect) with incubation time (Figure 9). The fluorescent intensity did not change significantly when LB extract was added to the Hb sample, similar to the blank control. The addition of LB extract was able to prevent large conformational changes arising from enhanced surface hydrophobicity of glycated Hb. Specific binding sites for ANS on the fructated Hb contributed to the observed hyperchromism of the fluorescent intensity [43]. It is likely that LB extract acts to limit access to ANS binding sites, prevents alterations to the protein tertiary structure, and reduces the formation of Hb-AGE adducts. Dissociation of heme moiety from Hb also exposes the hydrophobic residues, contributing to association between heme-globin and ANS. LB extract seems to protect the hydrophobic moieties from exposure to ANS upon

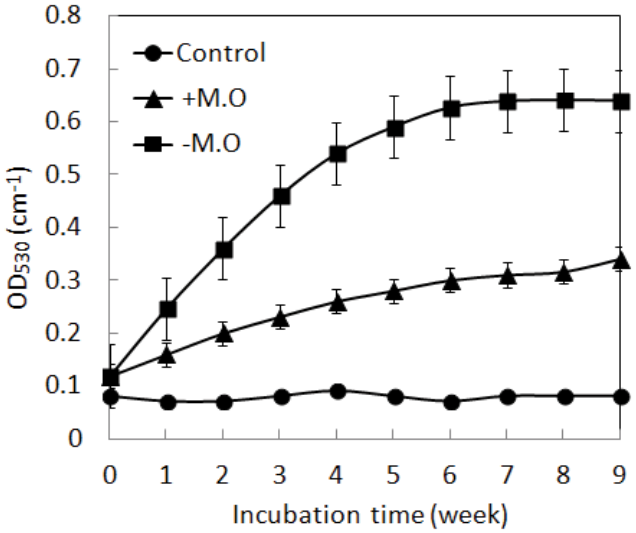

Figure 3: Time evolution of changes in the secondary structure of Hb as measured by Congo red assay at a wavelength of 530 nm.

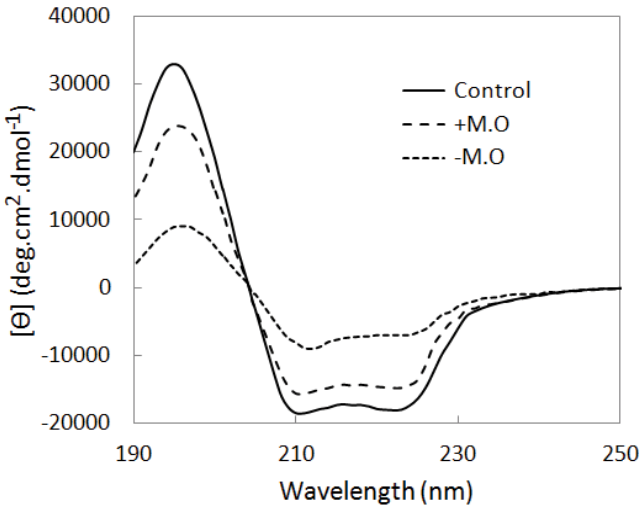

Figure 4: Far-UV CD spectra of Hb. All samples were incubated for 9 weeks in PBS (100 mM, pH 7.4) at 37°C for native Hb (control), in the presence (+MO) and absence of (-MO) of LB extract. The CD data was expressed as molar ellipticity (deg cm^2 dmol^{-1}).

Hb fructation.

Auto-fluorescent signals are observed only after the Maillard reaction. This is consistent with the various adducts generated during the advancement of Hb glycation [45]. The results from the autofluorescent intensity at 308/345 nm ($\lambda_{ex}/\lambda_{em}$) confirmed that fructation increases the Hb-AGE formation (Figure 10). These changes were reduced by the addition of LB extract. The presence of redox catalysts decreased AGE formation. This is conceivable because fructose can generate free-radicals and fructosamines can degrade by oxidation and form AGEs in the later stages (Figure 10). The results in Figure 10 provide evidence for accelerated consumption of ROS or reactive dicarbonyl intermediates and agree with the results in Figures 1 and 4.

Advanced glycation end products (AGEs) arise from the reaction of sugars with side chains and the N-terminus of proteins. They

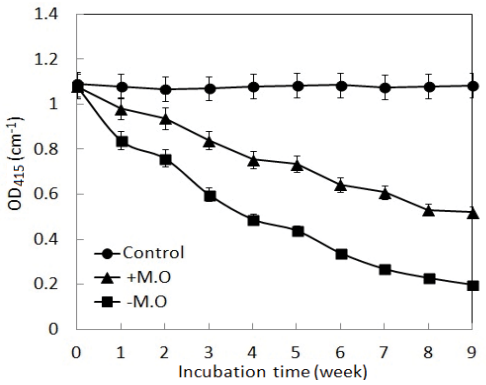

Figure 5: Kinetic changes in Soret peak measured at 415 nm wavelength during fructation. Absorbance spectra of Hb measured as a function of incubation with 0.05 M fructose at 37°C. HB was incubated with or without the LB extract in the absence of fructose in the control samples. Error bars are based on three independent measurements.

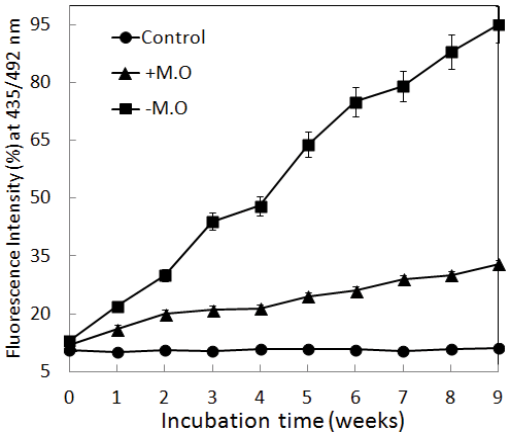

Figure 6: Amyloid fibrillation by Hb (0.75 mM) measured in PBS (100 mM pH 7.4) at 37°C. Kinetics of amyloid fibril formation was measured by increasing fluorescent intensity of ThT at 492 nm (λ_{em}=435 nm). Changes in ThT fluorescent intensity of the samples incubated in the absence (-MO) and presence (+MO) of LB extract. Hb was incubated with or without the balm extract in the absence of fructose in the control samples.

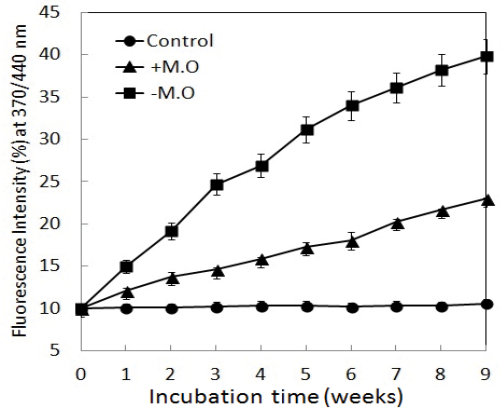

Figure 7: Fluorescent intensity of Hb-AGE as a function of incubation of native-Hb with 50 mM fructose in the absence (-MO) and presence (+MO) of LB extract. Changes in excitation and emission ($\lambda_{ex}/\lambda_{em}$) wavelength of 370/440 nm were recorded with and without the balm extract. Hb was incubated with or without the balm extract in the absence of fructose in the control samples.

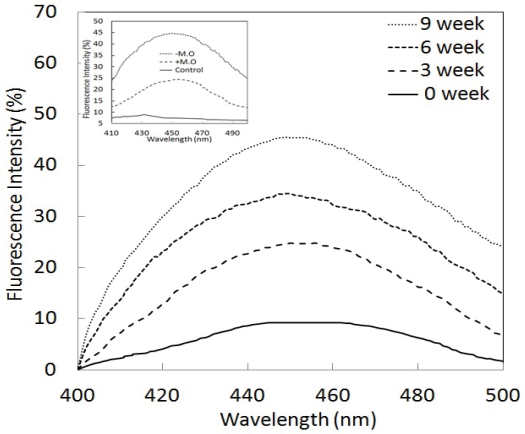

Figure 8: Fluorescent spectra of HSA-AGEs derived from fructose. Maximum fluorescent emissions were recorded in the wavelength range of 400–500 nm (after excitation at 370 nm) for control and test samples in the absence and presence of LB extract for the 9 week incubation time. The inset spectrum shows a fluorescent intensity in the wavelength range of 410–500 nm in ascending order of time.

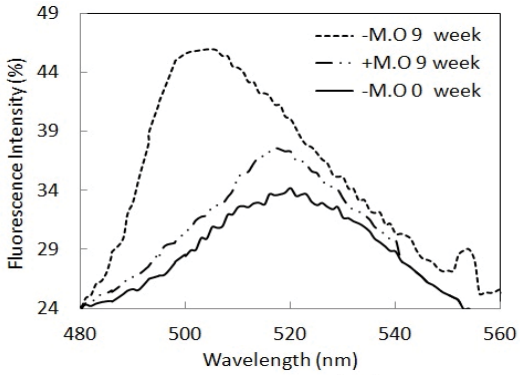

Figure 9: Fibrillation evolution of Fr-Hb as assessed by ANS fluorescent intensity (through excitation and emission wavelength of 370 nm and 480-522 nm, respectively) over the 9 week incubation with and without LB extract. Spectrum of ANS alone is also included.

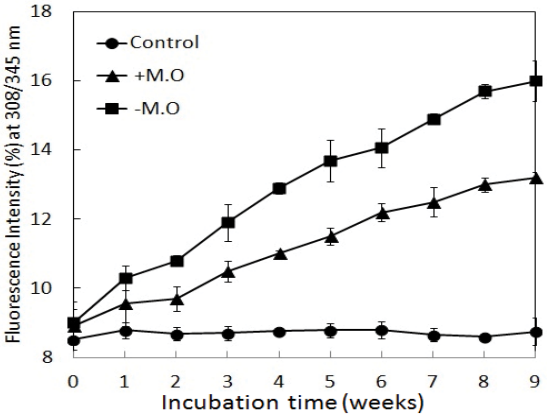

Figure 10: Effect of modification of lysine residues on the structure of Hb. Evolution of intensity of fluorescent signal was recorded at an emission wavelength of 345 nm and of 308 nm after excitation. Fluorescent intensity did not change significantly in the absence of fructose (control) over the 9 week incubation. The spectral changes imposed by fructose (-MO) declined to a remarkable extent in the presence of LB extract (+MO).

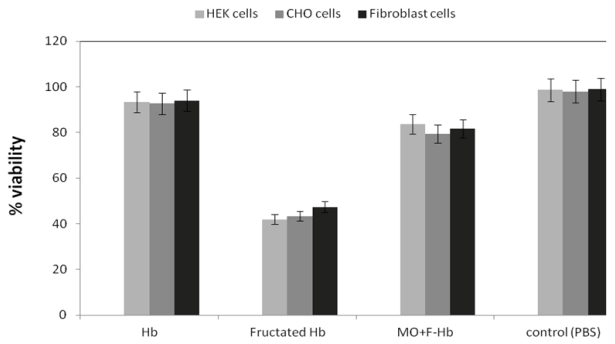

Figure 11: % Viability=(Test absorbance/Control absorbance) × 100%.

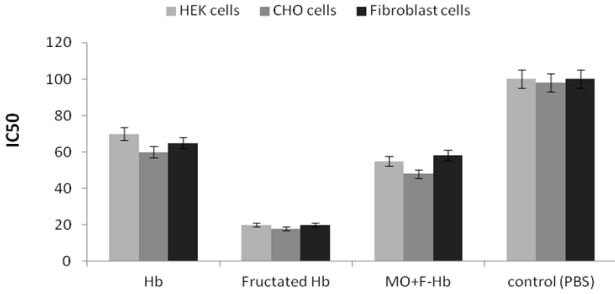

Figure 12: The inhibitory concentration (IC_{50}): (Concentration (µL) of sample needed to inhibit cell growth by 50%) calculated by %=(Blank-test/blank) × 100%

are thought to be involved in the pathogenesis of several diseases by inducing oxidative stress, inflammation, and cell death and are presumably mediated through activation of the receptor of AGE (RAGE). The cell viability of the AGEs was tested to address the question of whether the cell damaging effect of AGE mitigates in presence of LB extract (Figure 11). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate (PMS), produces a formazan product that has an absorbance maximum between 490 nm and 500 nm in phosphate-buffered saline. glycated Hb significantly ($P>0.05$) inhibit mitochondrial respiration as measured by the MTS assay (Figure 11).

IC50 of Hb and of glycated Hb in the presence and absence of LB extract shows that glycated Hb in less concentration can decrease cell viability ($P<0.05$) (Figure 12). This mitigates by applying LB extract [46,47].

Conclusion

Results demonstrate that the multi-target mode of LB action in Hb fructation has potential therapeutic activity for the prevention of AGE-induced complications. It can be concluded that LB antioxidant activity directs its anti-glycation properties. This suggests the potential of LB to mitigate multiple complications stemming from oxidative stress and protein glycation in conformational disorders such as diabetes and ageing.

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