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Dual effect of Caffeine and Curcumin as Antioxidants on Human Hemoglobin in the Presence of Methyl Tert-butyl Ether (MTBE)

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ABSTRACT

Extensive use of methyl tert-butyl ether (MTBE) has raised significant threats to the environment through pollution of environmental resources including ground waters. This compound could accumulate in the blood stream through inhalation of contaminated air since MTBE has a high affinity for blood proteins. The interaction of blood proteins such as human hemoglobin (Hb) with MTBE results in conformational and likely functional changes. The main mechanism for harmful effects of MTBE on Hb is through production of reactive oxygen species (ROS). In this regard, the present work was proposed to study the possible antioxidant potential of two dietary antioxidant agents, curcumin and caffeine, on the reduction of MTBE damage on Hb. Different spectroscopic methods including fluorescence, UV-Vis, circular dichroism, chemiluminescence, and molecular docking were used to study the interactions of curcumin and caffeine with Hb in the presence of MTBE. Our results showed caffeine could decrease the aggregation and ROS effects of MTBE on Hb. However, in the presence of curcumin the MTBE mediated aggregation of Hb was enhanced. These opposing effects of curcumin and caffeine as antioxidants were mainly contributed to the high iron chelating activity of curcumin. Thus, the complex formation between curcumin and heme further enhanced ROS production capability of MTBE.

Keywords: Antioxidants, Caffeine, Curcumin, Methyl tert-butyl ether (MTBE), Hemoglobin, ROS effect

INTRODUCTION

Methyl tert-butyl ether (MTBE), as a gasoline additive, is mainly used to improve octane number of gasoline and reduce the atmospheric concentrations of carbon monoxide. Overuse of MTBE has raised significant threats to the environment by contamination of environmental resources, especially ground waters [1]. According to the U.S. Geological Survey (USGS) report, MTBE concentration in contaminated water resources varied from 0.1 to 17.800 μg l^{-1} [2]. Although, there is no report on the carcinogenic effect of MTBE in humans, the long-term exposure of mice and rats to this compound resulted in lymphoma, leukemia, and kidney tumors [3-5]. MTBE could accumulate in the bloodstream through inhalation of contaminated air because of its high affinity for blood proteins [6]. In blood, as a result of blood proteins interactions with MTBE, these proteins may undergo some structural changes or denaturation, and in this way some toxic compounds could be generated [7].

One of the proposed mechanisms for toxic effects of MTBE on proteins is the production of ROS [8]. In the presence of ROS, proteins undergo structural denaturation and form protein aggregates, as reported in the case of Alzheimer's, Parkinson's and other neurodegenerative diseases [9,10]. Normally the enzymatic (superoxide dismutase, glutathione peroxidase, and catalase) and non-enzymatic antioxidants (vitamin E, vitamin C and

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glutathione) present in the body protect body from harmful effects of ROS compounds [11,12]. However, under conditions where excess ROS produced, including the presence of MTBE, the consumption of natural antioxidants could be beneficial. Thus, the possible interactions of these antioxidants with proteins may provide novel mechanistic insight into development of protective modalities.

Curcumin is a polyphenolic compound, and as a dietary yellowish orange pigment is extracted from the rhizome of the herb Curcuma longa [13]. Curcumin has broad therapeutic potential, such as antioxidant [14], anti-inflammatory [15], neuroprotective [16], and antiviral/antimicrobial [17] properties, and is used as an herbal medicine for centuries [18]. In recent years there is extensive research concerning the therapeutic applications of curcumin in a wide variety of diseases including cancer [19], diabetes [20], Parkinson's [21] and Alzheimer's diseases [22,23]. Despite these beneficial effects of curcumin, there are some reports regarding its adverse effects on protein structures upon its interaction with proteins, such as partial unfolding of Hb [24,25] and its inhibitory effects on cytochrome enzymes [26,27].

Caffeine (1,3,7-Trimethylxanthine) occurs naturally in tea leaves and coffee beans, and is used as a mild central nervous system stimulant in many popular drinks, especially tea and coffee. Caffeine has high solubility in lipids and can readily cross the blood-brain barrier and enter brain, for this reason, this compound is used in various therapeutic applications for neurodegenerative diseases [28-30] such as Parkinson's [31] and Alzheimer's diseases [32]. Caffeine also lowers the risk of type 2 diabetes [33,34]. Also, there are some reports regarding antioxidant activity of caffeine [35], and it is used in the reduction of chemotherapy-induced cytotoxicity [36] and lowering the risk of endometrial cancer [37]. Despite the useful biological activities of caffeine in treatment of many diseases, caffeine could have potential harmful effects [38], such as unfolding of bovine hemoglobin [39].

Human hemoglobin (Hb) as an iron-containing respiratory blood protein is mainly found in the red blood cells of vertebrates, and is responsible for transport of oxygen from the lungs to different tissues. The Hb molecule with molecular weight of 64,500 is made up of two α-chains and two β-chains, which are non-covalently linked in the erythrocytes as a tetramer. Each α chain contains 141 amino acid residues in its structure, and each β chain consists of 146 amino acid residues, and there is a heme prosthetic group in the center of each subunit [23].

In our previous work, we reported that the major mechanism for the harmful effects of MTBE on human hemoglobin (Hb) is related to the production of ROS as a result of the interaction of Hb with MTBE [8]. In this regard, the current study was proposed to determine the effect of dietary antioxidants, curcumin and caffeine, on Hb integrity upon interaction with MTBE.

MATERIALS AND METHODS

Materials

Luminol (5-amino-2,3-dihydro-1,4-phthalazine), caffeine, curcumin, MTBE, hydrogen peroxide (H_2O_2, 30% solution standardized by a UV-Vis spectrometer at 240 nm), and all phosphate buffer salts were purchased in analytical grade from Merck (Darmstadt, Germany). Gold(III) chloride hydrate ($HAuCl_4$) was purchased from Sigma (St. Louis, MO, USA). All buffer solutions were prepared with double distilled water except curcumin. Curcumin solution was first prepared in methanol, and then diluted in phosphate aqueous buffer. Due to the insolubility of curcumin in the aqueous buffer solution and its high solubility in methanol, curcumin was solved in methanol [40]. It must be noticed that methanol itself is not toxic but its metabolite products by the aldehyde dehydrogenase (ALDH) in the liver such as formic acid are toxic [41], therefore presence of methanol is problematic in *in vivo* studies, whereas none of our experiments are not in-vivo.

Hemoglobin Preparation

Human hemoglobin was extracted from the blood of healthy donors according to the previously reported method [42]. Blood was centrifuged to remove plasma components. The packed red cells were washed by adding 10 volumes of an isotonic saline solution (0.9% NaCl) and centrifuged at 4 °C for 15 min at 10,000 rpm. After removing the supernatant, five volumes of phosphate buffer (200 mM, pH 7.4) was added to the sample and centrifuged at 5,000 rpm for 15 min. The washed packed cells were lysed with 5 volume of deionized water and centrifuged at 4 °C for

10 min at 18,000 rpm. In this step, stroma was discarded. The Hb solution was then brought to 20% saturation with ammonium sulfate, left standing for 15 min, and centrifuged at 2 °C for 1 h at 14,000 rpm. The recovered supernatant was then dialyzed in phosphate buffer (50 mM, pH 7.4) at 4 °C for 48 h, which was changed every seven hours.

Ethical Approval

All experiments were performed in compliance with the relevant laws and institutional guidelines. Ethical approval was obtained from ethics committee of University of Tehran (reference number: 142/173928) for collection of blood samples. Furthermore, the informed consent was obtained from everyone participated in this project and blooding was performed by medical doctor.

Methods

ROS measurements. For determination of the ROS production capability of MTBE in interaction with Hb, and also the ROS elimination ability of caffeine and curcumin antioxidants in interaction with Hb, a chemiluminescence spectroscopy based technique was used [46]. Experimental details of this test were as follow: 5 µl of $HAuCl_4$ solution (dissolved in deionized water) and 5 µl Luminol solution (dissolved in100 mM sodium carbonate buffer; pH 11) were injected into the buffer, Hb or mixture of Hb with MTBE, curcumin or caffeine samples. The chemiluminescence intensity was then immediately measured in a chemiluminescence spectrophotometer (Synergy H4 Hybrid Reader; BioTek, USA) at the wavelength of 425 nm.

The luminescence emission intensity increases with the concentration of ROS. This has been investigated and verified using hydrogen peroxide as a species of ROS. Also, the luminescence intensity diagram with the concentration of hydrogen peroxide is plotted and used as a basis for measuring the antioxidant effect of curcumin. In the presence of curcumin, the intensity of luminescence emission decreased, indicating the antioxidant effect of curcumin.

Aggregation measurements. The thermal aggregation of hemoglobin (3.1 µM in 50 mM phosphate buffer) in the presence or absence of MTBE, curcumin or caffeine were studied by measuring the relative turbidity at the wavelength of 360 nm and temperature of 58 °C by Varian UV-Vis spectrophotometer (Carry 100 Bio). We have done measurements in 58 °C/331 K as it has been approved that 331 K is the highest temperature in which structural changes of Hb is 100% reversible. Going 2 K higher in temperature reduces reversibility from 100% to 64%. It must be noticed that the aggregation tests were done immediately after addition of MTBE, curcumin, and caffeine into the Hb solution. The aggregation kinetic data were interpreted using a model described by Wang and Kurganov [43]. In the case of first order kinetic fitting of this model, the effective first order aggregation rate constant, k_1 and the aggregation lag time (t_0) could be extracted from the slope and x-intercept of a fitted tangent line to the linear part of $\ln[(\tau_{lim} - \tau)/\tau_{lim}]$ *vs*. t plot, respectively, given by the following equation:

$$\ln[(\tau_{lim} - \tau)/\tau_{lim}] = k_1 (t_0 - t) \quad (1)$$

where τ is turbidity at the time of t, τ_{lim} is the limiting value of turbidity as time tends to infinity.

Circular Dichroism (CD) spectropolarimetry. One of the most commonly used techniques in assessing the secondary structural content of proteins is Circular dichroism (CD) spectroscopy. In the present study, the secondary structure changes of hemoglobin samples in the presence of MTBE, curcumin, and caffeine were determined in the far-UV region (195-260 nm) using a JASCO J-810 spectropolarimeter. These were done using a 1 mm quartz cell with the step size of 1 nm at 25 °C with three accumulations. CD results were represented in mean residue ellipticity $[\theta_\lambda]$ using the following relation:

$$[\theta_\lambda] = \frac{MW\theta_{obs}}{10ncl} \quad (2)$$

where θ_{obs} is the observed CD signal (millidegree), MW is the protein molecular mass (g mol^{-1}), *n* is the number of amino acid residues, *c* is the concentration of protein (mg ml^{-1}) and *l* is the path length (cm). The CDNN CD Spectra Deconvolution Software (version 2.1) was used to obtain protein secondary structure contents [44]. The concentration of all hemoglobin samples for CD analysis was set at 3.1 µM in phosphate buffer (50 mM). In CD experiments, after about 5 min from the injection of

MTBE, curcumin, and caffeine into the Hb solution, the CD spectra of the samples were recorded at room temperature.

Heme degradation analysis. Fluorescence spectroscopy is one of the most commonly used techniques to study the heme degradation products of Hb produced by its interaction with other materials. As reported by Nagababu and Rifkind [45], when Hb interact with ROS it undergoes a series of heme degradation process which some products of these process are fluorescent and can be detected by fluorescence technique. In the present work, a fluorescence technique was used for determination of the possible heme degradation products of Hb during its interaction with MTBE, curcumin, and caffeine. For this test, given values of MTBE, caffeine, or curcumin solutions at different final concentrations were added into the of 0.5 ml of Hb solution with a concentration of 3.1 μM in 50 mM phosphate buffer at pH 7.4 and after about 5 minutes, the room-temperature fluorescence spectra of the samples were recorded using a fluorescence spectrophotometer (Cary Eclipse, Varian Co.) at excitation (Ex) wavelength of 321 nm. Because in this wavelength, based on the caffeine excitation spectrum, caffeine has minimum excitation intensity and dose not interfere with other molecules in the medium. The final concentration of MTBE, caffeine, or curcumin in Hb solution could be funded in the related results section. The fluorescent emission (Em) spectra were scanned from 330 to 600 nm. All of the fluorescence experiments were performed at a temperature of 25 °C with the Ex and Em slits width of 10 nm.

Molecular docking. After obtaining the structure from the protein data bank we did 50 ns of molecular dynamics simulations to have a hemoglobin structure with optimum positioning of the amino acid residues. MD followed by a structural clustering gave us the final coordinates file of the Hb to perform molecular docking using the autodock package. Molecular docking was performed using AutoDock 4.2 and AutoDockTools version 1.5.6 with standard parameters [47]. Crystal structure of human Hb was obtained from protein data bank (pdb code 1GZX), ligand structures were constructed, and energy was minimized using PRODRG online server [48]. All calculations were performed with the use of a PC with Intel Core i7 Processor. Images were generated with PMV [47].

RESULTS

ROS Test Results

The antioxidant activity of caffeine and curcumin was measured by the ROS test and the results are summarized in Table 1. MTBE is an organic compound having ether functional group between methyl and *tert*-butyl groups which doesn't produce any ROS in buffer solution. From the results of this table it can be deduced that the ROS compounds of H_2O_2 in buffer were efficiently removed by the caffeine and curcumin antioxidants, and among these antioxidants, curcumin has more antioxidant activity.

Aggregation Analysis

The degree of protein aggregation was obtained *via* a turbidity measurement at the wavelength of 360 nm and elevated temperatures. This is a useful technique for measurement of the protein resistance against thermal denaturation. There are three phases in a typical thermal aggregation curve: a lag phase, a logarithmic phase, and a plateau phase. In lag phase step, the protein undergoes some denaturation in its structure, and these unfolded proteins start to aggregate in logarithmic phase. With the increase of the protein stability, duration of the lag phase increases and the slope of logarithmic phase decreases and *vice versa* [49, 50]. As discussed in our previous work, the presence of MTBE in the vicinity of Hb could stimulate ROS production, and with the increasing of the ROS amount (due to the increasing of MTBE), the aggregation of Hb increases [8]. According to the literature the oxidative conditions by reducing the number of hydrogen bonds leading to protein unfolding (decreasing the lag time). This subsequently results in the acceleration of the protein aggregation (increasing the slope of the logarithmic phase) [51,52]. In this regard, the current study was proposed to reduce the destruction effects of the MTBE on Hb by using some antioxidant agents. Figure 1 shows the effect of MTBE, caffeine and curcumin addition on the Hb aggregation.

Figure 1a shows that the aggregation rate of Hb increases and the aggregation lag time decreases in the presence of MTBE, curcumin, and mixture of MTBE and curcumin. Curcumin is an effective antioxidant agent [53] and could remove ROS generated by the MTBE interaction with Hb. However, MTBE interaction with Hb leads to

Table 1. ROS Measurements Using Chemiluminescence Spectroscopy. All Samples were Prepared at 25 °C in 50 mM Phosphate Buffer at pH 7.4

Sample	ROS (μM)
Buffer	0
Buffer + 10 μM H_2O_2	9.539
Buffer + 150 μM MTBE	0
Buffer + 100 μM Caffeine	0
Buffer + 100 μM Curcumin	0
Buffer + 10 μM H_2O_2 + 100 μM Caffeine	1.326
Buffer + 10 μM H_2O_2 + 100 μM Curcumin	0.853

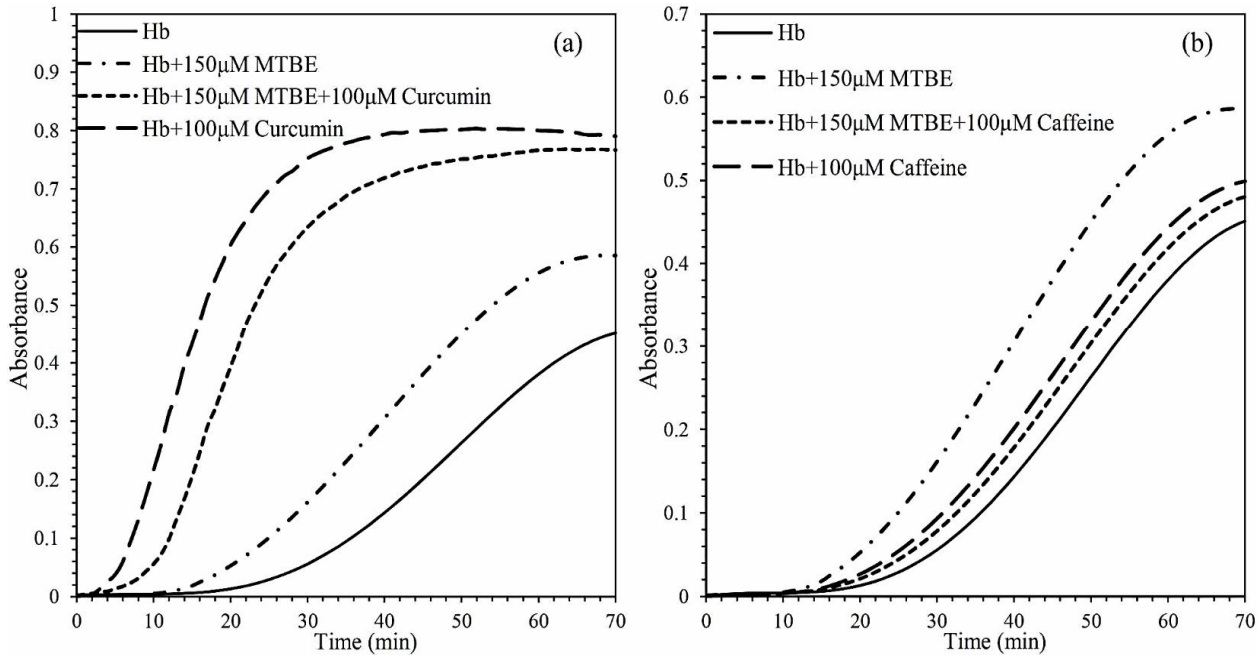

Fig. 1. Aggregation rate of Hb in the presence of (a) MTBE and curcumin, and (b) MTBE and caffeine, at temperature of 58 °C and wavelength of 360 nm.

protein aggregation. As reported in the literature, curcumin is a bidental ligand and can form a complex with transition metal ions [54-56]. Thus, curcumin could lead to the structural instability of proteins that have these types of metal ions in their structure.

Figure 1b indicates the aggregation rate of Hb in the presence of MTBE, caffeine, and a mixture of MTBE and caffeine. The aggregation lag time decreased, when caffeine or MTBE + caffeine interacted with Hb. Thus, caffeine is a suitable antioxidant for reducing the destruction effect of

Table 2. Aggregation Rate Parameters of Hb in the Presence of MTBE, Curcumin and Caffeine at Temperature of 331 K

Sample	Lag time (min)	k_1
Hb	10.74	0.0023
Hb + MTBE (150 μM)	7.85	0.0038
Hb + caffeine (100 μM)	9.35	0.0037
Hb + curcumin (100 μM)	1.27	0.0119
Hb + caffeine (100 μM) + MTBE (150 μM)	10.11	0.0036
Hb + curcumin (100 μM) + MTBE (150 μM)	1.39	0.0051

Table 3. Secondary Structure Contents of Hb at Different Conditions

Sample	Alpha-helix	Antiparallel	Parallel	Beta-turn	Random coil
Hb	59.71% ± 2.4	2.35% ± 1.35	4.40% ± 0.56	11.85% ± 0.35	21.82% ± 3.3
Hb + MTBE (150 μM)	54.25% ± 1.71	3.03% ± 1.34	5.14% ± 0.55	12.51% ± 0.57	25.12% ± 1.91
Hb + caffeine (100 μM)	57.16% ± 2.22	2.6% ± 1.28	4.67% ± 0.61	12.08% ± 0.44	23.49% ± 2.43
Hb + curcumin (100 μM)	58.03% ± 2.51	2.58% ± 1.23	4.88% ± 0.25	11.83% ± 1.95	22.7% ± 4.14
Hb + caffeine (100 μM) + MTBE (150 μM)	58.12% ± 2.72	2.48% ± 1.24	4.60% ± 0.59	12.17% ± 0.13	22.68% ± 3.17
Hb + curcumin (100 μM) + MTBE (150 μM)	47.65% ± 1.11	4.08% ± 1.65	6.48% ± 0.15	13.35% ± 0.89	28.48% ± 1.96

MTBE on Hb. All of the aggregation parameters resulted from the aggregation tests are summarized in Table 2.

CD Analysis

CD analysis was used for precise evaluation of the secondary structural changes of Hb under different conditions, and the results are summarized in Table 3. Figure 2a shows that the presence of MTBE mainly induced the conversion of the alpha helix to the random coils. In the presence of curcumin, there was a negligible change in the secondary structure contents of Hb. However, when the mixture of MTBE and curcumin interact with Hb, there was a remarkable reduction in secondary structure of Hb. Thus, the presence of curcumin increased the destruction effects of MTBE on the Hb secondary structure.

According to the results of Fig. 2b, it can be concluded that in the presence of caffeine Hb undergoes some secondary structural changes in such a way that its alpha helix structures were converted to the random coils. However, in comparison with curcumin, MTBE + caffeine reduced the destructive effects on the Hb secondary structure contents.

Heme Degradation

A series of heme degradation tests were performed to assess the heme degradation capability of caffeine, curcumin, and MTBE during interactions with Hb. Figure 3a shows that caffeine itself has an emission

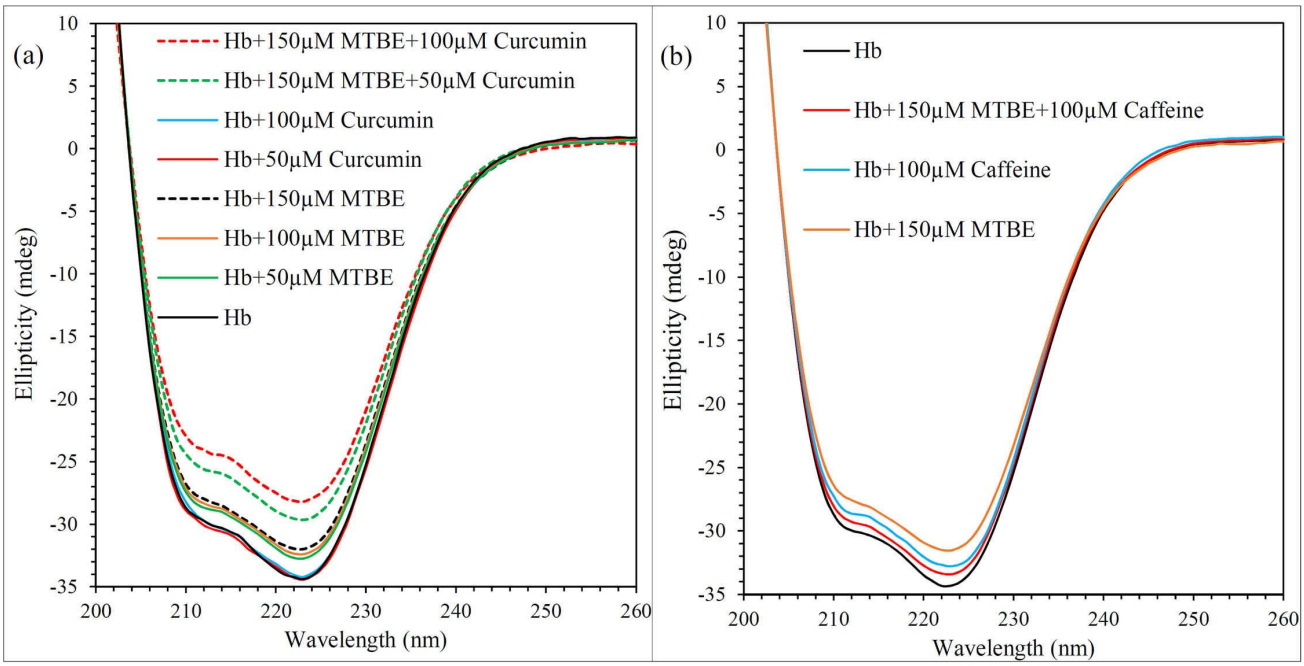

Fig. 2. Circular dichroism spectra of Hb in the presence of (a) MTBE and curcumin and (b) MTBE and caffeine.

spectrum at about 385 nm in excitation with 321 nm. These spectra were also observed in the interaction of curcumin with Hb (Fig. 3b). However, due to maximum absorption of Hb in this region (Fig. 3f), a local minimum was seen in the emission spectra of caffeine at about 415 nm. Thus, the interaction of caffeine with Hb did not produce much heme degradation products. The interaction of MTBE with Hb generated a new emission band whose intensity increased with increasing concentration of MTBE. Due to maximum absorption of Hb in this region (Fig. 3f), this spectrum was divided to two splits at about 415 nm (Fig. 3c). Based on our previous work, the main mechanism for the harmful effects of MTBE on Hb is related to the production of ROS from hem degradation products of Hb due to its interaction with MTBE [8]. Therefore, heme degradation tests of Hb interaction with MTBE in the different concentrations of caffeine were performed to determine the effective concentration of caffeine. Figure 3d shows with the addition of caffeine to Hb and MTBE solution the amounts of heme degradation products was decreased. Furthermore, the addition of caffeine with a concentration of 120 μM protects hemoglobin from hem degradation. Therefore, the effective concentration of caffeine is about 100 μM. To survey any possible interactions of caffeine with heme prosthetic group of Hb, the emission spectra of caffeine were obtained in the presence of hemin. Figure 3e shows these emission spectra are very similar to that of caffeine. The only difference was the presence of a local minimum at about 385 nm, which coincides with maximum absorption of hemin (Fig. 3f). Thus, caffeine has no interactions with the heme group of Hb. Furthermore, Hb and hemin had no emission at 350-600 nm in excitation with 321 nm.

Figure 4a shows that curcumin has an emission specrtum at about 510 nm at excitation of 321 nm. In the interaction of curcumin with Hb, a new emission spectrum was observed at about 360-390 nm. According to the results of Fig. 4e, for the interaction of curcumin with hemin, this new emission spectrum results from the interaction of curcumin with heme group. As reported in the literature, curcumin can form a complex with iron metal ions. Thus, this new emission band can be related to the formation of a complex between curcumin and heme prosthetic group of Hb. Figure 4d demonstrates that the presence of curcumin cannot reduce the amounts of hemedegradation products from the interaction of MTBE with Hb, and its presence facilities the complex formation between heme and curcumin.

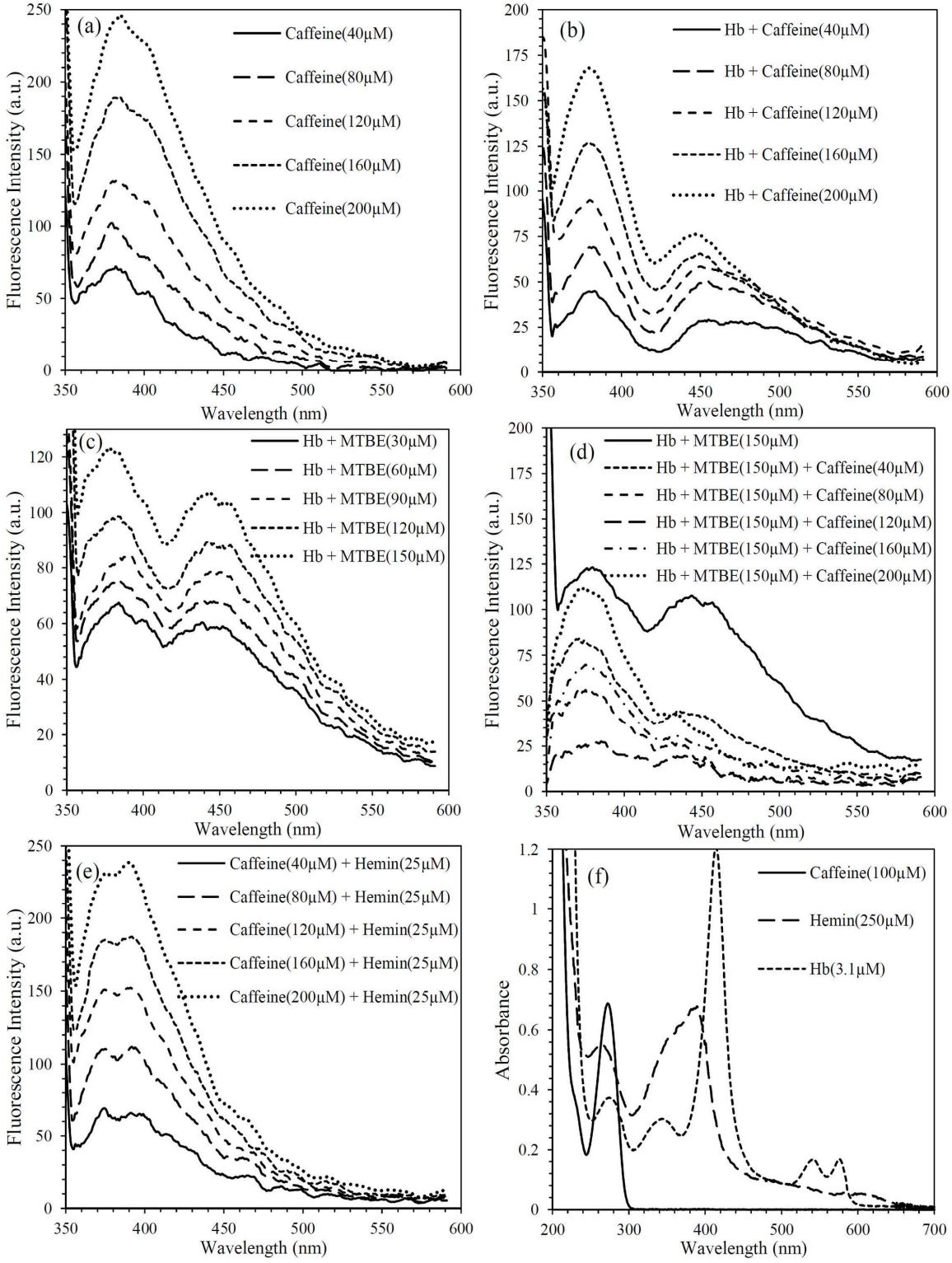

Fig. 3. Fluorescence emission spectra at λ_{ex} = 320 for (a) caffeine, (b) mixture of Hb and caffeine, (c) mixture of Hb and MTBE, (d) mixture of Hb, MTBE, and caffeine, (e) mixture of Hemin and caffeine, and (f) Absorption spectra of Hb, Hemin, and caffeine.

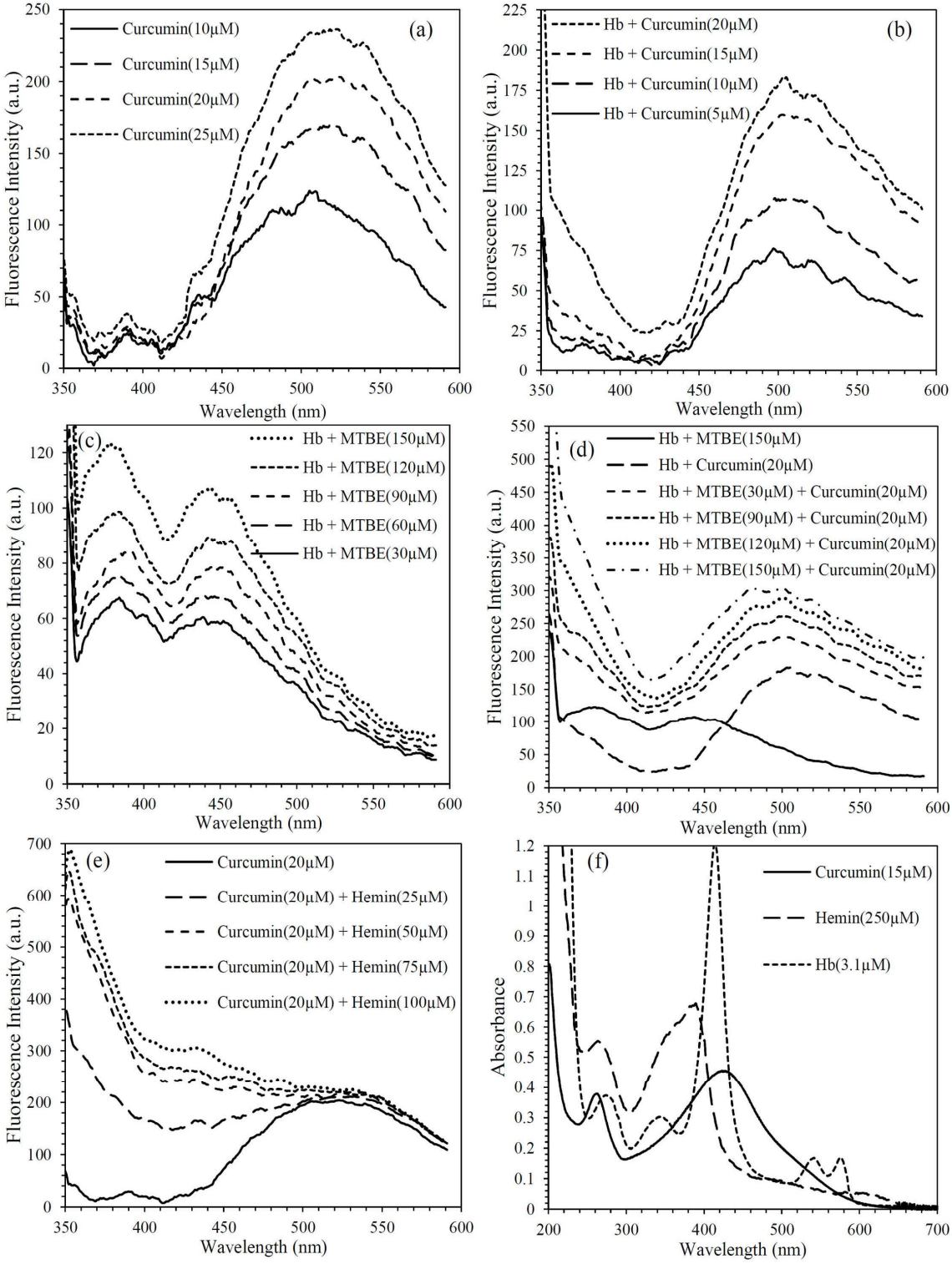

Fig. 4. Fluorescence emission spectra at λ_{ex} = 320 for (a) curcumin, (b) mixture of Hb and curcumin, (c) mixture of Hb and MTBE, (d) mixture of Hb, MTBE, and curcumin, (e) mixture of Hemin and curcumin, and (f) absorption spectra of Hb, Hemin, and curcumin.

Molecular Docking

In order to have a clear understanding of the mechanism behind the different antioxidant behavior of caffeine and curcumin molecules, molecular docking studies were carried out. A broad range of binding packets, with almost the same binding energies, were noted in the case of caffeine molecule (Fig. 5a). Figure 5b shows caffeine molecule was attached to the bottom of a deep cleft between two adjacent subunits in hemoglobin. The conformation related to the lowest binding energy, along with interacting amino acid residues of Hb, are shown in Fig. 5c. Amino acids of Hb with possible interaction with caffeine are also shown in Fig. 5c. This binding conformation, besides those which are shown as green spheres around the protein (Fig. 5a), may alter protein conformation. This provided a condition for slight generation of heme degradation products that was confirmed by CD and fluorescence spectroscopic studies.

Interaction of curcumin with hemoglobin was also studied using molecular docking. Figure 5d shows the conformation that is related to the best binding energy between curcumin and hemoglobin. Figure 5e shows that the curcumin molecule was attached and well fitted inside a cavity between the two heme groups at the surface of the hemoglobin. Arginine 183 and one of the heme groups from hemoglobin formed two hydrogen bonds with curcumin (Fig. 5f). The experimental CD and fluorescence spectroscopic results, and previous reports [56,57] indicate curcumin has high iron chelating activity. The curcumin special interaction site at the position close to the heme groups (as obtained from molecular docking) support the notion that binding of curcumin to heme group result in unfolding of Hb. This results in exposure of the heme groups and heme degradation products as a consequence of these interactions. Generation of heme degradation products increased ROS production [58], and reactions of ROS compounds with protein result in protein unfolding [59].

DISCUSSION

Oxidative stress induces oxidative denaturation and deactivation of biological macromolecules such as nucleic acids and proteins [59]. The human body has developed some efficient defense mechanisms to deal with excess production of ROS under oxidative stress conditions including scavenging of ROS compounds, blocking ROS production pathways, or using enzymatic and nonenzymatic antioxidant defenses produced in the body [60]. As discussed in our previous work, the presence of MTBE in a Hb solution results in the production of ROS, which their subsequent interaction with Hb leads to protein unfolding and instability [8,61]. For this reason in the current work, caffeine and curcumin were selected as dietary antioxidant agents to reduce the harmful effects of MTBE on Hb through consumption of ROS. The measurements of antioxidant activity of caffeine and curcumin revealed that curcumin has more antioxidant activity (Table 1). The results of aggregation studies confirmed that caffeine is more effective than curcumin in reducing the destructive effects of MTBE on thermal aggregation of Hb. In contrast, the presence of curcumin enhanced the destructive effect of MTBE on aggregation of Hb. From the secondary structure viewpoint, between curcumin and caffeine antioxidants, again caffeine was a better choice for decreasing MTBE adverse effect on Hb. According to the results of CD spectroscopy, upon interaction of Hb with curcumin, the α helix secondary structure of Hb was decreased, which could be related to hydrophobic interactions between curcumin and Hb [24]. According to the heme degradation results, caffeine did not have a significant heme degradation effect on Hb, but curcumin did due to its easy interaction with heme group of Hb and chelating of iron ion [53]. These caused the amino acids in Hb and the heme groups to be gradually exposed and led to heme degradation and production of more ROS. By production of more ROS, due to the synergic effects of curcumin and MTBE on Hb, the denaturation of Hb was enhanced in the presence of these compounds. The iron chelating capability of curcumin is well documented in the literature [56,57], and our molecular docking results confirmed this effect. Thus, the observed opposite effects between the two antioxidants, caffeine and curcumin, could be related to the iron chelating activity of curcumin. Incubating Hb with curcumin promotes protein aggregation due to the induction of conformational changes. On the other side, MTBE also triggers Hb aggregation. Considering these two similar effects, makes it reasonable

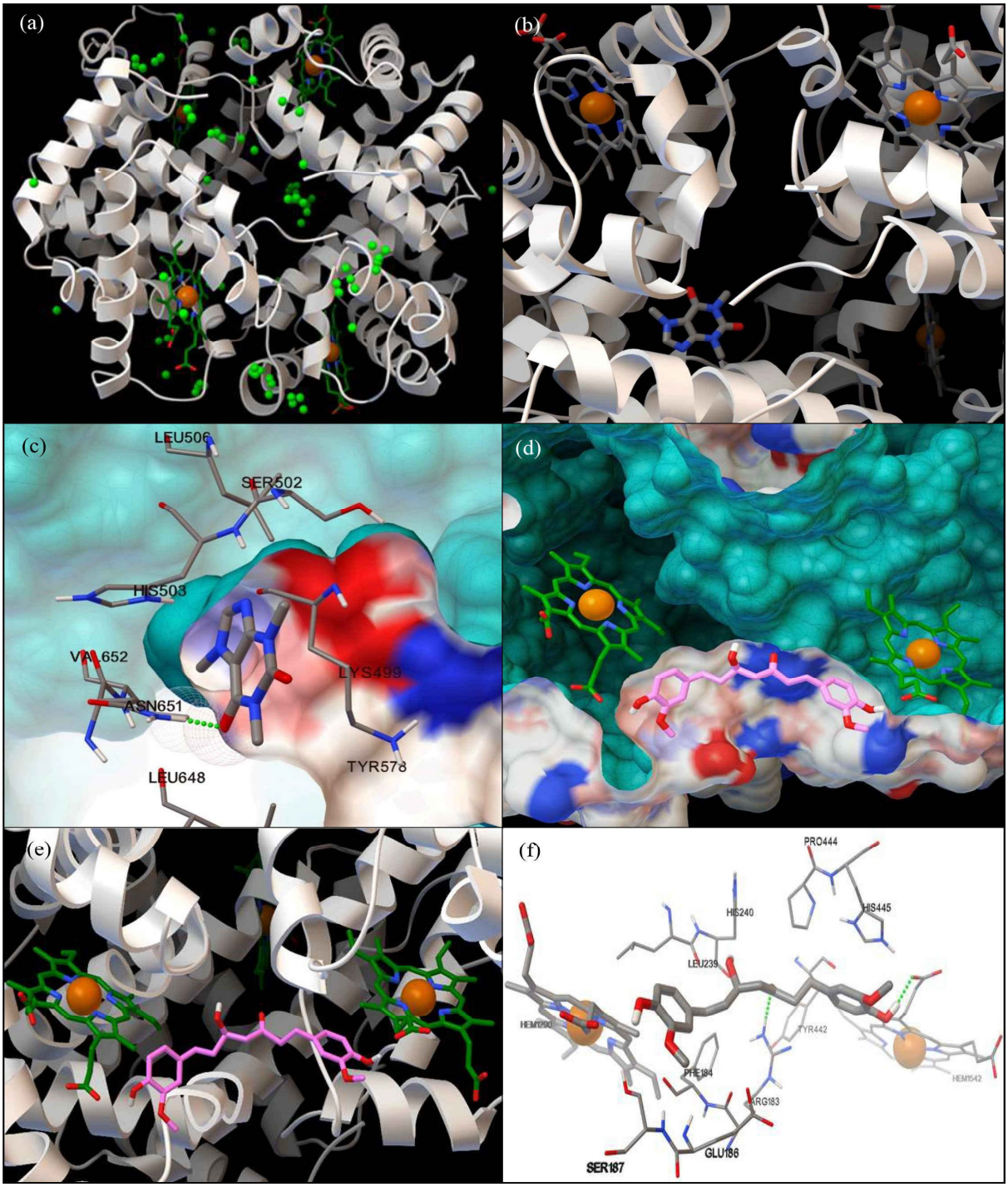

Fig. 5. (a) Possible interaction positions of caffeine with Hb (shown as green spheres), (b) position of caffeine in the conformation with lowest binding energy, (c) interacting amino acid residues of Hb with caffeine in best binding position, (d) 3D representation from curcumin interaction site with Hb with lowest binding energy, (e) curcumin interaction site with Hb with regarding heme groups, and (f) interacting amino acid residues of Hb with curcumin in best binding position.

that co-incubation with curcumin and MTBE will cause a synergic effect on aggregation. It is worth mentioning that, in the presence of MTBE, curcumin plays an important role as a radical scavenger and at the same time suppresses the aggregation process by intercalating in hydrophobic parts of the aggregates.

Protein malfunction contributes to pathogenesis of a wide range of diseases, and this condition happen as conformational instability which is triggered by various destabilizing conditions and preventive strategy to confront with protein aggregation is very important in bio-industry and medicine [62].

CONCLUSIONS

In summary, in the current study two dietary antioxidant agents (*i.e.* curcumin and caffeine) were evaluated for their ability to reduce the harmful effects of MTBE on Hb through inactivation of ROS. Our results indicated that between curcumin and caffeine antioxidants, only caffeine could decrease the destruction effect of MTBE on Hb through consumption of ROS. In contrast, the presence of curcumin enhanced the destructive effects of MTBE on Hb. Generally it is thought that the antioxidants reduce protein aggregation and ROS production. However, because of iron chelating capability curcumin for the heme prosthetic group of Hb, it acts contradictory and enhances the toxic effects of MTBE on Hb, which results in the production of more heme degradation compounds and consequently more ROS generation. Thus, in selection of the antioxidant agents for ROS inactivation purposes, their possible interactions with biological molecules must be carefully considered, especially in the case of antioxidants with high metal ion chelating activity.

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