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RESEARCH ARTICLE

Diverse Effects of Different "Protein-Based" Vehicles on the Stability and Bioavailability of Curcumin: Spectroscopic Evaluation of the Antioxidant Activity and Cytotoxicity *In Vitro*

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Abstract: ***Background*:** Curcumin is a natural polyphenolic compound with anti-cancer, anti-inflammatory, and anti-oxidation properties. Low water solubility and rapid hydrolytic degradation are two challenges limiting use of curcumin.

***Objective*:** In this study, the roles of the native/modified forms of Bovine Serum Albumin (BSA), β-lactoglobulin (β-lg) and casein, as food-grade biopolymers and also protein chemical modification, in stabilizing and on biological activity of curcumin were surveyed.

***Methods*:** In this article, we used various spectroscopic as well as cell culture-based techniques along with calculation of thermodynamic parameters.

***Results*:** Investigation of curcumin stability indicated that curcumin binding to the native BSA and modified β-lg were stronger than those of the modified BSA and native β-lg, respectively and hence, the native BSA and modified β-lg could suppress water-mediated and light-mediated curcumin degradation, significantly. Moreover, in the presence of the native proteins (BSA and casein), curcumin revealed elevated *in vitro* anti-cancer activity against MCF-7 (human breast carcinoma cell line) and SKNMC (human neuroblastoma cell line). As well, curcumin, in the presence of the unmodified "BSA and β-lg", was more potent to decrease ROS generation by hydrogen peroxide (H_2O_2) whereas it led to an inverse outcome in the presence of native casein. Overall, in the presence of the protein-bound curcumin, increased anti-cancer activity and decreased ROS generation by H_2O_2 *in vitro* were documented.

***Conclusion*:** It appears that "water exclusion" is major determinant factor for increased stability/efficacy of the bound curcumin so that some protein-curcumin systems may provide novel tools to increase both food quality and the bioavailability of curcumin as health promoting agent.

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1. INTRODUCTION

According to the International Food Information Council (IFIC) "functional food" is defined as "food that has health promotion properties beyond basic nutrition". Based on this fact, various researches have been devoted on developing novel methods for entrapment of functional ingredients (phytochemicals; especially polyphenols) into foods. Phenolic compounds are the most abundant antioxidants in human diet. These secondary metabolites (of the plants) are categorized into 15 major classes and have attracted great interest for their various bioactivities [1]. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6- heptadiene-3,5-dione), as the main component of Curcuma longa rhizomes (Figure **1**), is one of the most important plant-derived polyphenols possessing chemopreventive and safety activities against malignancy [2]. Also, due to its advantageous characteristics and yellow-orange color it has long been consumed in Asia as a dye and food additive. In the past decade, several other medicinal effects have been reported for curcumin including anti-inflammatory, anti-oxidant, anti-cystic fibrosis, anti-proliferative, anti-angiogenic, and wound healing properties [3]. Nowadays, it also has culinary uses as spice, and as preservative and industrial food dye (E-100).

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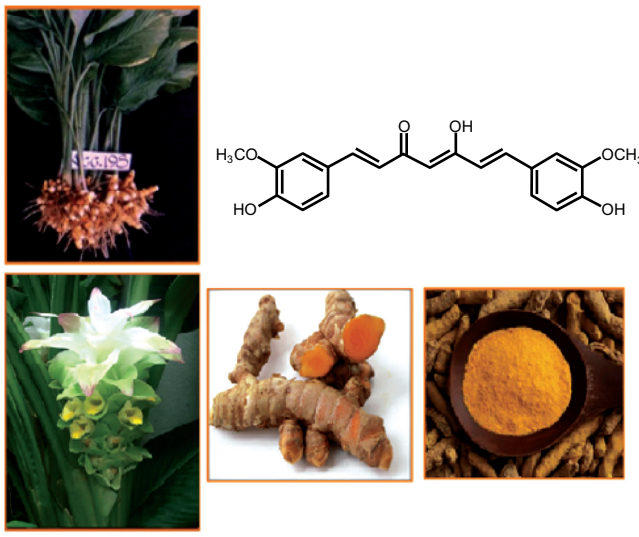

Figure 1. (Top) *Curcuma Longa* with flower and rhizome plus dried turmeric. Turmeric (*Curcuma longa* L.), belonging to the family of Zingiberaceae, is a perennial herb native to India where its rhizome is used as a yellow colorant curry spice and traditional medicine. (Bottom) Chemical structure of curcumin. The active principle in turmeric was identified as a group of polyphenolic compounds, namely curcumin (74-78%), demethoxy curcumin (15-18%) and bisdemethoxy curcumin (4-6%) commonly referred to as "curcumin".

Due to low intrinsic toxicity of curcumin for healthy (normal) cells, it has been generally recognized as safe (GRAS) and numerous clinical studies have been devoted on developing curcumin into the therapeutic agents [2b, 4]. So, it could be considered as a extremely safe compound even at very high doses [2b, 4], up to 8-12 g/day [5]. Preclinical studies revealed the potential of curcumin for carcinogenesis inhibition toward different cell lines originated from cervix, ovary, colon, stomach, pancreas, breast, prostate, liver, bone marrow, and oral epithelium. The potential of induction of apoptosis in cancer cells without cytotoxic effects on normal cells makes it as a promising candidate (or molecular scaffold) for development of new generation of anti-cancer drugs [2a]. Despite its non-toxic nature, high efficacy, and well-known mechanism of action, from the chemical viewpoint, there are various issues (limitations) which should be solved to use curcumin as a routine treatment compound. The low solubility in water (about 11 ng/mL) is one of these issues which significantly limited its bioavailability *in vivo* [3b]. The extremely low oral bioavailability of curcumin consequently limits its approval as a therapeutic agent [5]. On the other hand, the rapid hydrolysis of curcumin (even at physiological pH) causes molecular fragmentation and degradation within 30 min [6]. Moreover, the recent studies illustrated that the β-diketone moiety can act as a specific substrate for a series of aldo-keto reductases and undergo a rapid *in vivo* decomposition [7].

Due to the drawbacks such as the poor solubility, bioavailability and also instability of curcumin, numerous studies have been conducted to develop methods to overcome these defects. These methods include emulsification, chemical modification, and encapsulation in polymer nanoparticles, cyclodextrins, hydrogels, surfactants micelles, lipid bilayers, and vesicles [8].

The main aim of the current study is to introduce albumin, casein and β-lg as food-grade amphiphilic materials to interact with curcumin. Serum albumins and caseins are commonly used transporting vehicles for proteins, hormones, drugs, and diagnostic agents. Casein micelles are relatively easy to purify/prepare, biodegradable, and have potential for high drug loading capacity. Serum albumin is the most abundant protein in the blood plasma with outstanding binding capacity [9]. β-lg is also the most abundant protein in whey, constituting 40% of the protein component and has capable of binding and transporting small hydrophobic molecules such as steroids, fatty acids, retinoids, vitamin D, cholesterol etc within its central cavity known as the calyx [10]. Both casein micelles and albumin delivery systems have been previously employed for administration of several hydrophobic drugs [11]. In the literature, there are a few reports on the interaction of curcumin with β-lg, casein and albumins [2b, 12] and its potential delivery to tumor cells [2b], implying that these proteins have the ability to solubilize/stabilize curcumin in aqueous medium and also have the ability to improve its antioxidant activity [2b, 3b, 13]. Since proteins are generally amphiphilic polymers, we assume that curcumin binding to the mentioned proteins as well as its stability, bioavailability, anti-oxidant activity and also cytotoxicity can be manipulated under the effect of modification (acetylation) of the carrier protein. Chemical modification induces structural changes in proteins and exposure of previously buried hydrophobic patches or rendering groups prone to various interactions, affecting the binding of hydrophobic and/or hydrophilic compounds, respectively [14]. In continuation to our earlier studies [15] and since, half-life is an important factor of curcumin efficacy, in this study; we also reported the mechanism of the enhanced stability of curcumin upon hydrophobic interaction with proteins. Furthermore, we demonstrated that stability enhancement of curcumin in the presence of proteins, originates in part from its stronger binding (high K_b) to the protein. Finally, in the presence of bound, encapsulated (and stabilized) curcumin, increased anti-cancer activity and decreased ROS generation by H_2O_2 *in vitro* were documented.

2. MATERIALS AND METHODS

2.1. Materials

BSA (essentially free fatty acid) fraction V was purchased from Applichem, pure curcumin and 2, 2-diphenyl-1-picryl hydrazyl (DPPH) were supplied from Merck (Darmstadt, Germany). Casein, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acetic anhydride, 2,5 dichlorofluorescin diacetate (DCF-DA) were from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture medium, penicillin–streptomycin and Fetal Bovine Serum (FBS) were purchased from Gibco (Gibco, Grand Island, NY, USA). The other reagents were of analytical grade without any further purification. All the solutions were freshly prepared in doubly-distilled water and, otherwise stated, all of the experiments were carried out in 20 mM sodium phosphate, pH 7.0

as the buffer at room temperature. All of experiments were done in triplicate.

2.2. Purification of β-lg from Bovine Milk

Bovine Milk β-lg was purified from fresh cow milk that was collected from a local dairy according to the method of Lozano *et al*. [16]. The purity of β-lg was determined by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and the purity was found more than 97%.

2.3. Protein Modification with Acetic Anhydride (Protein Acetylation)

A specified concentration (10% w/v) of the three protein solutions (β-lg, BSA and Casein) in water was prepared individually, at 30°C, and the pH of 7.5-8.5. Acetic anhydride was added to the solution at the level of 0.5 g/g of protein over 30-90 min [17]. The obtained solution was dialyzed against 20 mM phosphate buffer pH 7.0 for 24 h and then the buffer was replenished at constant time intervals (6 h). The content of free amino groups in the protein was determined by the TNBS method to evaluate the lysine modification extent [18].

2.4. Solvent- and Light- Mediated Curcumin Degradation

A specified amount of curcumin was added to methanol to obtain a stock solution with concentration of 5 mM. 20 µl of the stock solution was added to 10 ml of the Phosphate Buffer Solution (PBS) at pH 7.0 (20 mM) and/or absolute ethanol to obtain the 10 µM curcumin concentration, in which the aggregation of curcumin could be negligible. All experiments were carried out in 72 h under normal conditions and at room temperature. For examining the curcumin stability in different mediums, samples were incubated in darkness and front of the direct light (100 w lamp). The maximum absorption of curcumin at 420 nm was recorded at different time intervals within 30 min using a PerkinElmer UV-vis spectrophotometer. The rate of degradation of curcumin in buffer aqueous and absolute ethanol in the absence and the presence of direct light was determined from the initial slope of the best-fit linear curve.

2.5. Effect of Temperature on Hydrolytic Stability of Curcumin

In order to examine the hydrolytic stability of curcumin at different temperatures, firstly a stock solution (1 mM) was prepared by addition of curcumin to methanol and 30 µl of the solution was added to 3 ml of the phosphate buffer solution to achieve a constant concentration of 10 µM curcumin in a quartz cell. Steady-state fluorescence measurements were performed with a Cary Eclipse (Varian) spectrofluorimeter equipped with multi-cell holder and Peltier temperature control. The samples were incubated at different temperatures (4, 25, 37 and 80 °C) in darkness, then at specified time intervals, the sample were transferred into cuvette and immediately the fluorescence spectra of 10 µM curcumin were recorded from 440 to 700 nm with the excitation wavelength at 420 nm. The slit widths were 10 and 20 for excitation and emission, respectively. The kinetic of curcumin degradation was obtained, based on the variation of the maximum emission, at different temperatures was recorded as a function of time. The decomposition rate of curcumin at each temperature was also estimated by calculating the initial slope corresponding to the best-fit linear curve.

2.6. Effect of Accompanying Proteins on the Curcumin Stability

All experiments were carried out within 1 h in the following conditions: pH 7.0, 37°C in 20 mM PBS. The protein stock solutions (native and modified) were prepared at the same concentrations (400 µM) for each protein in PBS. The mixture of curcumin/protein solutions with 1:4 molar ratios was prepared, separately for each protein. The fluorescence spectra of 10 µM curcumin in 37°C were recorded from 440 to 700 nm with the excitation wavelength of 420 nm. The slit widths were 10 and 20 for excitation and emission, respectively. The rate and kinetic of curcumin degradation was evaluated in the presence and absence of proteins at 37°C.

2.7. Effect of Proteins on Curcumin Stability in the Presence and Absence of Direct Light

Curcumin solution (10 µM) in buffer and natural and modified protein solutions in a concentration of 40 µM at pH 7 was stored in two series of glass bottles (with and without aluminum cover) and placed in a bright room with a 100 watt lamp. The degradation of curcumin within 22 h was recorded in the absence and the presence of direct light. Absorbance readings were performed at 425 nm using a UV–Vis spectrophotometer (PerkinElmer) at 25°C and 30 min intervals, for 22 h and the hydrolytic degradation rate of curcumin in each medium was determined [19].

2.8. Intrinsic Steady-state Fluorescence Measurements

Intrinsic Steady-state fluorescence measurements were carried out on a Cary Eclipse (Varian) spectrofluorimeter equipped with multi-cell holder and Peltier temperature control. Firstly, curcumin was co-incubated with each protein, individually for 1 min, then the fluorescence emission spectra of each protein solution (1 µM) in the absence and presence of the various concentrations of curcumin (0-2 µM) were separately recorded. The excitation and emission wavelengths were set at 290 and 310-700 nm, respectively and the excitation and emission slit widths were set at 5 and 10 nm.

2.9. Determination of Binding Constant Values and Number of Binding Sites

The modified Stern-Volmer equation was used to correlate the extent of fluorescence quenching of the macromolecule and the quencher concentration (Eq. 1).

$$\log ((F_0 - F)/F) = \log K_b + n \log [Q] \quad \text{Eq. 1}$$

Where K_b denotes the binding constant of the quencher with the fluorescent macromolecule, n is the number of binding sites per macromolecule and Q is the quencher (curcumin) concentration. F_0 and F are the fluorescence intensities of the macromolecule in the absence and presence of a quencher, respectively [20]. The values of K_b and n could be obtained by calculating the y-intercept and slope of the plot of $\log[(F_0-F)/F]$ *vs* $\log[Q]$ [21].

2.10. Thermodynamic Analyses of the Binding Processes

In order to understand the binding mode, the thermodynamic parameters (enthalpy and entropy) of the complexes were determined. For this purpose, the plot of ln*K vs* 1/*T* was obtained a according to the Van't Hoff equation (Eq. 2).

$$\ln K_b = (-\Delta H^\circ/RT) + (\Delta S^\circ/R) \quad \text{Eq. 2}$$

where K_b and R represent the binding constant and gas constant, respectively [18]. The thermodynamic parameters were assessed from the linear relationship between ln K_b and the reciprocal absolute temperature. The free energy change (ΔG°) based on the assumption of a nearly constant ΔH° can be calculated from the Gibbs equation (Eq. 3).

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad \text{Eq. 3}$$

2.11. Effect of Proteins on the Antioxidant Activity of Curcumin

The DPPH radical scavenging method was performed in this study to examine the antioxidant activity of curcumin and its complexes with the native and modified proteins [22]. A solution of 10 mM DPPH in methanol and a solution of 5 mM curcumin in methanol were used as stock solutions. A solvent mixture of 30% methanol in distilled water was used for all the samples. The specified volume of DPPH stock solution was added to the solvent mixture to determine the absorption of DPPH alone. An equal volume of DPPH was added to a small quantity of curcumin stock solution and each of curcumin-protein solutions, separately. In order to compare the antioxidant activity of these solutions, the values of maximum absorption of DPPH at 515 nm were recorded using a PerkinElmer UV-vis spectrophotometer at different time intervals within 30 min at 4°C in all the samples.

2.12. Cell Culture and *in vitro* Anti-cancer Activity of Curcumin

MCF-7 and SKNMC cells were cultured in Dubblico modified Eagle medium (DMEM) in addition to 10% fetal bovine serum by 1% antibiotic/penicillin–streptomycin. Cells were maintained in incubators at 37°C under 95% relative humidity and 5% CO_2. Anti-cancer activity of curcumin (and curcumin-protein complexes) was examined by MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenases of live cells to a purple formazan product. Briefly, MCF-7 and SKNMC cells were seeded in 96-well microtiter plates at a density of 20,000 cells per well in a final volume of 200 µl medium and pre-incubated for 24 h. Media were replaced with protein-free DMEM (40 µM) and then, curcumin at different concentrations (0, 5, 10, 15. 20, 30, 40 and 50 µM) in DMSO was added to each well and incubated for 24 h at 37°C. Other cells were untreated as negative control. After 24 h, cell culture media were aspirated and cells were incubated with 150 µl MTT solution (0.5 mg/mL in DMEM medium with 10% FBS) for 4 h at 37°C. Subsequently, MTT solution was carefully aspirated and the formazan crystals formed were dissolved in 100 µl DMSO per well. The absorbance was measured with a microplate reader at 570 nm (Bio-Tek, ELX 800, USA). Cell viability was expressed as a percentage of MTT reduction, assuming that the absorbance of untreated cells was 100%.

2.13 Statistical Analyses

In the present study, all the reported data were presented as mean±S.D, or data shown are representative example of three independent experiments and standard deviations were approximately within 5% of the experimental values. Statistical comparisons were made using GraphPad Prism version 3.00 for Windows, (GraphPad Software, San Diego, California, USA, www. graphpad.com).

2.14. Determination of Intracellular ROS

Intracellular ROS was monitored by using the fluorescent dye 2,5 dichlorofluorescin di-acetate (DCFH-DA) [23]. Intracellular ROS can oxidize DCFH-DA to the highly fluorescent compound di-chlorofluorescein (DCF). DCF fluorescence was detected at an excitation wavelength of 480 (or 488) nm and an emission wavelength of 540 (or 525) nm using a fluorescence microplate reader. PC12 cells were seeded in 9-well microtiter plates at a density of 20,000 cells per well in a final volume of 700 µl medium and pre-incubated for 24 h. After which the media were replaced with protein-free DMEM (40 µM) and then, were pretreated with nontoxic concentration (1 µM) of curcumin (or curcumin-protein complex) for 6 h. Then the cells were incubated with H_2O_2 (125 µM) for an additional 16 h. To produce oxidative stress, H_2O_2 was freshly prepared from 30% stock solution prior to each experiment. Then, DCFH-DA (10 µM) was added into the medium for a further 45 min at 37°C. The cells were washed twice with PBS, collected by pipetting and the fluorescence intensity of 2,7-dichlorofluorescin formed by reaction of DCFH-DA with intracellular ROS of more than 10,000 viable cells was analyzed by fluorescence microplate reader (BioTek ELX, USA).

3. RESULTS AND DISCUSSION

Despite the various pharmaceutical and therapeutic activities of curcumin, as a hydrophobic polyphenolic compound, its administration is limited due to its low water solubility and stability, [2a, 6]. So preparing a promising delivery system for carrying the curcumin to the targeted organ could be of a major importance. These appropriate systems could be selected among novel delivery systems based on incorporation into nanoparticles, cyclodextrins, micelles, and proteins [3b, 8a]. In this investigation, the interaction of curcumin with the native and modified forms of three important (and structurally unrelated) proteins, serum albumin and β-lg and milk casein, and their effects on curcumin stability, bioavailability and antioxidant property have been studied.

3.1. Effect of Solvent, Light and Temperature on the Stability of Curcumin

The effect of the type of solvent on the stability (mainly the degradation rate of curcumin) was examined by using aqueous (water) and organic (absolute ethanol) solvents (Figure **2**). This figure indicates that curcumin did not degrade in the presence of the non-aqueous solvent (ethanol), while its decomposition in the aqueous media was accelerated, confirming contribution of the water to curcumin degradation. Moreover, it was observed that light could accelerate the decomposition of curcumin and the degradation rate

of this compound, especially in the aqueous medium. Mondal *et al*., also reported that in aqueous solutions curcumin degradation is light sensitive [24].

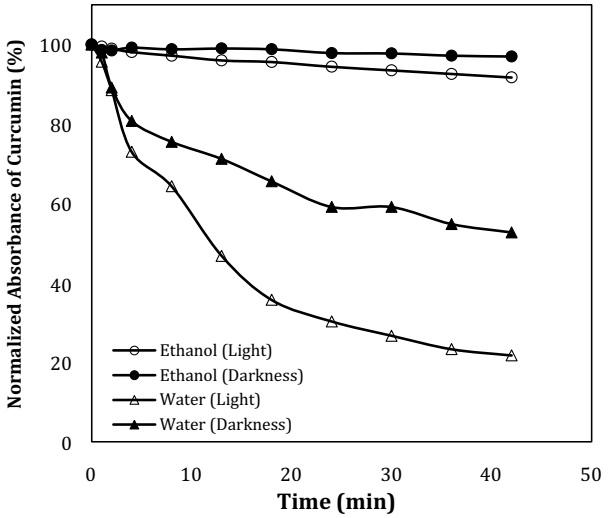

Figure 2. The comparison of curcumin stabilityin solvents of water/ethanol under condition of light and darkness (at 420 nm).

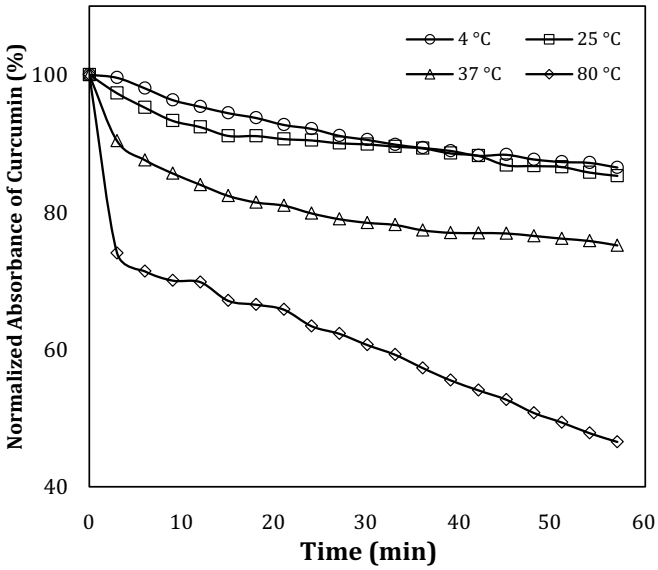

Figure 3. The stability of curcumin was investigated at 4, 25, 37 and 80°C temperatures. The experiments performed in 20 mM sodium phosphate buffer, pH 7.0 for 60 min.

Curcumin stability is also temperature-dependent. Curcumin showed the weak fluorescence signal of in phosphate buffer (at ~550 nm), while the degradation products did not fluoresce when excited at 420 nm. Therefore, the decrease in emission signifies the decrease of curcumin concentration. To investigate the effect of temperature on the stability of curcumin, the kinetic profiles corresponding to decomposition of curcumin at different temperatures were obtained by plotting of the maximum emission intensity against time and the results of the degradation profile of curcumin are illustrated in Figure **3**. As shown in the figure, the degradation rate of curcumin ([10 μM]) at both 4°C and 25°C are almost the same and are is lower than those at higher temperatures. Furthermore, increasing the temperature to 37°C caused relativity more curcumin degradation rate in the aqueous medium. Even at 80°C, more than 40% of initial curcumin population remained intact. So, temperature appears to play critical role in curcumin degradation.

3.2. Curcumin Degradation is Generally Postponed in the Presence of Proteins

In order to investigate the protective effects of the native and modified proteins on the degradation kinetics of curcumin, the kinetic profile of water-mediated curcumin degradation was examined in the absence and the presence of each protein. According to the results (Figure **4**, Supplementary Fig **S1**), the degradation of curcumin in phosphate buffer was found in association with the substantial reduction in the fluorescence emission intensity. The results exhibited up to 57% reduction in maximum emission intensity (at t = 60 min) compared to the initial value at t = 0 min (Figure **4** and Supplementary Figure **S1**). A considerable degradation of curcumin was also identified in the presence of the native β-lg and modified BSA and casein (Figure **4**, Supplementary Figure **S1** parts D, E and F), where, respectively, the 28, 33 and 42% reduction in maximum emission intensity where observed in comparison to that of the original value. In contrast, the reduction of maximum intensity of curcumin was negligible in the presence of the native BSA and casein and modified β-lg, as shown in Figure **4** (and Supplementary Figure **S1** parts B, C and G, respectively). The native BSA and modified β-lg improves the curcumin stability while the modified BSA and native β-lg increases the curcumin degradation rate, significantly. Additionally, by plotting the maximum emission intensity of spectra against time, the kinetic profiles of curcumin decomposition were obtained (Figure **4**, Supplementary Figure **S1** part H). The ratio of the degradation rate of curcumin in phosphate buffer to that in the protein solutions indicates that the degradation has been slow approximately 6.8, 2.2 and 1 times in the presence of the native BSA, modified β-lg and native casein, respectively. These results obviously indicated the intrinsic potential of the native BSA and casein and the modified β-lg in stabilizing of curcumin. In contrast to these results, Sneharani *et al.,* observed that native β-lg improves the stability of curcumin. In the presence of β-lg remarkable increase of curcumin half-life from 30.8 to 206 min was observed [10d]. The delay in the degradation of curcumin in the presence of the β-lg was attributed to its binding to the protein. It was also reported earlier that BSA and α_{S1}-Casein could help stabilizing of curcumin through the same mechanism [3b, 15a, 25].

3.3. Protective Effect of the Proteins Against Water-mediated Degradation of Curcumin in the Presence and Absence of Direct Light

As shown previously, by our research group [15a] hydrolysis is the main process in the degradation of curcumin in buffer solution in the presence and absence of direct light. Based on the analyses of HPLC and Mass spectra, it has

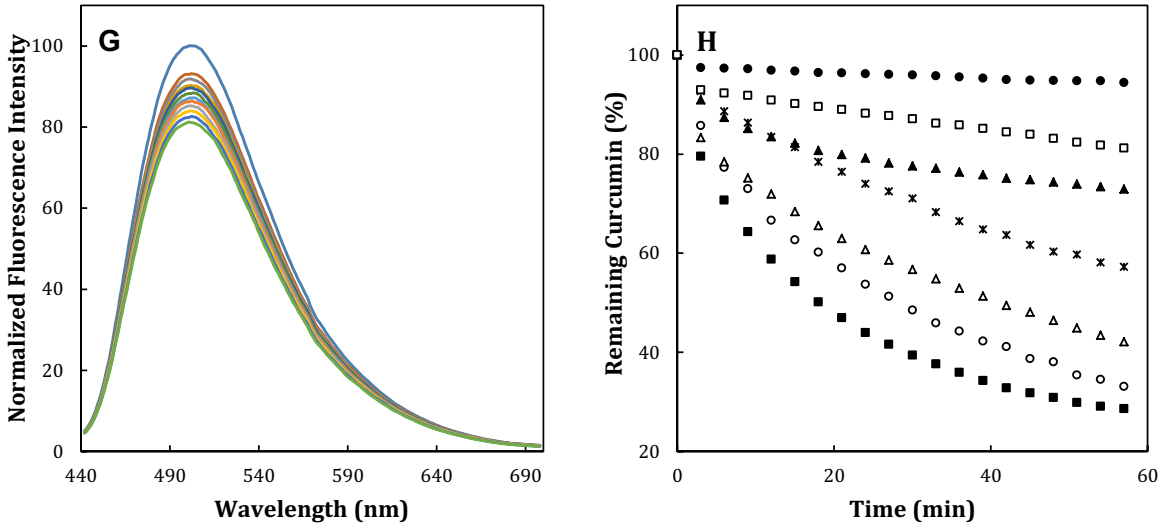

Figure 4. (Left) Fluorescence spectra of curcumin in 20 mM sodium phosphate buffer pH 7.0 in the presence of β-lg. Fluorescence spectra of curcumin in 20 mM sodium phosphate buffer pH 7.0 in the absence and presence of different proteins are also shown in Supplementary Figure **S1.** The concentrations of proteins and curcumin were 40 and 10 μM, respectively. (**Right**) curcumin stability, based on its fluorescence, in the presence of different proteins (BSA (●), Casein (▲), β-lg (■), modified BSA (○), modified Casein (Δ) and modified β-lg (□)) and in buffer alone (×) at 37°C.

been reported that at the first stage of degradation, curcumin is partially deprotonated, and then is fragmented into trans-6-(40-hydroxy-30-methoxyphenyl)-2,4- dioxo-5-hexanal, Further fragmentation of the obtained products could result in the smaller molecules such as vanillin, feruloyl methane, and ferulic acid. Despite the pure curcumin, these molecules contribute negligibly to the absorption at 420 nm [3b]. In addition, the results implied the negligible absorption of proteins at this wavelength. Therefore, the decreased absorbance at 420 nm could be mainly attributed to the decrease of intact curcumin population. In the current study, the kinetic of curcumin degradation was investigated in phosphate buffer with or without the native (unmodified) and modified proteins in the presence and absence of direct light. In the phosphate buffer solution, the degradation was found in association with a significant decrease in the absorption at ~400-600 nm range confirmed by fluorescence analyses.

In Figure **5**, the time dependent decays of curcumin in the buffer and protein solutions is illustrated, which shows linear behavior and hence fits to a pseudo-first-order model and was found in agreement with previous reports [3b, 26]. At pH 7.0, free curcumin possessed biphasic degradation behavior in phosphate buffer solution; initially at the first three hours, a rapid degradation rate of ~16 and 9.6 % hr^{-1} in the presence and absence of direct light was observed followed by a noticeably decrease in degradation rate. In the presence of the native and modified BSA, native and modified casein as well as the native and modified β-lg, however, the rates of degradation (under the effect of direct light) changed to ~5.9, 20.8, 11.6, 13.5, 18.9 and 6.7 % hr^{-1}, respectively. The calculated degradation rates for free curcumin and "curcumin-protein" complexes are in the following order: 5.9 % hr^{-1} (native BSA) < 6.7 % hr^{-1} (modified β-lg) < 11.6 % hr^{-1} (native casein) < 13.5 % hr^{-1} (modified casein) < 16 % hr^{-1} (free curcumin) < 18.9 % hr^{-1} (native β-lg) < 20.8 % hr^{-1} (modified BSA).

In darkness, the same observations were made. In the presence of the native and modified BSA, native and modified casein as well as the native and modified β-lg, however, the degradation rates changed to ~0.1, 18.1, 2.7, 3.8, 17.3 and 1.6 % hr^{-1}, respectively. The degradation rates are in the following order: 0.1 % hr^{-1} (native BSA) < 1.6 % hr^{-1} (modified β-lg) < 2.7 % hr^{-1} (native casein) < 3.8 % hr^{-1} (modified casein) < 9.6 % hr^{-1} (free curcumin) < 17.3 % hr^{-1} (native β-lg) < 18.1 % hr^{-1} (modified BSA).

Regarding the ratio of degradation rates in phosphate buffer to those in protein solutions, and in agreement with earlier stability analyses (Figure **4**), it could be concluded that these proteins, especially the native BSA and modified β-lg are pioneers in diminishing rate of curcumin degradation, both in light conditions and in darkness for 22 hr. The relative reductions in degradation rates at light were approximately 2.72, 1.39, 2.39 and 1.19 in the presence of the native BSA and casein, modified β-lg and casein, respectively. Moreover, in the absence of light, the relative reductions of degradation rates were surprisingly 89.5, 3.5, 5.8 and 2.5 in the presence of the native BSA and casein, modified β-lg and casein, respectively. The stability improvement could be probably due to the strong binding of the curcumin to the protein vehicles. This stimulated us to examine the interaction between curcumin and the proteins.

3.4. Intrinsic Protein Fluorescence: Determination of Association Constants and Number of Binding Sites

Fluorescence quenching of (macro-)molecules gives useful details about ligand binding to specific sites on the macromolecule [27]. So, fluorescence quenching experiments of the native/modified proteins were carried out at 277, 298 and 310 K. Figure **6** shows the fluorescence quenching spectra of the proteins in the presence of curcumin at 310 K. The strong fluorescence emission at wavelengths about 326-346 nm

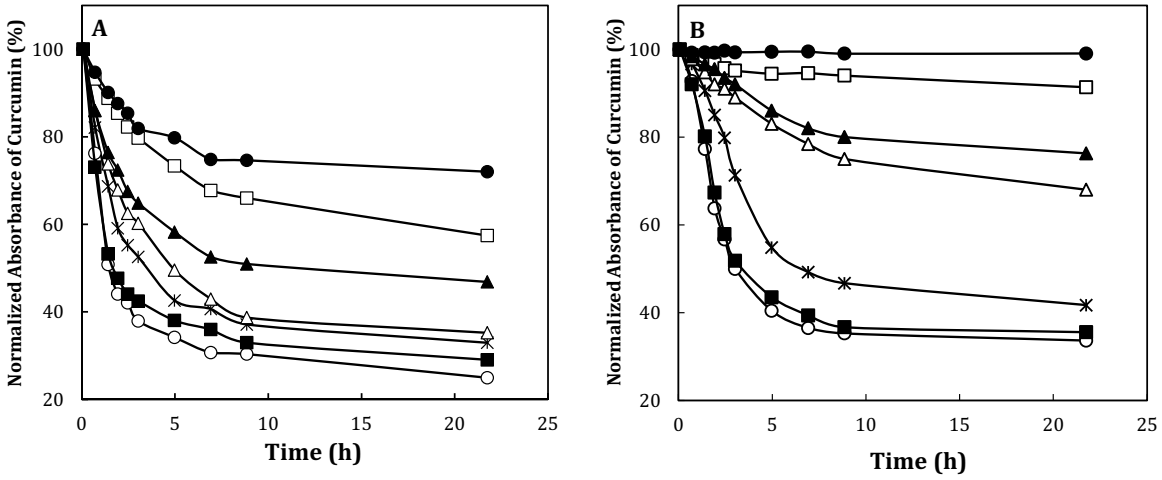

Figure 5. Effect of different proteins; native BSA (●), native casein)▲(, native β-lg (■), modified BSA (○), modified Casein (△) and modified β-lg (□) on the curcumin stability against direct light (A) and dark (B), at 25 °C, buffer alone (pH 7, asterisk).

upon excitation wavelength at 295 nm was observed for both native and modified proteins. According to the molecular structure of curcumin (Figure **1**), it could interact with polar as well as non-polar side chains due to presence of hydroxylic and hydrophobic moieties in its structure. So, due to changes of interacting side chains as well as conformational variations, differential bindings of curcumin to the native and (acetic anhydride-mediated) modified proteins are anticipated. Addition of curcumin to the protein solutions caused quenching of intrinsic fluorescence intensity with the formation of a new peak at higher wavelengths (Figure **6**). As indicated in this figure, upon addition of curcumin, the intrinsic fluorescence spectra of all the native and modified proteins enhanced regularly with or without shift to shorter/longer wavelengths. This indicates an interaction between the ligand and the proteins and also suggests that curcumin located at the vicinity of the tryptophanyl side chains. Also, these observations may suggest that binding of curcumin to the proteins is accompanied with altering the polarity of the microenvironment of the proteins' fluorophores, probably originated from conformational changes. Additionally, due to appearance of some new peaks, this may be attributed to a Foerster type energy transfer from the fluorophores (Trps) to the quencher (curcumin). The appearance of a new isosbestic point also demonstrates a simple equilibrium binding system [28].

The binding constant (K_b) and average number of binding sites (n) in the complex of curcumin-protein could be calculated by modified Stern-Volmer equation [20, 29]. Plot of log $[(F_0–F)/F]$ *vs*. log [Q] gives the values of K_b and n (Figure **7**). The binding constant is a criterion for efficiency of the carrier systems; mainly, lower K_b values indicate a short half-life or poor distribution, while the higher K_b values (as the indicator of strong binding) show the decreased concentrations of free drug in body fluids [30]. The proteins exhibit high binding constants on the order of 10^5 M^{-1} (Table **1**). The K_b values of curcumin complexes with the native forms of BSA, casein and β-lg was found in agreement with the values reported in the previous studies [2b, 12, 31]. The n values at different temperatures were found around unity, which indicated the presence of only one binding site on the native and modified albumin, casein and β-lg. Moreover, the number of binding sites on all the proteins did not significantly change, upon modification.

As it can be observed in Table **1**, K_b value of the complex of the modified BSA and curcumin is about 200 times lower than the value of corresponding unmodified one, demonstrating the very loose binding of curcumin to the modified BSA. Reciprocally, different observations were made for the β-lg and casein. The K_b values for the unmodified and modified caseins were approximately the same, while the k_b value for β-lg increased significantly (~100 times) upon the protein modification. Based on the above mentioned results (Figure **4**), it could be assumed that increasing the rate of curcumin degradation is highly attributed to the loose binding of the ligand to the modified BSA. This indicates the low protective effect of the modified BSA against hydrolytic curcumin degradation. The same observation for the modified β-lg is reminiscent of positive correlation between curcumin stability and K_b vales, so that the larger the association constant of "curcumin-protein" complex, the higher is the curcumin stability. Moreover, to find the possible correlation between water-mediated and light-induced curcumin degradation extents/rates *versus* "curcumin-protein" binding constants in the absence or presence of accompanying proteins, we performed Pearson's correlation analyses. As indicated in Figure **8** & **9A**, with increase of K_b values a relative increase in curcumin stability is documented. The correlation coefficients between curcumin degradation extent/rate and K_b values in direct contact with light and in the dark were "r^2=0.558" and "r^2=0.362", respectively, so curcumin protection by proteins is mainly originates from its isolation from bulk water environment and the underlying mechanism of curcumin stabilization appears to be "decreased water activity". It also appears that factors other than binding association values (such as type of interactions between curcumin and protein) affect the stability of the bound curcumin.

Curcumin possesses ideal structure features of ligands, including a flexible backbone, hydrophobic nature and sev-

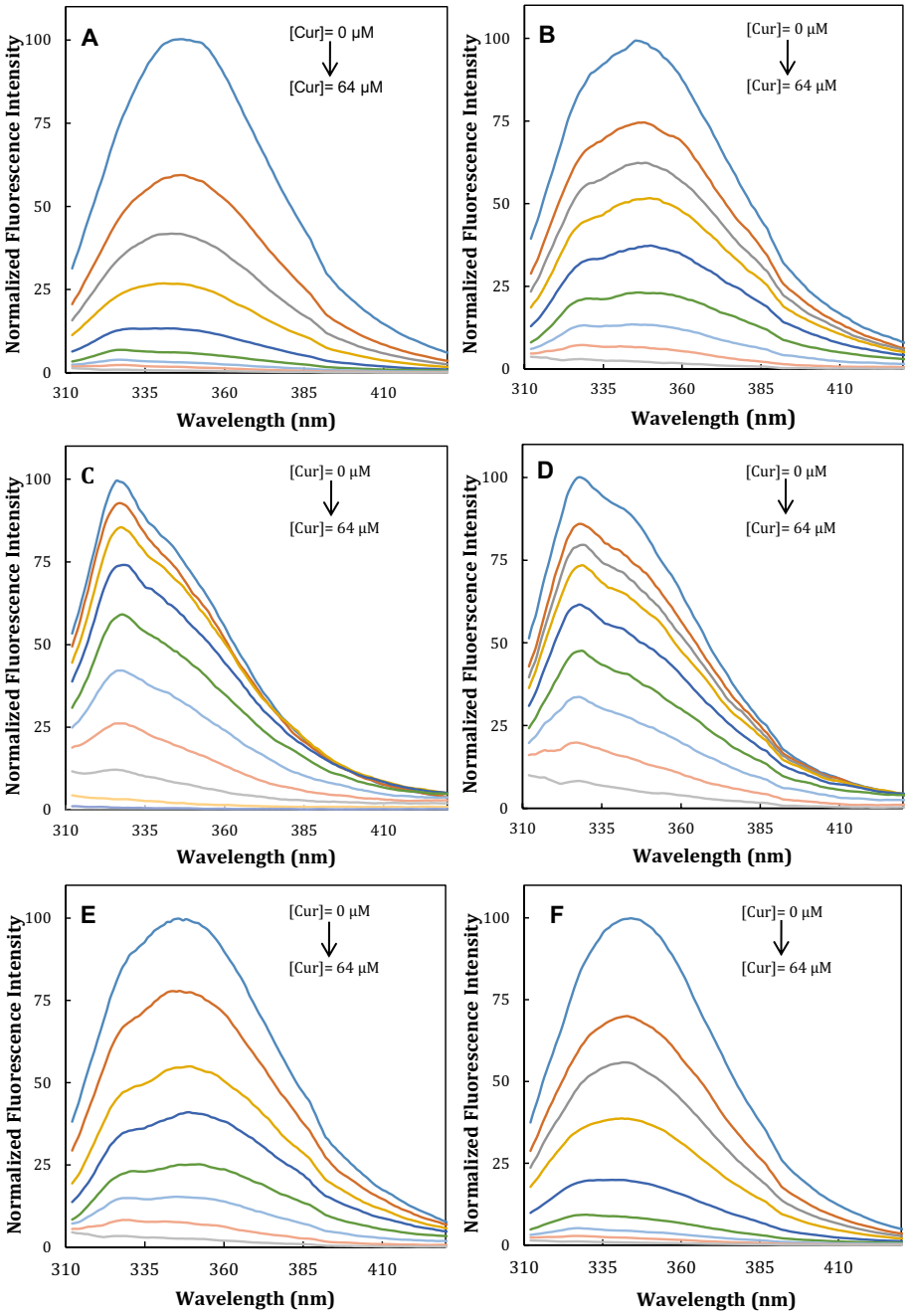

Figure 6. Curcumin-mediated quenching of the native BSA (**A**), native casein (**B**), native β-lg (**C**), modified BSA (**D**), modified casein (**E**), and modified β-lg (**F**) fluorescence at 310 K in 20 mM sodium phosphate buffer pH 7.0. Each data point was the mean of three independent determinations.

eral hydrogen bond donors and acceptors. So, regarding the structure of the ligand, the forces acting between curcumin and the proteins mainly include hydrogen bonding, van der Waals and hydrophobic interactions which vary for different proteins. Thus, it possibly seems that protective effects of β-lg against hydrolytic degradation of curcumin originate from factors other than enhanced stability of "drug β-lg" complex. These findings illustrated that the native BSA and modified β-lg as a natural biopolymer can be a good matrices for increasing of the curcumin stability in water, whereas the native β-lg may not be a suitable carrier.

3.5. Mode of Curcumin Binding to the Proteins and Nature of Binding Forces

Essentially, drug could bind the protein molecules because of the existence of non-covalent interactions (hydrogen bonds and van der Waals interactions, electrostatic forces, and hydrophobic interactions) between these molecules. The thermodynamic parameters of protein reaction (binding constant (K_b), thermodynamic parameters, $\Delta H°$ (enthalpy change) and $\Delta S°$ (entropy change)) are the major criteria for identifying the binding mode. Ross and Subramanian have

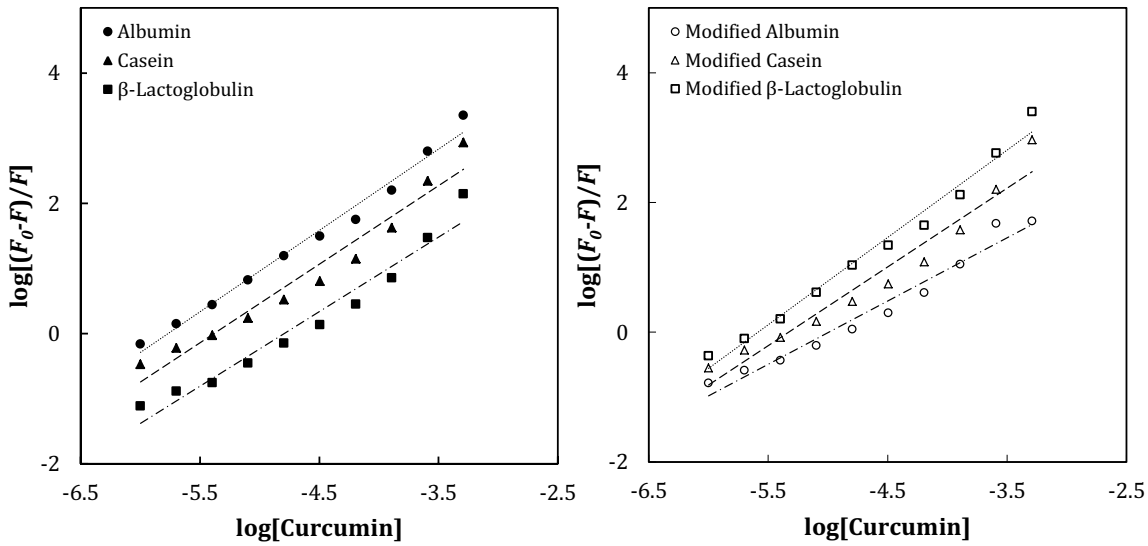

Figure 7. Double-logarithmic plot of the curcumin quenching effect on the native BSA (●), native casein (▲), native β-lg (■) (**A**), modified BSA (○), modified Casein (Δ) and modified β-lg (□) (**B**) fluorescence at 310 K in 20 mM sodium phosphate buffer pH 7.0. K_b and n values are obtained from the y-intercepts and slopes of the plots, respectively.

Table 1. Binding constants of Protein-Curcumin Complexes and number of curcumin binding sites on each protein.

System	R^2	$K_b \times 10^{-5}$ (M^{-1})	N
Unmodified Albumin	R^2=0.985	160.694±3.21	1.249±0.02
Modified Albumin	R^2=0.966	0.735±0.02	0.975±0.015
Unmodified Casein	R^2=0.953	30.903±1.242	1.206±0.032
Modified Casein	R^2=0.95	29.717±0.895	1.214±0.041
Unmodified B-LG	R^2=0.956	3.090±0.064	1.145±0.034
Modified B-LG	R^2=0.982	334.195±3.341	1.347±0.042

characterized the sign of the thermodynamic parameters associated with various types of interactions [32]. The temperature-dependent experiments were done to obtain the thermodynamic parameters of "curcumin-protein" interactions. The binding parameters of the complexes were calculated using van't Hoff plot (data not shown) followed by the Gibbs equation. The values of ΔH° and ΔS° are raised from the slope and y-intercept of the Van't Hoff plots, respectively, and ΔG◦ from the Gibbs equation: $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ and are listed in Table **2**. The Gibbs free energy change (ΔG°) for all complexes were negative, which indicates the spontaneous nature of the curcumin binding process.

It has been reported that when ΔH◦ is negative and ΔS◦ is positive, the electrostatic force dominates the interaction; meanwhile, when both ΔH◦ and ΔS◦ are negative, van der Waals interactions and hydrogen bonds dominate the reaction and when both of them are positive, hydrophobic interactions dominate the binding process [32-33]. The sign and magnitude of the thermodynamic parameters of the complexes (including the native BSA, native casein, modified casein and modified β-lg) were obtained and we found that both ΔH◦ and ΔS◦ are positive, which indicated that the hydrophobic interactions are the dominant intermolecular force in successful stabilizing the complex and the binding is entropy-driven. Reciprocally, the ΔH◦ and ΔS◦ values for curcumin binding to the modified BSA were both negative, which indicated that the binding is mainly enthalpy-driven and van der Waals interactions as well as hydrogen bonding are the dominant intermolecular forces for stabilizing the "curcumin-modified BSA" complex. Interestingly, the negative sign of the large ΔH◦ value and the positive sign of ΔS◦ corresponding to curcumin binding to the unmodified β-lg show that this binding is also enthalpy-driven and the electrostatic force is the dominant intermolecular force for stabilizing curcumin-unmodified β-lg complex. It is noteworthy that hydrophobic interactions between curcumin and the employed macromolecules (albumin, casein) are both main contributors to the highly stable "ligand-protein" interaction and provide less deleterious environment with lower water-activity for curcumin on the carrier protein. Furthermore, the obtained values for n (at different temperatures for different proteins) were found to be ~1 indicating that only one cur-

Table 2. Thermodynamic Parameters of curcumin binding to different carrier proteins.

System	ΔH° (kJ·mol^{-1})	ΔS° (J mol^{-1}·K^{-1})	ΔG° (kJ·mol^{-1})
Unmodified Albumin	27.184±0.815	0.212±0.002	-38.368±1.151
Modified Albumin	-34.218±0.684	-0.047±0.001	-19.645±0.589
Unmodified Casein	30.000±1.25	0.204±0.006	-33.245±0.665
Modified Casein	12.316±0.492	0.140±0.005	-31.099±0.622
Unmodified B-LG	-27.151±0.814	0.007±0.001	-29.428±1.765
Modified B-LG	9.317±0.094	0.161±0.006	-40.516±1.62

cumin binding site exists on the protein monomers. It also could be observed from the results (Table **S1**) that increasing the temperature from 277 to 310 K caused increasing the binding constant and the average number of binding sites of the unmodified BSA, unmodified casein and modified casein, and also decreased the binding constant and the average number of binding sites of the modified BSA and unmodified β-lg.

3.6. Effect of Protein on the Antioxidant Activity of Curcumin

Recently many studies have been devoted on well-understanding the mechanism of antioxidant activity of curcumin. It has been well approved that the phenolic hydrogen atoms of curcumin is almost entirely responsible for its antioxidant capability but the exact mechanism is not clear yet. Recently, the Sequential Proton Loss Electron Transfer (SPLET) and Hydrogen Atom Transfer (HAT) mechanisms have been proposed for the antioxidant capability of curcumin, as a chain-breaking antioxidant [34]. Based on these mechanisms, curcumin can donate hydrogen from either enolic (HAT mechanism) or phenolic groups (SPLET mechanism). The SPLET mechanism is favored for polar environments (such as methanol solvent as has been used in this study) and suggests the donation of a hydrogen atom from the phenolic hydroxyl group and formation of a curcumin phenoxyl radical. This is mainly resulted from the formation of a curcumin radical fragment. So, it could be resulted that in ionizing solvents, such as methanol as has been used in this study) electron-deficient radicals could react with curcumin *via* a rapid SPLET process [31]. In this study, the radical scavenging activity of curcumin was evaluated by DPPH scavenging method. DPPH radical could be converted to the non-radical form by reacting with an electron or hydrogen donor. Ever increase in the amount of non-radical form of DPPH could cause decreasing the maximum absorbance intensity of DPPH. Figure **8** shows the variation of the maximum intensity of DPPH at 515 nm as a function of time for curcumin-protein complexes in comparison with free curcumin at 4°C. A glance at figure indicates that all curves consisted two decreasing steps, which is consistent with the previously reported DPPH study [22]. Immediately after mixing DPPH with free curcumin and "curcumin-protein" complexes samples, the relative intensity of DPPH often has an abrupt decrease within ~5-20 hr, followed by much more gradual change of the relative intensity until steady state is reached. As it can be seen in Figure **8**, the complex of curcumin with the modified BSA was the most potent complex in DPPH scavenging activity, in comparison to the free curcumin as well as the other complexes. It should be worth noting that the higher antioxidant activity of "curcumin-modified BSA" complex does not approve that the modified BSA potentiates the scavenging activity of curcumin.

There was also significant difference between the scavenging activity of free curcumin and "curcumin-native BSA" complex. The percentage of un-reacted DPPH in the presence of the "native BSA–curcumin" complex was significantly higher than that in the presence of curcumin alone, which indicates the lower antioxidant activity of the BSA-bound curcumin. This result was also observed in the study carried out by Yang *et al*., which resulted that the lower activity of in the presence of BSA could be attributed to the restraining effect of BSA on curcumin to donate the hydrogen atom [35]. The other complexes were found to be less potent in DPPH radical scavenging in comparison to the pure curcumin suggesting that curcumin in these complexes possesses less hydrogen-donating ability.

Overall, the antioxidant activities of curcumin in the different systems are in the following order: native BSA < modified β-lg < native casein < modified casein < native β-lg < free curcumin < modified BSA taking the above statement into account and regarding the curcumin stability data and binding constant values (Figure **8**, Table **1**), it can be concluded that the degradation of curcumin in aqueous media is an autoxidation process and is also the direct consequence of its action as an antioxidant.

According to Figure **9B**, a linear negative correlation between K_b values and antioxidant activities was obtained with correlation coefficient r^2=0.594. The unmodified and modified BSA, unmodified casein as well as the modified β-lg appears to create hydrophobic microenvironment with lower water activity (water concentration much less than 55M).

At these conditions, radical chain reaction, mediated by stable incorporation of oxygen into curcumin, is unlikely, so that radical scavenging (degradation) of curcumin is postponed in the presence of accompanying proteins. This process is in full agreement with significant increased stability of curcumin (along with decreased antioxidant activity) within

organic media (Figure **2**). It may be also inspired that curcumin is a successful antioxidant within aqueous conditions; so that it's lower stability contributes to its low bioavailability at target sites in Polypharmacology.

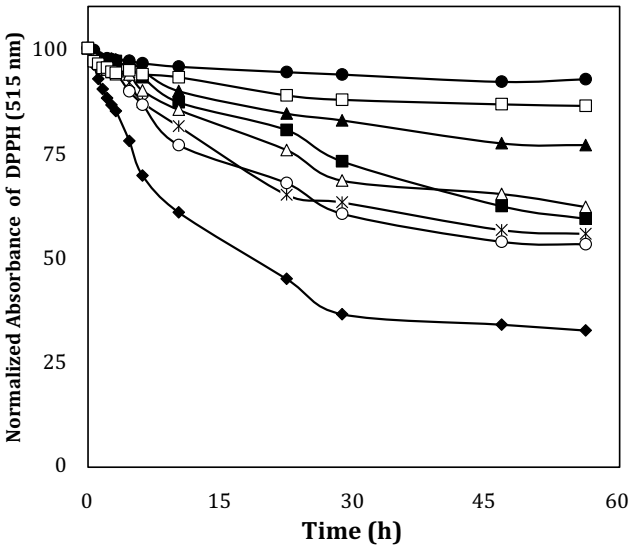

Figure 8. The antioxidant activity of curcumin alone (⁕) and curcumin in the presence of native BSA (●), native casein)▲(, native β-lg (■), modified BSA (○), modified Casein (Δ) and modified β-lg (□) was obtained by measuring of DPPH absorbance at 515 nm, at 4 °C, For 60 h. The concentrations of proteins and curcumin were 6 and 1 μM, respectively, and the concentration was DPPH 0.1 mM. (⧫); curcumin in the presence of Ascorbic acid at equal concentration.

3.7. Cytotoxic Effects of Free Curcumin and "Curcumin-protein" Systems Against Cancerous Cells

Curcumin is able to interact with different biomacromolecules in the biological systems [36]. According to previous reports on inhibitory effects of curcumin on the growth of human tumors [2b, 5, 36] and also to evaluate the efficacy of protein delivery systems, two human cancerous/non-cancerous cell lines (MCF-7 and SKNMC) were exposed to a number of equivalent concentrations of free and protein-bound curcumin. After treatment for 24 h, the cell viability (cytotoxicity) was quantified using MTT assay. As demonstrated in Figure **10**, both free curcumin and "curcumin-protein" systems showed dose-dependent cytotoxicities. Also, at higher curcumin concentrations, the anti-cancer activity of "curcumin-albumin" systems increased significantly compared with curcumin alone-both for SKNMC and MCF-7 cells. Moreover, "curcumin-casein" interaction potentiated cytoxicity of curcumin as the viability of the SKNMC cells has reached from 88% to 67% after exposure to 44 μM of curcumin and "curcumin–casein" (Figure **10**). Exposure of MCF-7 cells to the curcumin for 24 h resulted in a concentration dependent decrease in cell viability, with approximate IC_{50} of 43 μM. Treatment with "curcumin-casein" and "curcumin-albumin" systems also shifted the curcumin concentration response curve to a lower IC_{50} values in a dose-dependent manner. The average IC_{50} values of with "curcumin-casein" and "curcumin-albumin" systems were 39 μ M and 21 μM, respectively, confirming that both albumin and casein significantly potentiate cytotoxicity of curcumin against MCF-7 as well.

Based on these findings, interaction between curcumin and casein and/or albumin was able to protect curcumin and also potentiate its successful delivery to cancer cells thus achieving lower cell viability and showed greater cytotoxicity (lower IC_{50} values) compared to free curcumin, after 24 h treatment. However, DMSO (vehicle) and/or the protein alone showed comparable cell viability to untreated cells indicating that they have no cytotoxic effects on the cells, at employed concentrations. Additionally, in agreement with stability data, curcumin in the presence of the native proteins (casein and BSA) displayed higher cell-killing activity indicating that control of degradation rate of curcumin (Fraction of intact curcumin population) may play an important role in the cytotoxic properties.

Cao *et al.,* [30] showed that the reversible polyphenol-albumin interaction either overwhelmingly improves the radical scavenging capacities of phenolic compounds or control delivery of the intact molecules and their metabolites to cells. In the current study, both albumin and casein (unmodified forms) displayed great binding constants toward curcumin. It appears that there is a positive correlation between binding affinity and biological activity of curcumin. According to the free drug hypothesis [11a], if curcumin is highly bound (reversibly) to the proteins, the fraction of intact curcumin molecules and consequently successful delivery of biologically active molecules enormously increased.

As observed earlier, β-lg was unable to reduce the extent of curcumin degradation. Moreover, the obtained cytotoxicity (against MCF-7 and SKNMC) for "β-lg-curcumin" system showed us the other side of intriguing pharmacology of curcumin and its degradation products. To date, and regarding various molecular targets of curcumin on or within the cells, there is no reliable evidence that the degradation products are less active and/or more active in some aspects, compared with intact curcumin. So, considering different molecular and metabolic behaviors of two tested cell lines, the observed differential cytotoxicity of "curcumin" and "β-lg-curcumin" systems against MCF-7/SKNMC cells is not surprising. The same observations were (almost) made for PC12 cells (Figure **11**).

As stated earlier, the increased stability enhances lifetime and gives intact/functional curcumin an additional opportunity to be up taken by target cells. Moreover, binding of curcumin to proteins may lead to its controlled release. In a phase I trial, the concentrations of curcumin in plasma and target tissues were as low as 11.1 nM and 1.3 μM, respectively, even with an oral administration of 3.6 g/day [7, 37]. This poor bioavailability is mainly due to degradation (Chemically/Biologically) of curcumin in gastrointestinal tract and plasma.

3.8. Determination of Intracellular ROS

In another attempt, to investigate the antioxidant effects of free curcumin and "curcumin-protein" complexes by biological evaluation, we tested the protective potential of curcumin systems against oxidative stress induced by hydrogen peroxide in PC12 cells. As anticipated, adding H_2O_2 to PC12

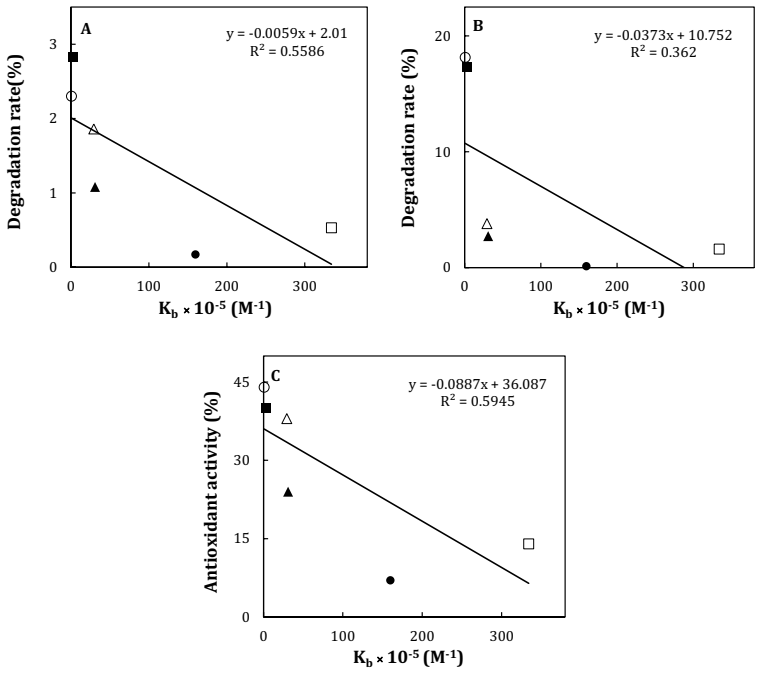

Figure 9. Negative correlation between K_b and stability of curcumin in the presence of native and unmodified proteins in light (**A**) and dark (**B**). Also Negative correlation between K_b and anti-oxidant acidity of curcuminin the presence of native and unmodified proteins (**C**) at 37 °C, buffer alone (pH 7, asterisk).

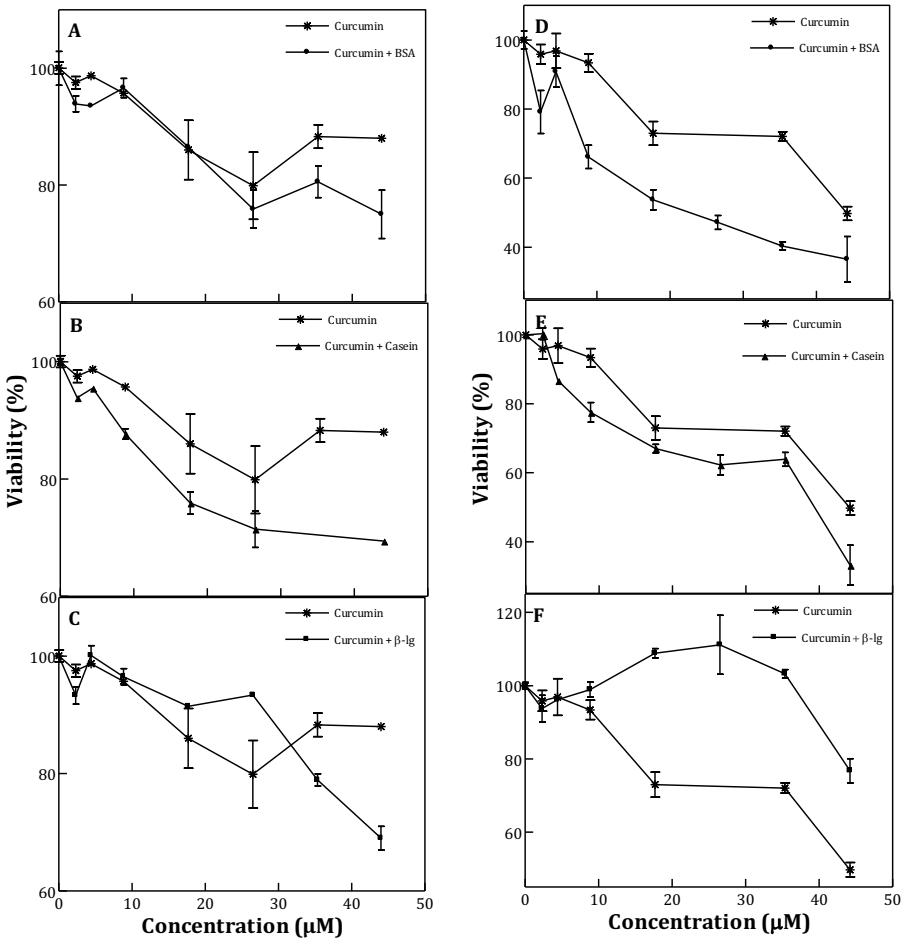

Figure 10. Cell viability of SKNMC (**A-C**) and MCF-7 (**D-F**) cells after exposure to different concentrations of curcumin alone and curcumin-native proteins complexes. Cell viability was determined by MTT assay. Data are expressed as the mean ± S.E.M of three separate experiments (n = 3).

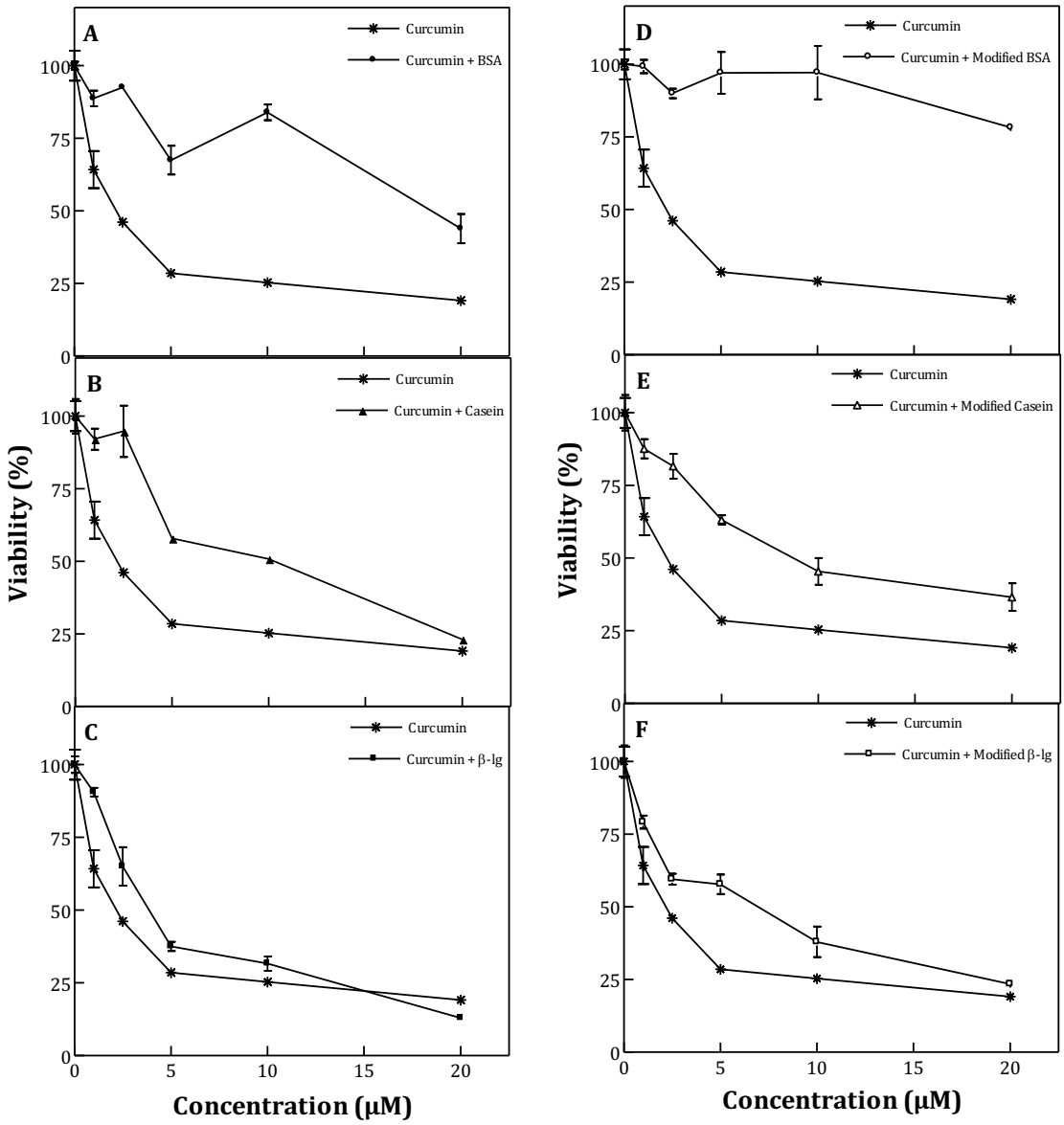

Figure 11. Cell viability of PC12 cells after exposure to different concentrations of free curcumin and curcumin-native proteins complexes (**A-C**) and curcumin-modified proteins complexes (**D-F**). Cell viability was determined using MTT assay. Data are expressed as the mean ± S.E.M of three separate experiments (n = 3).

cells caused a significant increase in ROS level. Then we evaluated whether curcumin and "curcumin-protein" complexes protect PC12 cells from oxidative damage caused by H_2O_2 *via* lowering levels of ROS. As demonstrated in the Figure **12**, and in agreement with earlier experiments, pretreatment of the cells with curcumin, curcumin-BSA and curcumin-β-lg complexes significantly reduced the ROS levels. Surprisingly, pretreatment with the "curcumin-BSA" and "curcumin-β-lg" equally reduced ROS significantly compared to that of curcumin. Unexpectedly, pretreatment with "curcumin-casein" complex caused significant increase of intracellular ROS levels. It has been previously reported that many polyphenols including flavonoids such as quercetin, rutin, apigenin, phenolics acids such as gallic acid, tannic acid, caffeic acid, as well as delphinidin, resveratrol, curcumin, gallocatechin and EGCG can cause oxidative strand breakage of DNA *in vitro* [38]. So, it is suggested that the anti-proliferative effects of some polyphenol antioxidants on cancer cells are partially due to their prooxidant actions. However, it has been proposed that this oxidative property depends on the amount of dissolved oxygen in the test medium [39]. We cannot reliably attribute this unexpected observation to the possible prooxidant activity of curcumin, induction of oxidative stress and diminished antioxidant capacity within cancer cells. There is another complexity for the unexpected results displayed by "curcumin-β-lg" system caused by unknown reason(s) although the certain Degradation Products (DPs) of curcumin retain the active phenolic hydroxyl, which is crucial for anti-oxidant activity of curcumin and these certain DPs are expected to have antioxidant potential according to the known structure-activity relationships of antioxidants [23].

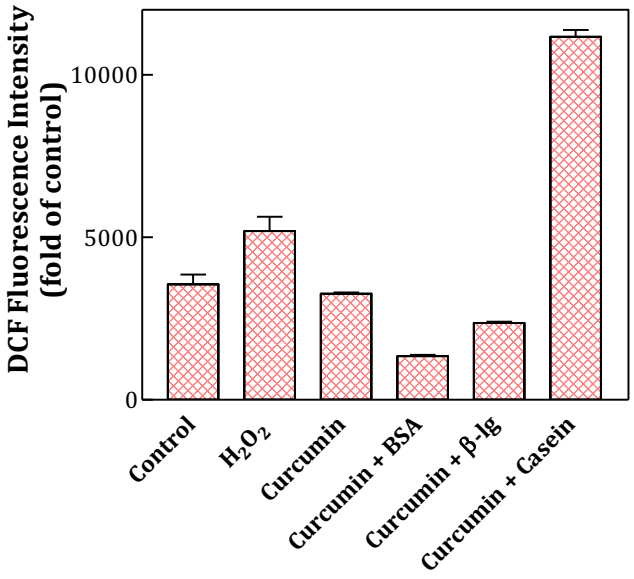

Figure 12. The effect of curcumin on H_2O_2-induced ROS generation. Column bar graph of mean cell florescent for DCF-DA. Data are expressed as the mean ± S.E.M of three separate experiments (n = 3). Control is cells that adding DMSO with them and curcmin control is cells without H_2O_2 and protein and H_2O_2 control is cells without curcumin and protein.

CONCLUSION

The data of the current work established some sort of correlation between protein "binding strength" and the stability/efficacy of curcumin; so that, generally, the higher the K_b values, the higher stability/radical scavenging activity and/or more effective cytotoxicity. For instance, both albumin and casein significantly potentiated toxicity of curcumin against MCF-7 cells. In agreement with stability data, curcumin in the presence of the unmodified "casein and BSA" displayed higher cell toxicity activity indicating that control of degradation rate of curcumin (Fraction of intact curcumin) may play an important role in the cytotoxic properties. As binding constant (K_b) value eventually determines stability as well as release extent of the bound curcumin, it is usually assumed that there is some sort of correlation between the levels of delivered intact curcumin and strength of protein-curcumin binding.

Contrary to our hypotheses and due to unknown reason(s), some unexpected observations, (implying "poor (or no) correlations between binding/stability and biological activity of curcumin"), were made. Actually, there are many complicated and diverse mechanisms involved in cell-protein interaction that are not yet sufficiently well understood, for example proteins may differ substantially in their half-lives; these issues can seriously limit the above mentioned correlation. The employed proteins (*e.g.* casein and BSA) prolong the effective time an increase the fraction of biologically active molecules, and with considering enhanced permeation and retention effect of albumin, successfully deliver intact curcumin molecules into the target cells, enhancing the drug efficacy. Adversely, if curcumin is very tightly bound (reversibly) to the proteins, the fraction of intact free curcumin molecules may be diminished and consequently successful delivery of biologically active molecules enormously decreased. Furthermore, this study showed that type of "curcumin-protein" interactions affect (at least in part) both the binding affinities and curcumin stability and then influence the displayed biological activity from curcumin.

ABBREVIATIONS

BSA	=	Bovine Serum Albumin
β-lg	=	β-lactoglobulin
H_2O_2	=	Hydrogen peroxide
TNBS	=	2,4,6-trinitrobenzene sulfonic acid
DPPH	=	2,2-diphenyl-1-picryl hydrazyl
MTT	=	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
DCF	=	Dichlorofluorescein
DCF-DA	=	2,5 dichlorofluorescin diacetate
DMEM	=	Dubblico Modified Eagle Medium
FBS	=	Fetal Bovine Serum
MCF-7	=	human breast carcinoma cell line
SKNMC	=	human neuroblastoma cell line
PC12	=	rat pheochromocytoma clonal cell line

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] (a) Rasouli, H.; Hosseini-Ghazvini, S.M.-B.; Adibi, H.; Khodarahmi, R. Differential α-amylase/α-glucosidase inhibitory activities of plant-derived phenolic compounds: A virtual screening perspective for the treatment of obesity and diabetes. *Food Funct.*, **2017**, *8*,

1942-1954. (b) Rasouli, H.; Farzaei, M.H.; Khodarahmi, R. Polyphenols and their benefits: A review. *Int. J. Food Prop*., **2017**, *20*, 1700-1741. (c) Siah, M.; Farzaei, M.H.; Ashrafi-Kooshk, M.R.; Adibi, H.; Arab, S.S.; Rashidi, M.R.; Khodarahmi, R. Inhibition of guinea pig aldehyde oxidase activity by different flavonoid compounds: An *in vitro* study. *Bioorg. Chem*., **2016**, *64*, 74-84.

[2] (a) Aggarwal, B.B.; Kumar, A.; Bharti, A.C. Anticancer potential of curcumin: Preclinical and clinical studies. *Anticancer Res*., **2003**, *23*, 363-398. (b) Sahu, A.; Kasoju, N.; Bora, U. Fluorescence study of the curcumin-casein micelle complexation and its application as a drug nanocarrier to cancer cells. *Biomacromolecules*, **2008**, *9*, 2905-2912.

[3] (a) Aggarwal, B.B.; Bhatt, I.D.; Ichikawa, H.; Ahn, K.S.; Sethi, G.; Sandur, S.K.; Natarajan, C.; Seeram, N.; Shishodia, S. Curcumin-Biological and Medicinal Properties. In: *Turmeric: The Genus Curcuma*, Ravindran P.N.; Babu, K.N.; Sivaraman K.; Eds.; CRC Press: Boca Raton, FL, **2006**, pp. 297-368. (b) Leung, M.H.; Kee, T.W. Effective stabilization of curcumin by association to plasma proteins: human serum albumin and fibrinogen. *Langmuir*, **2009**, *25*, 5773-5777.

[4] (a) Hsu, C.-H.; Cheng, A.-L. Clinical studies with curcumin, In: The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease. Aggarwal, B.B.; Surh, Y.-J.; Shishodia S.; Eds.; Springer: Berlin, Germany, **2007**; pp. 471-480. (b) Hsieh, C.-Y. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res*., **2001**, *21*, 2895-2900.

[5] Krishnakumar, I.; Ravi, A.; Kumar, D.; Kuttan, R.; Maliakel, B. An enhanced bioavailable formulation of curcumin using fenugreek-derived soluble dietary fibre. *J. Funct. Foods*, **2012**, *4*, 348-357.

[6] Wang, Y.-J.; Pan, M.-H.; Cheng, A.-L.; Lin, L.-I.; Ho, Y.-S.; Hsieh, C.-Y.; Lin, J.-K. Stability of curcumin in buffer solutions and characterization of its degradation products. *J. Pharm. Biomed. Anal*., **1997**, *15*, 1867-1876.

[7] Liang, G.; Shao, L.; Wang, Y.; Zhao, C.; Chu, Y.; Xiao, J.; Zhao, Y.; Li, X.; Yang, S. Exploration and synthesis of curcumin analogues with improved structural stability both *in vitro* and *in vivo* as cytotoxic agents. *Bioorg. Med. Chem*., **2009**, *17*, 2623-2631.

[8] (a) Canamares, M.; Garcia-Ramos, J.; Sanchez-Cortes, S. Degradation of curcumin dye in aqueous solution and on Ag nanoparticles studied by ultraviolet-visible absorption and surface-enhanced Raman spectroscopy. *Appl. Spectrosc*., **2006**, *60*, 1386-1391;(b) Mohanty, C.; Sahoo, S. K. The *in vitro* stability and *in vivo* pharmacokinetics of curcumin prepared as an aqueous nanoparticulate formulation. *Biomaterials*, **2010**, *31*, 6597-6611.

[9] Shahlaei, M.; Rahimi, B.; Nowroozi, A.; Ashrafi-Kooshk, M. R.; Sadrjavadi, K.; Khodarahmi, R. Exploring binding properties of sertraline with human serum albumin: Combination of spectroscopic and molecular modeling studies. *Chem. Biol. Interact*., **2015**, *242*, 235-246.

[10] (a) Kunwar, A.; Barik, A.; Pandey, R.; Priyadarsini, K.I. Transport of liposomal and albumin loaded curcumin to living cells: An absorption and fluorescence spectroscopic study. *Biochim. Biophys. Acta*, **2006**, *1760*, 1513-1520. (b) Mohammadi, F.; Bordbar, A.-K.; Divsalar, A.; Mohammadi, K.; Saboury, A.A. Interaction of curcumin and diacetylcurcumin with the lipocalin member β -lactoglobuli. *Protein J*., **2009**, *28*, 117-123. (c) Peters T.Jr. Serum albumin. In: Advances in Protein Chemistry. Anfinsen, C.B.; Edsall, J.T.; Richards F.M.; Eds.; Elsevier: Amsterdam, **1985**, *Vol. 37*, pp. 161-245. (d) Sneharani, A.H.; Karakkat, J.V.; Singh, S.A.; Rao, A.A. Interaction of curcumin with β-lactoglobulin stability, spectroscopic analysis, and molecular modeling of the complex. *J. Agric. Food Chem*., **2010**, *58*, 11130-11139.

[11] (a) Zamboni, W.C. Liposomal, nanoparticle, and conjugated formulations of anticancer agents. *Clin. Cancer Res*., **2005**, *11*, 8230-8234. (b) Portnaya, I.; Cogan, U.; Livney, Y.D.; Ramon, O.; Shimoni, K.; Rosenberg, M.; Danino, D. Micellization of bovine β-casein studied by isothermal titration microcalorimetry and cryogenic transmission electron microscopy. *J. Agric. Food Chem*., **2006**, *54*, 5555-5561.

[12] Mohammadi, F.; Bordbar, A.-K.; Divsalar, A.; Mohammadi, K.; Saboury, A.A. Analysis of binding interaction of curcumin and diacetylcurcumin with human and bovine serum albumin using fluorescence and circular dichroism spectroscopy. *Protein J*., **2009**, *28*, 189-196.

[13] (a) Mitra, S.P. Binding and stability of curcumin in presence of bovine serum albumin. *J. Surf. Sci. Technol*., **2007**, *23*, 91. (b) Esmaili, M.; Ghaffari, S.M.; Moosavi-Movahedi, Z.; Atri, M.S.; Sharifizadeh, A.; Farhadi, M.; Yousefi, R.; Chobert, J.-M.; Haertlé, T.; Moosavi-Movahedi, A.A. Beta casein-micelle as a nano vehicle for solubility enhancement of curcumin; food industry application. J. *LWT- Food Sci. Technol*., **2011**, *44*, 2166-2172.

[14] (a) Kulmyrzaev, A.A.; Levieux, D.; Dufour, É. Front-face fluorescence spectroscopy allows the characterization of mild heat treatments applied to milk. Relations with the denaturation of milk proteins. *J. Agric. Food Chem*., **2005**, *53*, 502-507. (b) Tayyab, S.; Haq, S.K.; Aziz, M.A.; Khan, M.M.; Muzammil, S. Effect of lysine modification on the conformation and indomethacin binding properties of human serum albumin. *Int. J. Biol. Macromol.,* **1999**, *26*, 173-180.

[15] (a) Mirzaee, F.; Kooshk, M.R.A.; Rezaei-Tavirani, M.; Khodarahmi, R. Protective effects of accompanying proteins on light-and water-mediated degradation of Curcumin. *J. Paramed. Sci.*, **2013**, *5*. (b) Kooshk, M.R.A.; Mansouri, K.; Nadi, M.M.; Khodarahmi, S.; Khodarahmi, R. *In vitro* anti-cancer activity of native curcumin and "Protein-Curcumin" systems: A perspective on drug-delivery application. *J. Rep. Pharm. Sci.,* **2013**, *2*, 66-74.

[16] Lozano, J.M.; Giraldo, G.I.; Romero, C.M. An improved method for isolation of β-lactoglobulin. *Int. Dairy J*., **2008**, *18*, 55-63.

[17] (a) Lawal, O.; Adebowale, K. The acylated protein derivatives of *Canavalia ensiformis* (jack bean): A study of functional characteristics. LWT-Food Sci.Tech., **2006**, *39*, 918-929. (b) Jangholi, A.; Ashrafi-Kooshk, M.R.; Arab, S.S.; Riazi, G.; Mokhtari, F.; Poorebrahim, M.; Mahdiuni, H.; Kurganov, B.I.; Moosavi-Movahedi, A.A.; Khodarahmi, R. Appraisal of role of the polyanionic inducer length on amyloid formation by 412-residue 1N4R Tau protein: A comparative study. *Arch. Biochem. Biophys*., **2016**, *609*, 1-19.

[18] Snyder, S.L.; Sobocinski, P.Z. An improved 2, 4, 6-trinitrobenzenesulfonic acid method for the determination of amines. *Anal. Biochem*., **1975**, *64*, 284-288.

[19] Hudson, E.A.; de Paula, H.M.C.; Ferreira, G.M.D.; Ferreira, G.M.D.; do Carmo Hespanhol, M.; da Silva, L.H.M.; Pires, A.C.d.S. Thermodynamic and kinetic analyses of curcumin and bovine serum albumin binding. *Food Chem*., **2018**, *242*, 505-512.

[20] Lakowicz, J. Principles of Fluorescent Spectroscopy. 2nd ed. Kluwer Academic/Plenum Press: New York, **1999**.

[21] Moradi, N.; Ashrafi-Kooshk, M.R.; Ghobadi, S.; Shahlaei, M.; Khodarahmi, R. Spectroscopic study of drug-binding characteristics of unmodified and pNPA-based acetylated human serum albumin: Does esterase activity affect microenvironment of drug binding sites on the protein? *J. Lumin.*, **2015**, *160*, 351-361.

[22] Goupy, P.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agri. Food Chem.*, **2003**, *51*, 615-622.

[23] Shen, L.; Ji, H.-F. Low stability remedies the low bioavailability of curcumin. *Trends Mol. Med.*, **2012**, *18*, 363-364.

[24] Mondal, S.; Ghosh, S.; Moulik, S.P. Stability of curcumin in different solvent and solution media: UV-visible and steady-state fluorescence spectral study. *J. Photochem. Photobiol.*, **2016**, *158*, 212-218.

[25] Nadi, M.M.; Kooshk, M.R.A.; Mansouri, K.; Ghadami, S.A.; Amani, M.; Ghobadi, S.; Khodarahmi, R. Comparative spectroscopic studies on curcumin stabilization by association to bovine serum albumin and casein: A perspective on drug-delivery application. *Int. J. Food Prop*., **2015**, *18*, 638-659.

[26] Leung, M.H.; Colangelo, H.; Kee, T.W. Encapsulation of curcumin in cationic micelles suppresses alkaline hydrolysis. *Langmuir*, **2008**, *24*, 5672-5675.

[27] Möller, M.; Denicola, A. Study of protein-ligand binding by fluorescence. *Biochem. Mol. Biol. Educ.*, **2002**, *30*, 309-312.

[28] Sahoo, B.K.; Ghosh, K.S.; Dasgupta, S. Molecular interactions of isoxazolcurcumin with human serum albumin: Spectroscopic and molecular modeling studies. *Biopolymers*., **2009**, *91*, 108-119.

[29] Goodsell, D.S.; Olson, A.J. Automated docking of substrates to proteins by simulated annealing. *Proteins*, **1990**, *8*, 195-202.

[30] Chen, T.; Cao, H.; Zhu, S.; Lu, Y.; Shang, Y.; Wang, M.; Tang, Y.; Zhu, L. Investigation of the binding of Salvianolic acid B to human serum albumin and the effect of metal ions on the binding. *Spectrochim. Acta A Mol. Biomol. Spectrosc.*, **2011**, *81*, 645-652.

[31] Li, M.; Ma, Y.; Ngadi, M.O. Binding of curcumin to β -lactoglobulin and its effect on antioxidant characteristics of curcumin. *Food Chem*., **2013**, *141*, 1504-1511.

[32] Ross, P.D.; Subramanian, S. Thermodynamics of protein association reactions: Forces contributing to stability. *Biochemistry*, **1981**, *20*, 3096-3102.

[33] (a) Khodarahmi, R.; Karimi, S.A.; Kooshk, M.R.A.; Ghadami, S.A.; Ghobadi, S.; Amani, M. Comparative spectroscopic studies on drug binding characteristics and protein surface hydrophobicity of native and modified forms of bovine serum albumin: Possible relevance to change in protein structure/function upon non-enzymatic glycation. *Spectrochim. Acta A Mol. Biomol. Spectrosc.*, **2012**, *89*, 177-186. (b) Hu, T.-Y.; Liu, C.-L.; Chen, J.-Y.; Hu, M.-L. Curcumin ameliorates methylglyoxal-induced alterations of cellular morphology and hyperpermeability in human umbilical vein endothelial cells. *J. Funct. Foods*, **2013**, *5*, 745-754.

[34] Litwinienko, G.; Ingold, K. Abnormal solvent effects on hydrogen atom abstraction. 2. Resolution of the curcumin antioxidant controversy. The role of sequential proton loss electron transfer. *J. Org. Chem*., **2004**, *69*, 5888-5896.

[35] Yang, M.; Wu, Y.; Li, J.; Zhou, H.; Wang, X. Binding of curcumin with bovine serum albumin in the presence of ι-carrageenan and implications on the stability and antioxidant activity of curcumin. *J. Agri. Food Chem*., **2013**, *61*, 7150-7155.

[36] Blasius, R.; Duvoix, A.; Morceau, F.; Schnekenburger, M.; Delhalle, S.; Henry, E.; Dicato, M.; Diederich, M. curcumin stability and its effect on glutathione S-transferase P1-1 mRNA expression in K562 cells. *Ann. NY Acad. Sci*., **2004**, *1030*, 442-448.

[37] Nishikawa, H.; Tsutsumi, J.; Kitani, S. Anti-inflammatory and anti-oxidative effect of curcumin in connective tissue type mast cell. *J. Funct. Foods*, **2013**, *5*, 763-772.

[38] (a) Azmi, A.S.; Bhat, S.H.; Hanif, S.; Hadi, S. Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: A putative mechanism for anticancer properties. *FEBS lett.*, **2006**, *580*, 533-538. (b) Elbling, L.; Weiss, R.-M.; Teufelhofer, O.; Uhl, M.; Knasmueller, S.; Schulte-Hermann, R.; Berger, W.; Micksche, M. Green tea extract and (–)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. *FASEB J.*, **2005**, *19*, 807-809.

[39] Agullo, G.; Gamet-Payrastre, L.; Fernandez, Y.; Anciaux, N.; Demigné, C.; Rémésy, C. Comparative effects of flavonoids on the growth, viability and metabolism of a colonic adenocarcinoma cell line (HT29 cells). *Cancer lett*., **1996**, *105*, 61-70.