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Caffeic acid phenethyl ester exhibiting distinctive binding interaction with human serum albumin implies the pharmacokinetic basis of propolis bioactive components

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ABSTRACT

Caffeic acid phenethyl ester (CAPE), as one of the major bioactive components present in propolis, exhibits versatile bioactivities, especially for its potent cytotoxic effects on several cancer cell models. To understand the pharmacokinetic characteristics of CAPE, the binding interaction between CAPE and human serum albumin (HSA) was investigated *in vitro* using multiple spectroscopic methods and molecular docking. The results reveal that CAPE exhibits a distinctive binding interaction with HSA comparing with other propolis components. The association constant K_A ($L\,mol^{-1}$) of the binding reaches 10^6 order of magnitude, which is significantly stronger than the other components of propolis. Based on the theory of fluorescence resonance energy transfer, the binding distance was calculated as 5.7 nm, which is longer than that of the other components of propolis. The thermodynamic results indicate that the binding is mainly driven by hydrogen bonds and van der Waals force. The docking and drugs (warfarin and ibuprofen) competitive results show that CAPE is located in the subdomain IIA (Sudlow's site I, FA7) of HSA, and Gln196 and Lys199 contribute to the hydrogen bonds. Circular dichroism spectra suggest an alteration of the secondary structure of HSA due to its partial unfolding in the presence of CAPE.

1. Introduction

Propolis is a distinct adhesive and resinous substance, commonly collected by honeybees (*Apis mellifera* L.) from resin present in cracks of tree barks and leaf buds. This resinous mixture is used for sealing holes in honeycombs, thereby protecting the entrance of honeycombs against intruders [1]. Propolis is known to have distinct biological and pharmacological properties, such as immunomodulatory, antitumor, antimicrobial, anti-inflammatory, antioxidant, *etc.* [2–4]. Though more than 300 compounds have been found in the propolis which include phenolic acids/cinnamic acid, caffeic acid and their esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols, and chalcones), terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes, and steroids [5]. whereas only some bioactive components of propolis were tested *in vitro* and *in vivo* [6].

Caffeic acid phenethyl ester (CAPE), as one of the most investigated components in propolis, has been widely reported to exhibit multiple and effective bioactivities against various pathologies such as anti-tumor [5], immunomodulatory [7], anti-inflammation [8], antioxidation [9], anti-microbial [10] and so on. CAPE shows significant cytotoxicity on tumors and virally transformed cells such as human leukemic cell line (HL-60) [9], colorectal cancer (CRC) cell line [11], and the breast cancer cell line [12,13]. Most importantly, CAPE has been shown to have very low toxicity to normal cells compared to carcinoma cells in the above studies. Therefore, CAPE can be considered as anticancer material [14]. However, a good *in vivo* stability is very crucial for a compound to be considered as a desired drug candidate. Although CAPE has been found to get hydrolyzed after six hours incubation in rats, human plasma does not affect its stability [15]. Nonetheless, the detailed transport mechanism of CAPE involved in the pharmacokinetic process of the human circulatory system remains unclear.

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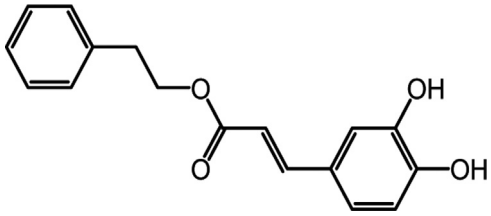

Fig. 1. Chemical structure of caffeic acid phenethyl ester (CAPE).

Human serum albumin (HSA), the most abundant protein in human plasma, is constituted by a single chain of 585 residues, with a molecular weight of 66,438 Da. HSA is composed of six subdomains and is comprised of three homologous domains (I: residues 1–195, II: residues 196–383, III: residues 384–585), each of the domains can further be divided into two subdomains (A and B) [16,17]. It has been suggested that HSA comprises two main distinct binding sites, which are located in the hydrophobic cavities of subdomains IIA and IIIA, respectively [18]. Moreover, the binding sites of HSA are labeled FA1-FA7 due to its multiple binding capabilities with long chain fatty acids (FAs). FA7 locating in subdomain IIA represents Sudlow's site I, the preferential binding site for bulky, heterocyclic anions (*e.g.*, warfarin), whereas the cavity hosting FA3 and FA4 locating in subdomain IIIA, contributes to Sudlow's site II, which is preferred by aromatic carboxylates with an extended conformation (*e.g.*, ibuprofen) [19].

HSA functions as a carrier for several endogenous and exogenous compounds, strongly affecting the pharmacokinetics of many drugs [20,21]. HSA also holds some ligands like flavonoids in a strained orientation, resulting in metabolic modifications of drugs [22,23]. Transportation as well as *in vivo* stability of a drug mainly relies on its interaction with HSA present in human plasma. There are several published reports available on the binding interactions between HSA and some functional compounds isolated from propolis, such as ferulic acid [24,25], caffeic acid [25,26], *p*-coumaric acid [26], cinnamic acid [26], and chlorogenic acid [27], *etc*. However, as one of the major biological active compounds in poplar-type propolis, the detailed transportation of CAPE in the human plasma have not yet been investigated until now. For optimizing operating conditions for the application of CAPE in pre-clinical therapeutic trials, it is necessary to elucidate the binding process of CAPE relying on HSA *in vitro*.

Hence, in the present study, we aim to probe the binding interaction between CAPE and HSA using multiple spectroscopic techniques as well as molecular docking analysis. Meanwhile the experimental results are compared with those propolis components interacting with HSA reported earlier in the literature.

2. Material and methods

2.1. Reagents and materials

Human serum albumin (purity > 99.0%), caffeic acid phenethyl ester (Fig. 1, purity > 97.0%) and two drugs, warfarin and ibuprofen (purity > 98.0%) used for competitive assay were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phosphate buffer solution (PBS, 0.1 M) buffer used as the dilution buffer of HSA was adjusted to pH 7.4. Water used throughout this experiment was prepared by Milli-Q water purification system (Millipore, USA). Other reagents used were of analytical grade.

2.2. Fluorescence quenching spectra

Fluorescence spectra of HSA with CAPE were measured using RF-5301 PC Spectrofluorimeter (Shimadzu, Japan) with a 1.0 cm quartz cell at various temperatures (285 K, 290 K, 295 K, 300 K, and 305 K). The widths of excitation and emission slit were set at 10.0 nm, excitation wavelength was 280 nm, and the emission spectra were recorded between 300 and 450 nm. The stock solution of HSA was diluted into working solution (5×10^{-7} M), then was titrated by the working CAPE (10^{-2} M). A 9012 type electronic thermostat water bath (PolyScience, USA) was used to stabilize the temperature of the interactions. Association constants of the HSA–CAPE complex were calculated from the fluorescence quenching data.

2.3. Synchronous fluorescence spectra

Synchronous fluorescence spectra of HSA were measured on RF-5301 PC Spectrofluorimeter with increasing concentrations of CAPE. The intervals between maximum excitation and emission wavelength are set as $\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm for tryptophan (Trp) and tyrosine (Tyr) residues, respectively. The spectra were recorded in range of 280–380 nm at room temperature.

2.4. Ultraviolet absorption spectra

Ultraviolet (UV) absorption spectra were measured on UV-1800 spectrophotometer (Shimadzu, Japan) from 190 to 400 nm equipped with a 1.0 cm quartz cell at room temperature. The operating parameter was the same as that in fluorescence spectra assay. The working concentration of CAPE was fixed at 5×10^{-7} M, which is equal to the concentration of HSA measured in fluorescence spectra.

2.5. Circular dichroism spectra

Circular dichroism (CD) spectra of HSA and that with CAPE were measured using a J-815 spectrometer (Jasco, Japan) with a 1.0 cm quartz cell. The spectra were recorded in range of 190–250 nm at a scan speed of 200 nm min^{-1} at room temperature, with the baseline subtracted though PBS buffer (pH 7.4). The molar ratio of HSA to CAPE was 0:1, 2.5:1, 10:1, and 15:1, respectively. The secondary structural information of HSA was calculated from the CD ellipticity of spectra data.

2.6. Molecular docking

The docking analysis between CAPE and HSA were stimulated by the Molegro Virtual Docker 5.0 (free trial) software. The three-dimensional (3D) crystal structure of HSA (PDB ID code: 1AO6) was downloaded from Protein Data Bank (PDB). Based on the developed grid-based cavity prediction algorithm, the potential binding sites of HSA were determined. The best binding pose of HSA–CAPE complex was obtained according to the searching algorithm of MolDock Optimizer and energetic evaluation of the complex with MolDock. The binding pose was then analyzed by Ligplot+ [28] and displayed by Pymol software [29].

2.7. Competitive binding assay

To determine the actual binding site of CAPE on HSA, the competitive binding assay was performed. The working solutions of warfarin and ibuprofen were first diluted into working solution (10^{-3} M) by methanol. Firstly, when the molar concentration ratio of CAPE and HSA in the experimental system was set 1:1, the working solutions of warfarin and ibuprofen were then titrated into the complex system, respectively. Secondly, according to the fluorescent decline of each drug to the CAPE–HSA complex, the molar concentration ratio of warfarin and HSA was set a gradient ratio of 1:1, 2:1, 3:1, and 4:1, the working solution of CAPE was then titrated into the corresponding gradient system, respectively. All

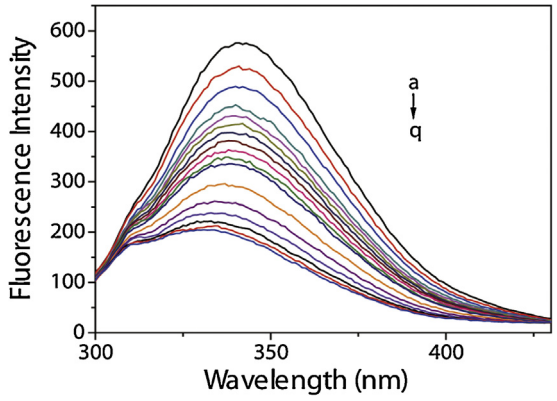

Fig. 2. Fluorescence quenching spectra of human serum albumin (HSA) with caffeic acid phenethyl ester (CAPE) titrating. c (HSA) = 5×10^{-7} M, c (CAPE) = 10^{-2} M; pH = 7.4; λ_{ex} = 280 nm. As CAPE titrated from *a* to *q* (final concentration is 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.30, 0.35, 0.40, 0.45, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5×10^{-6} M, respectively), the fluorescence intensity of HSA significantly quenched and the maximum emission peak of HSA has blue shift with the increasing concentration of CAPE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the binding constants under the corresponding gradient system were calculated and compared to determine the competitive effects and binding sites.

3. Results and discussion

3.1. Fluorescence quenching spectra

The fluorescence quenching spectra of HSA in absence and presence of CAPE are shown in Fig. 2. HSA exhibits a strong fluorescence emission (λ_{em}) around 310 nm, when excited at 280 nm (λ_{ex}). As evident from the figure, the fluorescence intensity of HSA gradually decreases with increase in CAPE concentration; however, there is neither any shift in λ_{em} nor any change in peak shape. These results indicate that CAPE interacts with HSA by generating non-fluorescent complex between them.

3.2. Binding mechanism

The binding modes of proteins quenched by organic molecules are commonly dynamic or static quenching [30]. The dynamic quenching means that the higher temperature results in faster diffusion and occurrence of larger amounts of collision. In contrast, static quenching causes to generate the stable complexes and results in the dissociation of complexes at higher temperatures.

Initially, we assumed that the fluorescence quenching of HSA in presence of CAPE occurs *via* dynamic quenching, which is commonly analyzed by the well-known Stern–Volmer equation (Eq. (1)) [31]:

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{sv} [Q] \quad (1)$$

where F_0 and F are the fluorescence intensities of fluorophore in absence or presence of a quencher at [*Q*] concentration, respectively. K_q is the quenching rate constant of the biomolecule, τ_0 is the lifetime of fluorophore (for the tryptophan fluorescence decay τ_0 is about 10^{-8} s) [32], K_{sv} is the Stern–Volmer dynamic quenching constant. According to this equation, if the quenching process is dynamic, F_0/F should be linear with respect to quencher concentration [*Q*], and the slope should be equal to the value of K_{sv}. All K_{sv} (L mol^{-1}) and K_q (L mol^{-1} s^{-1}) of CAPE binding with HSA at different temperatures were calculated (Table 1).

Table 1
Fluorescence quenching constants (in equation of Stern–Volmer and Lineweaver–Burk) for the interaction between CAPE and HSA.

T (°K)	K_{sv} (L mol^{-1})	K_q (L mol^{-1} s^{-1})	r	K_A (L mol^{-1})	r
290	1.53×10^6	1.53×10^{14}	0.9983	2.76×10^6	0.9545
295	1.41×10^6	1.41×10^{14}	0.9993	2.15×10^6	0.9786
300	1.31×10^6	1.31×10^{14}	0.9992	1.08×10^6	0.9949
305	1.27×10^6	1.27×10^{14}	0.9982	9.29×10^5	0.9948
310	1.09×10^6	1.09×10^{14}	0.9989	8.88×10^5	0.9604

As shown in Fig. 3A, the values of K_{sv} decrease with increase of temperature, indicating that the quenching of HSA fluorescence intensity in presence of CAPE occurs *via* static quenching. Static quenching usually occurs because of the generation of a non-fluorescent complex that dissociates at higher temperature [33].

Assuming the fluorescence quenching behavior of HSA in presence of CAPE as a static quenching process, the association constants (K_A) and the number of binding sites (n) can be obtained from the regression curve based on the following Lineweaver–Burk equation (Eq. (2)) [34]:

$$\frac{1}{(F_0 - F)} = \frac{1}{F_0} + \frac{1}{K_A F_0 [Q]} \quad (2)$$

where F_0 is the fluorescent intensity of fluorophore in absence of quencher, and F is the fluorescent intensity of fluorophore in presence of quencher at [*Q*] concentration. K_A is the apparent association constant. K_A at different temperatures were obtained from the plot $1/(F_0 - F)$ *versus* $1/[Q]$ (Fig. 3B).

Interestingly, we found that these binding association constants (K_A) are nearly in the order of 10^6 L mol^{-1} (Table 1), the values of which are significantly higher than those of the other known components of propolis, such as ferulic acid [24,25], caffeic acid [25,26], *p*-coumaric acid [26], cinnamic acid [26], and chlorogenic acid [27] *etc*. The K_A values of these are generally in the order of 10^3–10^5 L mol^{-1}) (Table 2). It is known that HSA often binds drugs

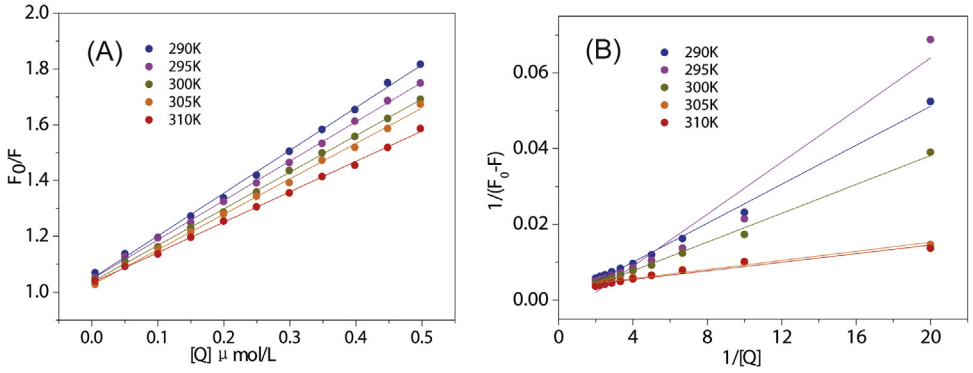

Fig. 3. Stern–Volmer plots (A) and Lineweaver–Burk equation plots (B) for fluorescence quenching data at five different temperatures (290 K, 295 K, 300 K, 305 K, and 310 K).

Table 2
Comparison of binding constants of some known propolis components and HSA based on the fluorescence quenching spectra.

Propolis components	K_A (L mol^{-1})	Binding distance with HSA	Binding to the cavity of HSA
CAPE	2.15×10^6 (in this study)	5.7	IIA
Caffeic acid	4.31×10^5 [25]	–	IIA[a]
	1.60×10^5 [26]	2.79	–
Ferulic acid	4.72×10^5 [25]	–	IIA[a]
	2.23×10^4 [24]	3.57	IIIA[b]
p-Coumaric acid	1.10×10^5 [26]	2.59	–
Chlorogenic acid	4.37×10^4 [24]	2.45	IIA[b]
	3.00×10^4 [27]	3.10	IIA
Cinnamic acid	4.00×10^3 [26]	1.87	–

[a] The binding site was predicted based on the fluorescence quenching of both the emission and synchronous spectra of HSA-ligand.
[b] The binding site was predicted based on the fluorescence quenching fraction (%) of HSA-ligand complex.

Table 3
Thermodynamics parameters and binding force for the interaction between CAPE and HSA.

T (°K)	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG (kJ mol^{-1})	Binding force
290	−46.67	−38.01	−35.65	Hydrogen bonds and van der Waals force
295			−35.46	
300			−35.27	
305			−35.08	
310			−34.89	

with association constants that range from 10^3 to 10^{10} L mol^{-1} [35]. HSA can bind highly hydrophobic drugs, thus diminishing the high clearance rates or poor distribution that might otherwise render these ineffective [36], while the low binding of drugs with HSA may result in high toxicity or metabolism [35]. Therefore, the higher affinity of CAPE with HSA would be helpful for its long duration staying in the human blood circulatory system as well as acting on the biological targets in nidus. Therefore, from the pharmacokinetic perspectives, this suggests that CAPE exhibits better transport properties in human plasma compared to the other components of propolis, a probable reason for the higher bioactivity exhibited by CAPE compared to the other components of propolis [37].

3.3. Thermodynamic analysis

The binding interactions between CAPE and HSA are known to be influenced by different factors, such as hydrophobic interaction, electrostatic force, hydrogen bond, and van der Waals interactions *etc*. These factors can be deduced from the following thermodynamic equations (Eqs. (3) and (4)) [21]:

$$\Delta G = -RT \ln K = \Delta H - T\Delta S \quad (3)$$

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

where ΔG, ΔH, and ΔS represent free energy change, enthalpy change, and entropy change, respectively. The apparent association constants K of CAPE with HSA at five different temperatures obtained from the Lineweaver–Burk equation were used for calculating ΔG, ΔH, and ΔS. If the temperature changed slightly in some ranges, enthalpy change was always regarded as a constant. The values of ΔH and ΔS were calculated based on Eq. (4), while the values of ΔG were calculated based on Eq. (3) (Fig. 4). Table 4 lists all the calculated values. Theoretically, when $\Delta H < 0$ or $\Delta H \approx 0$ and $\Delta S > 0$, hydrophobic and electrostatic forces play as the main forces of interaction; when $\Delta H < 0$ and $\Delta S < 0$, hydrogen bond and van der Waals force act as the main force of interaction; and hydrophobic interaction plays the main role when $\Delta H > 0$ and $\Delta S > 0$ [30]. In the present study, we found $\Delta H < 0$ and $\Delta S < 0$, indicating that the binding processes are exothermic and entropy driven.

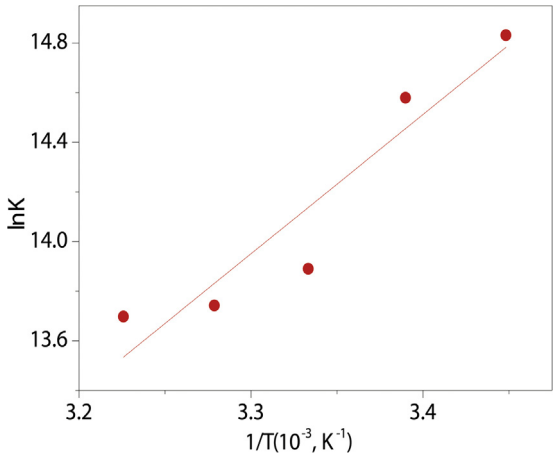

Fig. 4. The linear regression plot of lnK *versus* 1/T based on the values of ΔH and ΔS at five different temperatures (290 K, 295 K, 300 K, 305 K, and 310 K).

Therefore, the acting forces are mainly driven by hydrogen bond and van der Waals force. Moreover, we found $\Delta G < 0$ for all of these binding interactions, suggesting that the binding interaction should be spontaneous.

3.4. Synchronous fluorescence spectra

The characteristics and the molecular environment in the vicinity of the both fluorescent amino acid residues of HSA, tyrosine and tryptophan, can be obtained using synchronous fluorescence (SF) spectroscopy, keeping the scanning interval $\Delta\lambda$ ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$) fixed at 15 and 60 nm, respectively [38]. In the present study, we employed SF spectroscopy for exploring the conformational changes of HSA at various CAPE concentrations. Fig. 5 shows increasing concentration of CAPE leads to a significant decrease (about 40%, from 525 to 320) in the fluorescence intensity of the tryptophan residues in HSA, while the intensity drops (about 19%, from 117 to 95) for tyrosine residues. It indicates that the fluorescence from tryptophan is more intense and gets more efficiently quenched than those from Tyr residues in presence of CAPE, implying that tryptophan might be closer to the binding site than tyrosine residues. Moreover, a weak blue shift (~2 nm) in emission maximum was also observed for tryptophan residues, indicating increased polarity of the binding microenvironment.

3.5. Binding distance

The intrinsic energy transfer from HSA to CAPE could be elucidated by the Förster resonance energy transfer (FRET) theory [39].

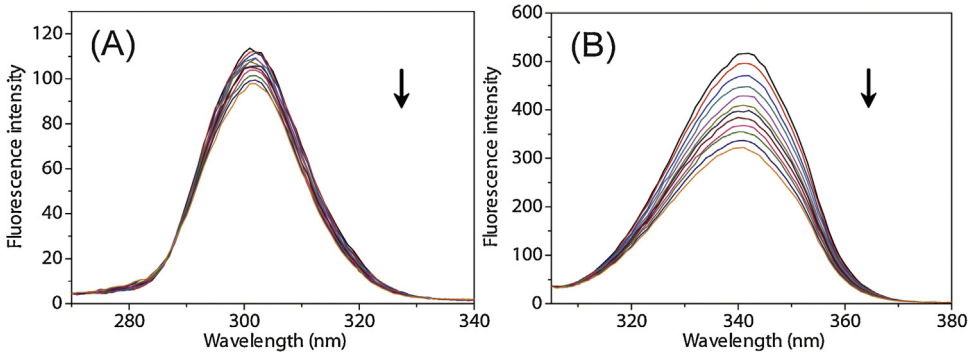

Fig. 5. Synchronous fluorescence spectra of human serum albumin (HSA) and caffeic acid phenethyl ester (CAPE). (A) = 15 nm (tyrosine). When CAPE is titrated, the fluorescence intensity decrease slightly, and the maximum emission wavelength and the shape of peak change scarcely. (B) = 60 nm (tryptophan). When CAPE is titrated, the fluorescence intensity decrease sharply, and the maximum emission wavelength has a slight blue shift from 343 to 340 nm.

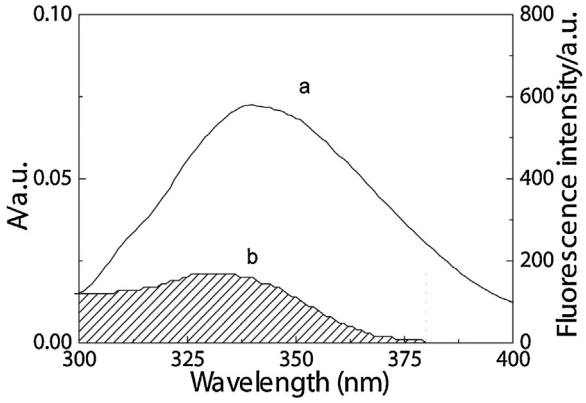

Fig. 6. Overlapping of fluorescence emission spectrum of human serum albumin (HSA) (a) and ultraviolet absorption spectrum of caffeic acid phenethyl ester (CAPE) (b). c (HSA) = c (CAPE) = 5×10^{-7} M; pH 7.4; λ_{ex} = 280 nm.

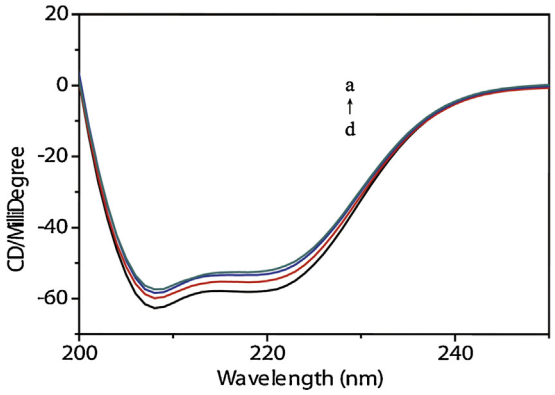

Fig. 7. Circular dichroism spectra of human serum albumin and caffeic acid phenethyl ester (CAPE). The final concentration of CAPE added from *a* to *d* is 0, 2.5, 10, and 15×10^{-6} M, respectively. The typical shoulder peaks of α-helix increased at 209 and 222 nm.

There are following three requirements for FRET to occur in a donor-acceptor system: (a) the relative orientation of donor and acceptor dipoles, (b) the extent of overlap of fluorescence emission spectrum of donor with the absorption spectrum of acceptor, and (c) the distance between donor and acceptor should be less than 10 nm. Here, we applied the FRET theory to demonstrate the static binding interaction between HSA (donor) and CAPE (acceptor). The efficiency of energy transfer (E) is related to the distance R_0 between donor and acceptor by the equations (Eqs. (5) and (6)) [40]:

$$E = \frac{R_0^6}{R_0^6 + r_0^6} = 1 - \frac{F}{F_0} \tag{5}$$

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^{-4} \phi J \tag{6}$$

where r_0 is the binding distance between donor and acceptor, and R_0 is the critical distance when the efficiency of transfer is 50%, K^2 is the spatial orientation factor of the dipole, n is the refractive index of the medium, ϕ is the fluorescence quantum yield of the donor. Here, J is the overlap integral of the fluorescence emission spectrum of the donor (HSA) with the absorption spectrum of the acceptor (CAPE) (Fig. 6), which can be calculated by the following equation (Eq. (7)):

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^{-4}\Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{7}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength λ, and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. In the present case, $K^2 = 2/3$, $N = 1.336$, $\phi = 0.2$. From Eqs. (5)–(7), we obtained the following values: $R_0 = 5.38$ nm, $E = 0.4191$, and $r_0 = 5.7$ nm. Due to $r_0 < 10$ nm, it indicates that the intrinsic energy transfer from HSA to CAPE is in accordance with the theory of FRET. Compared with other propolis components (Table 3), the calculated binding distance of CAPE with HSA seems longer. It might be resulted from their different binding sites with HSA, which will be discussed in the following section of molecular docking.

3.6. Circular dichroism

Circular dichroism (CD) measurements were performed to monitor the secondary structure changes induced by the interaction of CAPE with HSA. The CD spectrum of HSA exhibits two negative bands at 208 and 222 nm, which are characteristics of the α-helical structure of a protein (shown in Fig. 7). The interaction between CAPE and HSA caused a slight decrease in ellipticity at all wavelengths of the far CD without any significant shift in the peak position. We found the α-helical ratio of free HSA to be 81.4%. The α-helical ratio of HSA decreased to 79%, 74.9%, and 69.2% on 1:5, 1:20, and 1:30 binding with CAPE, respectively, indicating alteration of the conformational structure of HSA in presence of the external CAPE molecule [41].

3.7. Molecular docking

Based on the crystal structure of HSA from PDB database (PDB ID code: 1AO6) [42], the Molegro Virtual Docker (MVD) software 5.0 (trial version) was used to perform the docking analysis. According to the best docking pose, CAPE is mainly predicted to be close

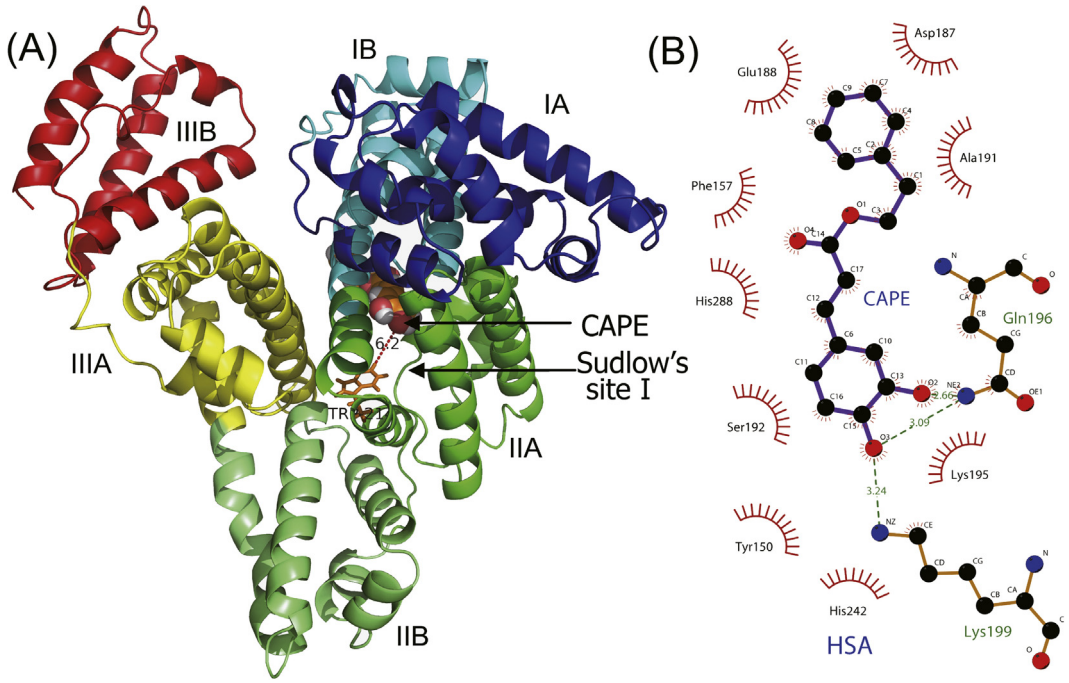

Fig. 8. (A) Predicted binding mode of caffeic acid phenethyl ester (CAPE) docked into human serum albumin (HSA). The docking analysis was processed by the Molegro virtual docker software 5.0 (trial version). According to the best docking pose, CAPE is clearly observed to be close to the Sudlow's site I located in subdomain IIA. Both CAPE and the main fluorescent amino acid Trp214 are showed in the same subdomain IIA, and the distance between them was predicted to be as 6.2 nm. (B). The hydrophobic interactions and hydrogen bonds between CAPE and the binding site of HSA. The nitrogen atoms of Gln196 and Lys199 residues in human serum albumin form two and one hydrogen bonds (shown by green dashed lines) with oxygen atoms in CAPE, respectively. There are other nine amino acid residues around CAPE forming the HSA binding cavity. In which Lys199 and Tyr150 of HSA binding with CAPE are two key residues positioned in the hydrophobic cavity of subdomain IIA (i.e., Sudlow's site I, FA7). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to the Sudlow's site I (FA7) located in HSA subdomain IIA (Fig. 8A), which is almost similar to that of the reported propolis components (Table 3). On the other hand, though both CAPE and the main fluorescent amino acid Trp214 exist in the same subdomain IIA, the distance between them was shown as 6.2 nm (Fig. 8A), which may be longer than those of other propolis compounds (Table 2) and is also in accordance with the calculated experimental value (5.7 nm) based on the FRET theory above. It implies that the different binding and transporting process of CAPE leads to the distinct and effective bioactivities comparing with other reported propolis components.

Nevertheless, based on the predicted detailed interaction of amino acids around CAPE (Fig. 8B), CAPE is located within one cavity composed of the 11 residues of HSA. The binding cavity is mainly composed of nine various amino acids, including two hydrophobic residues (Phe157 and Ala191), two hydrophilic residues (Tyr150 and Ser192), two acidic residues (Asp187 and Glu188), and three alkaline residues (Lys195, His242, and His288) (Fig. 8B), Furthermore, we also found two polar residues, Gln196 and Lys199, totally forming three hydrogen bonds with CAPE (Fig. 8B, three green dashed lines). Remarkably, Lys199 and Tyr150 of HSA binding with CAPE were reported to be two key residues positioned in the hydrophobic cavity of subdomain IIA (i.e., Sudlow's site I, FA7) of HSA [16].

Table 4
Binding constants declines under gradient molar concentration ratio of warfarin and HSA.

[Warfarin]:[HSA]	K (L mol^{-1})	r
0:1	2.76×10^6	0.9545
1:1	1.47×10^6	0.9901
2:1	1.35×10^6	0.9949
3:1	1.31×10^6	0.9946
4:1	0.98×10^6	0.9978

The findings of the docking study are in agreement with the multispectral and thermodynamic experimental data, which may deepen the theoretical understanding of the CAPE pharmacokinetic properties as well as the development of the clinical application as an effective adjuvant of chemotherapy [12,43].

3.8. Determination of binding site based on competitive assay

Warfarin and ibuprofen are suggested to exhibit the specific binding sites on HSA, namely Sudlow's site I and II, respectively [44]. When warfarin and ibuprofen were first titrated into the CAPE–HSA system with equal molar concentration, respectively, the fluorescence quenching of warfarin to CAPE–HSA complex showed more significant than that of ibuprofen (Fig. S1). Hence, when a serial of molar concentration gradient ratio of warfarin and HSA was set 1:1, 2:1, 3:1, and 4:1, the corresponding binding constants of CAPE to the warfarin–HSA were measured and calculated according to the Lineweaver–Burk equation (Eq. (2)) (Table 4). It was evident that the K_A values declined with the molar ratio of warfarin–HSA rising (Fig. 9), implying that CAPE effectively competed with warfarin on the binding site of HSA. Thus, the Sudlow's site I in subdomain IIA was suggested to be the main binding site for CAPE on HSA, which is in accordance with the deduction of docking analysis above. The location site binding with HSA was also observed with other drugs, like some antituberculosis [45], implying that some drugs containing heterocyclic anions structure may prefer to binding with subdomain IIA of HSA.

4. Conclusions

In this study a distinctive binding interaction of CAPE, one propolis bioactive component, with human serum albumin (HSA) was investigated *in vitro* by using multiple spectroscopic methods

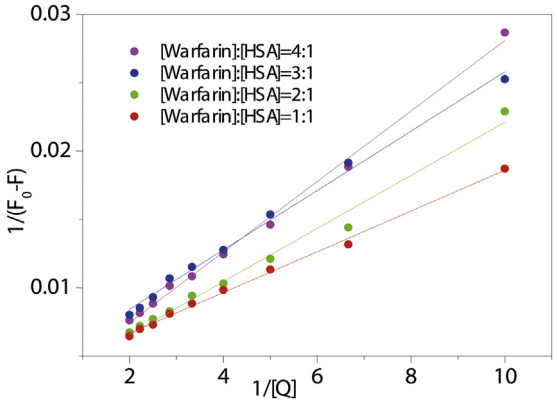

Fig. 9. Determination of CAPE binding site on HSA based on competitive assay of warfarin. It is evident that the larger of the molar ratio of warfarin–HSA (a serial of gradient ratio as 1:1, 2:1, 3:1, until 4:1), the higher of the corresponding slopes (being a negative correlation with the binding constants of CAPE to the warfarin–HSA system). It implies that CAPE effectively competes with warfarin on the binding site, Sudlow's site I of subdomain IIA of HSA.

and molecular docking. The relevant pharmacokinetic and thermodynamic parameters of the interactions were calculated and compared with other propolis components reported before. CAPE had a significantly strong ability (K_A (L mol^{-1}) as 10^6 order of magnitude) to quench the intrinsic fluorescence of HSA *via* a static quenching. The binding constant is evidently stronger than those of all propolis bioactive components.

On the basis of the theory of FRET, the binding distance was calculated as 5.7 nm, which is supported by the corresponding docking analysis and seems longer than those of other propolis components. The docking results indicate that CAPE may locate in the Sudlow's site I in HSA subdomain IIA, a common site of other propolis components. Thermodynamic parameters indicated that the interaction was driven mainly by hydrogen bond and van der Waals force, and CD results suggested that the secondary structure of HSA altered due to its partial unfolding in the presence of CAPE.

In conclusion, we hope that the findings in this study would be helpful for further understanding transportation of bioactive components of propolis *in vivo*, which will provide with some suggestions about the therapeutic trials based on the transporting pharmacokinetic properties. All the trials would also evoke inspirations for the development of discovery and screening of natural bioactive medicine like propolis based on the pharmacokinetic basis in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.01.040.

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