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Screening, Identification, and Potential Interaction of Active Compounds from *Eucommia ulmodies* Leaves Binding with Bovine Serum Albumin

Yuping Zhang,[†] Mijun Peng,[‡] Liangliang Liu,[†] Shuyun Shi,[*,†,§] and Sheng Peng[‡]

[†]School of Chemistry and Chemical Engineering, Central South University, Changsha 410083, People's Republic of China

[‡]Key Laboratory of Hunan Forest Products and Chemical Industry Engineering, Jishou University, Zhangjiajie 427000, People's Republic of China

[§]Institute of Clinical Pharmacology, Central South University, Changsha 410078, People's Republic of China

ABSTRACT: The aqueous extract of *Eucommia ulmoides* leaves has been commonly known as Du-zhong tea as a functional health food for the treatment of hypertension, hypercholesterolemia, and fatty liver. This study developed a centrifugal ultrafiltration−high-performance liquid chromatography (HPLC) method for screening and identification of bioactive compounds in *E. ulmoides* leaves binding with bovine serum albumin (BSA). Six active compounds were screened, isolated, and elucidated by their ultraviolet (UV), electrospray ionization−mass spectrometry (ESI−MS), and nuclear magnetic resonance (NMR) data as geniposidic acid (**1**), caffeic acid (**2**), chlorogenic acid (**3**), quercetin-3-*O*-sambubioside (**4**), rutin (**5**), and isoquercitrin (**6**). The interaction between active compounds and BSA was investigated in the absence and presence of other compounds by quenching the intrinsic BSA fluorescence. The results indicated that the structures significantly affected the binding process. The values of binding constants for compounds **2**−**6** were in the range of 10^5−10^6 mol L^{-1}, while geniposidic acid (**1**) hardly quenching the BSA intrinsic fluorescence. However, the quenching process of geniposidic acid was easily affected in the presence of other active compounds. The formation of the geniposidic acid−phenylpropanoid (flavonoid) complex could increase the binding affinity of geniposidic acid with BSA; however, the increased steric hindrance of the complex may make phenylpropanoid or flavonoid dissociate from BSA and then decrease their affinities.

KEYWORDS: *Eucommia ulmoides leaves, BSA, centrifugal ultrafiltration, HPLC, binding affinity, bioactive compound*

INTRODUCTION

Eucommia ulmoides (also called Du-zhong, belonging to Eucommiaceae) is a commonly used tonic herb in Asia, which can elicit pharmacological effects on coronary blood flow, obesity, diuresis, hypertension, lipid metabolism, and diabetes.[1−4] As mentioned in Chinese Pharmacopoeia, the medicinal parts of *E. ulmoides* are its bark and leaves.[5] The aqueous extract of *E. ulmoides* leaves, which has been commonly known as Du-zhong tea, has long been known as a functional health food for the treatment of hypertension, hypercholesterolemia, and fatty liver.[3] Up to now, many efforts have been made on the identification of chemical constituents and pharmacological activities of *E. ulmoides* leaves. The major phytochemicals were iridoids, phenylpropanoids, flavonoids, and triterpenes,[6,7] and the extracts of *E. ulmoides* leaves have recently been reported to exhibit a wide range of biological activities, such as antioxidant activity,[8,9] glycation inhibitory activity,[10] and anti-obesity activity.[11] However, most of the pharmacological activities were conducted on the complex extracts, and how each of these components contributed to the effects was not clearly understood probably because of the commercially unavailable compounds. It is widely accepted that the active compounds in natural products will produce a synergistic effect or antagonistic action;[12,13] therefore, the screening, identification, and potential interaction of active compounds are very important, not only for the quality control of natural products but also for elucidating the therapeutic principle, which lead to the safety of clinic application.

Serum albumin is the major soluble protein in the circulatory system, which has many physiological functions, such as maintaining the osmotic pressure and pH of blood and functioning as a carrier transporting a great number of endogenous and exogenous compounds, including fatty acids, amino acids, drugs, and pharmaceuticals.[14] The drug−serum albumin interaction plays a dominant role in drug disposition and efficacy. The study of the interaction between drugs and serum albumin is not only important to provide salient information about the nature of drugs and pharmacokinetics but is also helpful to explain the relationship between the structures and functions of drugs. Up to now, most papers have reported the binding process between drugs and serum albumin.[15−19] Bovine serum albumin (BSA), which has structural homology with human serum albumin (HSA),[20] is composed of three structurally homologous, predominantly helical domains (I, II, and III), each containing two subdomains (A and B), and the principal regions of drug-binding sites on albumin are located in hydrophobic cavities in sub-domains IIA and IIIA, which exhibit similar chemistry properties.[21] There-

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fore, the drug−serum albumin interaction always causes interference with the binding of other drugs as the result of the overlap of binding sites or conformational changes.[22−25] It is therefore the detailed investigations of drug−drug serum albumin interactions that play a dominant role for the interpretation of drug−drug interactions.

The conventional activity-guided isolation process for complex extracts is a time-consuming, labor-intensive, and expensive process, and it is often inefficient for the direct screening of bioactive compounds from natural complex mixtures.[26] The recent developed centrifugal ultrafiltration technology uses both centrifugation force and a semi-permeable membrane to retain suspended solids and compounds of high molecular weight, while the liquid and low-molecular-weight compounds are allowed to pass through the membrane depending upon the nominal molecular weight cut-off of the membrane.[27] Therefore, centrifugal ultrafiltration has been successfully applied for screening and analysis of active compounds from complex mixtures binding with biomacromolecules, such as BSA, liposome, and rat plasma protein, because of its fast speed, easy operation, and high reliability.[28−33]

As part of our ongoing effort on the identification of active compounds from natural products,[34−36] the present work developed a centrifugal ultrafiltration−high-performance liquid chromatography (HPLC)−diode array detector (DAD)−tandem mass spectrometry (MS/MS) method for the screening and identification of active compounds from the aqueous extract of *E. ulmoides* leaves. Moreover, the detected active compounds were isolated directly by preparative HPLC, and the chemical structures of the active compounds are elucidated by their ultraviolet (UV), electrospray ionization−mass spectrometry (ESI−MS), and nuclear magnetic resonance (NMR) data. The binding affinities and potential interaction of active compounds with BSA have also been investigated using the quenching fluorescence methods. This is the first report on the screening, identification, and potential interaction of main active compounds from *E. ulmoides* leaves binding with BSA.

■ MATERIALS AND METHODS

Chemicals and Reagents. BSA was purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, methanol, and acetic acid used for HPLC was of chromatographic grade (Merk, Darmatadt, Germany). Geniposidic acid, caffeic acid, chlorogenic acid, quercetin-3-*O*-sambubioside, rutin, and isoquercitrin, with the purity over 99%, were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Distilled water was further purified by a Milli-Q (18.2 MΩ) system (Millipore, Bedford, MA). The other chemicals used for the extraction, separation, and binding process, such as ethanol, petroleum ether (60−90 °C), ethyl acetate, *n*-butanol, Tris buffer, NaCl, and HCl, were all of analytical grade and purchased from Chemical Reagent Factory of Hunan Normal University (Hunan, China), which were used without further purification. D101 macroporous resin was purchased from the Chemical Plant of Nankai University (Tianjin, China), which was a cross-linked polystyrene co-polymer.

The *E. ulmoides* leaves was collected from Zhangjiajie, Hunan Province, China, in 2010, and identified by one of the authors, Prof. Mijun Peng. A voucher specimen (EULZJJ2010) was deposited at the School of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan, China.

Apparatus and Instruments. HPLC−DAD−MS/MS was performed on an Acquity UPLC system (Waters Corp., Milford, MA) with a cooling autosampler and column oven enabling temperature control of the analytical column. The target compounds were separated by a reversed-phase SunFire C_{18} (250 × 4.6 mm inner diameter, 5 μm, Milford, MA) column. Triple−quadrupole tandem mass spectrometric detection was carried out on a Micromass Quattro micro API mass spectrometer (Waters Corp., Milford, MA) with an electrospray ionization (ESI) interface. The ESI source was set in negative ionization mode. The following settings were applied to the instrument: capillary voltage, 3.00 kV; cone voltage, 40.0 V; extractor voltage, 3.00 V; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas flow, 750 L h^{-1}; cone gas flow, 50 L h^{-1}; and dwell time, 0.05 s. Nitrogen was used as the desolvation and cone gas. Mass detection was performed in full-scan mode for m/z in the range of 160−800. All data were acquired and processed by MassLynx NT 4.1 software with the QuanLynx program (Waters Corp., Milford, MA).

The preparative HPLC experiments were performed on a self-assembled instrument, which was composed of a P3000 delivery pump, a 2PB00C sample injection pump, UV3000 variable wavelength detector with detection monitored at 254 nm, and a SCJS-3000 ChemStation. A preparative column (500 × 80 mm inner diameter) packed with 5 μm ODS C_{18} (Fuji, Japan) was used for the preparative separation. The flow rate was 25 mL min^{-1} at 20 °C.

The NMR spectra were recorded at 25 °C on a Varian Inova-400 (Varian Corporation, Palo Alto, CA) NMR spectrometer using a 5 mm pulsed-field gradient ^{1}H indirect-detection probe. The acquisition parameters were as follows: the 90° pulse width was 5.7 μs; the spectral window was 7000 Hz; the acquisition time was 2.2 s; and the delay between transients was 0.5 s. The reference compound tetramethylsilane (TMS) was used as the internal standard for the determination of chemical shifts.

All fluorescence spectra were recorded on a F-2000 spectrofluorimeter equipped with 1.0 cm quartz cells and a 150 W xenon lamp (Hitachi, Tokyo, Japan). An excitation wavelength of 280 nm was used. The excitation and emission slit width were both set at 2.5 nm. The weight measurements were performed on an AY-120 electronic analytical weighing scale with a resolution of 0.1 mg (Shimadzu, Japan). The pH value was measured in a pHS-3 digital pH meter (Shanghai, China).

Preparation of Solutions. Tris-HCl buffer solution (0.1 mol L^{-1} Tris, pH 7.4) containing 0.1 mol L^{-1} NaCl was prepared to keep the pH value and maintain the ionic strength of the solution. The working solution of BSA (1×10^{-4} moL L^{-1} or 3.0 mg mL^{-1}) was prepared by dissolving it in Tris-HCl buffer solution. The stock solutions of isolated active compounds (8×10^{-4} mol L^{-1}) were prepared by dissolving them in a small amount of ethanol and then diluting in Tris−HCl buffer solution (the ethanol content was about 2%), respectively.

About 6.0 g of the dried *E. ulmoides* leaves was extracted by deionized water at 95 °C for 3 times. All of the filtrates were combined and concentrated to dryness under reduced pressure by rotary evaporation at 60 °C to give syrup (592.4 mg). A total of 30.0 mg of residue was dissolved in 10.0 mL of deionized water and filtered through a 0.45 μm membrane. All of the solutions were stored in a refrigerator at 4 °C for further experiments.

Centrifugal Ultrafiltration−HPLC−DAD−MS/MS Screening and Identification. The aqueous extract of *E. ulmoides* leaves (3.0 mg mL^{-1}) was added with BSA solution (3.0 mg mL^{-1}) (1:1, v/v) and then reacted for 1.0 h at 25.0 °C for a fully balanced combination between the compounds in *E. ulmoides* leaves and BSA. The mixtures were then incubated at 37.0 °C in a water bath for 1.0 h. Then, the mixture was centrifuged at 8000 rpm for 10 min. The solution was filtered through a 0.45 μm membrane, and 20 μL solutions were injected for HPLC−DAD−MS/MS analysis. The mobile phase consisted of solvents A (0.4% acetic acid in water) and B (acetonitrile), which was programmed as follows: 0−10.0 min, 12.0% B; 10.0−16.0 min, 12.0−18.0% B; and 16.0−37.0 min, 18.0% B. The flow rate was 0.8 mL min^{-1}, while the temperature was set at 20 °C. Spectra were recorded from 200 to 400 nm (peak width of 0.2 min and data rate of 1.25 s^{-1}), while the chromatogram was acquired at 254 nm, and the mass spectrum was acquired in negative ionization mode.

Preparative Isolation and Identification of Target Active Compounds. Fresh *E. ulmoides* leaves were dried at 55 °C for 3 days before grinding. About 600 g of the powder was extracted by deionized water at 95 °C for 3 times. All of the filtrates were combined and concentrated to dryness under reduced pressure by rotary evaporation at 60 °C to give syrup (69.2 g). A mass of 65.0 g of syrup were then suspended in water and submitted to a liquid−liquid fraction using ethyl acetate and *n*-butanol with increasing polarities. This procedure produced ethyl acetate (3.74 g) and *n*-butanol (10.48 g) fractions. To enrich the targeted components, the *n*-butanol fraction was subsequently suspended in water and carried out by D101 column chromatography (20.0 × 100.0 cm, containing 3.0 kg of D101 macroporous resin). At first, water was used to elute the resin until the elution was nearly colorless, and then increasing concentrations of ethanol were used to give four main fractions: fractions A−D (fraction A, 10.0% ethanol elution, 0.69 g, which contained compound **1** and some impurities; fraction B, 20.0% ethanol elution, 0.23 g; fraction C, 30.0% elution, 2.41 g, which mainly contained compounds **2** and **3**; and fraction D, 40.0% elution, 2.08 g, which mainly contained compounds **4**−**6**). The fractions were all concentrated to dryness. Fraction A was redissolved in 10% ethanol aqueous solution and further enriched on an activated carbon column (3.5 × 70.0 cm, with the total volume of the column being about 500.0 mL). A 50.0% ethanol aqueous solution was first used to elute the impurities, and then an 80.0% ethanol aqueous solution was used to elute the target compound to give fraction A-2, 0.42 g, which mainly contained compound **1**. Fractions A-2, C, and D were further purified by preparative HPLC. In the preparative HPLC, a linear gradient elution of solvents A (0.4% acetic acid in water) and B (acetonitrile) was used according to the polarity of every fraction. Elution of fraction A-2 with 13.0% B for 20.0 min yields pure compound **1** (0.19 g). Elution of fraction C started with 14.0% B for 15.0 min, then from 14.0 to 20.0% for 20.0 min, and then 25.0% for 10.0 min, to yield pure compounds **2** (0.13 g) and **3** (0.74 g). Fraction D was submitted to isocratic conditions of 22.0% for 15.0 min and linear gradient from 22.0 to 25.0% B in 40 min, affording pure compounds **4** (0.76 g), **5** (0.11 g), and **6** (0.14 g). All of the fractions eluted were monitored by UV light at 254 nm. The collected fractions were evaporated to dryness in vacuum, while small portions of water were added occasionally to avoid acid glycoside hydrolysis, and the residues were lyophilized. The samples were redissolved in methanol for recrystallization. Six compounds were separated, and their chromatographic purities were determined by HPLC as higher than 98.5%. Characterization of the target isolated compounds was accomplished by their spectroscopic spectra, mass data, and NMR spectra.

Fluorescence Spectra of Active Compounds Binding with BSA. Appropriate amounts of 8.0×10^{-4} mol L^{-1} active compounds were added to 11 flasks (5.0 mL), and then 300.0 μL of BSA solution was added and diluted to 5.0 mL with Tris-HCl buffer. The final concentrations of the active compound in BSA solution were 0.0, 4.0, 8.0, 12.0, 16.0, 20.0, 24.0, 28.0, 32.0, 36.0, and 40.0 μmol L^{-1}. The resultant mixtures were then incubated at 298 K for 1.0 h. After 1.0 h of incubation, the fluorescence emission spectra were scanned in the range of 290−450 nm and the fluorescence intensity at 340 nm was measured. All of the experiments were repeated in triplicate and found to be reproducible with the experimental error (<1%).

Fluorescence Spectra of Geniposidic Acid Binding with BSA in the Presence of Other Active Compounds. A total of 300 μL of BSA solution and 37.5 μL of caffeic acid, chlorogenic acid, quercetin-3-*O*-sambubioside, rutin, or isoquercitrin were added to 11 flasks (5.0 mL). After reaction for 1.0 h, appropriate amounts of 8.0×10^{-4} mol L^{-1} geniposidic acid were added and then diluted to 5 mL with Tris-HCl buffer. The final concentrations of geniposidic acid were 0.0, 4.0, 8.0, 12.0, 16.0, 20.0, 24.0, 28.0, 32.0, 36.0, and 40.0 μmol L^{-1}, and the concentration of caffeic acid, chlorogenic acid, quercetin-3-*O*-sambubioside, rutin, or isoquercitrin was 6 μmol L^{-1}, which was the same as that for BSA. The resultant mixtures were then incubated at 298 K for 1.0 h. After 1.0 h of incubation, the fluorescence emission spectra were scanned in the range of 290−450 nm and the fluorescence intensity at 340 nm was measured. All of the experiments were repeated in triplicate and found to be reproducible with the experimental error (<1%).

Data Analysis. The binding constants (K) and binding sites (n) are calculated by the double-logarithm equation[37]

$$\log[(F_0 - F)/F] = \log K + n \log[Q] \quad (1)$$

where F_0 and F denote the steady-state fluorescence intensities of serum albumin without and with the existence of the quencher, respectively, and $[Q]$ is the concentration of the quencher with units of mol L^{-1}.

RESULTS AND DISCUSSION

Optimization of HPLC Analysis. The whole separation of all target compounds is a crucial and challenging task for HPLC analysis. To the best of our knowledge, the *E. ulmoides* leaves were rich in iridoids, phenylpropanoids, and flavonoids. Because acid is known to achieve better separation for compounds with hydroxyl groups by reducing the tailing of the peaks, acid should be added into the mobile phase.[38] In the course of optimizing the separation conditions, the system conditions, including the mobile phase system (acetonitrile−water and methanol−water with different concentrations of acetic acid), gradient program (gradient time, gradient shape, and initial composition of the mobile phase), column temperature, and detection wavelength (relatively higher absorption), were investigated. The final results showed that best resolution, shortest analysis time, and lowest pressure variations were achieved when a gradient elution mode composed of solvents A (0.4% acetic acid in water) and B (acetonitrile) was programmed as follows: 0−10.0 min, 12.0% B; 10.0−16.0 min, 12.0−18.0% B; and 16.0−37.0 min, 18.0% B. The flow rate was 0.8 mL min^{-1}; the column temperature was set at 20.0 °C; and 254 nm was selected as the detection wavelength. Under the optimum gradient elution, the compounds in the aqueous extract of *E. ulmoides* leaves reached almost baseline separation (Figure 1a).

Interaction of *E. ulmoides* Leaves Extract with BSA. The centrifugal ultrafiltration−HPLC−DAD−MS/MS method could be used for a rapid screening of active compounds binding with biomacromolecules from complex mixtures, particularly for natural products with a minimum of sample

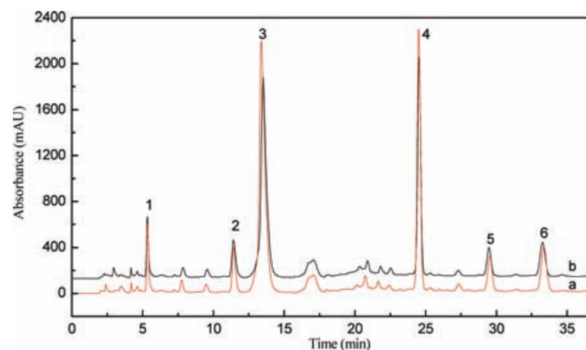

Figure 1. HPLC chromatograms of the aqueous extract of *E. ulmoides* leaves with BSA: (a) before the interaction with BSA and (b) after the interaction with BSA. HPLC conditions: column, reversed-phase SunFire C_{18} (250 × 4.6 mm inner diameter, 5 μm); mobile phase, consisting of solvents A (0.4% acetic acid in water) and B (acetonitrile), which was programmed as follows: 0−10.0 min, 12.0% B; 10.0−16.0 min, 12.0−18.0% B; and 16.0−37.0 min, 18.0% B; flow rate, 0.8 mL min^{-1}; UV wavelength, 254 nm; and column temperature, 20 °C.

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preparation.[39] The peak areas of active compounds will be reduced or disappear in the HPLC chromatogram because the active compound−biomacromolecule complex was retained during the ultrafiltration process, and for those without activities, the peak areas have almost no change because they freely passed through the filter membrane. The chromatogram of *E. ulmoides* leaves extraction after the interaction with BSA was shown in Figure 1b, which presented peak areas of six compounds (**1**−**6**) reduced obviously. Therefore, six compounds (**1**−**6**) in aqueous extraction of *E. ulmoides* leaves have the potent ability to bind with BSA. The retention times and UV and MS/MS data of the active compounds were presented in Table 1.

Table 1. Spectral Data of the Main Active Compounds in *E. ulmoides* Leaves

number	t_R (min)	UV (λ_{max}, nm)	proposal ions	molecular formula	structure assignment
1	5.35	238	$[M - H]^-$ = 373 $[M - Glc - H]^-$ = 211 $[M - Glc{-}CO_2 - H]^-$ = 211	$C_{16}H_{22}O_{10}$	geniposidic acid
2	11.43	243, 300 sh, 328	$[M - H]^-$ = 179 $[M - CO_2 - H]^-$ = 135	$C_9H_8O_4$	caffeic acid
3	13.51	242, 300 sh, 325	$[M - H]^-$ = 353 $[M -$ caffeic acid $- H]^-$ = 191	$C_{16}H_{18}O_9$	chlorogenic acid
4	24.51	254, 350	$[M - H]^-$ = 595 $[M - Glc{-}Xyl - H]^-$ = 301	$C_{26}H_{28}O_{16}$	quercetin-3-*O*-sambubioside
5	29.54	255, 353	$[M - H]^-$ = 609 $[M - Glc{-}Rha - H]^-$ = 301	$C_{27}H_{30}O_{16}$	rutin
6	33.36	255, 351	$[M - H]^-$ = 463 $[M - Glc - H]^-$ = 301	$C_{21}H_{20}O_{12}$	isoquercitrin

Structural Elucidation of the Active Compounds. Compound identification relied first on UV spectra and reasonable molecular formulas obtained from HPLC−DAD−MS/MS analysis and a comparison of these data to the metabolites previously reported from *E. ulmoides*.[6,7] Three types of target active compounds in *E. ulmoides* leaves could be elucidated by their UV spectra and from the biogenetic point of view. Compound **1** presented a UV spectrum characteristic for simple iridoid with a single absorption band at about 238 nm, which corresponded to an α,β-unsaturated system,[40] and the relatively shorter retention time suggested the presence of glycoside group. Compounds **2** and **3** had a similar type of UV spectrum with a maximum absorbance at about 245 and 325 nm with a broad absorption shoulder at about 300 nm, presumably corresponding to phenylpropanoids.[41] Compounds **4**−**6** had two maximum absorption UV bands at about 255 and 351 nm, which were the typical spectra of flavonoid derivatives.[36] All three types of compounds are known to exist in *E. ulmoides* and possess a variety of biological activities. Compounds **1**−**6** were then unambiguously characterized and identified as geniposidic acid (**1**), caffeic acid (**2**), chlorogenic acid (**3**), quercetin-3-*O*-sambubioside (**4**), rutin (**5**), and isoquercitrin (**6**) (Figure 2) by comparison of their ^{1}H NMR, ^{13}C NMR, and 2D NMR data to earlier published data.[36,42−44]

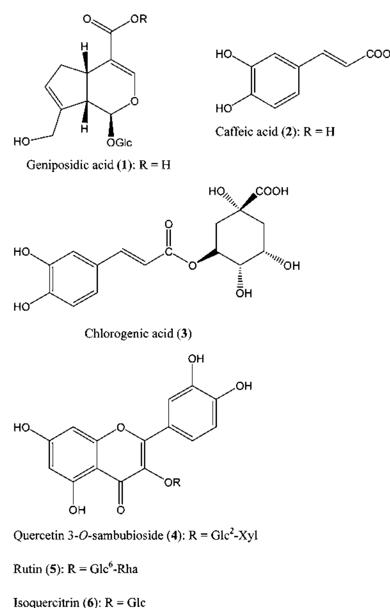

Figure 2. Chemical structures of active compounds isolated from *E. ulmoides* leaves.

Fluorescence Quenching of BSA by Active Compounds. BSA has three linearly arranged, structurally distinct, homologous domains (I−III), and each domain is composed of two sub-domains (A and B). The specific sites that bind with BSA are sites I and II, which are located in hydrophobic cavities in the IIA and IIIA sub-domains.[45] When the fluorescence emission spectra of BSA are measured with a series of concentrations of the quencher by fixing the excitation wavelength at 280 nm, the fluorescence emission peak of BSA at 340 nm gives the information of tryptophan residues.[46] Therefore, fluorescence quenching can be considered as a technique for measuring binding constants.

Figure 3 shows the representative fluorescence spectra of geniposidic acid, chlorogenic acid, and rutin (the fluorescence spectra for other active compounds were not shown here). The fluorescence intensities of BSA are almost the same with the addition of geniposidic acid and just fluctuated in the small range, which can be suggested that geniposidic acid hardly interacted with BSA. However, the fluorescence intensity of BSA attenuated gradually with the increasing concentration of chlorogenic acid or rutin, which indicated that the interaction had happened between chlorogenic acid or rutin and BSA. About 61.0 and 65.8% of the fluorescence intensities of BSA were quenched by adding 40 μmol L^{-1} chlorogenic acid and rutin, respectively (calculated from Figure 3). The results indicated that the quenching effect of active compounds on BSA fluorescence highly depended upon the structures. In addition, an obvious red shift of the maximum emission wavelength (λ_{em}, 10 nm) of BSA fluorescence occurred for chlorogenic acid; however, a slight blue shift of λ_{em} (5 nm) was observed for rutin. The emission of indole may be blue shifts if the group was buried within a native protein, and its emission may shift to longer wavelengths when the protein is unfolded.[23] Therefore, the shift results suggested that the environment of the trypophan and tyrosine residues was changed and the fact that chlorogenic acid and rutin were situated at close proximity to the tryptophan and tyrosine residues for the quenching to occur.

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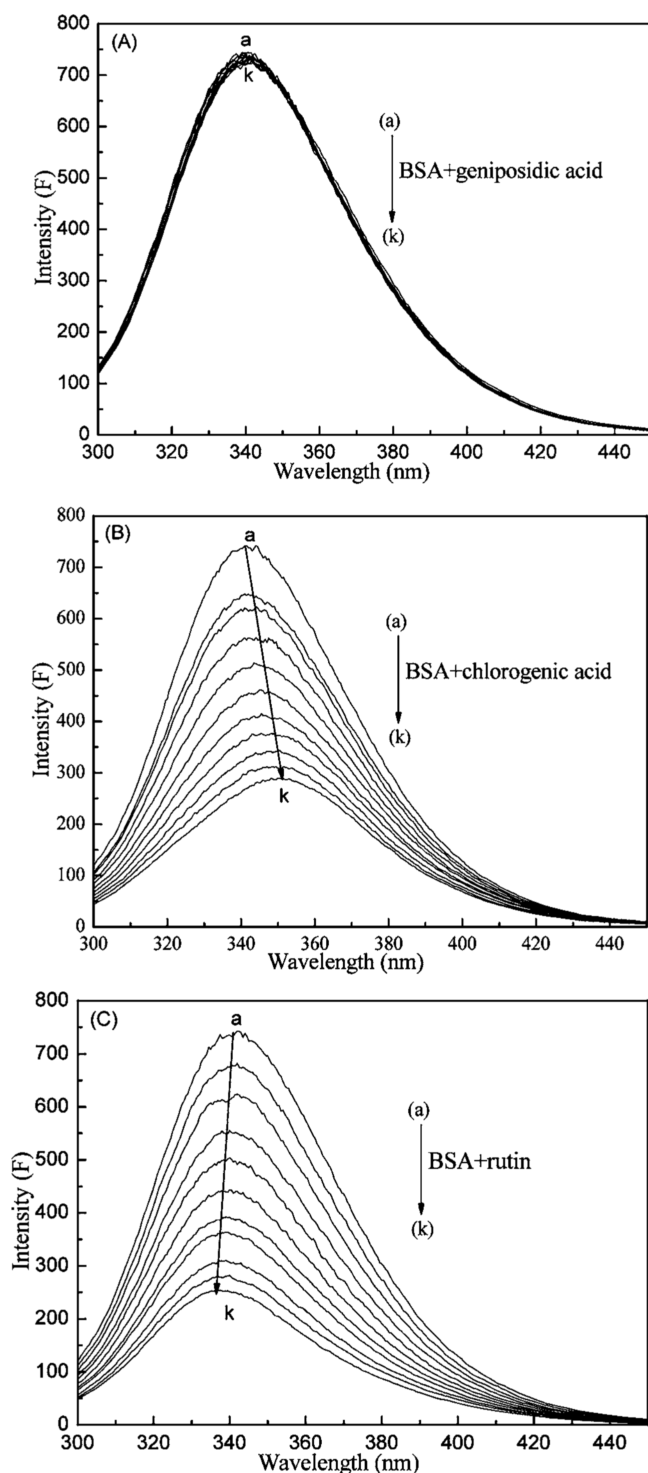

Figure 3. Fluorescence quenching spectra of BSA at various concentrations of geniposidic acid, chlorogenic acid, and rutin. Conditions: λ_{ex}, 280 nm; c_{BSA}, 6.0 μmoL L^{-1}; $c_{geniposidic\ acid} = c_{chlorogenic\ acid} = c_{rutin}$ (a → k), 0.0, 4.0, 8.0, 12.0, 16.0, 20.0, 24.0, 28.0, 32.0, 36.0, and 40.0 μmol L^{-1} for (A) geniposidic acid, (B) chlorogenic acid, and (C) rutin; and T, 298 K.

The binding constants (K) and binding sites (n) can be calculated by the double-logarithm equation (eq 1). Plots of $\log(F_0 - F)/F$ versus $\log[Q]$ for caffeic acid, chlorogenic acid, quercetin-3-O-sambubioside, rutin, and isoquercitrin to BSA are shown in Figure 4. It is clear that the double-logarithm curves for all of the active compounds were linear. The corresponding calculated K (n) between caffeic acid, chlorogenic acid, quercetin-3-O-sambubioside, rutin, or isoquercitrin and BSA were 3.06×10^5 (1.112), 5.04×10^5 (1.256), 1.27×10^5 (1.114), 1.59×10^6 (1.353), and 1.01×10^6 (1.285), respectively. The values of binding constants were in the range of 10^5–10^6 mol L^{-1}, which agreed with common BSA–ligand binding.[15−19]

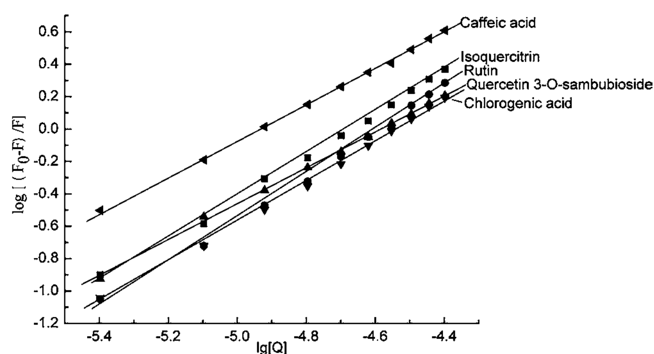

Figure 4. Double-logarithm curves of caffeic acid, chlorogenic acid, quercetin-3-O-sambubioside, rutin, and isoquercitrin quenching BSA fluorescence at 298 K.

The single geniposidic acid hardly interacted with BSA; however, geniposidic acid in the complex extract could react with BSA from the analysis of the results in the centrifugal ultrafiltration–HPLC experiment. How did the existence of phenylpropanoids or flavonoids enhance the binding affinity of geniposidic acid with BSA? It could be hypothesized that geniposidic acid interacted with the phenylpropanoid–BSA complex or flavonoid–BSA complex by hydrogen bonds, which enhanced its binding affinity.

Effect of Chlorogenic Acid on Geniposidic Acid Binding with BSA. The degree of ligand binding to serum albumin may have consequences for the rate of clearance of metabolites and for their delivery to cells and tissues, and the interactions of ligand–serum albumin depend upon surrounding circumstances. There are many kinds of compounds with different molecular weights and polarities existing in the natural product. Therefore, the binding process with BSA would influence each other, which then resulted in an altered binding degree, and then the suppressing, inductive, and synergistic effects were probably produced. The increased binding affinities of geniposidic acid may be consistent with two interpretations.[22−25] The first reason may be that a conformational change of BSA happened because of binding with different sites, and the second reason probably was a newly formed complex between geniposidic acid and other active compounds by hydrogen bonds that act as the new species to quench the fluorescence of BSA.

The fluorescence spectra of BSA with the addition of geniposidic acid in the presence of chlorogenic acid were shown in Figure 5 (the presence of other active compounds has a similar spectrum, which was not shown here). Surprisingly, when geniposidic acid was continuously added to the BSA solution (6 μmol L^{-1}) containing chlorogenic acid (6 μmol L^{-1}), the fluorescence of BSA increased with a slight blue shift of λ_{em} from 343 to 341 nm. The results indicated that the environment around BSA was changed after the addition of geniposidic acid. The increased fluorescence may be explained that geniposidic acid could interact with chlorogenic acid–BSA by hydrogen bonds to form the geniposidic acid–chlorogenic acid–BSA complex, which increased the binding affinity of

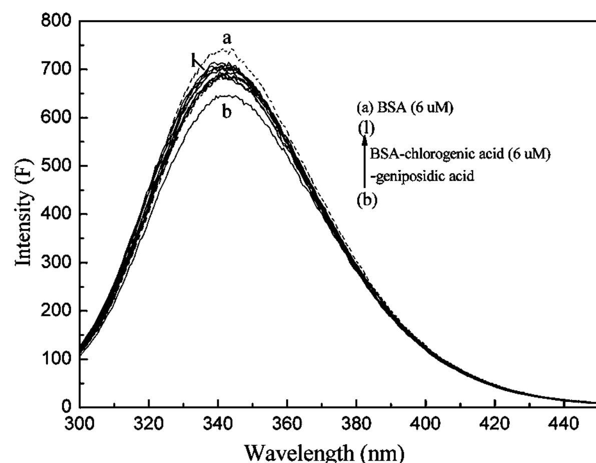

Figure 5. Fluorescence quenching spectrum of BSA at various concentrations of geniposidic acid in the presence of chlorogenic acid. Conditions: λ_{ex}, 280 nm; c_{BSA} = $c_{chlorogenic\ acid}$ = 6.0 μmoL L^{-1}; $c_{geniposidic\ acid}$ (b → j), 0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 μmoL L^{-1}; and *T*, 298 K.

geniposidic acid. However, the formation of the geniposidic acid−chlorogenic acid complex increased the molecular size, and then steric hindrance may take place, which could weaken the capacity to penetrate into BSA. Therefore, some chlorogenic acid was dissociated from BSA, which was well-consistent with a red shift of λ_{em} of fluorescence of BSA with the concentration of chlorogenic acid increasing, and then the binding affinity of chlorogenic acid was decreased.

The results above indicated that the binding affinities of compounds in natural complex mixtures could be changed because of the existence of the interaction and influence of each other, which may then affect the activity. Moreover, the centrifugal ultrafiltration−HPLC method was a useful approach in the fast screening of potential active compounds from natural products with high possibility.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +86-731-88879616. E-mail: shuyshi@gmail.com.

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Notes

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