



## ORIGINAL ARTICLE

# Investigation on the differences of four flavonoids with similar structure binding to human serum albumin



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**Abstract** Flavonoids are structurally diverse and the most ubiquitous groups of polyphenols distributed in the various plants, which possess intensive biological activities. In this study, the interaction mechanisms between four flavonoids containing one glucose unit with similar molecular weight isolated from the Tibetan medicinal herb *Pyrethrum tatsienense*, namely, apigenin-7-*O*- $\beta$ -D-glucoside(**1**), luteolin-7-*O*- $\beta$ -D-glucoside(**2**), quercetin-7-*O*- $\beta$ -D-glucoside(**3**), quercetin-3-*O*- $\beta$ -D-glycoside(**4**), and human serum albumin(HSA), were investigated by fluorescence, UV–vis absorbance, circular dichroism, and molecular modeling. The effects of biological metal ions  $Mg^{2+}$ ,  $Zn^{2+}$ , and  $Cu^{2+}$  on the binding affinity between flavonoids and HSA were further examined. Structure–activity relationships of four flavonoids binding to HSA were discussed in depth and some meaningful conclusions have been drawn by the experiment data and theoretical simulation. In addition, an interesting phenomenon was observed that the microenvironment of the binding site I in HSA has hardly changed in the presence of **4** differentiating from the other three flavonoids on the basis of conformation investigations.

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## 1. Introduction

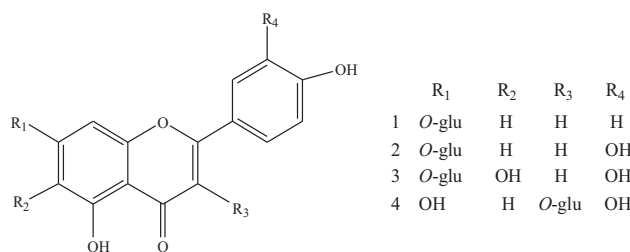
Human serum albumin (HSA), the most abundant protein in blood serum with a concentration of 0.63 mM, is the major soluble transport protein in the circulatory system for reversibly binding a large diverse of metabolites and organic compounds such as

unesterified fatty acids, hormones, and metal ions [1]. HSA not only plays a significant role in absorption, distribution, metabolism and excretion of drug, but also influences the drug toxicity and stability during the chemotherapeutic process in the circulatory systems [2]. It is well known that the functions of various drugs are strongly affected by the protein–drug interactions in blood plasma. The deep knowledge of the mechanism of the drug–HSA interaction is of great importance in understanding the process of drug transportation, the prediction of the free drug concentrations, and the clinical application. Consequently, the study on the binding of drug to HSA may provide some useful information of structure

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**Fig. 1** The chemical structures of the four flavonoids.

features that determine the therapeutic effect of drug, and has become an important research field in life sciences [3].

Flavonoids are widely distributed in plants and are also present in considerable amounts in *Pyrethrum tatsienense*, which could be regarded as the most representatively secondary metabolites of this Tibetan medicinal herb in China [4]. Research on flavonoids has witnessed a remarkable interest during the last decades, due to high efficacy and low toxicity of these compounds that make them viable alternatives to conventional medicines [5]. Among therapeutic properties of flavonoids, we mention antioxidant [6], anti-cancer, anti-AIDS, anti-inflammatory, anti-neoplastic, as well as neuro-protective activities [7,8]. It can be predicted that a huge number of biochemical signaling pathways and, therefore, physiological and pathological processes, can be affected by flavonoids [9]. Thus, the structure–activity relationship of flavonoids binding to HSA is especially important in further evaluating the transportation and distribution of flavonoids in blood.

Spectral methods are powerful tools for the study of drug binding to proteins since they allow noninvasive measurements of substances in low concentrations [3]. In this work, four flavonoids (Fig. 1), namely, apigenin-7-*O*- $\beta$ -D-glucoside (1), luteolin-7-*O*- $\beta$ -D-glucoside(2), quercetin-7-*O*- $\beta$ -D-glucoside(3), and quercetin-3-*O*- $\beta$ -D-glucoside(4), were isolated from *P. tatsienense* by our group [10]. The mechanism and characteristics of interaction between the above flavonoids (containing one glucose unit with similar molecular weight) and HSA were first investigated systematically by spectroscopic and molecular modeling methods. The effects of some metal ions, the number of phenolic hydroxyl and location of glycosylation in flavonoids on the binding affinity between the flavonoids and HSA were discussed in depth. We hope that this study will be helpful for realizing the transportation and distribution of flavonoids *in vivo* at the molecular level, which is also in some degree beneficial to the in-depth understanding of therapeutic effects of *P. tatsienense* herb.

## 2. Experimental

### 2.1. Materials and apparatus

HSA was obtained from Sigma Chemical Company(USA). Four flavonoids were isolated from *P. tatsienense* (Bur. et Franch.) Ling. Their purities were over 98% by normalization of the peak areas detected by HPLC-UV. Steady-state fluorescence measurements were carried out through a F2500 spectrophotometer (Hitachi, Japan). UV–vis and CD measurements were performed with a UV1000 UV–vis spectrophotometer (Techcomp, China) and Chirascan spectropolarimeter (Applied Photophysics Ltd., England), respectively.

### 2.2. Spectroscopic measurement

Four flavonoids were dissolved in ethanol to obtain  $1.20 \times 10^{-3}$  M stock solution, respectively. A Tris–HCl buffer (0.10 M, pH=7.4)

containing 0.10 M NaCl was selected to keep the pH value constant and to maintain the ionic strength of the solution. Fluorescence measurements were carried out keeping the concentration of HSA fixed at  $4.0 \times 10^{-7}$  M and that of drugs varied from 0 to  $2.88 \times 10^{-5}$  M. The excitation wavelength was 280 nm and the intrinsic fluorescence emission spectra of HSA were recorded at three different temperatures (25, 31 and 37 °C). Absorption spectra were recorded at 0.5 nm intervals keeping the concentration of HSA fixed at  $1.0 \times 10^{-5}$  M and that of drugs varied from 0 to  $4.0 \times 10^{-5}$  M. CD spectra were recorded at 0.5 nm intervals under constant nitrogen flush keeping the concentration of HSA fixed at  $2.00 \times 10^{-6}$  M and the mole ratio of the drugs to HSA varied from 0:1 to 20:1.

The fluorescence spectra of HSA were also recorded in the presence of some metal ions, which contained  $Zn^{2+}$ ,  $Mg^{2+}$ , and  $Cu^{2+}$  at 25 °C in the range of 280–600 nm at excitation wavelength of 280 nm. In the system, the overall concentrations of HSA and the metal ions were fixed at  $6.0 \times 10^{-4}$  M.

The crystal structure of HSA was taken from the Brookhaven Protein Data Bank (entry codes 1h9z). The potential 3D structure of HSA was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The initial structure of all the molecules was generated by software Sybyl-X1.1. Geometries of the ligands were optimized using the Tripos force field with Gasteiger–Hückel charges. Surfex program was applied to calculate the possible conformations of the ligand binding to protein in which the lattice water remained.

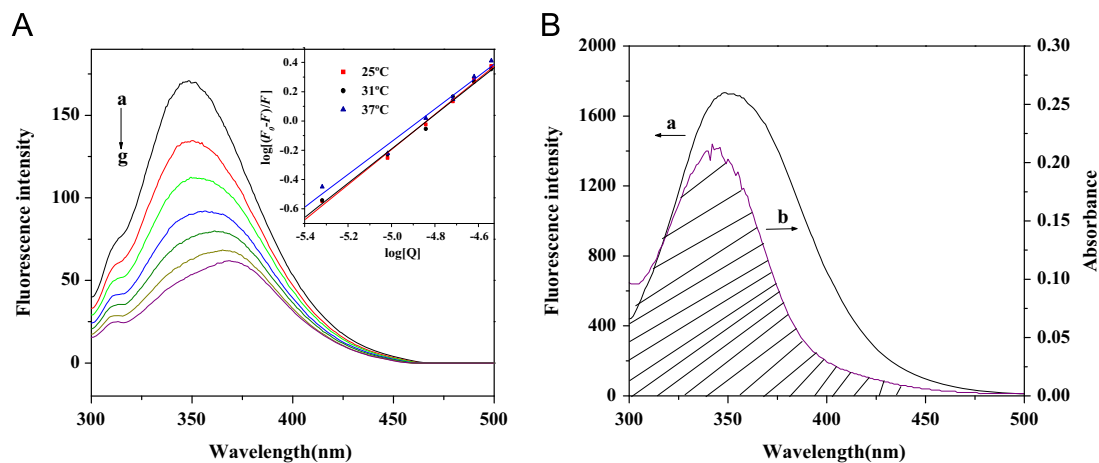
## 3. Results and discussion

### 3.1. Fluorescence quenching of HSA by the four flavonoids

As shown in Fig. 2A, upon addition of the flavonoids into HSA solution, the fluorescence intensity of HSA at around 347 nm regularly decreased and the fluorescence intensity decreased tardily in each titration curve, indicating that the flavonoids could interact with HSA and that HSA binding site was gradually saturated. Furthermore, the maximum wavelength of HSA shifted about from 347 to 366 nm after the addition of flavonoids 1, 2 and 3, but the shift of maximum emission wavelength of HSA after addition of flavonoid 4 was not observed. A red shift of the emission peak could be deduced that the Trp-214 residue of HSA was placed in a more hydrophilic environment [11], namely, the polarity of microenvironment around Trp-214 of HSA was increased after addition of flavonoids 1, 2 and 3.

In order to further confirm the possible quenching mechanism of the four flavonoids binding to HSA, the fluorescence quenching constants were usually analyzed by the Stern–Volmer equation [12] and the results are listed in Table 1. The values of  $K_q$  decreased with rising temperature, and were larger than the limiting diffusion constant  $K_{diff}$  ( $2.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ ) [13], which suggested that the possible quenching mechanism was a static quenching process accompanied with the formation of HSA–flavonoid complexes, while dynamic collision could be negligible.

Because the concentrations of flavonoids were far greater than those of HSA, the logarithm equation [14,15] used to calculate the binding constant  $K_a$  and the number of binding site  $n$  was reasonable for a static quenching process. The values of  $K_a$  and  $n$  were obtained from the intercept and slope of the plots (shown in the inset of Fig. 2A), and are listed in Table 1, respectively. At 25 °C,  $K_a$  was found as  $6.56 \times 10^5$ ,  $7.74 \times 10^5$ ,  $2.85 \times 10^6$ ,  $5.97 \times 10^5 \text{ M}^{-1}$ , and  $n$



**Fig. 2** (A) Effects of flavonoid (**1**) on the fluorescence spectra of HSA in Tris–HCl buffer of pH 7.40 at 25 °C. The inset shows Double-lg plots for flavonoid (**1**) at different temperatures. (B) Spectral overlap of HSA fluorescence (a) with the absorption spectra (b) of flavonoid (**1**).  $c(\text{HSA})=c(\text{flavonoid})=1.0 \times 10^{-5}$  M.

**Table 1** Binding parameters of HSA with the four flavonoids at different temperatures.

Flavonoid	$T$ (°C)	$K_{sv}$ ( $\times 10^5 \text{ M}^{-1}$ )	$K_q$ ( $\times 10^{13} \text{ M}^{-1} \cdot \text{s}^{-1}$ )	$n$	$K_a$ ( $\text{M}^{-1}$ )	$\Delta H$ (kJ/mol)	$\Delta S$ (J mol/K)	$\Delta G$ (kJ/mol)
<b>1</b>	25	0.8479	0.8479	1.20	$6.56 \times 10^5$	−65.06	−99.05	−33.18
	31	0.8477	0.8477	1.17	$4.64 \times 10^5$			−32.98
	37	0.8414	0.8414	1.12	$2.81 \times 10^5$			−32.34
<b>2</b>	25	0.8569	0.8569	1.22	$7.74 \times 10^5$	−36.75	−10.55	−33.59
	31	0.8003	0.8003	1.19	$5.83 \times 10^5$			−33.55
	37	0.7577	0.7577	1.17	$4.36 \times 10^5$			−32.17
<b>3</b>	25	1.3854	1.3854	1.29	$2.85 \times 10^6$	−54.46	−58.94	−36.82
	31	1.3492	1.3492	1.26	$2.00 \times 10^6$			−36.68
	37	1.3204	1.3204	1.24	$1.53 \times 10^6$			−36.11
<b>4</b>	25	0.8149	0.8149	1.20	$5.97 \times 10^5$	−52.11	−63.96	−32.95
	31	0.7775	0.7775	1.17	$4.50 \times 10^5$			−32.90
	37	0.7705	0.7705	1.12	$2.64 \times 10^5$			−32.17

was 1.20, 1.22, 1.29, 1.20 for **1**-HSA, **2**-HSA, **3**-HSA and **4**-HSA system, respectively. The values of  $K_a$  were proportional to the number of binding sites  $n$ , which confirmed the method used here was suitable to study the interaction between the four flavonoids and HSA.  $n$  was approximately equal to 1, which indicated that the four flavonoids could be stored and carried by HSA under physiological conditions via forming the mole ratio 1:1 complexes. The binding intensity between the four flavonoids and HSA was  $\mathbf{3} > \mathbf{2} > \mathbf{1} > \mathbf{4}$ , which suggested that the binding affinity was affected by the number of phenolic hydroxyls, and especially the link location of glucose in the flavonoids. Although flavonoids **3** and **4** had the same number of phenolic hydroxyls, the latter was more favorable for releasing from combinative state in blood serum than the former. These results showed the differences of the four flavonoids in the treatment of oxidative injury and related diseases. The facts of  $\Delta G < 0$ ,  $\Delta H < 0$  and  $\Delta S < 0$  indicated that binding of the four flavonoids to HSA was a spontaneous inter-molecular reaction. The negative  $\Delta H$  and  $\Delta S$  values were frequently taken as enthalpy driven while entropy was unfavorable for it, and the binding process belonged to the exothermic process. Therefore, hydrogen bond and Van der Waals forces played a major role in the binding process [16].

### 3.2. Energy transfer from HSA to drugs

According to Förster's non-radiative energy transfer theory (ERET) [17], if an acceptor can absorb the emitted fluorescence from a donor, energy may transfer from the donor to the acceptor. The overlapping between the HSA emission spectra and the flavonoid absorption spectra (Fig. 2B) means that the non-radiative energy transfer occurred obviously between the four flavonoids and HSA. Thus, the quenching mechanism in the flavonoid–HSA binary systems was a combination of static quenching and non-radiative energy transfer (Table 2). Using the FRET, the donor-to-acceptor distance  $r$  was 1.21, 1.26, 1.20 and 1.29 nm for the binding of flavonoids **1**, **2**, **3** and **4** with HSA, respectively, which was less than 8 nm, indicating that the energy transferring from HSA to the flavonoids occurred with high possibility [18]. The order of distance  $r$  between the flavonoids and Trp-214 residue of HSA was  $\mathbf{3} < \mathbf{2} < \mathbf{1} < \mathbf{4}$ , which was just the opposite to the order of binding constants  $K_a$ . These results showed that the donor-to-acceptor distance  $r$  was related to  $K_a$ . In other words, the larger the binding constants, the smaller  $r$  would be in the flavonoid–HSA system.

**Table 2** Energy transfer parameters of binding reaction between the flavonoids and HSA.

Flavonoid	$J(\times 10^{-10} \text{ cm}^3/\text{M})$	$E$	$R_0$ (nm)	$r$ (nm)
<b>1</b>	1.63	0.32	1.12	1.28
<b>2</b>	1.47	0.35	1.10	1.26
<b>3</b>	1.90	0.44	1.15	1.20
<b>4</b>	1.60	0.30	1.12	1.29

### 3.3. Molecular modeling studies

In addition to providing experimentally originated spectroscopic parameters, we also applied the molecular modeling methodology, which offers a molecular level explanation to estimate the participation of specific chemical groups and their interactions in flavonoid–HSA complex stabilization. Crystal structure of HSA shows that HSA is a heart-shaped globular protein consisting of a single polypeptide chain with 585 amino acid residues, which contains three repeating similar homologous domains numbered I (residues 1–195), II (196–383) and III (384–585). Each domain is composed of two sub-domains (A and B). The principal binding sites with different specificities are referred to site I and site II [19], and located in the hydrophobic cavities of sub-domains IIA (Trp-214 residue located in sub-domain IIA) and IIIA, respectively. According to the literature [20,21], site I (warfarin binding pocket) is regarded as the binding site of four flavonoids during the process of molecular modeling.

Fig. 3 shows the most possible interaction mode between the four flavonoids and HSA. The calculated binding Gibbs free energies  $\Delta G$  were  $-43.09$ ,  $-46.36$ ,  $-47.26$ , and  $-37.22$  kJ/mol for **1**-HSA, **2**-HSA, **3**-HSA, and **4**-HSA systems, respectively. According to the formula  $\Delta G = -RT \ln K_a$ , the order of  $K_a$  obtained from molecular modeling was consistent with fluorescence results. The interactions of the four flavonoids with binding site were dominated by hydrogen bonds observed from Fig. 3. There were three hydrogen bonds between **1** and Arg-222(1.80 Å), His-242(2.47 Å), Arg-257(1.87 Å) of HSA, seven hydrogen bonds between **2** and Lys-199(2.45 Å, 2.68 Å), Trp-214(2.27 Å), His-242(2.00 Å), Arg-257(2.42 Å), Ala-258(2.32 Å), Ala-291(2.62 Å) of HSA, eight hydrogen bonds between **3** and Lys-199(2.56 Å), Phe-211(1.86 Å), Arg-222(2.39 Å, 2.57 Å), His-242(1.96 Å), Arg-257(2.35 Å, 2.54 Å), Ala-291(1.91 Å) of HSA, and six hydrogen bonds between **4** and Lys-199(2.18 Å), Arg-222(1.79 Å, 2.01 Å, 2.72 Å), His-242(1.82 Å), Ala-291(2.11 Å) of HSA. These data indicate that the greater the number of hydrogen bonds is, the larger the binding constants  $K_a$  are for flavonoids **1**, **2** and **3**. These results well agree with those of fluorescence studies described above. It is demonstrated that the formation of hydrogen bond decreases the hydrophilicity and increases the hydrophobicity to stabilize the flavonoid–HSA system. Although flavonoids **3** and **4** have the same number of phenolic hydroxyls,  $K_a$  of **4** is the smallest. The reason may be that the link location of glucose is at the 7 position of A ring in **4** differentiating from the other three flavonoids.

On the other hand, docking of flavonoids **1**, **2** and **3** with HSA creates a hydrophobic environment near Trp-214 shown in Fig. 3A–C, which provides a good structural basis to explain the efficient fluorescence quenching of HSA in the presence of these three flavonoids. And the B ring of these three flavonoids has

formed effective  $\pi$ - $\pi$  stacking with indole ring of Trp-214 resulting in the decrease of electron density of indole ring, which reasonably explains the blue shift of the maximum peaks in UV and CD spectra (vide infra) of HSA after addition of flavonoids **1**, **2** and **3**. It is important to note that the Trp-214 residue of HSA is far from **4** shown in Fig. 3D, which results in poor fluorescence quenching of HSA emission in the presence of **4**. Thus, the acting forces are mainly hydrogen bond and Van der Waals forces between the four flavonoids and HSA, which are in accordance with the results of fluorescence experiment.

### 3.4. Conformation investigations

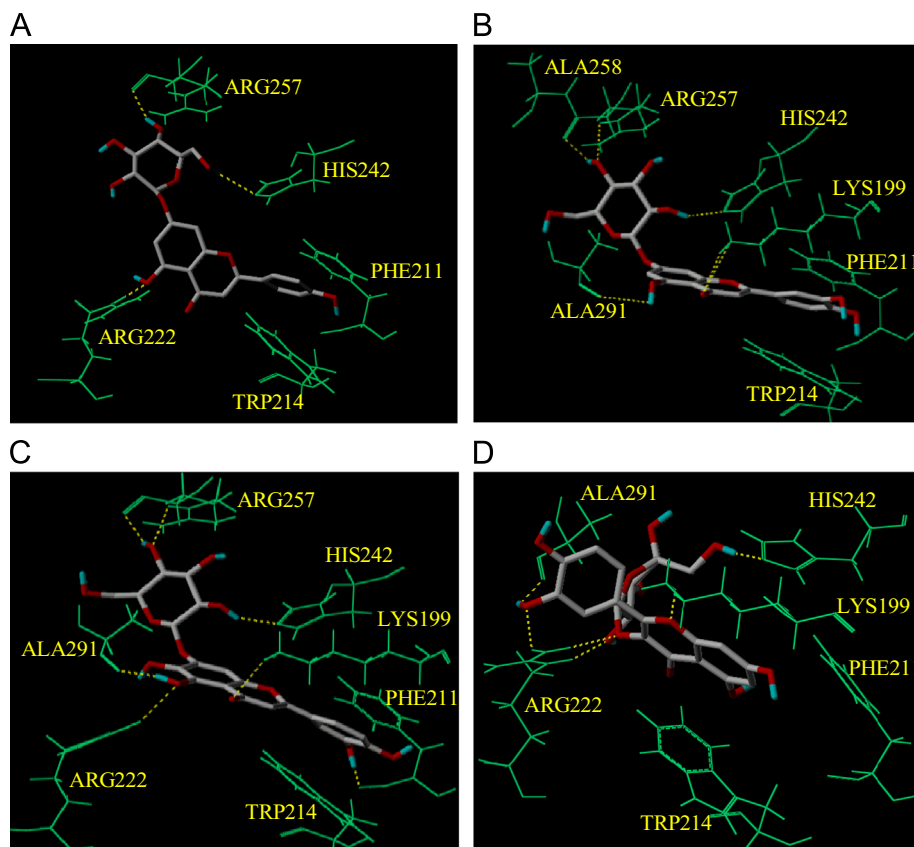
To further estimate whether any conformational changes of HSA molecules occurred in the binding reaction, UV–vis and CD spectra of HSA were measured. UV–vis spectra (Fig. 4A) show that the absorption peaks of HSA at 279 nm had an obvious blue shift toward short wavelength with the increasing addition of flavonoids **1**, **2**, and **3**, while the maximum absorption peak of **4** had little shift. The reason may be that the conjugation around the chromophore Trp-214 of HSA decreases due to the specific interaction between flavonoid **1** (**2**, **3**) and HSA. But the binding of **4** with HSA had little effect on the secondary structure of HSA, which was in accordance with the results of fluorescence and CD(vide infra). In addition, the absorption peak of flavonoids **1**, **2** and **3** had almost no longer shifted toward short wavelengths when the mole ratio of the flavonoid to HSA reached about 20, which suggests at this point the concentrations of the flavonoid around HSA are basically saturated.

CD spectra (Fig. 4B) exhibit that the bands intensity of HSA at 208 nm decreased in the presence of flavonoid **1**, indicating that the  $\alpha$ -helix contents of HSA decreased. The  $\alpha$ -helix contents of HSA were calculated to be 69.50% in free HSA, 66.76% for **1**, 66.74% for **2**, 64.47% for **3**, and 66.95% for **4** in bound form at mole ratio of the flavonoid to HSA 20:1 based on the literature [1]. The decreasing degree of  $\alpha$ -helix contents was  $3 > 2 > 1 > 4$ , which was in accordance with the binding constants  $K_a$ . Moreover, the band intensity at 208 nm in the system of HSA and **1** (**2**, **3**) had a weak red shift toward long wavelength, suggesting the emergence of a partial  $\beta$ -turn in **1** (**2**, **3**)–HSA systems except in **4**-HSA system [22], which was consistent with the fluorescence results. A small decrease of  $\alpha$ -helix percentage and no significant shift of the peaks indicated that the three-dimensional structure of HSA had a little change and HSA was also predominantly  $\alpha$ -helix after addition of the flavonoids [23], which displayed slight unfolding of the constitutive polypeptides of HSA and increased the exposure of some hydrophobic regions previously buried [24].

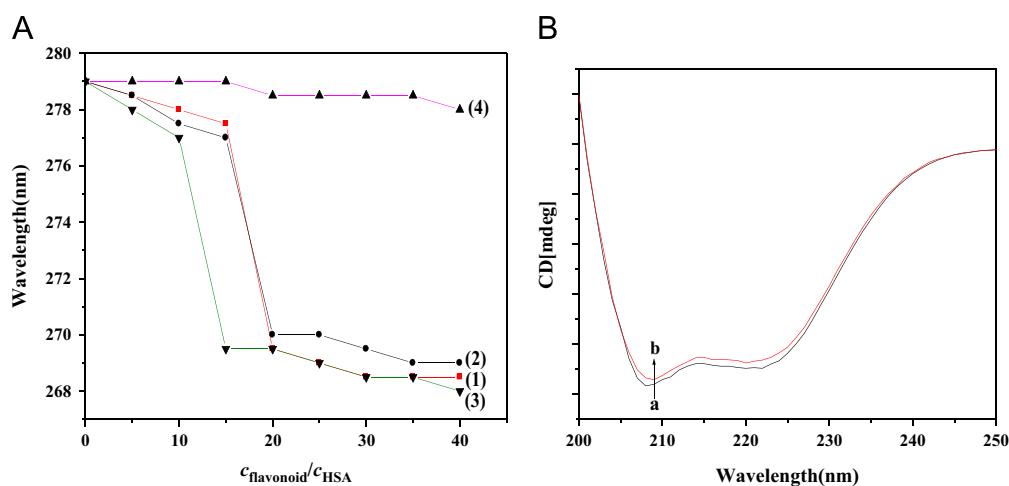
### 3.5. Effect of co-ions on the interaction of the four flavonoids with HSA

In plasma, there are some essential metal ions, which may affect the binding properties of drugs and serum albumins, because many drugs are good coordination groups of metal ions that can bind to protein. In this study, the effects of some biological metal ions  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Cu}^{2+}$  on the interaction between the flavonoid and HSA were investigated at 25 °C (Table 3).

$K_a'$  of **2** increased while  $K_a'$  of **3** decreased when  $\text{Cu}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Zn}^{2+}$  coexisted in HSA solution. The reasons may be that **3** with two pairs of two adjacent hydroxyls in A and B ring can form the stable chain complexes with  $\text{Cu}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Zn}^{2+}$ , which cannot effectively bind to HSA due to the large volume of



**Fig. 3** Interaction mode between HSA and the flavonoids (1) (A), (2) (B), (3) (C), and (4) (D), respectively. The hydrogen bonds between the four flavonoids and HSA are represented using yellow dashed line. The structures of the four flavonoids are represented using stick model, and the key residues of HSA are represented using line model.



**Fig. 4** (A) The relationship of absorbance spectra maximum wavelength about 279 nm and the mole ratio between the four flavonoids and BSA.  $c(\text{HSA})=1.0 \times 10^{-5}$  M,  $c(\text{flavonoids})=0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 (\times 10^{-5})$  M, respectively. (B) Circular dichroism spectra of flavonoid (1)–HSA system at in Tris–HCl buffer of pH 7.40. Concentration ratios of flavonoid to HSA are 0:1 and 20:1 (from curve a to b).

complexes. It was speculated that the metal ions might play an “ion bridging” role among two adjacent phenolic hydroxyls [25]. But **2** with a pair of two adjacent phenolic hydroxyls in B ring can form the stable complexes (mole ratio of metal ion to flavonoid is 1:2) with  $\text{Cu}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Zn}^{2+}$ , which can bind to the hydrophobic

site of HSA due to the hydrophobicity of complexes [26]. Although **4** has a pair of two adjacent hydroxyls, the link location of glucose is at the 3 position of C ring, which is unfavorable to the effective formation of complexes because of the steric effect of glucose. In a word, the presence of biological metal ions may

**Table 3** Binding constants and sites of HSA with the flavonoids and metal ions.

Flavonoid	Metal ions	<i>n</i>	$K_a'(\times 10^5 \text{ M}^{-1})$	$K_a'/K_a$
1	Cu <sup>2+</sup>	1.07	4.33	0.66
	Zn <sup>2+</sup>	1.03	1.98	0.29
	Mg <sup>2+</sup>	1.18	13.20	2.01
2	Cu <sup>2+</sup>	1.18	11.20	1.45
	Zn <sup>2+</sup>	1.16	9.83	1.27
	Mg <sup>2+</sup>	1.31	50.90	6.57
3	Cu <sup>2+</sup>	1.10	4.05	0.14
	Zn <sup>2+</sup>	1.08	4.58	0.16
	Mg <sup>2+</sup>	1.16	1.54	0.54
4	Cu <sup>2+</sup>	1.08	4.69	0.79
	Zn <sup>2+</sup>	1.21	19.20	3.22
	Mg <sup>2+</sup>	1.09	5.90	0.99

shorten or prolong the storage time of flavonoids in blood plasma and enhance or weaken its maximum effects, and modulate flavonoid delivery to cells *in vitro* and *in vivo* [27].

#### 4. Conclusions

In this work, the interaction of four flavonoids isolated from the Tibetan medicinal herb *P. tatsienense* with HSA has been investigated by several spectroscopic techniques and molecular modeling. The fluorescence results showed that the intrinsic fluorescence of HSA was quenched through static quenching and non-radiative energy transfer. The binding affinity of flavonoids to HSA was  $3 > 2 > 1 > 4$ , which was in agreement with the result of molecular modeling. The results of CD and UV-vis spectra showed that the formation of flavonoid-HSA complexes induced changes in different degrees in the protein secondary structure.

The above studies have shown that the following relationship may exist between the structure and binding interaction of the four flavonoids with HSA: (1) the increase of phenolic hydroxyls among flavonoids is favorable for the drug binding to HSA, (2) the link location of glucose in flavonoids takes a more effect on the binding affinity than the number of phenolic hydroxyls, (3) the location of glucose in flavonoids has a significant effect on the microenvironment of binding site in HSA, and (4) flavonoid with two pairs of two adjacent phenolic hydroxyls in A and B ring cannot effectively bind to HSA when some metal ions coexist in HSA due to the formation of chain complexes. This work provides important structure-activity data for flavonoids binding to HSA, which serves as valuable clues for understanding the pharmacodynamic response and therapeutic effects of flavonoids and Tibetan medicinal herb *P. tatsienense*.

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