

**Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> AND Mn<sup>2+</sup> REDUCE THE AFFINITIES OF FLAVONE, GENISTEIN AND KAEMPFEROL FOR HUMAN SERUM ALBUMIN *IN VITRO***

FAN YANG\*, JIE WANG, CHUNXI LIU, XIAOCHEN XU, WENJUN LI,  
ZHIHUA XIA and JIANBO XIAO

*College of Life and Environment Science, Shanghai Normal University, Shanghai 200234, PR China*

**Abstract** - Flavone (Fl), genistein (Gen) and kaempferol (Kol) were studied for their affinities towards human serum albumin (HSA) in the presence and absence of Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup>. The fluorescence intensities of HSA decreased with increasing concentration of the three flavonoids. Kaempferol resulted in a blue-shift of the  $\lambda_{em}$  of HSA from 336 to 330 nm; flavone showed an obvious red-shift of the  $\lambda_{em}$  of HSA from 336 to 342 nm; genistein did not cause an obvious blue-shift or red-shift of the  $\lambda_{em}$  of HSA. However, the extents of  $\lambda_{em}$ -shifts induced by the flavonoids in the presence of metal ions were much bigger than that in the absence of metal ions. Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> reduced the quenching constants of the flavonoids for HSA by 14.6% to 60.7% , 28% to 67.9%, 3.5% to 59.4%, 23.2% to 63.7% and 14% to 65%, respectively. The affinities of flavone, genistein and kaempferol for HSA decreased about 10.84%, 10.05% and 3.56% in the presence of Pb<sup>2+</sup>, respectively. Cu<sup>2+</sup> decreased the affinities of flavone, genistein and kaempferol for HSA about 14.04%, 5.14% and 8.89%, respectively. Zn<sup>2+</sup> decreased the affinities of flavone, genistein and kaempferol for HSA about 3.79%, 0.55% and 3.58%, respectively. Mg<sup>2+</sup> decreased the affinities of flavone, genistein and kaempferol for HSA about 16.94%, 2.94% and 7.04%, respectively. Mn<sup>2+</sup> decreased the affinities of flavone, genistein and kaempferol for HSA about 14.24%, 3.66% and 4.78%, respectively.

**Key words:** flavone, genistein, kaempferol, human serum albumin, Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>

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## INTRODUCTION

Flavonoids are the most important polyphenols in plants such as fruits, vegetables, nuts, and tea (Ghosh et al., 2009; Sepehr et al., 2009). Investigation of flavonoids from dietary sources has attracted great interest for their nutritional and medicinal effects on humans. Most of their biological activities are likely related to the antioxidant abilities of flavonoids (Krafczyk et al., 2010; Perez-Fons et al., 2010). The structural differences between flavones significantly affects their absorption, metabolism, and bioactivities (Wen, 2006; Wolfe and Liu, 2008). Flavonoids have wide clinical uses.

Human serum albumin (HSA), the most abundant protein in the serum, is the most important drug carrier protein. HSA aids in the transport, distribution, and metabolism of many endogenous and exogenous ligands, including fatty acids, amino acids, metals ions, and numerous pharmaceuticals, and contributes significantly to the colloid osmotic pressure of blood (Carter and Ho, 1994; He and Carter, 1992; Liu et al., 2010).

Human serum albumin (HSA), the most prominent protein in the plasma, binds different classes of ligands at multiple sites. HSA provides a depot for many compounds, affects pharmacokinetics

of many drugs. The studies on the interaction between plasma proteins (serum albumin) and small molecule-drugs have been an interesting field of research in life science, chemistry and clinical medicine. The affinities of drugs for proteins affect the concentrations of free drug and consequently contribute towards the intensity of their biological actions in vivo (Yazhou et al., 2007). Furthermore, there are also many metal ions in blood plasma. They participate in many biochemical processes. Some plasma proteins usually act as sequestration agents of metal ions (Kraghshansen, 1981) and have a variety of metal sites with different specificities. The phenomenon of HSA molecular conformational alterations caused by metal ion-HSA binding has been observed. The binding of metal ions such as  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Co}^{2+}$  to serum albumins has been widely reported (Laussac and Sarkar, 1984; Liang and Shen, 1994; Liang et al., 1998; Liang et al., 2001; Sadler and Viles, 1996; Shen et al., 2000; Xiao et al., 2009). Interactions between bovine serum albumin (BSA) and HSA and daunorubicin (Zhou et al., 1992), 5-fluorouracil (Zhou et al., 1994) in the presence of metal ions has also been reported.

Many metal ions can form complexes with drug molecules, thereby affecting some of the characters of the drug. It is reasonable to expect that metal ions affect the interaction of clinical drugs with HAS in a ternary system of drug-protein-metal ion, and thus the distribution, pharmacological properties and metabolism of the drug. Therefore, it is necessary to investigate the interactions of proteins and drugs in the presence of metal ions. The study of binding phenomena will be important in providing basic information on the pharmacological actions, biotransformation, and bio-distribution of drugs.

In this paper, we discovered that certain metal ions lowered the affinities of flavonoid-protein compounds. Flavone, genistein and kaempferol were studied for their affinities for HSA in the presence and absence of  $\text{Pb}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ .

## MATERIALS AND METHODS

### *Reagents*

Flavone, genistein and kaempferol (99.0%) were obtained from Tongtian Co. (Shanghai, China) and used without further purification. Working solutions of flavonoids ( $1.0 \times 10^{-3}$  mol/L) were prepared by dissolving the flavonoids in methanol. HSA was purchased from Sigma Co. (MO, USA). The working solution of HSA ( $1.0 \times 10^{-5}$  mol/L) was prepared in PBS buffer and stored in the refrigerator prior to use. The working solutions of  $\text{Pb}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  ( $1.0 \times 10^{-2}$  mol/L) were prepared by dissolving  $\text{Pb}(\text{CH}_3\text{COO})_2$ ,  $\text{CuSO}_4$ ,  $\text{Zn}(\text{CH}_3\text{COO})_2$ ,  $\text{MgSO}_4$  and  $\text{MnSO}_4$  in double-distilled water. All other reagents and solvents were of analytical reagent grade and were used without further purification unless otherwise noted. All aqueous solutions were prepared in fresh double-distilled water.

### *Apparatus*

Fluorescence experiments were performed on a Varian Cray/E spectrofluorophotometer (Palo Alto, USA). UV-vis absorption spectra were carried out on a Varian Cary 50 UV-vis spectrometer (Palo Alto, USA).

### *Fluorescence spectra*

The fluorescence spectra were recorded in the wavelength range 310–450 nm after excitation at 280 nm when the HSA samples were titrated with the metal ions or flavonoids. The slit widths, scan speed, and excitation voltage were kept constant in each data set. Quartz cells (1 cm path length) were used for all measurements. Titrations were performed manually by using trace syringes. In each titration, the fluorescence spectrum was collected with  $1.0 \times 10^{-5}$  mol/L of HSA. The experiments were repeated and found to be reproducible within experimental errors.

### Data process

Fluorescence quenching was described by the Stern-Volmer equation:

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

where  $F_0$  and  $F$  represent the fluorescence intensities of fluorophore in the absence and in the presence of quencher,  $K_q$  is the quenching rate constant of the bimolecule,  $K_{SV}$  is the dynamic quenching constant,  $\tau_0$  is the average lifetime of the fluorophore without quencher, and  $[Q]$  is the concentration of quencher.

In many instances, the fluorophore can be quenched both by collision and by complex formation with the same quencher. In this case, the Stern-Volmer plot exhibits an upward curvature which is concave towards the y-axis at high  $[Q]$ ;  $F_0/F$  is related to  $[Q]$  (Xiao et al., 2008a; Xiao et al., 2008b, Xiao et al., 2009):

$$F_0/F = (1 + K_D [Q]) (1 + K_S [Q]) \quad (2)$$

where  $K_D$  and  $K_S$  are the dynamic and static quenching constants, respectively.

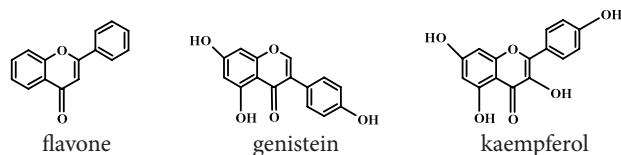
The binding constants were calculated according to the double-logarithmic equation (Bi et al., 2004; Jiang et al., 2003; Lu et al., 2001; Zhou et al., 1994):

$$\log_{10}(F_0 - F)/F = \log_{10} K_a + n \log_{10} [Q] \quad (3)$$

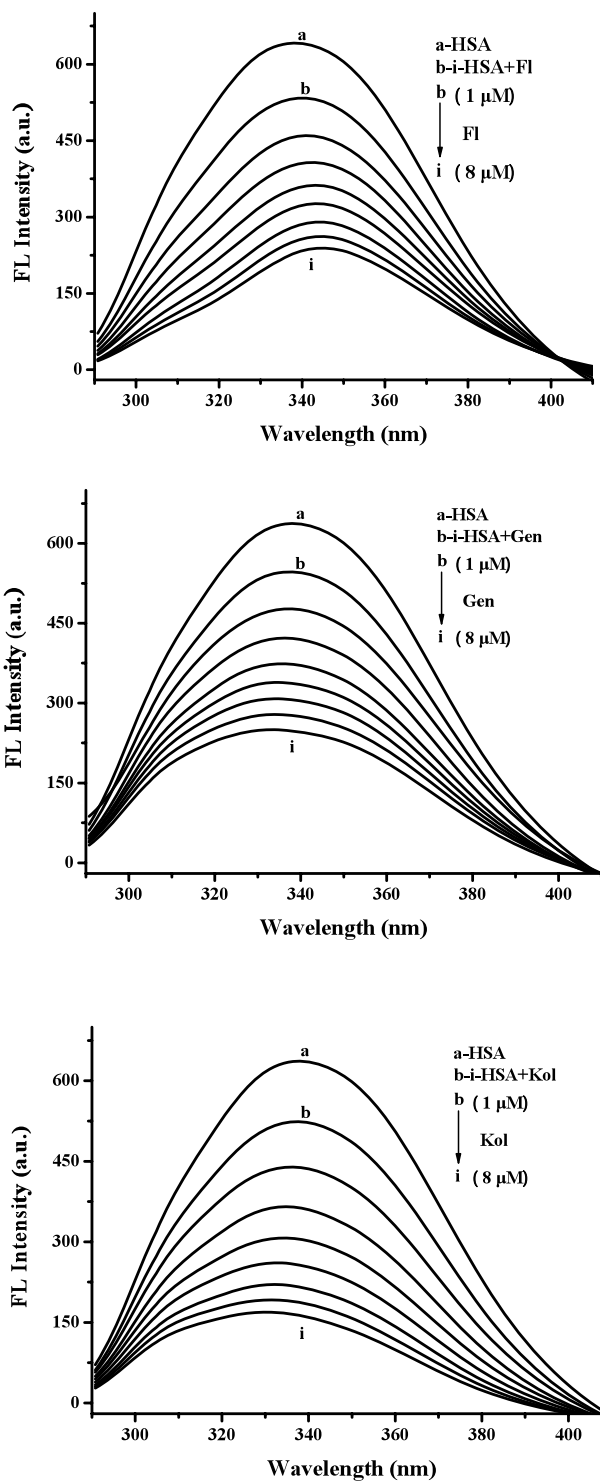
where  $K_a$  is the binding constant and  $n$  is the number of binding sites per HSA.

## RESULTS AND DISCUSSION

### Quenching of HSA fluorescence by flavonoids



**Fig. 1.** Structures of flavone (Fl), genistein (Gen) and kaempferol (Kol).



**Fig. 2.** The quenching effects of flavone (Fl), genistein (Gen) and kaempferol (Kol) on the intensity of HSA fluorescence.  $\lambda_{ex}=280$  nm; HSA,  $1.00 \times 10^{-5}$  mol/L; a-i: 0.00, 1.00, 2.00, 8.00 ( $10^{-6}$  mol/L) of flavonoids.

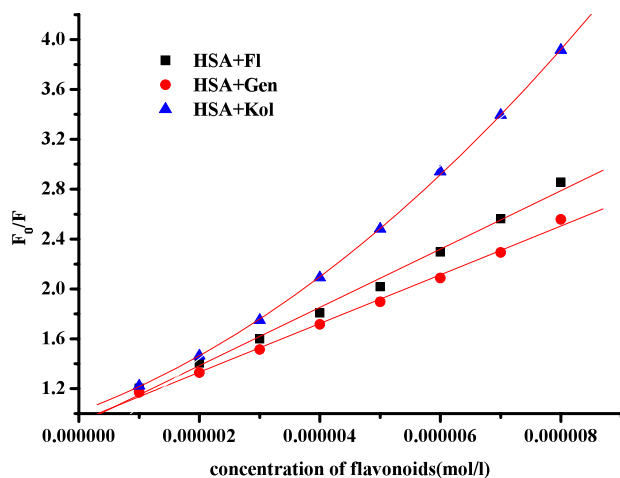


Fig. 3. The Stern-Volmer plots for HSA fluorescence quenching by flavone (Fl), genistein (Gen) and kaempferol (Kol).

Upon addition of flavone, genistein and kaempferol to the HSA solution, the fluorescence quenching of HSA is observed (Fig. 2). The fluorescence intensity attenuated gradually with increasing concentration of these three flavonoids. Approximately 64.99%, 60.7%, 74.50% of fluorescence intensities were quenched when the concentration of the flavonoids reached to 8.0  $\mu\text{mol/L}$  (Fig. 2). The extent of the fluorescence attenuation is in the order: genistein < flavone < kaempferol. In addition, kaempferol resulted in a blue-shift of the  $\lambda_{\text{em}}$  of HSA from 336 to 330 nm; flavone showed an obvious red-shift of the  $\lambda_{\text{em}}$  of HSA from 336 to 342 nm; genistein did not cause an obvious blue-shift or red-shift of the  $\lambda_{\text{em}}$  of HSA. The obvious shift of the maximum  $\lambda_{\text{em}}$  of HSA is indicative of changes in the immediate environment of Trp residues (typically, the polarity of Trp residues and the hydrophobicity of the hydrophobic cavity of has). The hydrophobic groups are in the interior of the tertiary structure and the polar groups are on the surface of native proteins. The emission of HSA may be blue-shifted if the indole group of Trp is buried within the native protein, and its emission may be red-shifted when the protein is unfolded. The result suggests that a greater change in the immediate environment of the Trp residues occurred and that flavonoid was in close proximity to or on the surface of the Trp residues. The buried indole group of Trp could be redeployed in a more hydrophobic envi-

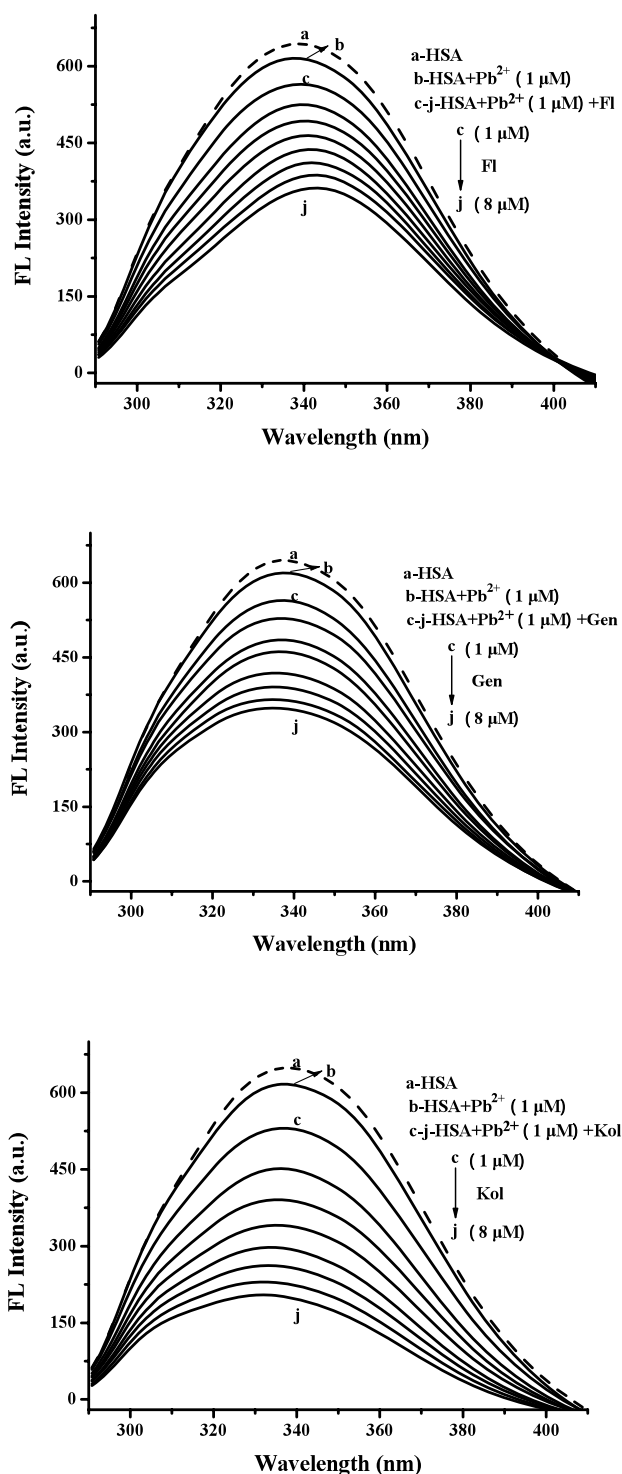
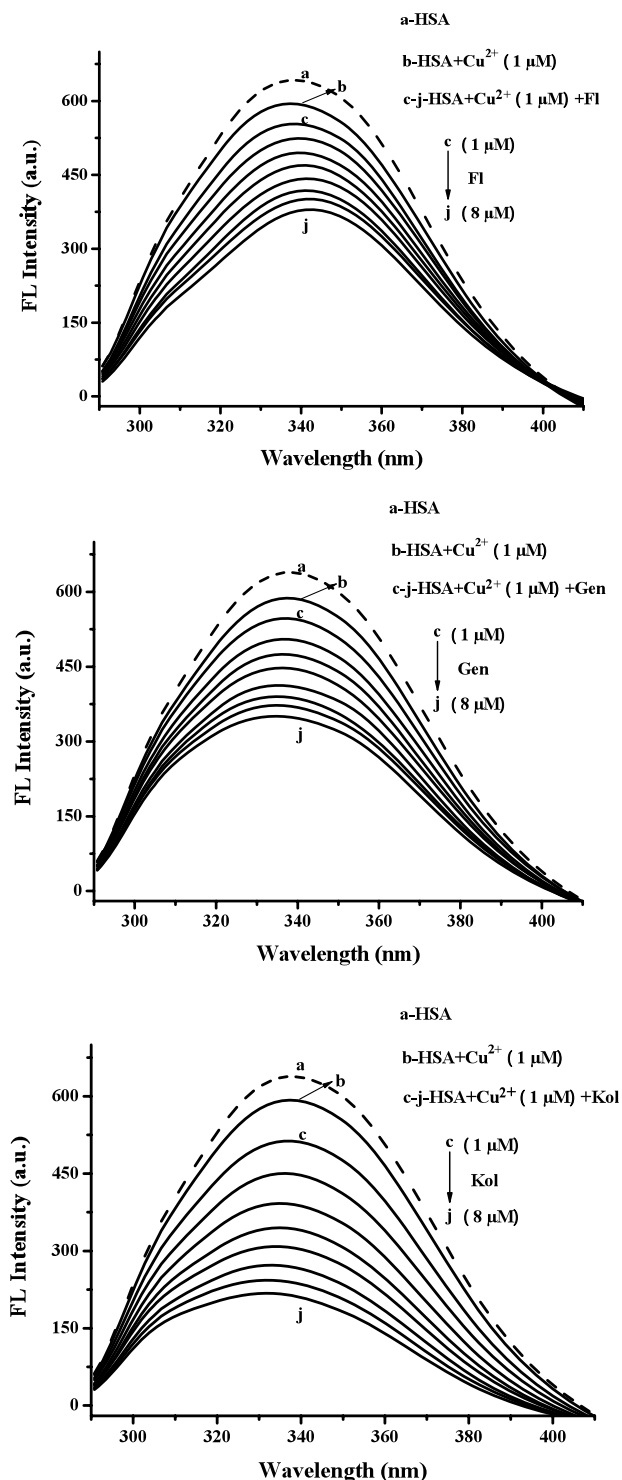
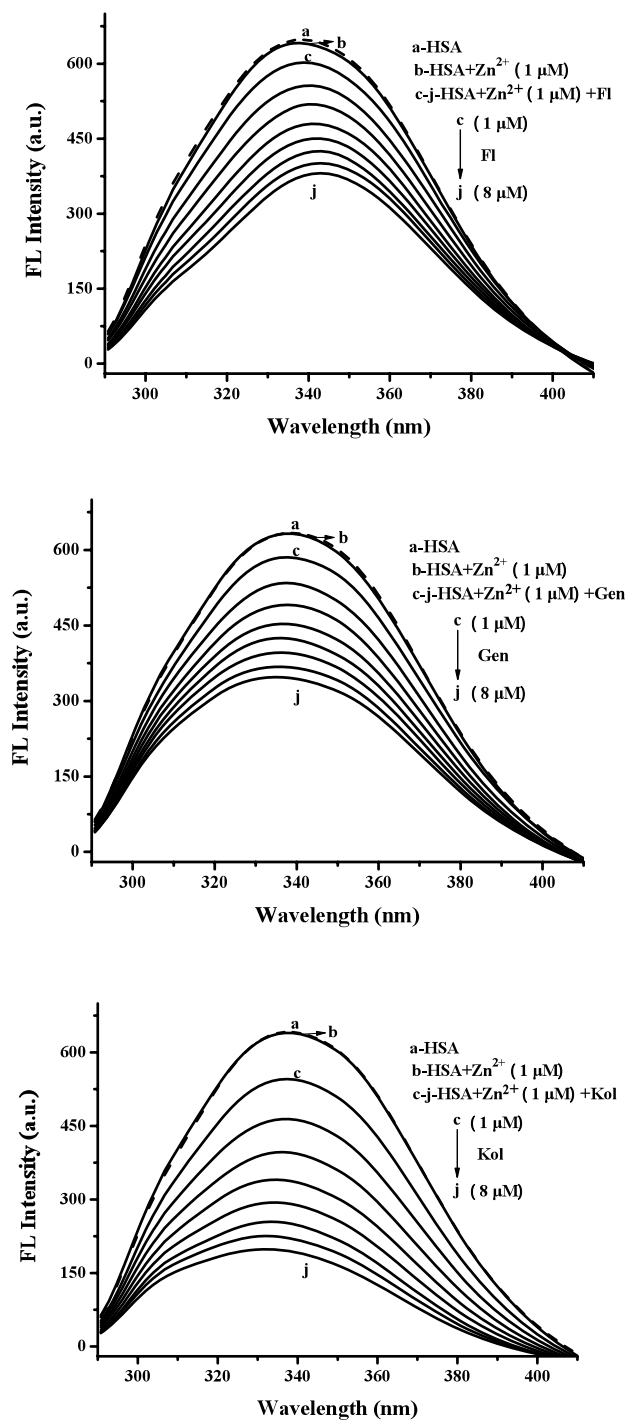


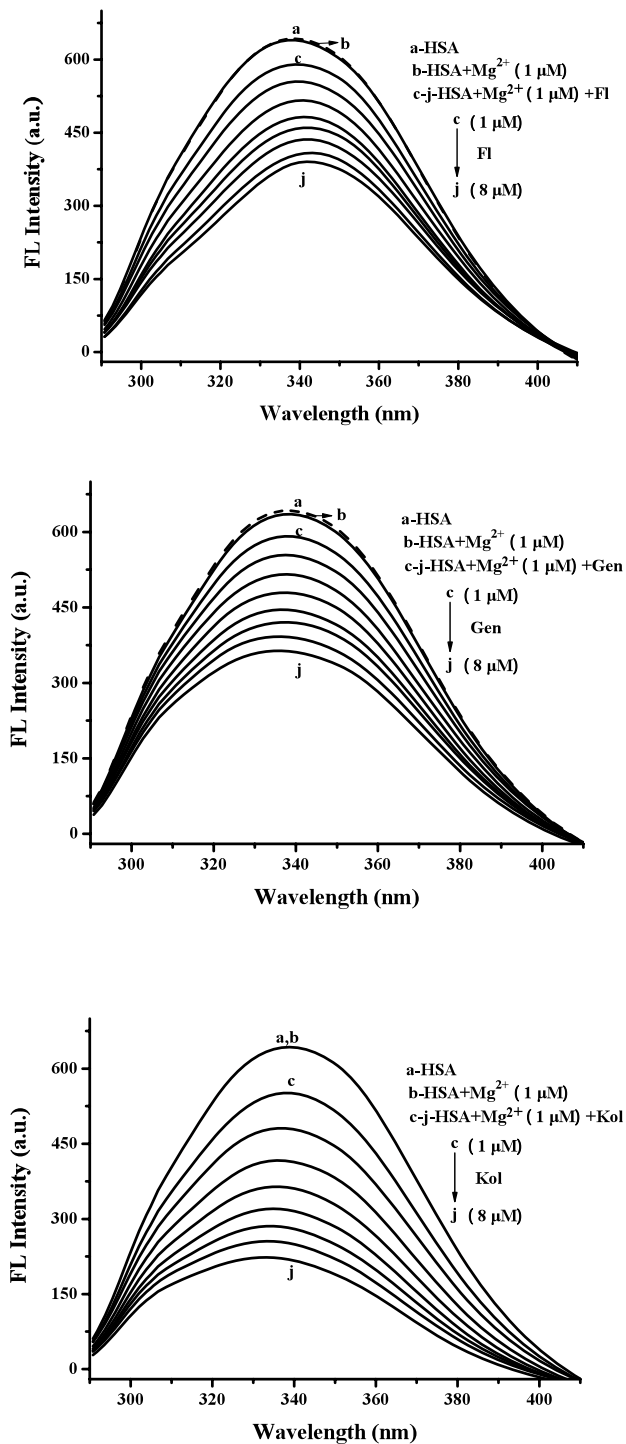
Fig. 4. The fluorescence quenching of HSA by flavone (Fl), genistein (Gen) and kaempferol (Kol) in the presence of  $\text{Pb}^{2+}$  ( $1.0 \times 10^{-6}$  mol/L),  $\lambda_{\text{ex}}=280$  nm; HSA,  $1.00 \times 10^{-5}$  mol/L; a-j: 0.00, 1.00, 2.00, 8.00 ( $10^{-6}$  mol/L) of flavonoids.



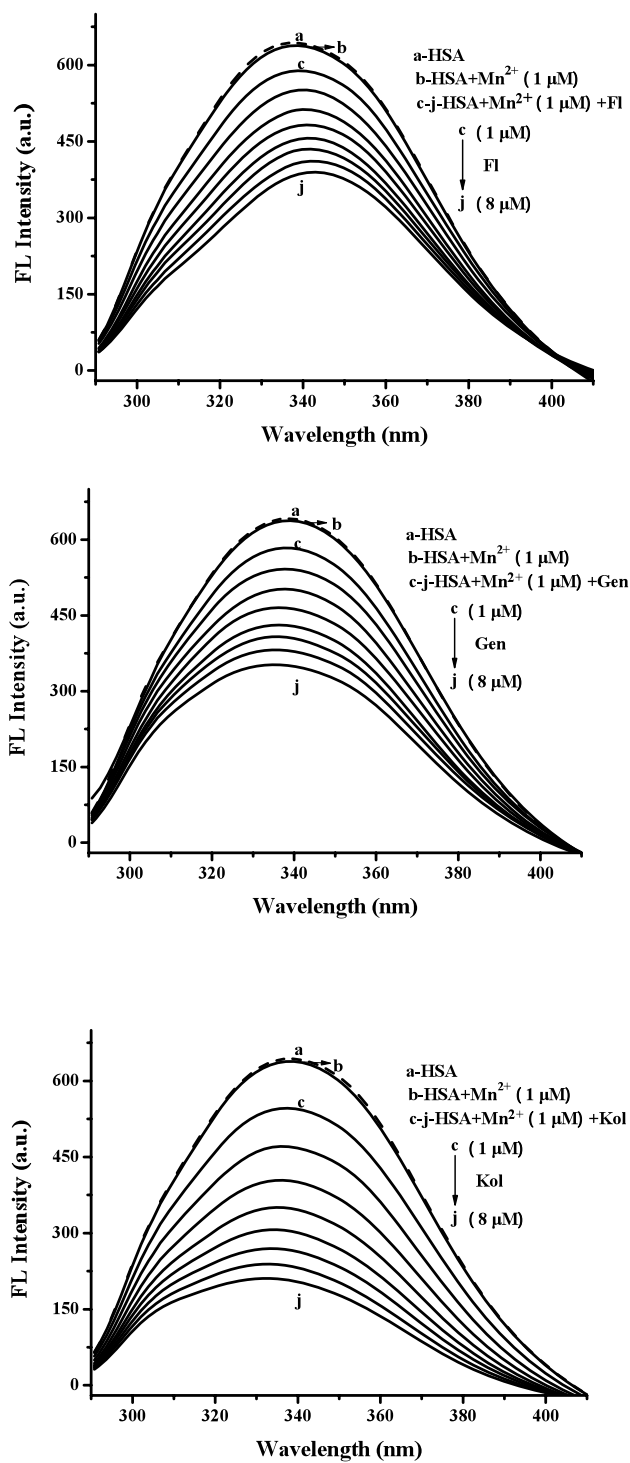
**Fig. 5.** The fluorescence quenching of HSA by flavone (Fl), genistein (Gen) and kaempferol (Kol) in the presence of Cu<sup>2+</sup> ( $1.0 \times 10^{-6}$  mol/L).  $\lambda_{\text{ex}}=280$  nm; HSA,  $1.00 \times 10^{-5}$  mol/L; a-j: 0.00, 1.00, 2.00, 8.00 ( $10^{-6}$  mol/L) of flavonoids.



**Fig. 6.** The fluorescence quenching of HSA by flavone (Fl), genistein (Gen) and kaempferol (Kol) in the presence of Zn<sup>2+</sup> ( $1.0 \times 10^{-6}$  mol/L).  $\lambda_{\text{ex}}=280$  nm; HSA,  $1.00 \times 10^{-5}$  mol/L; a-j: 0.00, 1.00, 2.00, 8.00 ( $10^{-6}$  mol/L) of flavonoids.



**Fig. 7.** The fluorescence quenching of HSA by flavone (Fl), genistein (Gen) and kaempferol (Kol) in the presence of  $Mg^{2+}$  ( $1.0 \times 10^{-6}$  mol/L).  $\lambda_{ex}=280$  nm; HSA,  $1.00 \times 10^{-5}$  mol/L; a-j: 0.00, 1.00, 2.00, 8.00 ( $10^{-6}$  mol/L) of flavonoids.



**Figure 8.** The fluorescence quenching of HSA by flavone (Fl), genistein (Gen) and kaempferol (Kol) in the presence of  $Mn^{2+}$  ( $1.0 \times 10^{-6}$  mol/L).  $\lambda_{ex}=280$  nm; HSA,  $1.00 \times 10^{-5}$  mol/L; a-j: 0.00, 1.00, 2.00, 8.00 ( $10^{-6}$  mol/L) of flavonoids.

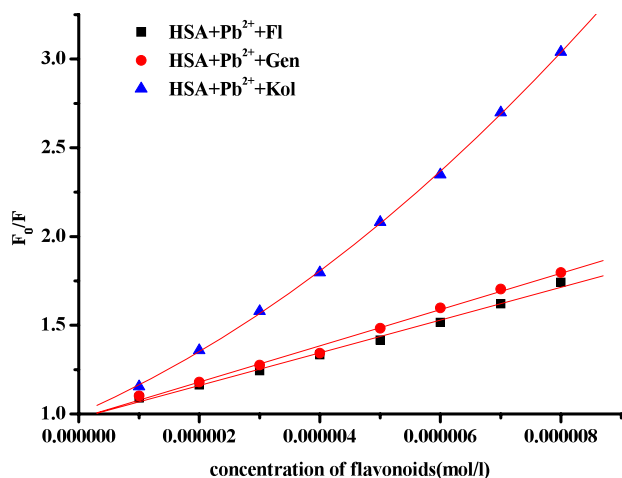


Fig. 9. The Stern-Volmer plots for HSA fluorescence quenching by flavone (Fl), genistein (Gen) and kaempferol (Kol) in the presence of Pb<sup>2+</sup>.

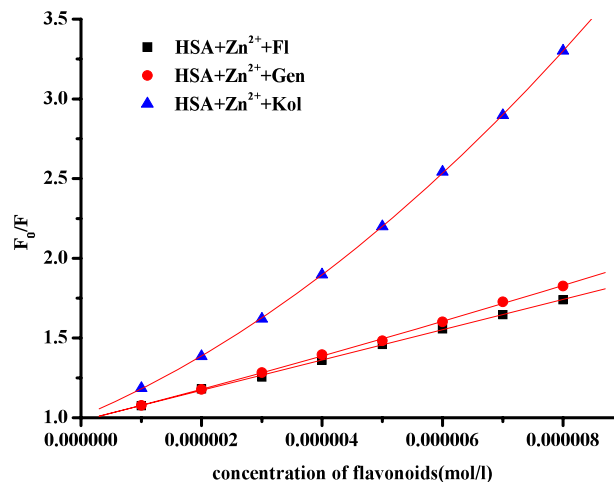


Fig. 11. The Stern-Volmer plots for HSA fluorescence quenching by flavones (Fl), genistein (Gen) and kaempferol (Kol) in the presence of Zn<sup>2+</sup>.

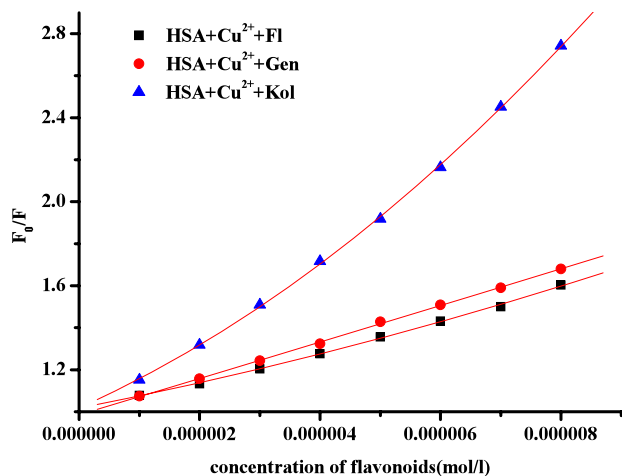


Fig. 10. The Stern-Volmer plots for HSA fluorescence quenching by flavones (Fl), genistein (Gen) and kaempferol (Kol) in the presence of Cu<sup>2+</sup>.

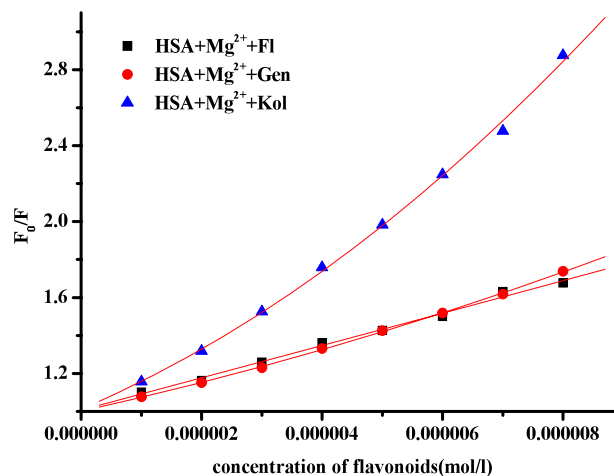


Fig. 12. The Stern-Volmer plots for HSA fluorescence quenching by flavones (Fl), genistein (Gen) and kaempferol (Kol) in the presence of Mg<sup>2+</sup>.

ronment after the addition of flavonoid. Thus, the molecular conformation of the protein was affected. This is in agreement with a recent study that showed that the tertiary structure of proteins changes upon binding of flavonoid. These results also indicated that the quenching effect of flavonoids on HSA fluorescence depended on the structures of flavonoid. Fig. 3 shows the Stern-Volmer plots for the HAS fluorescence quenching by flavone, genistein and kaempferol. The Stern-Volmer plots largely deviated from

linearity toward the y-axis at high flavone, genistein and kaempferol concentrations, which indicated that both dynamic and static quenching were involved for flavonoids on HSA fluorescence.

#### *Fluorescence quenching of HSA induced by flavonoids in the presence of Fe<sup>2+</sup> and Co<sup>2+</sup>*

When flavone, genistein and kaempferol was continuously added to the HSA solution ( $1.0 \times 10^{-5}$  mol/L) containing  $1 \mu\text{mol/L}$  of Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup>

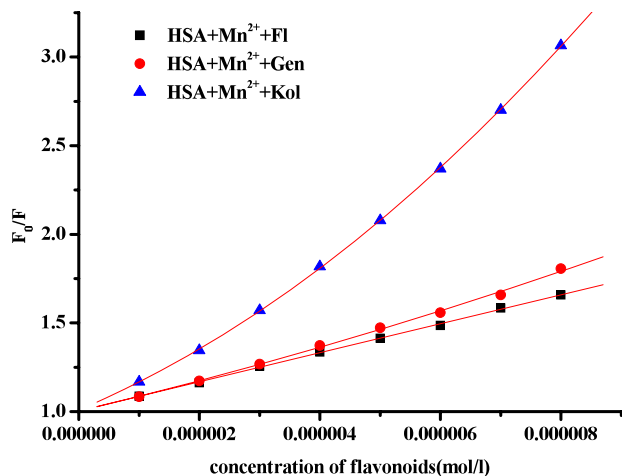


Fig. 13. The Stern-Volmer plots for HSA fluorescence quenching by flavones (Fl), genistein (Gen) and kaempferol (Kol) in the presence of  $Mn^{2+}$ .

and  $Mn^{2+}$ , further attenuation in the fluorescence of HSA was observed (Figs. 4, 5, 6, 7, 8). When flavone, genistein and kaempferol at the final concentration of  $8.00 \mu M$  was added to HSA- $Pb^{2+}$  system, the fluorescence intensities decreased by 42.62%, 44.34%,

and 67.11%, respectively. In addition, the obvious blue-shifts of the maximum emission of HSA with the addition of these three flavonoids were observed in the presence of five metal ions; the extents of the shifts induced by the flavonoids in the presence of five metal ions were obvious. The  $\lambda_{em}$  wavelengths of HSA induced by flavone, genistein and kaempferol changed from 336 nm to 342 nm, 334 nm, and 330 nm in the presence of  $Pb^{2+}$ , respectively. The  $\lambda_{em}$  wavelengths of HSA induced by flavone, genistein and kaempferol changed from 336 nm to 342 nm, 334 nm, and 332 nm in the presence of  $Cu^{2+}$ , respectively. The  $\lambda_{em}$  wavelengths of HSA induced by flavone, genistein and kaempferol changed from 336 nm to 340 nm, 334 nm, and 332 nm in the presence of  $Zn^{2+}$ , respectively. The  $\lambda_{em}$  wavelengths of HSA induced by flavone, genistein and kaempferol changed from 336 nm to 336 nm, 336 nm, and 336 nm in the presence of  $Mg^{2+}$ , respectively. The  $\lambda_{em}$  wavelengths of HSA induced by flavone, genistein and kaempferol changed from 336 nm to 340 nm, 332 nm, and 330 nm in the presence of  $Mn^{2+}$ , respectively. Figs. 9, 10, 11, 12, 13 show the Stern-Volmer plots for HSA

Table 1. The Stern-Volmer quenching constants (K<sub>SV</sub>) of flavonoids for HSA in the absence and presence of  $Pb^{2+}$ ,  $Cu^{2+}$ ,  $Zn^{2+}$ ,  $Mg^{2+}$  and  $Mn^{2+}$  at 300.15 K.

	K <sub>SV</sub> (L/mol)		
	flavone	genistein	kaempferol
Free	$2.34 \times 10^5$	$1.96 \times 10^5$	$3.14 \times 10^5$
$Pb^{2+}$	$0.92 \times 10^5$	$1.02 \times 10^5$	$2.68 \times 10^5$
$Cu^{2+}$	$0.75 \times 10^5$	$0.88 \times 10^5$	$2.26 \times 10^5$
$Zn^{2+}$	$0.95 \times 10^5$	$1.09 \times 10^5$	$3.03 \times 10^5$
$Mg^{2+}$	$0.85 \times 10^5$	$0.94 \times 10^5$	$2.41 \times 10^5$
$Mn^{2+}$	$0.82 \times 10^5$	$1.01 \times 10^5$	$2.70 \times 10^5$

Table 2. Apparent static binding constants (K<sub>a</sub>) for the flavonoid-HSA system in the absence and presence of  $Pb^{2+}$ ,  $Cu^{2+}$ ,  $Zn^{2+}$ ,  $Mg^{2+}$  and  $Mn^{2+}$  at 300.15 K.

	flavone			genistein			kaempferol		
	lgK <sub>a</sub>	n	R	lgK <sub>a</sub>	n	R	lgK <sub>a</sub>	n	R
Free	5.61069	1.05309	0.99849	5.58718	1.06213	0.99922	6.79185	1.24686	0.9978
$Pb^{2+}$	5.00229	1.01257	0.99778	5.02548	1.00824	0.99588	6.55039	1.22801	0.99946
$Cu^{2+}$	4.823	0.99437	0.99731	5.28489	1.06872	0.99984	6.18782	1.17125	0.99904
$Zn^{2+}$	5.39778	1.08286	0.99896	5.55638	1.10587	0.9996	6.5489	1.2187	0.99843
$Mg^{2+}$	4.6602	0.94779	0.99642	5.42285	1.09305	0.99909	6.31359	1.19089	0.99869
$Mn^{2+}$	4.81167	0.97983	0.99964	5.38276	1.07747	0.99965	6.46692	1.21183	0.99861



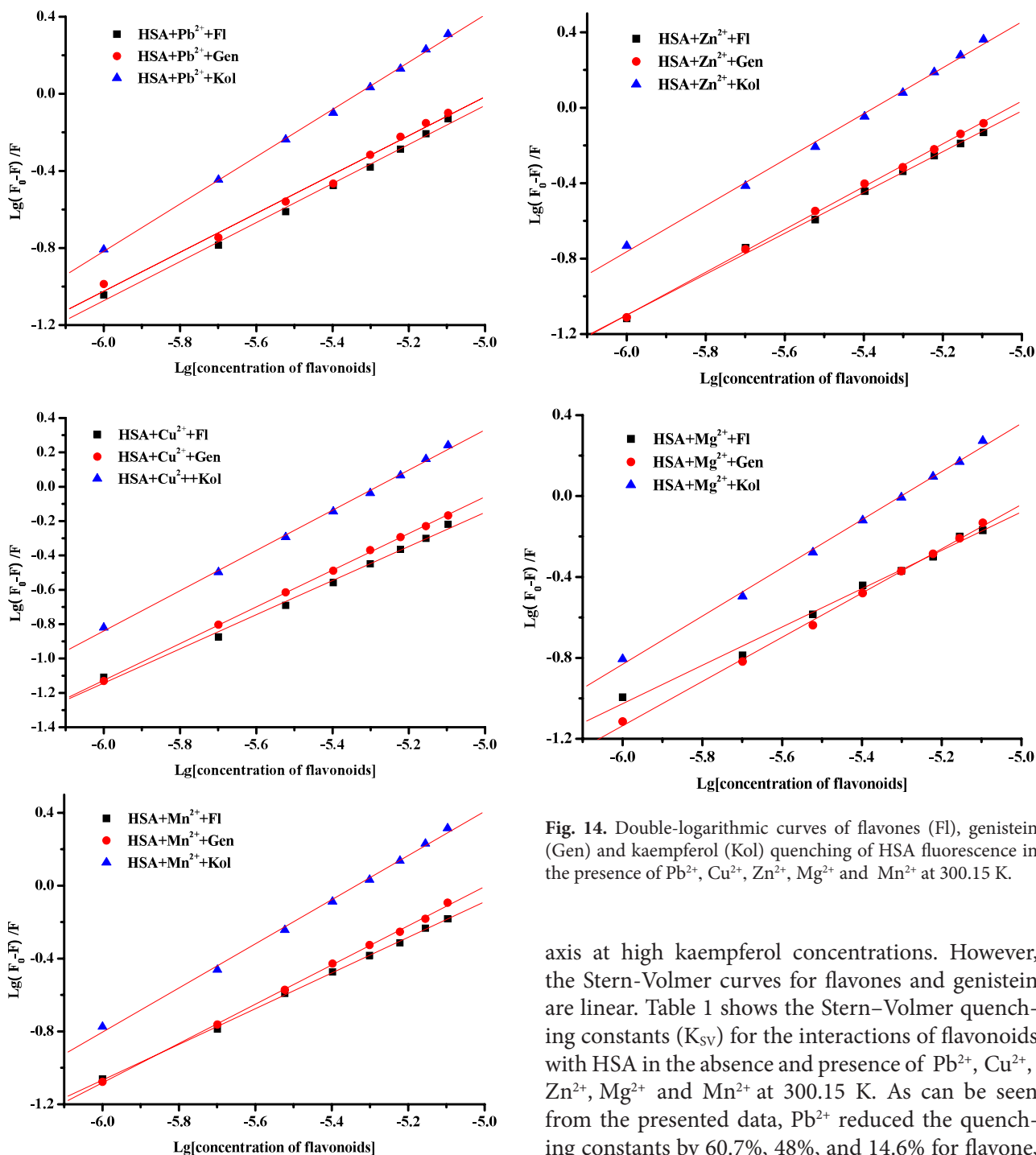


Fig. 14. Double-logarithmic curves of flavones (Fl), genistein (Gen) and kaempferol (Kol) quenching of HSA fluorescence in the presence of Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> at 300.15 K.

fluorescence quenching by flavonoids in the presence of Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup>, respectively. As can be seen from the curves, the Stern-Volmer plots largely deviated from linearity toward the y-

axis at high kaempferol concentrations. However, the Stern-Volmer curves for flavones and genistein are linear. Table 1 shows the Stern-Volmer quenching constants (K<sub>SV</sub>) for the interactions of flavonoids with HSA in the absence and presence of Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> at 300.15 K. As can be seen from the presented data, Pb<sup>2+</sup> reduced the quenching constants by 60.7%, 48%, and 14.6% for flavone, genistein and kaempferol, respectively. Cu<sup>2+</sup> reduced the quenching constants by 67.9%, 55.1%, 28% for flavone, genistein and kaempferol, respectively. Zn<sup>2+</sup> reduced the quenching constants by 59.4%, 44.4%, 35% for flavone, genistein and kaempferol, respec-

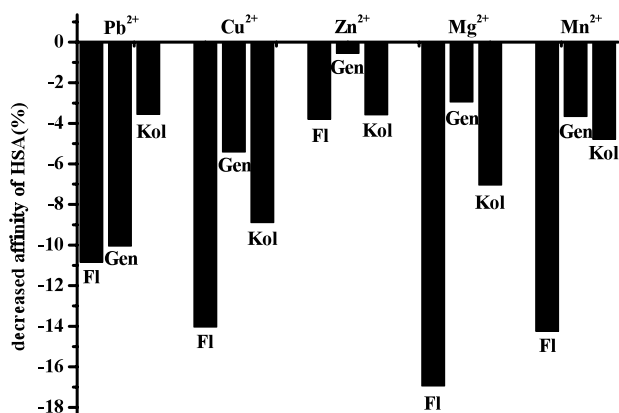


Fig. 15. Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> improved the affinities of flavone(Fl), genistein(Gen) and kaempferol(Kol) for HSA.

tively. Mg<sup>2+</sup> reduced the quenching constants by 63.7%, 52%, 23.2% for flavone, genistein and kaempferol, respectively. Mn<sup>2+</sup> reduced the quenching constants by 65%, 48.5%, 14% for flavone, genistein and kaempferol, respectively. The effects of Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> on the quenching constants of flavonoids for HSA reveal that the flavonoid structure and metal ion together, affected the binding interactions between flavonoids and protein.

#### Effect of Fe<sup>2+</sup> and Co<sup>2+</sup> on the affinities of flavonoids for HSA.

Fig. 14 shows the double-logarithmic curves of flavone, genistein and kaempferol quenching HSA fluorescence in the presence of five metal ions at 300.15 K. Table 2 has the corresponding calculated results. As shown in Fig. 15, Pb<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> improved the affinities of flavone, genistein and kaempferol for HSA *in vitro*. Pb<sup>2+</sup> only slightly reduced the affinities of flavone, genistein and kaempferol – by 10.84%, 10.05% and 3.56%, respectively. Cu<sup>2+</sup> reduced the affinities of apigenin, chrysin □daidzein and quercetin by 14.04%, 5.41% and 8.89%, respectively. Zn<sup>2+</sup> reduced the affinities of apigenin, chrysin □daidzein and quercetin by 3.79%, 0.55% and 3.58%, respectively. Mg<sup>2+</sup> reduced the affinities of apigenin, chrysin □daidzein and quercetin by 16.94%, 2.94% and 7.04%, respectively. Mn<sup>2+</sup> reduced the affini-

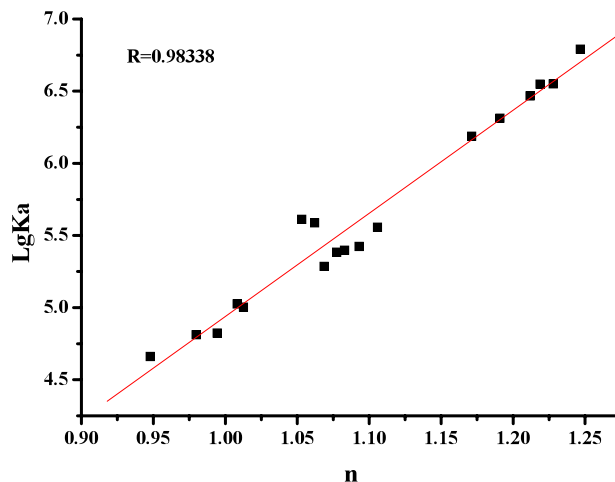


Fig. 16. The relationship between the affinities ( $\log_{10}K_a$ ) and the number of binding sites ( $n$ ) between flavones and HSA.

ties of apigenin, chrysin, daidzein and quercetin by 14.24%, 3.66% and 4.78%, respectively.

#### Relationship between the binding constants ( $K_a$ ) and the number of binding sites ( $n$ ).

The obtained values for  $n$  (1.08~1.31) correspond to the binding sites with high affinity. The existence of the low affinity sites was not studied in this work. Using the measured values of unbound drug fraction, the calculated number of binding sites increased with increasing concentrations of the compounds. The number of binding sites ( $n$ ) is different from the number of molecules actually bound to the sites. The number of molecules bound to the binding sites of a biological macromolecule follows a binomial distribution if the number of binding sites is fixed. When binding to the receptor with  $n$  sites is considered, with  $K_d = 1/K_a$  the dissociation binding constant (affinity of the binding site), it is necessary to have the ligand concentration roughly equal to  $10^4 K_d$  to occupy 90% of the binding sites. The low affinity site ( $K_d$  equal to or greater than  $1000 \mu\text{M}^{-1}$ ) was practically unoccupied (compared to the binding sites with high affinity), and thus was not detected at the low concentrations of flavonoids used in the experiments. If the number of low affinity sites is significant (for instance 10 with  $K_d = 1000 \mu\text{M}^{-1}$ ), binding to them will be comparable to binding to a single high affi-

ity site. This is because the increase in the quantity of sites leads to an increase of bound ligands. Thus, the number of binding sites increasing with increasing binding constant can be used to evaluate the models. The relationship between the  $\log_{10}(K_a)$  and the number of binding sites ( $n$ ) between flavonoids and serum albumins is shown in Fig. 16. The values of  $\log_{10}K_a$  are proportional to the number of binding sites ( $n$ ). This result confirms the equation (1) used here and is suitable to study the interaction between flavones and HSA.

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