

# Interaction between *Plectranthus barbatus* herbal tea components and human serum albumin and lysozyme: Binding and activity studies

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**Abstract.** Anti-cholinesterase and antioxidant active constituents of *Plectranthus barbatus* aqueous extract were found in plasma of rats after its administration – rosmarinic acid, luteolin and apigenin. The aim of the present work is to determine if the extract components can interact with human plasma proteins, namely albumin and lysozyme. Protein intrinsic fluorescence analysis showed that the plant phenolic compounds may bind to albumin, the main transport protein in plasma, and to lysozyme. The estimated thermodynamic parameters suggest that the main intermolecular interaction is hydrophobic association. FTIR analysis of the protein amide bands showed that the plant extract components do not alter the secondary structure of either albumin or lysozyme, however the rate of hydrogen–deuterium exchange suggests that tertiary structure changes might have occurred. An increase of hydrogen deuterium exchange suggests that rosmarinic acid may bind to the fatty acid binding sites in albumin, while luteolin and apigenin may bind to the drug binding sites. The plant extract components also inhibit lysozyme activity with IC<sub>50</sub> values around 100 μM. Therefore *P. barbatus* herbal tea, rosmarinic acid, luteolin and apigenin interact and may be transported by albumin and lysozyme. The inhibition of lysozyme activity may be an additional mechanism for its anti-inflammatory activity.

Keywords: Human serum albumin, lysozyme, spectroscopy, *Plectranthus barbatus*, rosmarinic acid

## 1. Introduction

Plasma proteins play an important role in transportation and deposition of substances in the circulatory system, such as fatty acids, hormones and medicinal drugs. Therefore it is important to reveal the interaction between drugs and proteins in the bloodstream as it may affect the bioavailability, distribution and elimination of pharmaceutical or nutraceutical active compounds. Albumin is the main protein of plasma, and its main function is the regulation of colloidal osmotic pressure and the binding and transport of substances in the bloodstream [14]. The interaction of human serum albumin with chemically synthesized drugs used in medicine may influence their bioavailability and their effectiveness, and so many recent studies have focused on these interactions [25]. The interaction between HSA and plant secondary metabolites traditionally taken by people as “natural medicines” has also been subject to

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many reports [19]. Lysozyme is also known to play a role in the transportation of drugs [9], although its main function is to hydrolyse peptidoglycans, which are found in bacterial cell walls (especially Gram-positive), as part of the innate immune system [11]. The most dramatic lysozyme-related conditions are caused by the decrease or lack of its activity [11], however its excessive activity is known to be related to allergic conditions and aggravation of inflammation in the immune response to pathogens [16,21,26].

*Plectranthus barbatus* (*Lamiaceae*) is drunk as an herbal tea to treat a wide range of diseases, and is also used in food recipes, particularly in South America, Africa and Eastern regions [15]. Previous work demonstrated that this tea has antiacetylcholinesterase as well as antioxidant activity [3], which is related to its main components: rosmarinic acid, flavonoid glucuronides and abietane diterpenoids. The enzyme inhibition was shown to occur after the administration of the extract by *in vitro* and *in vivo* models [4,18]. After the oral administration of the plant extract to rats, its main component, rosmarinic acid, was found in the plasma, as well as the flavonoids apigenin, luteolin and acacetin, in both the glucuronated and as aglycon forms [4].

The aim of the present study was to find if *P. barbatus* water extract compounds and metabolites that were previously found circulating in the plasma can interact with human albumin and lysozyme, by binding and being transported by these proteins, or affecting lysozyme activity.

## 2. Materials and methods

### 2.1. Instruments and chemicals

Human serum albumin (HSA), lysozyme from human milk (EC 3.2.1.17), lyophilized *Micrococcus lysodeikticus*, apigenin, luteolin and rosmarinic acid were obtained from Sigma. Trizma base and K-phosphate buffer salts were obtained from Merck.

### 2.2. Plant extract

The aqueous *P. barbatus* extract, prepared as a decoction, was the same used in previous studies [18]. Briefly, 10 g of ground fresh leaves boiled for 10 min at 100°C, in 100 ml of distilled water and filtered through grade 1 Whatman paper. The extract was lyophilised and the yield of extraction was approximately 140 mg of extract/g of plant.

The extract was analysed by HPLC and MS and its composition was as previously reported [18].

### 2.3. Fluorescence measurements

The fluorescence quenching study on HSA was performed in 3 ml solutions containing  $1 \times 10^{-8}$  M HSA in Tris-HCl buffer (0.20 M, pH 7.4) containing 0.10 M NaCl and the appropriate quantities of quencher – *P. barbatus* extract, rosmarinic acid, luteolin or apigenin [28].

Lysozyme intrinsic fluorescence quenching was studied in 3 ml solutions containing  $0.02 \text{ mg} \cdot \text{ml}^{-1}$  and the appropriate amount of quencher in K-phosphate buffer (0.01 M, pH 7.4). All samples were pre-incubated for 1 h at 37°C [28].

All fluorescence measurements were performed on a LS55 spectrofluorometer (Perkin-Elmer, UK) in a 1 cm quartz cell. Excitation and emission bandwidths were set to 10 nm. Emission spectra were recorded from 300 to 500 nm, under excitation wavelength of 280 nm. The fluorescence intensity was measured at 335 nm under the same excitation wavelength, at different temperatures (293, 298 and 303 K).

## 2.4. FTIR measurements

FTIR measurements were carried out at 21°C using a Bruker Vector 22 Spectrometer. Each spectrum was acquired by averaging between 32 and 300 scans in the spectral range of 400–4000  $\text{cm}^{-1}$  at 4  $\text{cm}^{-1}$  resolution.

Fifty microliters of reaction mixture were transferred into a  $\text{CaF}_2$  infrared cell, fitted with a 50  $\mu\text{m}$  path Teflon spacer and placed in the spectrometer. Spectra were acquired every 2 min for 1 h. For each sample a spectrum was acquired after 2 h, averaging 300 scans.

The hydrogen–deuterium exchange was initiated when HSA was dissolved in TRIS buffer, 50 mM pH 7.4, containing 25 mM NaCl, prepared in deuterium oxide, and the compounds in study in a final concentration of 500  $\mu\text{M}$  HSA, 500  $\mu\text{M}$  of standard compounds or 10 mg/ml of *P. barbatus* extract.

For the measurements with lysozyme, the hydrogen–deuterium exchange was started when lysozyme was dissolved in K-phosphate buffer 10 mM pH 7.4, prepared in deuterium oxide, containing lysozyme in the final concentration of 2 mM, standard compounds in the same concentration or *P. barbatus* extract in the final concentration of 10  $\text{mg} \cdot \text{ml}^{-1}$ .

The FTIR spectra recorded were analysed using the Bruker OPUS software. FTIR spectra of the buffer were recorded under identical conditions and the OPUS software was used to subtract the spectrum of the buffer from the spectrum of the protein in buffer using previously described procedures [5]. Subsequently, second-derivative analysis was carried on the absorbance spectra to reveal the overlapping amide I components (see [5]).

## 2.5. Lysozyme activity measurements

Lysozyme activity was measured with an adaptation of a method previously described [11]. Five hundred microliters of *M. lysodeikticus* suspension in K-phosphate buffer (0.10 M, pH 7.4) were added to 500  $\mu\text{l}$  of a solution containing lysozyme (0.4 mg) and an appropriate amount of inhibitor – *P. barbatus* extract, rosmarinic acid, luteolin or apigenin – in the same buffer. The decrease in absorbance was followed at 570 nm for the first five minutes of reaction and compared with the appropriate controls (without inhibitor and without enzyme). All assays were done in triplicate and  $\text{IC}_{50}$  values were calculated.

## 3. Results

### 3.1. Binding of *P. barbatus* to albumin and lysozyme

Both lysozyme and human serum albumin (HSA) showed excitation spectra with a maximum wavelength at 280 nm and emission spectra with a maximum at 335 nm, under excitation at 280 nm. Figure 1 shows the emission fluorescence spectra of HSA (1a) and Lysozyme (1b) with the addition of increasing concentrations of *P. barbatus* plant extract. The intensity of the intrinsic fluorescence of both proteins decreased significantly – was quenched – with the increase of concentration of the plant extract, which acted as a quencher.

The dominant fluorophore in these proteins is the indole group of the tryptophan residues, and the fluorescence quenching by the plant extract is caused by the interaction between the plant compounds and the protein in the vicinity of the tryptophan residues [12]. The emission spectrum of the indole may be shifted towards lower wavelengths (blue shifted) if the group is buried within the native protein,

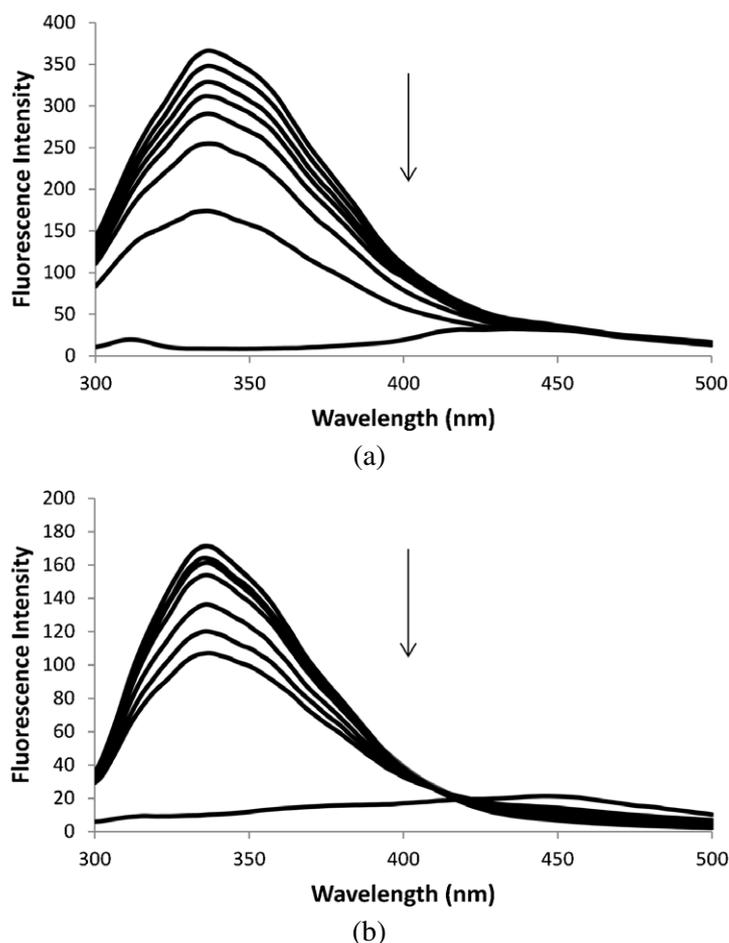


Fig. 1. Fluorescence emission spectra of HSA (a) and lysozyme (b) with the addition of *P. barbatus* aqueous extract. Arrow points to increasing concentrations of *P. barbatus* plant extract, ranging 0; 0.5; 0.75; 1; 2.5; 5; 7.5; 100  $\mu\text{g} \cdot \text{ml}^{-1}$ .

or its emission may be shifted towards larger wavelengths (red shifted) when the protein is unfolded [12]. These effects were not observed in the present study (Fig. 1a and b), suggesting that, although the flavonoids may bind to the proteins in close proximity of the tryptophan residues for the fluorescence quenching to occur, they do not change the tryptophan residue exposition by altering the secondary or tertiary structure of the protein.

The fluorescence data were analysed by the Stern–Volmer equation (Eq. (1)), which allows one to calculate the Stern–Volmer quenching constant ( $K_{SV}$ ) and the quenching rate constant ( $K_q$ ) of the fluorescence quenching reaction:

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

where  $F_0$  and  $F$  are the steady state fluorescence in the absence and presence of quencher, respectively,  $[Q]$  the concentration of quencher, and  $\tau_0$  the average lifetime of the protein fluorescence in the absence of quencher.

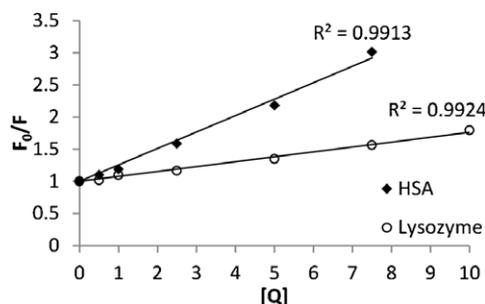


Fig. 2. Stern–Volmer plots of HSA and lysozyme with *P. barbatus* aqueous extract. [Q] is the concentration of *P. barbatus* in  $\mu\text{g} \cdot \text{ml}^{-1}$ .

The Stern–Volmer plots for the fluorescence quenching of HSA and Lysozyme by *P. barbatus* extract can be found in Fig. 2, where it is shown that the fluorescence quenching by the plant extract follows the behaviour of single-compound binding for both proteins. As the extract is composed of many compounds, the concentration is expressed in  $\mu\text{g} \cdot \text{ml}^{-1}$  and the  $K_{SV}$  values cannot be calculated in  $\text{M}^{-1}$ , just estimated in  $\text{l} \cdot \text{mg}^{-1}$ . The molarity of the *P. barbatus* extract was estimated, based on the concentration of rosmarinic acid, luteolin glucuronide and apigenin glucuronide as 1.1122 mmol per gram of plant extract, calculated by the HPLC chromatogram peak areas [18]. The protein intrinsic fluorescence quenching was also analysed for the main component of the plant extract, rosmarinic acid, and for luteolin and apigenin, which were also found as aglycons in the plasma of rats after the administration of *P. barbatus* herbal tea [4].

The values for the Stern–Volmer quenching constant ( $K_{SV}$ ) and the quenching rate constant ( $K_q$ ) for HSA and Lysozyme in the presence of rosmarinic acid, apigenin or luteolin are shown in Table 1. The estimated  $K_{SV}$  and  $K_q$  for the *P. barbatus* extract were, respectively, 461,518  $\text{M}^{-1}$  and  $4.61 \times 10^{13} \text{M}^{-1}\text{s}^{-1}$  for HSA, and 68,513  $\text{M}^{-1}$  and  $6.85 \times 10^{12} \text{M}^{-1}\text{s}^{-1}$  for lysozyme, which were approximate to the values found for the extract's main component, rosmarinic acid. The maximum scatter collision quenching constant ( $K_q$ ) value of various quenchers with a biopolymer is reported to be  $2 \times 10^{10} \text{l} \cdot \text{M}^{-1}\text{s}^{-1}$  [13]. As the  $K_q$  values obtained in the present work were higher, ranging from  $10^{11}$  to  $10^{13}$ , than the  $K_q$  obtained for a scatter mechanism, it is implied that the quenching was not initiated by dynamic collision but rather originated by the formation of a complex.

### 3.2. Analysis of binding equilibria

Fluorescence quenching data also allows one to determine the equilibrium constants between free and bound molecules, as well as the number of binding sites on a macromolecule, by using Eq. (2) [9],

$$\log([F_0 - F]/F) = \log K_b + n \log[Q], \quad (2)$$

where  $F_0$ ,  $F$  and  $Q$  have the same meaning as in Eq. (1),  $K_b$  is the binding constant and  $n$  is the number of binding sites. As shown in Fig. 3, the binding of *P. barbatus* extract follows the behaviour of a single molecule binding to a macromolecule that is modelled by Eq. (2), in both cases of HSA and lysozyme. Similarly to the Stern–Volmer relationship, the  $K_b$  value could not be determined in  $\text{M}^{-1}$  to the plant extract, just be expressed in terms of its concentration in  $\text{mg} \cdot \text{l}^{-1}$ , and estimated based on concentration of rosmarinic acid, luteolin glucuronide and apigenin glucuronide as  $5.84 \times 10^5$  and  $7.92 \times 10^5 \text{M}^{-1}$  for

Table 1

Binding parameters ( $K_{SV}$ ,  $K_q$ ,  $K_b$ ,  $n$ ) and thermodynamic parameters ( $\Delta H^0$ ,  $\Delta S^0$ ,  $\Delta G^0$ ) for the binding of *P. barbatus* extract, rosmarinic acid (RA), luteolin (Lut) and apigenin (Api) to HSA and to lysozyme ( $R^2 > 0.99$  to all linear regressions), rates of amide II/amide I variation and  $IC_{50}$  values for the inhibition of lysozyme activity

	T(K)	Human serum albumin				Lysozyme			
		<i>P. barbatus</i>	RA	Lut	Api	<i>P. barbatus</i>	RA	Lut	Api
$K_{SV}$ ( $M^{-1}$ )	293	–	245,192	194,500	28,029	–	87,179	4191.9	5344.4
	298	0.5133 (a)	355,856	250,748	8090.5	0.0762 (a)	98,081	3381.4	3805.1
	303	–	482,757	326,653	5886.5	–	143,377	3533.7	3250.8
$K_q$ ( $M^{-1}s^{-1}$ )	293	–	2.45E+13	1.95E+13	2.8E+12	–	8.72E+12	4.19E+11	5.34E+11
	298	5.13E+7 (b)	3.56E+13	2.51E+13	8.09E+11	7.62E+6 (b)	9.81E+12	3.38E+11	3.81E+11
	303	–	4.83E+13	3.27E+13	5.89E+11	–	1.43E+13	3.53E+11	3.25E+11
$K_b$ ( $M^{-1}$ )	293	–	1.32E+08	3.03E+08	3.07E+04	–	3,069,729	2760.57	7084.35
	298	0.6493 (a)	3.96E+08	1.12E+09	1.85E+06	0.8806 (a)	205,541.7	3498.64	642.98
	303	–	7.79E+08	6.92E+09	1.61E+09	–	84,781.29	4926.06	363.74
$n$	293	–	1.4998	1.5849	1.0101	–	1.1132	0.9551	0.9935
	298	1.4643	1.5796	1.5403	1.4283	1.4667	1.0673	0.9839	0.8253
	303	–	1.6267	1.6267	2.0964	–	0.9617	0.9789	0.7814
$\Delta H^0$ ( $kJ \cdot mol^{-1}$ )	–	–	131.16	230.67	96.35	–	–265.66	42.69	–219.90
$\Delta S^0$ ( $J \cdot mol^{-1}K^{-1}$ )	–	–	603.68	948.93	338.72	–	–784.91	211.45	–679.24
$\Delta G^0$ ( $kJ \cdot mol^{-1}$ )	293	–	–45.551	–47.576	–25.172	–	–36.389	–19.302	–21.598
	298	–	–49.048	–51.619	–35.757	–	–36.389	–20.218	–16.021
	303	–	–51.577	–57.081	–53.413	–	–28.588	–21.420	–14.855
Rates of amide II/amide I intensity ratios (mAU/min)	298	–0.979	–1.071	–0.071	–0.715	–1.425	–1.820	–0.086	–0.449
$IC_{50}$ ( $\mu M$ )	298	–	–	–	–	$9.02 \pm 0.40$ (c)	$97.2 \pm 3.9$	$106.5 \pm 9.6$	$91.0 \pm 1.4$

Notes: The rates of amide II/amide I variation were determined in the presence of similar amount of RA, Api and Lut, or  $10 \text{ mg} \cdot \text{ml}^{-1}$  of *P. barbatus* extract. Without ligand the rates of amide II/amide I variation were  $-0.716$  for HSA and  $-1.815$  for lysozyme. For *P. barbatus* extract the values are expressed in  $l \cdot \text{mg}^{-1}$  (a),  $l \cdot \text{mg}^{-1}\text{s}^{-1}$  (b) and  $\text{mg} \cdot l^{-1}$  (c).

HSA and lysozyme, respectively. The fluorescence quenching effects by rosmarinic acid, luteolin and apigenin standards were analysed independently, and the values of  $K_b$  and  $n$  can be found in Table 1. The  $n$  value estimated for the interaction of *P. barbatus* extract with HSA was within the range of the one found for the isolated compounds, while the  $K_b$  value was lower, suggesting that the extract components may interfere with the binding of each other to HSA. For lysozyme, the plant extract seemed to bind to more than one binding site ( $n = 1.5$ , Table 1) while for the isolated compounds  $n$  is approximately 1 (Table 1), which may be the cause of the higher  $K_b$  value estimated for the plant extract than for the isolated compounds (Table 1).

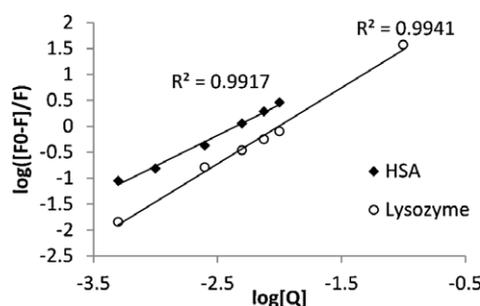


Fig. 3. Plots of  $\log([F_0 - F]/F)$  vs.  $\log[Q]$  for HSA and lysozyme with *P. barbatus* aqueous extract.  $[Q]$  is the concentration of *P. barbatus* in  $\text{g} \cdot \text{l}^{-1}$ .

The values for  $K_b$  and  $n$  for the three compounds to HSA increased with the temperature, suggesting that temperature may increase the binding of the molecules to two sites in the HSA structure. The binding of luteolin showed higher  $K_b$  values than apigenin, which agrees with the results of Xiao and coworkers [28] for the binding of flavonols to bovine serum albumin, where it is reported that the binding constants increased with the number of hydroxyl groups in the B-ring of flavonoids.

A decrease in  $K_b$  and  $n$  was observed for lysozyme with rosmarinic acid and apigenin, suggesting that temperature may be unfavourable to the binding in these cases. For the binding of luteolin with lysozyme, the value of  $n$  remained similar through the changes of temperature, while the  $K_b$  value increased. This suggests that the catechol group that is present in luteolin, but not in apigenin, may play an important role in stabilizing the complex flavonoid–protein at higher temperatures. Apigenin, in spite of decreasing  $K_b$  with temperature, showed a higher  $K_b$  with a smaller temperature than luteolin, suggesting that the lack of catechol group may increase the hydrophobic interaction between the flavonoids and the proteins. This interaction, although it may be stronger than the weak polar interactions made by the catechol group, is more susceptible to be broken by increasing the energy in the system. At higher temperatures, closer to body temperature, the binding affinities of luteolin to HSA and lysozyme are higher than the ones for the binding of apigenin, as was shown by Xiao and coworkers [27] for total plasma proteins extracted from rat blood, which demonstrates that the hydroxyl group in position 3' of the B ring in the flavonoid structure has great influence in flavonoid affinity to the proteins.

### 3.3. Determination of interaction forces between *P. barbatus* extract metabolites and HSA and lysozyme

The thermodynamic parameters enthalpy change ( $\Delta H^\circ$ ) and entropy change ( $\Delta S^\circ$ ) of the interaction of the plant phenolic compounds and the proteins allow to elucidate the nature of the bond between the plant extract components and the HSA/Lysozyme macromolecules. For this purpose, the variation of the binding constant  $K_b$  with temperature was studied at three temperatures: 293, 298 and 303 K.

The thermodynamic parameters were determined by the van't Hoff equation (Eq. (3)), considering that the enthalpy change does not vary significantly with temperature [9]. The free energy change ( $\Delta G^\circ$ ) was determined by Eq. (4):

$$\ln K_b = -\Delta H^\circ / RT + \Delta S^\circ / R, \quad (3)$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ, \quad (4)$$

where  $R$  is the gas constant and  $T$  – the temperature. The values of the thermodynamic parameters of the interaction of rosmarinic acid, apigenin and luteolin with HSA and Lysozyme can be found in Table 1.

The negative values of  $\Delta G^\circ$  indicate that the binding processes occurred spontaneously in all studied cases.

From the view-point of water structure, a positive  $\Delta S^\circ$  value is typical evidence of hydrophobic interaction [29]. In the case of HSA, both  $\Delta H^\circ$  and  $\Delta S^\circ$  values were positive (Table 1), suggesting that hydrophobic association is the dominant form of interaction of the tested plant phenolics with HSA [22]. Previous reports on the interaction of apigenin with HSA [30], and luteolin with bovine serum albumin [29], also showed positive values for  $\Delta S^\circ$ . However, Yang and coworkers [29] presented a negative value for  $\Delta H^\circ$ , which may be due to a strengthening of the interaction by other forces, such as van der Waals or hydrogen bonds, introduced by hydrophobic effect in higher temperature conditions than the interval used in the present work [22].

The values of  $\Delta H^\circ$  and  $\Delta S^\circ$  in the interaction of rosmarinic acid and apigenin with Lysozyme were negative, suggesting van der Waals interactions between phenolic compounds and certain domains of lysozyme [22]. In these cases, the major contribution to  $\Delta G^\circ$  arises from the  $\Delta H^\circ$  term rather than from  $\Delta S^\circ$ , which implies that the binding processes are enthalpy driven. In the interaction of luteolin and lysozyme, both  $\Delta H^\circ$  and  $\Delta S^\circ$  values were positive, which points to hydrophobic association as the main form interaction between the molecules. The difference in thermodynamic parameters found between the apigenin and luteolin may have been caused by the higher polarity and solubility in water of luteolin [10], that arises from the higher hydroxylation of the B-ring. The higher affinity to water of luteolin may cause a weaker hydrophobic association that did not induce the electrostatic interactions in our interval of temperatures [22].

#### 3.4. Determination of protein structure changes caused by *P. barbatus* extract and its plasma metabolites by FTIR

FTIR spectroscopy may provide additional evidence on the interaction between a protein and a ligand. The protein secondary structure is reflected in the amide I band, in the region  $1600\text{--}1700\text{ cm}^{-1}$  (mainly C=O stretch), and in the amide II band,  $\approx 1540\text{ cm}^{-1}$  (C–N stretch coupled with NH bending mode). Amide I is more sensitive to secondary structure changes than amide II [6,24,28], hence its analysis is more useful for the study of structural changes induced by diverse factors.

The FTIR spectra of HSA and Lysozyme, obtained by subtracting the absorption of the buffer solution, are shown in Fig. 4a and b, for HSA and Lysozyme, respectively, in the absence and in the presence of *P. barbatus* extract, rosmarinic acid, luteolin or apigenin. The second derivatives are shown in Fig. 4c and d for both HSA and lysozyme, respectively. The position of the amide I band in region  $1651\text{--}1653\text{ cm}^{-1}$  indicates the presence of predominantly  $\alpha$ -helical structure in these two proteins which is consistent with their known X-ray structures. As it can be seen in the absorbance spectra and second derivatives, the amide I band showed the same peak and shape in the presence and absence of the ligands for both proteins. As the amide I is composed of the sum of  $\beta$ -turn,  $\beta$ -sheet and  $\alpha$ -helix absorbance contributions [7,10], it can be concluded that the presence of *P. barbatus* extract or its metabolites did not interfere with the secondary structure of HSA or lysozyme.

FTIR spectroscopy also allows one to determine the rate of hydrogen–deuterium exchange in proteins (for a review see [10]). Following the rate of hydrogen–deuterium exchange of a protein molecule in deuterium oxide medium may give information about solvent accessibility in various parts of the molecule,

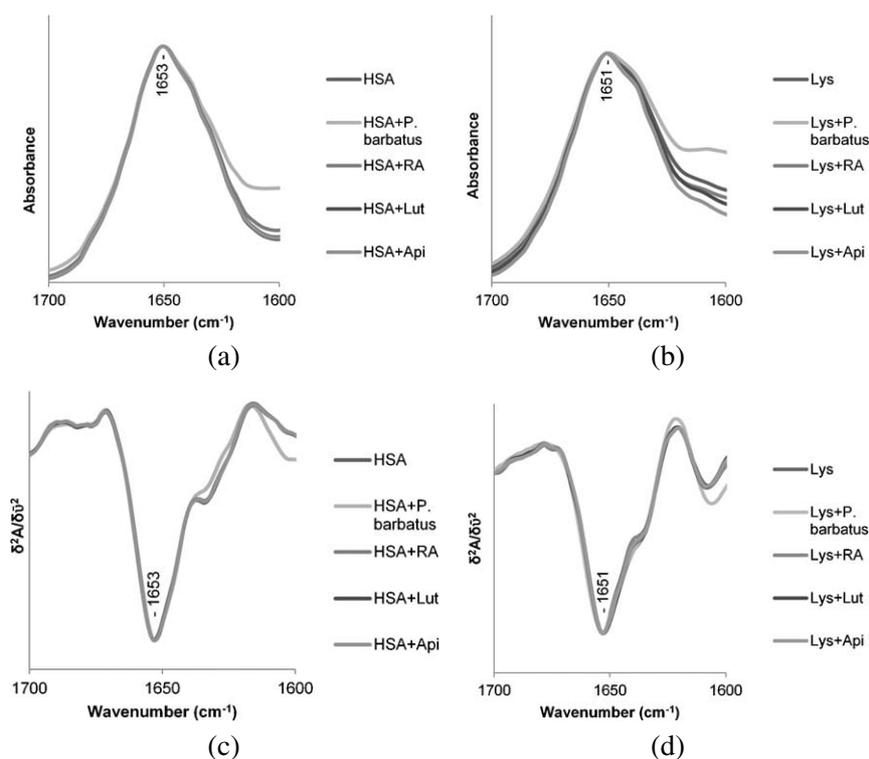


Fig. 4. FTIR spectra of the proteins alone, with *P. barbatus* extract, rosmarinic acid (RA), luteolin (Lut) and apigenin (Api). The absorbance spectra obtained for HSA are shown in (a) and the second derivatives are in (c). The absorbance spectra obtained for lysozyme are shown in (b) and the second derivatives are in (d).

which reflects the tertiary structure of the protein [5]. The hydrogen–deuterium exchange can be followed by a decrease in amide II band during the first moments of contact of the protein with deuterium oxide [6].

When *P. barbatus* extract ( $10 \text{ mg} \cdot \text{ml}^{-1}$ ) was in contact with the proteins under study, the rate of hydrogen/deuterium exchange increased by 36.2% for HSA and decreased by 21.5% for lysozyme (Fig. 5). The effects of rosmarinic acid, luteolin and apigenin were analysed separately for each of the compounds in the same concentration of the protein. Rosmarinic acid caused a 49.6% increase in the rate of hydrogen–deuterium exchange of HSA, while the increase in lysozyme was negligible (0.3%). The flavonoids luteolin and apigenin caused a decrease in hydrogen–deuterium exchange in both HSA and lysozyme. The highest decrease was observed for luteolin (90.1% and 95.3% for HSA and lysozyme, respectively), while apigenin caused a 75.3% decrease in the hydrogen–deuterium exchange rate of lysozyme, and a negligible decrease in HSA (0.1%) (Fig. 5). There was no apparent difference in the overall shape and position of amide I bands, as seen from both the absorbance and second-derivative spectra (Fig. 4), of HSA and lysozyme in the presence of *P. barbatus* extract. This suggests that the interaction does not alter the secondary structure of these two proteins. Therefore, the differences found in the rate of hydrogen–deuterium exchange may be due, predominantly to changes in the tertiary structure of the proteins [6].

The decrease of lysozyme hydrogen–deuterium exchange by *P. barbatus* extract may be due to its flavonoid components, since its main component, rosmarinic acid, does not affect the hydrogen–

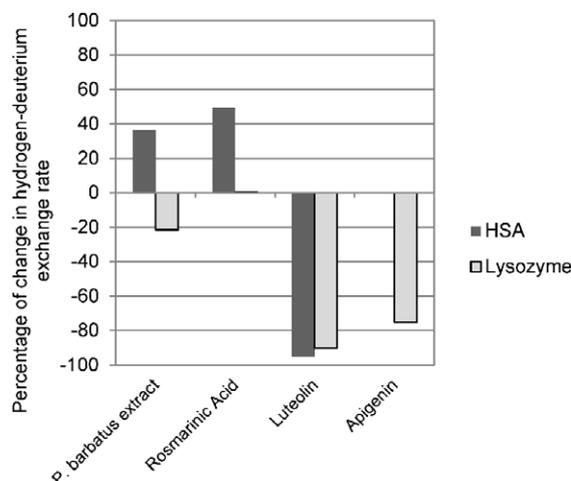


Fig. 5. Percentage of change in protein (HSA and lysozyme) hydrogen–deuterium exchange rate, determined from the analysis of the amide II band, in the presence of *P. barbatus* extract, rosmarinic acid, luteolin or apigenin, in comparison with the hydrogen–deuterium exchange rate of the protein alone.

deuterium exchange of lysozyme. The increase in the rate of hydrogen–deuterium exchange in HSA when in contact with *P. barbatus* extract appears to be due to rosmarinic acid, as it is the major component, while the flavonoids play a smaller role in altering the global hydrogen–deuterium exchange.

### 3.5. Effect of *P. barbatus* extract on lysozyme activity

Lysozyme is a glycoside hydrolase able to hydrolyse the peptidoglycan that constitutes the cell walls of bacteria such as *Micrococcus* sp. [11].

The aqueous extract of *P. barbatus* inhibited lysozyme activity, with an  $IC_{50}$  value of  $9.02 \mu\text{g} \cdot \text{ml}^{-1}$  (Table 1). The  $IC_{50}$  values for the inhibition of lysozyme by rosmarinic acid, apigenin and luteolin were similar and around  $100 \mu\text{M}$ , as it is shown in Table 1.

## 4. Discussion

The structure of human serum albumin, alone and when bound to diverse molecules, has been determined using X-ray crystallography (for a review see [2]). In this study we have demonstrated using fluorescence spectroscopy that flavonoids including rosmarinic acid alter the tertiary structure of HSA. Furthermore, our FTIR spectroscopic analysis reveals significant reduction in amide proton hydrogen–deuterium exchange rate for HSA complexed to flavonoids. This is indicative of a decrease in the accessibility of the exchangeable hydrogens to the solvent (deuterium oxide). However, we did not detect any changes in the secondary structure of HSA, through the analysis of the amide I band, as a consequence of interaction with the flavonoids. This finding is in good agreement with the results of X-ray crystallographic studies which have consistently shown that there are no gross changes in the secondary structure of HSA associated with binding ligands although changes in tertiary structure such as rigid-body-rotation of domains have been detected (for a review see [2]). The latter changes in tertiary structure may explain the differences observed in the fluorescence spectra and hydrogen–deuterium exchange rates.

Of the different compounds tested, luteolin induced the highest decrease of hydrogen–deuterium exchange and it also presented the highest values for the binding constants and lowest  $\Delta G^\circ$  values, suggesting a higher stability for the HSA-luteolin complex compared to the HSA-apigenin complex. The increased stability of the protein associated with the bonding between the flavonoids and the HSA molecule makes it more difficult for the amide protons to be substituted with deuterium. Furthermore, the interaction may result in the shielding of certain segments of the protein from being accessible to solvent. All this explains the significant reduction in hydrogen–deuterium exchange upon binding of the flavonoids to HSA. Rosmarinic acid, like luteolin, had no effect on the secondary structure of HSA. However, unlike luteolin, rosmarinic acid increased the hydrogen–deuterium exchange of HSA, suggesting that the nature of its interaction with the transport protein is different. Our results suggest that hydrophobic regions of the protein becomes accessible to solvent due to alterations in the protein tertiary structure [17] without any changes in the secondary structure. The nature of the change could be a large rigid-body rotation of the subunits that have been shown to occur on fatty acid binding to the main fatty acid binding sites in HSA, as was extensively reviewed by Curry [2]. X-ray crystallographic analysis has shown that fatty acid binding results in a HSA molecule that is 10 Å wider than the defatted HSA. It is thus possible that whilst the binding may reduce hydrogen–deuterium exchange for molecules that are interacting with rosmarinic acid but a much larger change such as widening of the molecule could expose a greater number of amino acid residues to solvent resulting in an overall increase in hydrogen–deuterium exchange. We propose that rosmarinic acid may induce a similar type of tertiary structural change as induced by binding of fatty acids to HSA. In five of the seven known fatty acid binding sites the lipid is anchored by the interaction of the carboxylic group with a basic or polar group at the pocket entrance. Some non-lipid acidic compounds known to interact with these binding sites, specifically by interacting with the carboxylic acid binding residues [2]. Rosmarinic acid and fatty acids both share a common functional group (carboxylic acid group) which is not the case for luteolin and this may explain as to why they behave differently in altering the structure of the HSA molecule.

Flavonoids have been reported to bind to the drug binding sites [19,30], which are different from the fatty acid binding sites. The drug binding sites 1 and 2 comprise largely apolar cavities with defines polar features located in sub-domains IIA and IIIA, respectively. Most drugs bind to the drug binding site 1, which has preference for flat aromatic compounds that fit between Leu238 and Ala291 in the centre of the pocket [2]. The planar structure of flavonoids may be responsible for their binding predominantly to the drug site 1, as it was reported for apigenin [30] and quercetin [19]. Usually small adjustments in the side chains in the site 1 cavity can happen upon the binding of the compounds. These changes are not so extensive that may lead to changes in the secondary structure of HSA [2], but may be the cause of the decrease in hydrogen–deuterium exchange rate in the binding of luteolin to HSA.

It is interesting that like with HSA, luteolin and apigenin interaction with lysozyme results in a decrease in amide proton hydrogen–deuterium exchange rate. This can be due to one or more of the following reasons:

- (i) due to the amide protons participating in stronger H-bonds within the secondary structural elements as a consequence of interaction with these compounds. This will make it more difficult to break the H-bonds for the deuterium substitution to occur;
- (ii) due to amide protons forming H-bonds to other groups including luteolin and apigenin making it more difficult for the hydrogen–deuterium exchange reaction to occur;
- (iii) or it could be due to movement of domains/regions of protein that results in a reduction in solvent accessibility so that deuterium oxide is unable to penetrate into the core of the protein to replace the amide protons with deuterium.

Similarly to HSA, luteolin induced a greater decrease in lysozyme hydrogen deuterium exchange (Table 1), and presented a higher binding constant and lower  $\Delta G^\circ$  value at room temperature, suggesting that the binding of luteolin to lysozyme is more stable at room temperature than the binding of apigenin.

Once again, like with what we saw for HSA, the interaction of rosmarinic acid with lysozyme is different to that observed for luteolin. Although, there is no significant increase in hydrogen–deuterium exchange, it at least does not reduce the hydrogen–deuterium exchange. This could be due to weaker interaction between rosmarinic acid and lysozyme or that the nature of the binding does not alter the tertiary structure in a way that would make the amide protons more vulnerable to hydrogen–deuterium exchange.

Although we have detected changes in hydrogen–deuterium exchange, no significant alteration in the secondary structure was detected. However, other studies concerning the interaction of flavonoids detected small secondary structure changes, as well as tertiary structure changes, by the flavonoids alpinetin and cardamonin [7]. Our findings reveal that alterations in protein secondary structure were not found with either luteolin or apigenin, by FTIR or fluorescence spectrometry, suggesting that the alterations in rate of hydrogen deuterium exchange is mainly due to changes in the tertiary structure.

As the proteins and the plant phenolic compounds seemed to interact by weak forces by fluorescence spectrometry, the bonds formed may be reversible and the compounds released under certain conditions. Studies with total plasma protein extracted from rat blood report that the affinity of flavonoids to the total protein may be even lower than to purified albumin, which may be due to presence of metallic ions such as  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Ca^{2+}$  and  $Mg^{2+}$  [27]. The weak and reversible interaction between these plant compounds and plasma proteins is in agreement with *in vivo* studies that show a decrease in the concentration of plant phenolic compounds in plasma, due to metabolism and excretion, as was observed in *P. barbatus* extract components [4], rosmarinic acid [1,4] and the flavonoids apigenin and luteolin [23]. Therefore it is important that the structural changes in plasma proteins when bound to compounds are not so drastic that their function may be compromised after the release of the compounds.

Lysozyme, apart from drug transport, has an important function related to the immune response process [11]. When degranulation occurs, after neutrophils reach the injured tissue by margination, adhesion and emigration, lysozyme is discharged from lysosomes of neutrophils and destroys not only the phagosomes but also damages the animal tissue itself, thus aggravating the response to inflammation [21,26]. Previous reports showed that phenolic compounds from *Leggera* species extracts decreased the release of lysozyme from the neutrophils to the serum [26]. In the present work, *P. barbatus* extract phenolic compounds inhibited directly lysozyme activity. It is long known that some flavonoids may act as lysozyme inhibitors [20]. Previous works also reported that plant extracts mainly composed of apigenin, luteolin and luteolin glucosides decreased allergy symptoms caused by egg-white lysozyme sensitization in rats [8]. The same anti-allergic effect was observed for *Perilla frutescens* water extract, and by its major component, rosmarinic acid [16].

As *P. barbatus* components bind to lysozyme with weak interactions, our data suggests that the enzyme does not undergo major conformational changes, the plant compounds are eliminated from plasma in a short period of time [4], and the antibacterial activity of lysozyme is not permanently compromised. Therefore, these compounds may be helpful in decreasing the damage caused by a high lysozyme activity, in response to pathogens or in allergic conditions.

*P. barbatus* extract has also proved to have antioxidant activity as a radical scavenger more powerful than the commercial antioxidant BHT [3]. This activity is mainly due to its main component, rosmarinic acid. The present results suggest that *P. barbatus* water extract may be useful to treat inflammatory conditions as its components and metabolites may be transported in circulatory system binding to albumin,

the most abundant transport protein, and to lysozyme, and decrease the inflammation process by two mechanisms: as free radical scavengers, and as lysozyme inhibitors.

In conclusion, protein intrinsic fluorescence quenching proved that the *P. barbatus* extract components and its metabolites found in rat plasma are able to bind to the human transport proteins albumin and lysozyme, allowing them to be carried in the bloodstream to organs where they can have a beneficial activity. The spectroscopic data suggest that the interaction of the plant phenolic compounds and the proteins in this study is made by weak interactions, causing some changes in protein tertiary structure, but not in the secondary structure. This suggests that the compounds may be released from the complexes they form with the proteins, without compromising the albumin or lysozyme function in plasma.

*P. barbatus* extract components and metabolites also inhibit lysozyme activity, which may be helpful in decreasing the aggravation of the inflammation caused by the immune system in response to pathogens and in allergies. This mechanism can be added to the already well known radical scavenger capacity of the extract components, making the anti-inflammatory activity of plant extract very promising.

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## References

- [1] S. Baba, N. Osakabe, M. Natsume and J. Terao, Orally administered rosmarinic acid is present as the conjugated and/or methylated forms in plasma, and is degraded and metabolized to conjugated forms of caffeic acid, ferulic acid and m-coumaric acid, *Life Sciences* **75** (2004), 165–178.
- [2] S. Curry, Lessons from the crystallographic analysis of small molecule binding to human serum albumin, *Drug Metabolism and Pharmacokinetics* **24** (2009), 342–357.
- [3] P.L. Falé, C. Borges, P.J.A. Madeira, L. Ascensão, M.E.M. Araújo, M.H. Florêncio and M.L.M. Serralheiro, Rosmarinic acid, scutellarein 4'-methyl ether 7-O-glucuronide and (16S)-coleon E are the main compounds responsible for the anti-acetylcholinesterase and antioxidant activity in herbal tea of *Plectranthus barbatus* ("falso boldo"), *Food Chemistry* **114** (2009), 798–805.
- [4] P.L.V. Falé, P.J.A. Madeira, M.H. Florêncio, L. Ascensão and M.L.M. Serralheiro, Function of *Plectranthus barbatus* herbal tea as neuronal acetylcholinesterase inhibitor, *Food and Function* **2** (2011), 130–136.
- [5] P.I. Haris, D. Chapman, R.A. Harrison, K.F. Smith and S.J. Perkins, Conformational transition between native and reactive center cleaved forms of  $\alpha_1$ -antitrypsin by Fourier transform infrared spectroscopy and small-angle neutron scattering, *Biochemistry* **29** (1990), 1377–1380.
- [6] P.I. Haris and F. Severcan, FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media, *Journal Molecular Catalysis B: Enzymatic* **7** (1999), 207–221.
- [7] W. He, Y. Li, J. Tang, F. Luan, J. Jin and Z. Hu, Comparison of the characterization on binding of alpinetin and cardamonin to lysozyme by spectroscopic methods, *International Journal of Biological Macromolecules* **39** (2006), 165–173.
- [8] E. Iwaoka, H. Oku, M. Iinuma and K. Ishiguro, Allergy-preventive effects of the flowers of *Impatiens textori*, *Biological and Pharmaceutical Bulletin* **33** (2010), 714–716.
- [9] J. Jin and X. Zhang, Spectrophotometric studies on the interaction between pazufloxacin mesilate and human serum albumin or lysozyme, *Journal of Luminescence* **128** (2010), 81–86.
- [10] H. Kim, H. Kim and S. Jung, Aqueous solubility enhancement of some flavones by complexation with cyclodextrins, *Bulletin of the Korean Chemical Society* **29** (2008), 590–594.
- [11] N.J. Laible and G.R. Germaine, Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: inhibition by chitin oligosaccharides, *Infection and Immunity* **48** (1985), 720–728.
- [12] J.R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd edn, Plenum Press, New York, 2006.
- [13] J.R. Lakowicz and G. Weber, Quenching of fluorescence by oxygen. A probe for structural fluctuations in macromolecules, *Biochemistry* **12** (1973), 4161–4170.

- [14] Y. Li, W.Y. He, H. Liu, X. Yao and Z. Hu, Daidzein interaction with human serum albumin studied using optical spectroscopy and molecular modeling methods, *Journal of Molecular Structure* **831** (2007), 144–150.
- [15] C.W. Lukhoba, M.S.J. Simmonds and A.J. Paton, *Plectranthus*: a review of ethnobotanical uses, *Journal Ethnopharmacology* **103** (2006), 1–24.
- [16] T. Makino, Y. Furuta, H. Wakushima, H. Fujii, K. Saito and Y. Kano, Anti-allergic effect of *Perilla frutescens* and its active constituents, *Phytotherapy Research* **17** (2003), 240–243.
- [17] V. Militello, C. Casarino, A. Emanuele, A. Giostra, F. Pullara and M. Leone, Aggregation kinetics of bovine serum albumin studied by FTIR spectroscopy and light scattering, *Biophysical Chemistry* **107** (2004), 175–187.
- [18] S. Porfírio, P.L.V. Falé, P.J.A. Madeira, M.H. Florêncio, L. Ascensão and M.L.M. Serralheiro, Antiacetylcholinesterase and antioxidant activities of *Plectranthus barbatus* tea, after *in vitro* gastrointestinal metabolism, *Food Chemistry* **122** (2010), 179–187.
- [19] H.M. Rawel, S.K. Frey, K. Meidtner, J. Kroll and F.J. Schweigert, Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence, *Molecular Nutrition and Food Research* **50** (2006), 705–713.
- [20] G. Rodney, A.L. Swanson, L.M. Wheeler, G.N. Smith and C.S. Worrel, The effect of a series of flavonoids on hyaluronidase and some other related enzymes, *Journal Biological Chemistry* **183** (1950), 739–747.
- [21] F. Ronca, L. Palmieri, P. Panicucci and G. Ronca, Anti-inflammatory activity of chondroitin sulphate, *Osteoarthritis and Cartilage* **6** (1998), 14–21.
- [22] P.D. Ross and S. Subramanian, Thermodynamics of protein association reactions: forces contributing to stability, *Biochemistry* **20** (1981), 3096–3102.
- [23] R. Shi, S. Qiao, D. Yu, X. Shi, M. Liu, X. Jiang, Q. Wang and L. Zhang, Simultaneous determination of five flavonoids from *Scutellaria barbata* extract in rat plasma by LC-MS/MS and its application to pharmacokinetic study, *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* **879**(19) (2011), 1625–1632.
- [24] R. Tantipolphan, T. Rades, A.J. McQuillan and N.J. Medicott, Adsorption of bovine serum albumin (BSA) onto lecithin studied by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy, *International Journal Pharmaceutics* **337** (2007), 40–47.
- [25] A. Varshney, P. Sen, E. Ahmad, M. Rehan, N. Subbarao and R.H. Khan, Ligand binding strategies of human serum albumin: How can the cargo be utilized?, *Chirality* **22** (2010), 77–87.
- [26] Y. Wu, C. Zhou, L. Song, X. Li, S. Snhi, J. Mo, H. Chen, H. Bai, X. Wu, J. Zhao, R. Zhang, X. Hao, H. Sun and Y. Zhao, Effect of total phenolics from *Laggera alata* on acute and chronic inflammation models, *Journal Ethnopharmacology* **108** (2006), 243–250.
- [27] J. Xiao, H. Cao, T. Chen, F. Yang, C. Liu and X. Xu, Molecular property-binding affinity relationship of flavonoids for common rat plasma proteins *in vitro*, *Biochimie* **93** (2011), 134–140.
- [28] J. Xiao, M. Suzuki, X. Jiang, X. Chen, K. Yamamoto, F. Ren and M. Xu, Influence of B-Ring hydroxylation on interactions of flavonols with bovine serum albumin, *Journal of Agricultural and Food Chemistry* **56** (2008), 2350–2356.
- [29] Y. Yang, Q. Hua, Y. Fan and H. Shen, Study on the binding of luteolin to bovine serum albumin, *Spectrochimica Acta A: Molecular Biomolecular Spectroscopy* **69** (2008), 432–436.
- [30] J. Yuan, Z. Lv, Z. Liu, Z. Hu and G. Zou, Study on interaction between apigenin and human serum albumin by spectroscopy and molecular modelling, *Journal of Photochemistry and Photobiology A: Chemistry* **191** (2007), 104–113.



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