REPORT

Molecular property–affinity relationship of flavanoids and flavonoids for HSA *in vitro*

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The relationship between the structural properties of selected dietary flavanoids and flavonoids and their affinities for HSA were investigated by fluorescence analysis. The binding process with HSA was strongly influenced by the structural differences of the compounds under study. Methylation of hydroxyl groups improved the affinities for HSA by 2–16-fold. Hydroxylation on rings A, B, and C also affected the affinity for HSA significantly. Glycosylation decreased the affinities for HSA by 1–3 orders of magnitude depending on the conjugation site and the class of sugar moiety. Hydrogenation of the C2 = C3 double bond also decreased the binding affinity. Galloylated catechins and pyrogallol-type catechins exhibited higher binding affinities for HSA than non-galloylated and catechol-type catechins, respectively. The affinities for HSA increased with increasing partition coefficients and decreased with increasing hydrogen bond donor and acceptor numbers of flava(o)noids, which suggested that the binding interaction was mainly caused by hydrophobic forces.

Keywords:
Affinity / Flavanoids / Flavonoids / HSA binding / Structure

1 Introduction

Dietary flavan derivatives are most important polyphenols in plant foods, such as, *e.g.* fruits, vegetables, nuts, and tea [1–5], as they are of great interest for their bioactivities, which are basically related to their antioxidative properties [5–8]. The structural differences between the various classes concern the chemistry of the ring C, as well as the number and distribution of hydroxyl groups and their substitutions on the rings A and B. These differences significantly affect their absorption, metabolism, and bio-activities *in vivo*. For instance, methylation of the free hydroxyl groups in the flavones dramatically increased their intestinal absorption and metabolic stability by preventing the formation of glucuronic acid and sulfate conjugates [9, 10]. Walle concluded that methylation appears to be a simple and effective way to increase the metabolic resistance and transport of flavonoids [11]. Courts and Williamson found that the C-glycoside aspalathin was methylated and glucuronidated *in vivo* in an intact form in human [12]. The flavonol moiety, *i.e.* the 2,3-double bond in conjugation with a 4-oxo group and a 3-hydroxyl group, as well as the 5,7-dihydroxylation at the A-ring has been found to be important structural features for significant antioxidant activity [13]. In addition to –OH moieties in the structural arrangements of flavonoids, the resonance of electrons between the rings A and B was reported to be essential for the antioxidant and biological activities of the compounds [14].

Recently, the interactions between flavonoids and proteins have attracted great interest [15–21]. They result in forming a

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Abbreviations: BCRP, breast cancer resistance protein; **EC,** epicatechin; **ECG,** epicatechin gallate; **EGC,** epigallocatechin; **EGCG,** epigallocatechin gallate; **GCG,** gallocatechin gallate; **TPSA,** topological polar surface area

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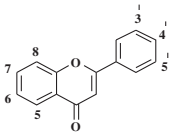
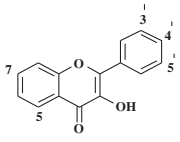
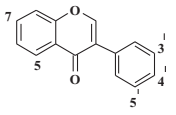
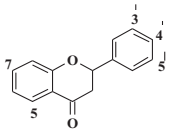
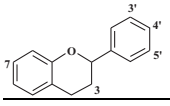
stable protein–flavonoid complex, which may be considered as a model for gaining general fundamental insights into flavonoid–protein binding [20, 21]. Most of the reports focused on the binding process, concerning the forces involved in binding, binding distance, energy transfer, and molecular modeling. Few reports, however, have focused on the structure–affinity relationship of flavonoids on the affinities for proteins. We have studied the effects of the hydroxylation on rings A [22] and B [23] of flavonols, as well as the glycosylation of flavonoids on binding to BSA [24]. The present work concerns the relationship between the molecular properties of dietary flava(o)noids and their affinities for HSA. Thirty-five flavonoids (Table 1) were studied.

2 Materials and methods

2.1 Apparatus and reagents

The fluorescence spectra were recorded on a *JASCO FP-6500* fluorometer (Tokyo, Japan). The pH measurements were carried out on a *Cole-Parmer PHS-3C* Exact Digital pH meter (IL, USA). HSA (~99%, lyophilized powder) and 7-hydroxyflavone (99.5%) were purchased from Sigma (MO, USA). Biochanin A, genistein, apigenin, puerarin, catechin, epicatechin (EC), and luteolin (99.0%) were purchased from Aladin (Shanghai, China). Flavone, chrysin, and baicalein (99.5%) were obtained commercially from Wako Pure

Table 1. Chemical structures of the various flavonoids and their affinities for HSA *in vitro*

Subclass	Name	Substitutions				
		OH	OCH_3	Others	lg K_a	*n*
Flavones	Flavone				4.92	1.052
	7-OH-flavone	7			6.55	1.221
	Chrysin	5,7			6.03	1.110
	Baicalein	5,6,7			5.96	1.138
	Baicalin	5,6		7-β-D-Glucuronide	5.66	1.090
	Apigenin	5,7,4′			6.80	1.265
	Luteolin	5,7,3′,4′			7.70	1.389
	Wogonin	5,7	8		6.37	1.235
	Hispidulin	5,7,4′	6		5.66	1.100
	Tangeretin		5,6,7,8,4′		4.55	0.913
	Nobiletin		5,6,7,8,4′,5′		6.56	1.288
Flavonols	Kaempferide	3,5,7	4′		7.03	1.309
	Kaempferol	3,5,7,4′			5.84	1.131
	Kaempferitrin	5,4′		3,7-Dirhamnoside	4.93	1.045
	Quercetin	3,5,7,3′,4′			6.61	1.267
	Myricetin	3,5,7,3′,4′,5′			6.50	1.177
	Galangin	3,5,7			5.89	1.131
	Fisetin	3,7,3′,4′			5.46	1.065
	Rutin	5,7,3′,4′		3-α-L-Rham-1,6-D-Glc	3.93	0.852
Isoflavones	Daidzein	7,4′			5.38	1.122
	Formononetin	7	4′		6.13	1.281
	Genistein	5,7,4′			4.68	0.955
	Genistin	5,4′		7-Glucoside	3.03	0.762
	Biochanin A	5,7	4′		4.97	0.987
	Tectorigenin	5,7,4′	6		5.41	1.046
	Puerarin	7,4′		8-C-Glucose	3.24	0.751
Flavanone	Naringenin	5,7,4′			4.44	0.922
	Naringin	5,4′		7-Neohesperidose	3.44	0.791
	Dihydromyricetin	3,5,7,3′,4′,5′			4.85	1.030
Flavanonol	GCG (2,3-*trans*)	5,7,3′,4′,5′		3-Gallate	3.81	0.845
	EGCG (2,3-*cis*)	5,7,3′,4′,5′		3-Gallate	4.47	0.975
	ECG (2,3-*cis*)	5,7,4′,5′		3-Gallate	4.32	0.904
	EC (2,3-*cis*)	3,5,7,4′,5′				
	EGC (2,3-*cis*)	3,5,7,3′,4′,5′				
	C (2,3-*trans*)	3,5,7,4′,5′				

Chemical Industries (Osaka, Japan). Kaempferide, kaempferol, tangeretin, nobiletin, quercetin, myricetin, daidzein, baicalin, wogonin, hispidulin, kaempferitrin, galangin, fisetin, genistin, dihydromyricetin, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG), gallocatechin gallate GCG, tectorigenin, and formononetin (>98.0%) were obtained commercially from Tongtian (Shanghai, China). The working solution of the flava(o)noids (1.0×10^{-3} mol/L) was prepared by dissolving each flava(o)noids with methanol. *Tris*-HCl buffer (0.20 M, pH 7.4) containing 0.10 mol/L NaCl was selected to keep the pH value and maintain the ionic strength of the solution. The working solutions of HSA (1.0×10^{-5} mol/L) was prepared with *tris*-HCl buffer and stored in refrigerator prior to use. All other reagents and solvents were of analytical grade and all aqueous solutions were prepared using newly double-distilled water.

2.2 Fluorescence spectra

Different concentrations of flava(o)noids solutions were transferred to 3.0 mL of HSA solution. The resultant mixture was subsequently incubated at 300.15 K for 0.5 h. The results of the time course experiments for the equilibration are not given here. The fluorescence intensity at 340 nm was determined under the excitation wavelength of 280 nm. The fluorescence emissions of these flava(o)noids within the range of 300–350 nm were not observed under the excitation wavelength of 280 nm. The flava(o)noids were stable during the fluorescence measurements, as shown by HPLC analyses (not given here).

3 Results and discussion

3.1 The binding constants K_a and the number of binding sites (*n*)

As representative examples, the fluorescence spectra of HSA after addition of kaempferide and kaempferol are shown in Fig. 1 (The fluorescence spectra of HSA quenched by other flava(o)noids are not given here). In these and all other cases, the fluorescence intensities of HSA decreased remarkably with increasing concentration of flava(o)noids. The obvious blue shifts of the maximum λ_{em} of HSA fluorescence were observed for kaempferol and kaempferide (The λ_{max} values of the first and last spectrum are given in Fig. 1). The molecular conformation of HSA was affected, which is in good agreement with the data of recent similar studies [15–24].

The binding constants were calculated according to the double-logarithm equation [20–25]:

$$\lg(F_0 - F) = \lg K_a + n \lg[Q] \quad (1)$$

where F_0 and F represent the fluorescence intensities of HSA in the absence and in the presence of flava(o)noids, K_a is the binding constant, n is the number of binding sites *per* HSA, and $[Q]$ is the concentration of flava(o)noids. Table 1 summarizes the results correspondingly calculated according to Eq. (1). The values of $\lg K_a$ are proportional to the number of binding sites (n) (Fig. 2), which indicates that Eq. (1) used here is suitable to study the interaction between flava(o)noids and HSA [26, 27].

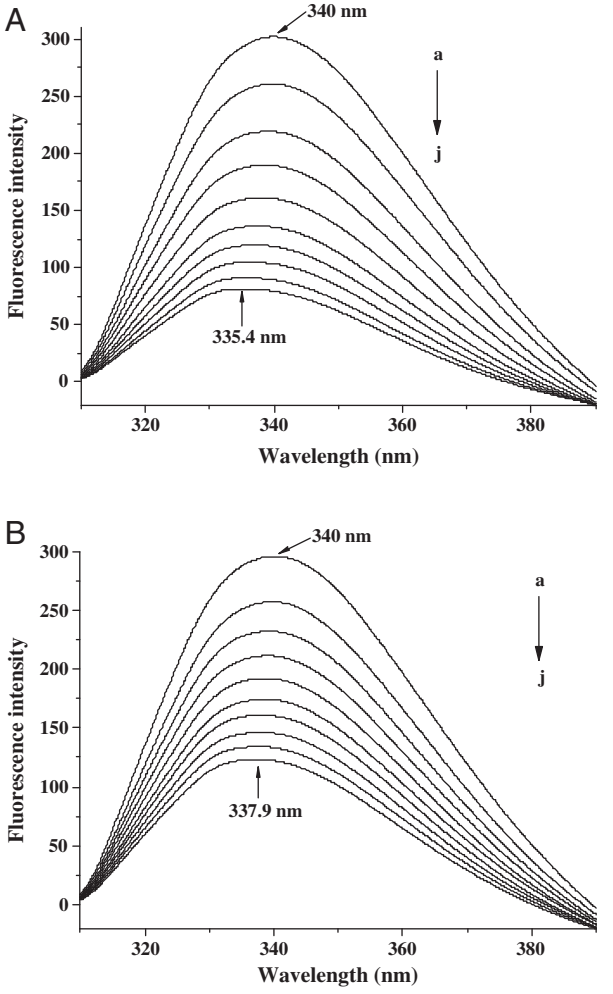


Figure 1. The quenching effect of kaempferide and kaempferol on HSA fluorescence spectra at 300.15 K. $\lambda_{ex} = 280$ nm; HSA, 10.00 μmol/L; a–j: 0.00, 1.00, 2.00 …. 9.00 ($\times 10^{-6}$ mol/L) of kaempferide (A) and kaempferol (B).

3.2 Effects of methylation of hydroxyl group in dietary flavonoids on the affinities for HSA

As shown in Fig. 3, the methylation of hydroxyl group in flavonoids enhanced the binding affinities for HSA. In general, the methylation of hydroxyl group in flavonoids enhanced their binding affinities for HSA by 2–16 times. Extremely, the affinity of kaempferide for HSA was found to be 16 times higher than that of kaempferol (Fig. 3). On the

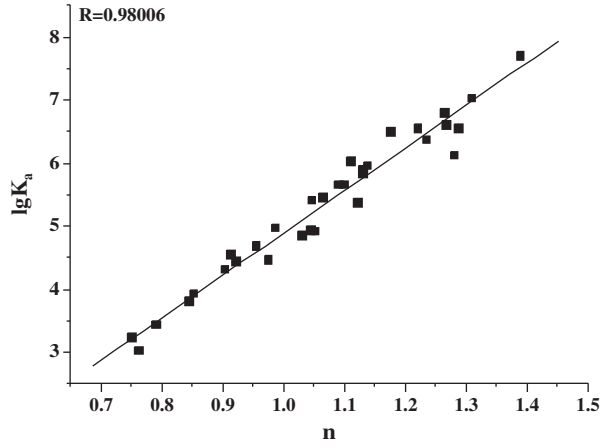


Figure 2. The relationship between the affinities (lg K_a) and the number of binding sites (n) between flava(o)noids and HSA.

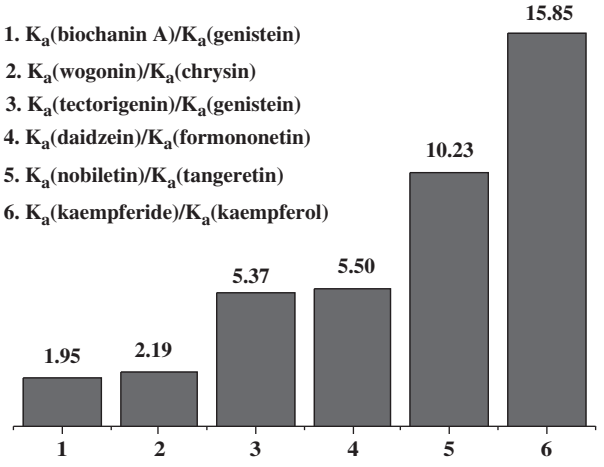


Figure 3. The methoxyl group increases the affinity of the flavonoids for HSA.

other side, the affinity of biochanin A for HSA was only two times higher than that of genistein (Fig. 3). This result supports that the methylation of flavonoids enhanced the transporting ability, which leads to facilitated absorption and greatly increased bioavailability. The methylation increases the hydrophobicity of flavonoids and the hydrophobic interaction plays an important role in binding flavonoids to proteins [28–30]. The capacity to penetrate into the tryptophan-rich hydrophobic regions of proteins, which are frequently buried in the interior of the folded proteins, was enhanced. The methylation of hydroxyl groups in flavonoids also results in increased membrane transport ability, which leads to facilitated absorption and greatly increased bioavailability [10] and results in derivatives with increased intrinsic ability to inhibit cancer cell proliferation [28]. In 2006, an epidemiological study provided strong evidence for the methylated flavones as potential cancer chemopreventive dietary agents [29]. As reported by others [30], the methylation of hydroxyl group at position 4 of flavonoids can substantially increase breast cancer resistance protein (BCRP) inhibition activity [30]. The methylation of hydroxyl groups at position 4′ (biochanin A and kaempferide) enhanced the BCRP inhibition activity when compared with genistein and kaempferol, respectively [30]. Recently Dou and co-workers reported that the methylated flavonoids possess a different mechanism of action compared with unmethylated flavonoids for anticancer activity [31]. The unmethylated flavonoids induced apoptosis through proteasome inhibition, while the methylated flavonoids did not induce apoptosis or proteasome inhibition and act mostly through inducing G0/G1 cell cycle arrest [31]. The potential therapeutic utility of the methylated flavones has also been advanced by finding that the methylation of flavonoids reduces the possibility of toxic side effects [10]. The oligomethoxyflavones occurring in plants are less utilized for human consumption compared with the polymethoxyflavones and polyhydroxyflavones.

3.3 Effects of hydroxylation of flavonoids on the affinities for HSA

Table 2 shows the effects of hydroxylation of flava(o)noids on the affinities for HSA *in vitro*. As seen from the data, hydroxylation on the rings A, B, and C of flava(o)noids significantly affected the binding affinities for HSA.

3.4 Hydroxylation on ring A of flavones

As illustrated in our previous report [22], it appears that the optimal number of hydroxyl groups introduced to ring A of flavones is one, as the highest binding was observed with 7-hydroxyflavone (containing only one hydroxyl group). In cases where more hydroxyl groups are introduced to positions at C-5, C-6, and/or C-7 of flavones, the affinities for serum albumins slightly decreased [22].

3.5 Hydroxylation on ring B of flavones

As shown in Table 2, the apparent binding constants (K_a) between flavones and HSA increased with the increasing numbers of hydroxyl groups on the B ring. The hydroxylation on position 4′ or 3′ of flavone significantly improves the binding affinity for HSA. The affinities of apigenin (5,7,3′) and luteolin (5,7,3′,4′) for HSA were about 5.9 and 7.9 times higher than that of chrysin (5,7) and apigenin (5,7,3′).

3.6 Hydroxylation on ring C of flavones

As shown in Table 2, the hydroxylation on the ring C of flavones decreased the binding affinities for HSA. The

Table 2. Effects of hydroxylation of flavonoids on the affinities for HSA *in vitro*

Class	Ring	Position	Example	Effect (times)
Flavone	A	7 H→OH	Flavone→7-OH-flavone	↑ 42.66
		5 H→OH	7-OH-flavone→chrysin	↓ 3.31
		6 H→OH	Chrysin→baicalein	No effect
	B	4′H→OH	Chrysin→apigenin	↑ 5.89
		3′H→OH	Apigenin→luteolin	↑ 12.59
	C	3 H→OH	Chrysin→galangin	↓ 1.38
			Apigenin→kaempferol	↓ 9.12
			Luteolin→quercetin	↓ 12.30
Flavonol	A	5 H→OH	Fisetin→kaempferol	↑ 2.39
	B	3′H→OH	Kaempferol→quercetin	↑ 5.89
		4′H→OH	Galangin→kaempferol	No effect
		5′H→OH	Quercetin→myricetin	No effect
Isoflavone	A	5 H→OH	Daidzein→genistein	↓ 5.01
			Formononetin→biochanin A	↓ 14.45

affinities of chrysin (5,7), apigenin (5,7,3′), and luteolin (5,7,3′,4′) for HSA are about 1.4, 9.1, and 12.3 times higher than those of galangin (3,5,7), kaempferol (3,5,7,3′), and quercetin (3,5,7,3′,4′) for HSA.

3.7 Hydroxylation of rings A and B of flavonols

In an earlier study we reported that the binding constants (K_a) and the number of binding sites (n) between flavonols and BSA increased with the increased hydroxyl groups on ring B [23]. Addition of another hydroxyl group on ring B of flavonols enhanced the affinity for BSA by 1 order of magnitude [23]. In this study, however, it was found that the hydroxylation on position 3′ of flavonol significantly improves the binding affinity for HSA and the hydroxylation on positions 4′ and 5′ of flavonol hardly affected their binding affinities for HSA. The affinity of quercetin (3′, 4′) for HSA was about six times higher than that of kaempferol (4′). The affinity of myricetin (3′, 4′, 5′) for HSA was almost the same as that of quercetin (3′, 4′) and the affinity of kaempferol (4′) for HSA was similar to that of galangin (no hydroxyl groups on ring B). Flavonols are the most prominent flavonoids in plants. The most prominent flavonols such as quercetin and kaempferol in foods exist with 5,7-dihydroxyl groups on ring A. Here, it was found that the hydroxylation on position 5 of flavonol slightly enhances the binding affinity for HSA by 2.4 times.

3.8 Hydroxylation on ring A of isoflavones

As shown in Table 2, the hydroxylation on position 5 of isoflavones decreased the binding affinity for HSA. The affinities of daidzein and formononetin for HSA were about five times and 14 times higher than that of genistein and biochanin A, respectively.

3.9 Comparing the affinities of flavonoid isomers with HSA

Previously, we have compared the affinities of flavonoid isomers with BSA and found the apparent binding constants (Ka) were determined as: flavone > isoflavone ≈ flavonol [32]. In this study, we can compare two isomer groups (apigenin, baicalein, genistein and luteolin, kaempferol). The binding constants (Ka) were determined as: apigenin > baicalein > genistein and luteolin > kaempferol. These results are in good agreement with data reported previously about the interaction between flavonoid isomers with BSA [32].

3.10 Effects of glycosylation of dietary flavonoids on the affinities for HSA

The dietary flavonoids in nature occur mostly as β-glycosides. The flavonols are found mainly as the 3- and 7-*O*-glycoside, although the 4′ position may also be glycosylated in some plants (Table 1). Dufour and Dangles determined the binding constants between the flavonol (quercetin) and its three-positioned glycosides with serum albumin and concluded that glycosylation of flavonoids could lower the affinity to albumins by 1 order of magnitude depending on the conjugation site [33, 34]. Most recently, we have reported the glycosylation of flavonoids lowered the affinity for BSA by 1–3 orders of magnitude depending on the conjugation site and the class of sugar moiety [24].

Herein, the effect of glycosylation of dietary flavonoids on the affinities for HSA was investigated. The sugar moieties are in three or seven positions of flavonoids. In our present study (Fig. 4), the glycosylation of flavonoids lowered the affinity for HSA by 1–2 orders of magnitude. The affinity of quercetin for HSA was about 478.6-fold higher than that of rutin, but the affinity of baicalein for HSA is only two times

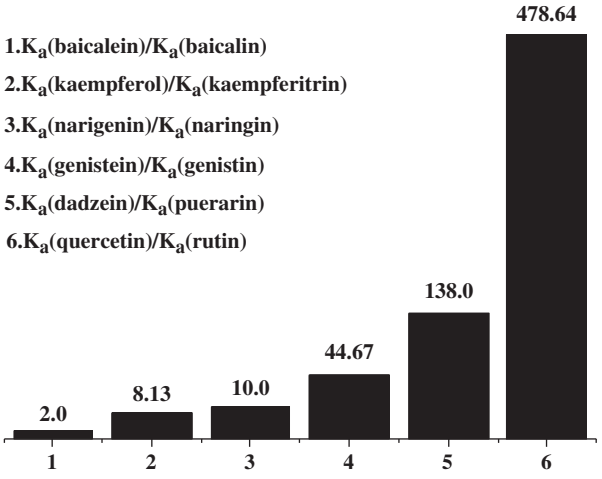


Figure 4. Glycosylation decreases the affinity of the flava(o)noids for HSA.

higher than that of baicalin. The glucopyranosylation (genistin) of genistein lowered the affinity for HSA by 44.67 times. Compared with the affinity of daidzein for HSA, the affinity of puerarin (daidzein-8-C-glucose) for HSA decreased 138 times. The decreasing affinity for HSA after glycosylation may be caused by the non-planar structure. After the hydroxyl group is substituted by a glycoside, steric hindrance may take place, which weakens the affinity for has [24].

3.11 Effects of hydrogenation of the C2 = C3 double bond of dietary flavonoids on the affinities for HSA

The C2 = C3 double bond in conjugation with a 4-oxo group plays a very important role for the affinity for HSA. It was found that hydrogenation of the C2 = C3 double bond of flavonoids decreased the binding affinties for HSA. As shown in Table 1, the affinities of apigenin and myricetin for HSA were about 250 times and 45 times higher than those of naringenin and dihydromyricetin, respectively. Previously, we have investigated the effect of hydrogenation of the C2 = C3 double bond in flavonoids on the affinities for BSA [35]. Hydrogenation of the C2 = C3 double bond for many flavonoids decreased the binding affinty for BSA by 2–4 orders of magnitude. Planarity of the C ring in flavonoids may be important for binding interaction with proteins, as the molecules with saturated C2-C3 bonds (flavanones and certain others) permit more twisting of the B ring with reference to the C ring. A C2 = C3 double bond increases the p-conjugation of the bond linking the B and C rings, which favors near-planarity of the two rings [36]. Molecules with near-planar structure easier enter the hydrophobic pockets in proteins.

3.12 Catechins

Catechins are the major polyphenols in green tea leaves. The major catechins of greenextract are (−)-catechin, (−)-epicatechin (EC), (−)-EGC, (−)-ECG, (−)-EGCG, and GCG. Recent studies have suggested that the catechins form complexes with HSA for transport in human blood, and their binding affinity for albumin is believed to modulate their bioavailability. Here, we determined the affinities between catechins and HSA by fluorescence quenching method with double logarithm regression curve. The binding constants ($\log_{10}K_a$) between ECG, EGCG, and GCG for HSA were 4.32, 4.47, and 3.81, respectively. However, EC, EGC, and catechin hardly quenched the fluorescence of HSA. It illustrates that the galloylated catechins have higher binding affinities with HSA than non-galloylated catechins and the pyrogallol-type catechins had higher affinities than catechol-type catechins. The presence of the galloyl moiety is the most decisive factor; the increasing hydroxyl groups on ring B increases the affinity for HSA. In our present study, the affinity of the catechin with 2,3-*trans* structure (GCG) for HSA was lower than that of the catechin with 2,3-*cis* structure (EGCG).

3.13 Relationship of partition coefficient and the affinity for HSA

The lipophilicity of the compounds under study was assessed by their partition coefficient values ($XLogP_3$) according to PubChem Public Chemical Database (http://pubchem.ncbi.nlm.nih.gov/). There is a relationship between the $XlogP_3$ values and lg K_a values for flava(o)noids (figure not shown). The linear regression equation using the Origin 7.5 software was $XlogP_3 = -0.604750 + 0.44618\lg K_a$ ($R = 0.45055$). The affinities of flavonoids increased with increasing partition coefficient. From this point, the binding interaction between flava(o)noids and HSA was mainly caused by hydrophobic forces. HSA consists of large number of NH, OH, and COOH groups, which indicates that HSA is a highly polar macromolecule.

To further investigate whether or not the hydrogen bond force plays an important role in binding flava(o)noids to HSA, the relationships of the hydrogen bond acceptor/donor numbers (*N*, data were from the Pubchem Public Chemical Database) of flava(o)noids with the affinities for HSA are shown in Fig. 5. The affinities for HSA obviously decreased with increasing hydrogen bond donor and acceptor numbers of flava(o)noids. These results support the above-mentioned conclusion that the hydrophobic interaction is the main force to bind flava(o)noids to HSA.

3.14 Relationship of topological polar surface area and the affinity for HSA

The topological polar surface area (TPSA) is defined as the sum of surfaces of polar atoms in a molecule. TPSA has

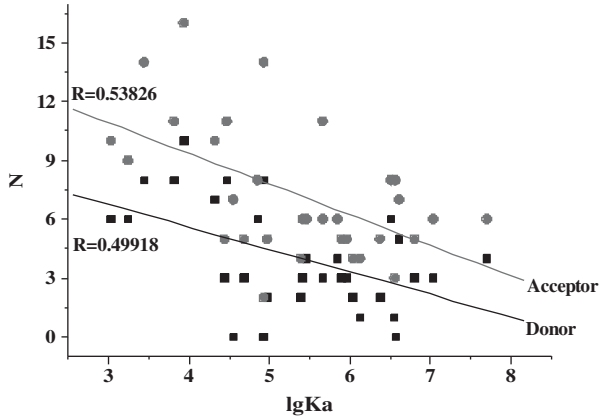


Figure 5. Relationships of the hydrogen bond acceptor/donor number of flava(o)noids (*n*) with the affinities for HSA. The hydrogen bond acceptor/donor numbers were taken from PubChem Public Chemical Database.

been shown to be a very good descriptor characterizing drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability, and blood–brain barrier penetration. Fernandes and Gattass used TPSA to analyze drug transport by multidrug resistance-associated protein 1 (MRP1/ABCC1) [37]. The compounds with high TPSA are transported while those with low TPSA are not. A strong correlation between TPSA and transport properties (K_m) was also found. In our present study, the relationship between TPSA and the binding affinity for HSA was studied. The TPSA values were obtained from PubChem Public Chemical Database [37]. There is a relation between the TPSA values and lg K_a values for flava(o)noids. TPSA values were found to decrease with the increasing lg K_a for flava(o)noids (figure was not shown here). The linear regression equation using the Origin 7.5 software was TPSA = 263.58031-26.90789 lg K_a ($R = 0.53291$). The flava(o)noids with low TPSA are bound tightly while those with high TPSA are not.

4 Concluding remarks

Some of the structural elements that influence the affinities of flava(o)noids for HSA are the following: (i) one or more hydroxyl groups in the B ring (*e.g.* 3′,4′-dihydroxylatedcatechol group) of flavonoids enhanced the binding affinity for HSA. However, the hydroxyl group in C-ring weakens the affinity; (ii) presence or absence of an unsaturated 2,3-bond in conjugation with a 4-carbonyl group, characteristic of flavonols structure, has been associated with stronger binding affinity with HSA; (iii) glycosylation decreases the affinities for HSA by 1–3 orders of magnitude depending on the conjugation site and the class of sugar moiety; (iv) methylation of hydroxyl groups enhanced the affinities for HSA by 2–16 times; (v) galloylated catechins and pyrogallol-type catechins exhibited higher binding affinities for HSA than non-galloylated and catechol-type catechins, respectively.

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