REPORT

Molecular structure-affinity relationship of natural polyphenols for bovine γ-globulin

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Scope: The aim of this study was to investigate the interaction between polyphenols and bovine γ-globulin.
Methods and results: The relationship between the structural properties of natural polyphenols and their affinities for bovine γ-globulin were investigated by fluorescence titration analysis. Methylation of hydroxyl groups on flavonoids weakened the affinities for γ-globulin by 1.20–38.0 times. Hydroxylation on rings A, B, and C of flavonoids also significantly affected the affinity for γ-globulin. Glycosylation of flavonoids slightly affected the affinity depending on the conjugation site and the class of sugar moiety. Hydrogenation of the C2═C3 double bond on flavonoids decreased the binding affinities. Galloylated catechins and catechol-type catechins exhibited higher binding affinities for γ-globulin than non-galloylated and pyrogallol-type catechins. The glycosylation of resveratrol decreased its affinity for γ-globulin.
Conclusion: The binding process with γ-globulin was strongly influenced by the structural differences of polyphenols.

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Affinity / γ-Globulin / Polyphenols / Protein binding / Structure

1 Introduction

Dietary flavonoids and stilbenes are important polyphenols in plant foods, such as fruits, vegetables, nuts, and tea [1–4], as they are of great interest for their bioactivities, which are basically related to their antioxidative properties [5–7]. The structural differences between the various classes of polyphenols significantly affect their absorption, metabolism, and bio-activities *in vivo* [8].

Polyphenols in plasma are bound to proteins such as HSA, a_1-acid glycoprotein, lipoproteins, and globulins to some degree. The polyphenols-protein interaction is reversible in that the polyphenols-protein complex can dissociate and release the free polyphenols [9]. Polyphenols and their metabolites rapidly exchange between free and bound forms within the circulation. The reversible binding to plasma proteins may have consequences for the delivery of the polyphenols and their metabolites to cells and tissues [10].

γ-Globulin is a class of globulins in the plasma of humans and other mammals that function as part of the body's immune system. γ-Globulin is capable of binding an extraordinarily diverse range of metabolites, drug, organic compounds, and relevant antigens [11]. Recently, the interactions between polyphenols with immuno-globulins have attracted great interest [12, 13]. Few reports, however, have focused on the structure-affinity relationship of polyphenols on their affinities for γ-globulin. The present work concerns about the relationship between the structure properties of natural polyphenols and their affinities for γ-globulin. Forty-two polyphenols (Table 1) were studied.

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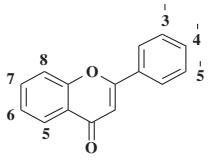
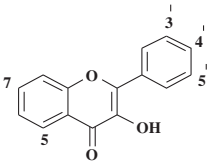
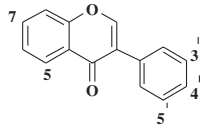
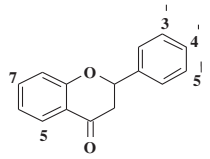
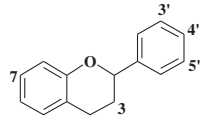
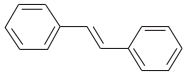
Abbreviations: C, catechin; **EC,** epicatechin; **EGC,** epigallocatechin; **EGCG,** epigallocatechin gallate; **GC,** gallocatechin; **GCG,** gallocatechin gallate

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

Table 1. Chemical structures of the various polyphenols and their affinities for γ-globulin *in vitro*

Subclass	Name	Substitutions				
		OH	OCH_3	Others	lgK_a	*n*
Flavones	Flavone				4.26	0.926
	7-Ohflavone	7			4.31	0.973
	Chrysin	5,7			4.38	0.954
	Baicalein	5,6,7			4.73	1.022
	Baicalin	5,6		7-β-D-Glucuronide	4.30	0.917
	Apigenin	5,7,4′			4.32	0.966
	Luteolin	5,7,3′,4′			4.84	1.021
	Hispidulin	5,7,4′	6		3.99	0.869
	Tangeretin		5,6,7,8,4′		5.00	1.026
	Nobiletin		5,6,7,8,4′,5′		4.79	0.983
Flavonols	Kaempferide	3,5,7	4′		4.32	0.965
	Kaempferol	3,5,7,4′			4.40	0.948
	Kaempferitrin	5,4′		3,7-Dirhamnoside	4.63	1.005
	Quercetin	3,5,7,3′,4′			4.47	0.957
	Quercitrin	5,7,3′,4′		3-*o*-β-D-Glucoside	3.44	0.797
	Myricetin	3,5,7,3′,4′,5′			4.75	1.041
	Galangin	3,5,7			3.86	0.888
	Fisetin	3,7,3′,4′			4.13	0.926
	Rutin	5,7,3′,4′		3-α-L-Rham-1,6-D-Glc	4.92	1.083
Isoflavones	Daidzein	7,4′			4.85	1.110
	Daidzin	4′		7-Glucoside	3.99	0.944
	Formononetin	7	4′		3.28	0.797
	Genistein	5,7,4′			4.93	1.073
	Genistin	5,4′		7-Glucoside	4.49	1.051
	Biochanin A	5,7	4′		4.11	0.927
	Tectorigenin	5,7,4′	6		4.17	0.920
	Puerarin	7,4′		8-C-glucose	4.77	1.083
Flavanone	Naringenin	5,7,4′			3.76	0.869
	Hesperitin	5,7,3′	4′		5.11	1.112
	Naringin	5,4′		7-Neohesperidose	4.50	1.007
	Narirutin	5,4′		7-α-L-Rham-1,6-D-Glc	4.42	0.997
	Dihydromyricetin	3,5,7,3′,4′,5′			4.17	0.952
Flavanonol	GCG (2,3-*trans*)	5,7,3′,4′,5′		3-Gallate	3.13	0.767
	EGCG (2,3-*cis*)	5,7,3′,4′,5′		3-Gallate	4.14	0.998
	ECG (2,3-*cis*)	5,7,4′,5′		3-Gallate	4.32	0.978
	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′				
	GC (2,3-*trans*)	3,5,7,3′,4′,5′				
Stilbene	Resveratrol	3,5,4′			5.57	1.222
	Polydatin	5,4′		3-Glucoside	4.63	0.995

carried out on a Cole-Parmer PHS-3C Exact Digital pH meter (IL, USA). γ-Globulin (~99%) and 7-hydroxyflavone (99.5%) were purchased from Sigma (MO, USA). Biochanin A, genistein, apigenin, puerarin, catechin (C), epicatechin (EC), and luteolin, daidzin, resveratrol, polydatin, hesperitin, and quercetin-3-*o*-β-D-glucoside (99.0%) were purchased from Aladin (Shanghai, China). Flavone, chrysin, and baicalein (99.5%) were obtained commercially from Wako Pure Chemical Industries (Osaka, Japan). Other polyphenol standards (>98.0%) were obtained commercially from Shanghai Tauto Biotech (Shanghai, China). The working solution of the polyphenol (1.0×10^{-3} mol/L) was prepared by dissolving each polyphenol with methanol. Tris-HCl buffer (0.20 mol/L, 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 γ-globulin (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

The fluorescence spectra were recorded in the wavelength range of 310–450 nm upon excitation at 295 nm when γ-globulin samples were titrated with polyphenols. Slit widths, scan speed, and excitation voltage were kept constant within each data set and each spectrum was the average of three scans. 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 the concentrations of γ-globulin at 1.0×10^{-5} mol/L. The results of the time course experiments for the equilibration are not given here. The fluorescence emissions of these polyphenols within the range of 300–400 nm were not observed under the excitation wavelength of 295 nm. The polyphenols were stable during the fluorescence measurements, as shown by HPLC analyses (not given here). Each fluorescence intensity determination was repeated and found to be reproducible within experimental errors. The binding experiments for the determination of binding constants for protein were repeated three times. The experimental errors were less than 4.0%.

3 Results and discussion

3.1 Quenching effect of polyphenols on γ-globulin fluorescence

As representative examples, the fluorescence spectra of γ-globulin after addition of tangeretin, chrysin, gallocatechin gallate (GCG), and naringenin are shown in Supporting Information (The fluorescence spectra of γ-globulin quenched by other polyphenols are not given here). Except for EC, epigallocatechin (EGC), gallocatechin (GC), and C, all polyphenols tested can quench the fluorescence of γ-globulin remarkably with increasing concentration of polyphenols. There are no obvious shifts of the maximum λ_{em} of γ-globulin fluorescence for polyphenols tested, which is different with the data of recent similar studies for BSA [14–17].

The quenching ratio (F/F_0) of γ-globulin fluorescence with addition of tangeretin, chrysin, naringenin, and GCG were shown in Fig. 1. The intensities of γ-globulin fluorescence decreased rapidly with the addition of tangeretin and chrysin. However, GCG and naringenin quenched the γ-globulin fluorescence slowly. About 8.0 μmol/L of tangeretin was found to lead to 37.5% γ-globulin fluorescence quenching and GCG only quenched 14.9% γ-globulin fluorescence. These results indicated that the quenching effect of polyphenols on γ-globulin fluorescence depended on the structures of polyphenols.

Fluorescence quenching was described by the Stern–Volmer equation [18]:

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

where F_0 and F represent the fluorescence intensities of γ-globulin in the absence and in the presence of polyphenols, K_q is the quenching rate constant, K_{SV} is the dynamic quenching constant, τ_0 is the average lifetime, and $[Q]$ is the concentration of polyphenols.

Figure 2 showed the Stern–Volmer plots for γ-globulin fluorescence quenching by tangeretin, chrysin, naringenin, and GCG. As seen from Fig. 2, the Stern–Volmer plots for tangeretin are linear. However, it was found that both dynamic and static quenching were involved for chrysin, GCG, and naringenin on γ-globulin fluorescence, which demonstrated by the fact that the Stern–Volmer plots slightly deviated from linearity toward the *y*-axis at higher polyphenols concentrations. In the linear range of Stern–Volmer regression curve, the average quenching constants (K_{SV}) for tangeretin, chrysin,

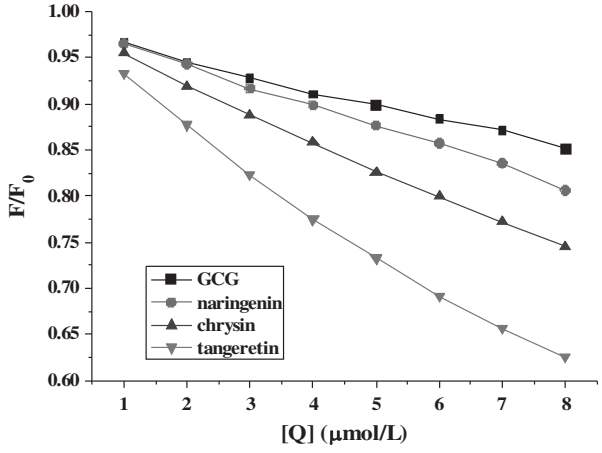


Figure 1. The quenching ratio (F/F_0) of γ-globulin fluorescence spectra with addition of polyphenols.

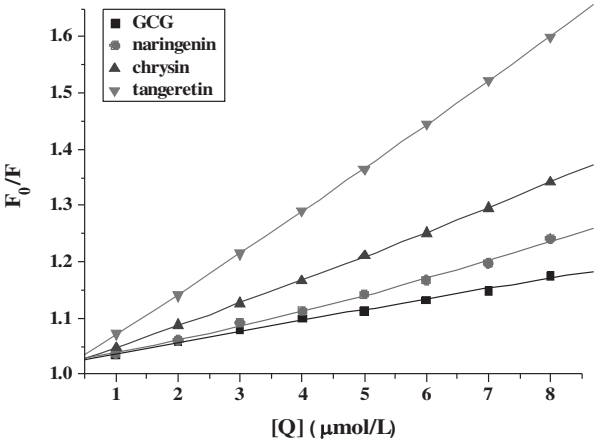


Figure 2. The Stern–Volmer plots for γ-globulin fluorescence quenching by polyphenols at 300.15 K.

naringenin, and GCG (having the lowest quenching effect, figures are not shown) at 300.15 K were determined as 7.32, 3.94, 2.59, and 2.14×10^5 L/mol, respectively.

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

The binding constants were calculated according to the double-logarithm equation [18]:

$$\lg[(F_0 - F)/F] = \lg K_a + n\lg[Q] \quad (2)$$

where F_0 and F represent the fluorescence intensities of γ-globulin in the absence and in the presence of polyphenols, K_a is the binding constant, n is the number of binding sites *per* γ-globulin, and [Q] is the concentration of polyphenols. Table 1 summarized the results correspondingly calculated results according to Eq. (2). The values of $\lg K_a$ were linear to the number of binding sites (n) (Fig. 3; $\lg K_a = -0.63043 + 0.51431n$, $R^2 = 0.94462$), which indicated that the Eq. (2) used here is suitable to study the interaction between polyphenols and γ-globulin [19, 20]. The magnitudes of apparent binding constants for γ-globulin were almost in the range of 10^3–10^5 L/mol, which were similar to recent report for γ-globulin by He *et al.* [12]. However, these data were much smaller than the affinities of polyphenols for BSA and HSA from our previous reports (10^4–10^8/M) [14–17].

3.3 Methylation of hydroxyl groups on flavonoids

As shown in Fig. 4, the methylation of hydroxyl group in flavonoids weakened the binding affinities for γ-globulin. In general, the methylation of hydroxyl group in flavonoids decreased their binding affinities for γ-globulin by 1.2–38.0 times. Extremely, the affinity of dadzein for γ-globulin was found to be 38 times higher than that of its methylated form (formononetin). On the other side, the affinity of keampferol for γ-globulin was almost the same as that of keampferide.

3.4 Hydroxylation of flavonoids

3.4.1 Hydroxylation on ring A of flavones

As illustrated in Table 2, the apparent binding constants (K_a) between flavones and γ-globulin increased with the increasing numbers of hydroxyl groups on the A-ring. It appears that the optimal number of hydroxyl groups introduced to the ring A of flavones is three, as the highest binding was observed with baicalein (3 hydroxyl groups on ring A).

3.4.2 Hydroxylation on ring B of flavones

As seen from the data in Table 2, hydroxylation on position 4′ of flavone significantly improves the binding affinity for γ-globulin. The affinity of luteolin (5,7,3′,4′) was about 3.31 times higher than that of apigenin (5,7,3′) for γ-globulin. However, the hydroxylation on position 3′ of flavone hardly affected the binding affinity for γ-globulin. The affinity of apigenin (5,7,3′) is almost the same as that of chrysin (5,7).

3.4.3 Hydroxylation on ring C of flavones

As shown in Table 2, it appears that the hydroxylation on the ring C of flavones decreased the binding affinities for γ-globulin. The affinities of chrysin (5,7) and luteolin

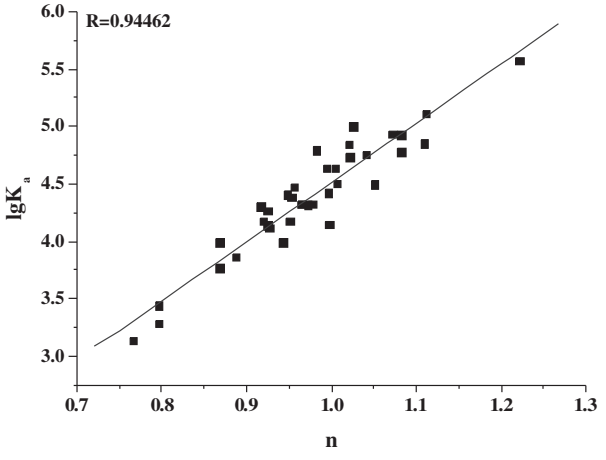


Figure 3. The relationship between the affinities ($\lg K_a$) and the number of binding sites (n) between polyphenols and γ-globulin.

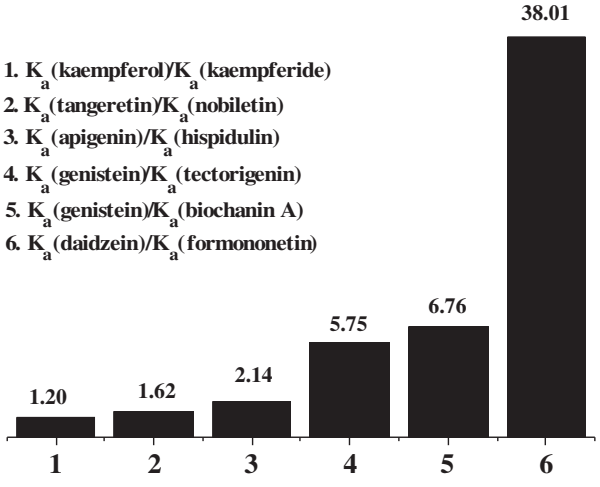


Figure 4. The methylation of hydroxyl group weakens the affinity of the flavonoids for γ-globulin.

Table 2. Effects of hydroxylation of flavonoids on the affinities for γ-globulin *in vitro*

Class	Ring	Position	Example	Effect (times)
Flavone	A	7 H→OH	Flavone→7-Ohflavone	↑ 1.12
		5 H→OH	7-Ohflavone→chrysin	↑ 1.17
		6 H→OH	Chrysin→baicalein	↑ 2.24
	B	3′H→OH	Chrysin→apigenin	No effect
		4′H→OH	Apigenin→luteolin	↑ 3.31
	C	3 H→OH	Chrysin→galangin	↓ 3.31
			Apigenin→kaempferol	No effect
			Luteolin→quercetin	↓ 2.40
Flavonol	A	5 H→OH	Fisetin→quercetin	↑ 2.14
	B	3′H→OH	Kaempferol→quercetin	↑ 1.17
		4′H→OH	Galangin→kaempferol	↑ 3.47
		5′H→OH	Quercetin→myricetin	↑ 1.91
Isoflavone	A	5 H→OH	Daidzein→genistein	↑ 1.17
			Daidzin→genistin	↑ 3.16
			Formononetin→biochanin A	↑ 6.60

(5,7,3′,4′) for γ-globulinare about 3.31 and 2.40 times higher than those of galangin (3,5,7) and quercetin (3,5,7,3′,4′) for γ-globulin. The affinity of apigenin (5,7,3′) for γ-globulin is almost the same as that of kaempferol (3,5,7,3′).

3.4.4 Hydroxylation of rings A and B of flavonols

Addition of another hydroxyl group on the ring B of flavonols slightly enhanced the affinity for γ-globulin. The affinity of kaempferol (4′) for γ-globulin is about 3.47 times higher than does galangin (no hydroxyl groups on ring B). The affinity of quercetin (3′,4′) for γ-globulin is about 1.17 times higher than that of kaempferol (4′). The affinity of myricetin (3′,4′,5′) for γ-globulin was about 1.91 times higher than that of quercetin (3′,4′).

3.4.5 Hydroxylation on ring A of isoflavones

As shown in Table 2, the hydroxylation on position 5 of isoflavones increased the binding affinity for γ-globulin. The affinities of genistein, genistin and biochanin A for γ-globulin were about 1.17, 3.16, and 6.60 times higher than those of daidzein, daidzin, and formononetin, respectively.

3.4.6 Comparing the affinities of flavonoid isomers with γ-globulin

The affinities of flavonoid isomers with γ-globulin were determined as: isoflavone > flavone > flavonol. In this study, we can compare two isomer groups (apigenin, baicalein, genistein and luteolin and kaempferol). The binding constants (K_a) were determined as: genistein > baicalein > apigenin and luteolin > kaempferol.

3.5 Glycosylation of flavonoids

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). Herein, the effect of glycosylation of dietary flavonoids on the affinities for γ-globulin was investigated. The sugar moieties are glucopyranose, glucuronic acid, rhamnose, rutinose, and glucose-rhamnose. In our present study (Fig. 5), the glycosylation of flavonoids slightly affected the affinity for γ-globulin depending on the conjugation site and the class of sugar moiety.

As shown in Fig. 5, the 7-glucopyranosylation of genistin, 8-C-glucosylation of daidzein and 7-β-D-glucuronidation of baicalein lowered the affinity for γ-globulin. The affinities of dadzein, baicalein, and genistein for γ-globulin were about 1.20, 2.70, and 2.75 times higher than do puerarin, baicalin, and genistin. However, the affinities of rutin, kaempferitrin, naringin, and narirutin for γ-globulin were higher than their unglycosylated forms.

3.6 Hydrogenation of the C2=C3 double bond of flavonoids

The C2=C3 double bond in conjugation with a 4-oxo group plays a very important role for the affinity for γ-globulin. It was found that hydrogenation of the C2=C3 double bond of flavonoids decreased the binding affinties for γ-globulin. As shown in Table 1, the affinities of apigenin and myricetin for γ-globulin were about 3.80 times higher than those of

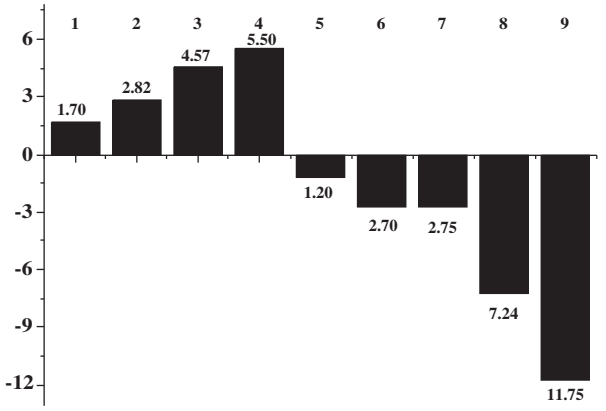


Figure 5. Effect of glycosylation on the affinity of the flavonoids for γ-globulin. 1. K_a(kaempferitrin)/K_a(kaempferol); 2. K_a(rutin)/K_a(quercetin); 3. K_a(narirutin)/K_a(narigenin); 4. K_a(naringin)/K_a(narigenin); 5. K_a(dadzein)/K_a(puerarin); 6. K_a(baicalein)/K_a(baicalin); 7. K_a(genistein)/K_a(genistin); 8. K_a(dadzein)/K_a(dadzin); 9. K_a(quercetin)/K_a(quercitrin).

naringenin and dihydromyricetin. Previously, we have investigated the effect of hydrogenation of the C2═C3 double bond in flavonoids on the affinities for BSA [21]. 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 maybe 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 [22]. Molecules with near-planar structure easier enter the hydrophobic pockets in proteins. As shown in Table 1, the combination of hydroxylation and methylation for naringenin will significantly improve its affinity for γ-globulin.

3.7 Catechins

Catechins are the major polyphenols in green tea leaves. The major catechins of green tea extract are (-)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate, (-)-epigallocatechin gallate (EGCG), GC and GCG. Recent studies have suggested that the catechins form complexes with proteins for transport in human blood, and their binding affinity for proteins is believed to modulate their bioavailability. Here, the affinities between catechins and γ-globulin were determined by fluorescence quenching method with double logarithm regression curve. The binding constants ($\lg K_a$) between epicatechin gallate, EGCG, and GCG for γ-globulin were 4.32, 4.14, and 3.13, respectively. However, EC, EGC, GC, and C hardly quenched the fluorescence of γ-globulin. It illustrates that the galloylated catechins have higher binding affinities with γ-globulin 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. In our present study, the affinity of C with 2,3-*trans* structure (GCG) for γ-globulin was much lower than that of C with 2,3-*cis* structure (EGCG).

3.8 Stilbenes

Stilbenes are important polyphenols with the C_6-C_2-C_6 structure. The typical natural stilbenes are resveratrol and its 3-glucoside, polydatin. The glycosylation of resveratrol obviously weakened the affinity for γ-globulin. The affinity of resveratrol for γ-globulin was about 8.71 times higher than that of polydatin. The decreasing affinity for protein after glycosylation may be caused by the non-planar structure. After the hydroxyl group is substituted by a glycoside, the steric hindrance may take place [17].

4 Concluding remarks

Some of the structural elements that influence the affinities of polyphenols for γ-globulin are the following: (i) one or more hydroxyl groups in the rings A and B (e.g. 3′,4′-dihydroxylatedcatechol group) of flavonoids enhanced the binding affinity for γ-globulin. 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 γ-globulin; (iii) glycosylation of flavonoids affected the affinities for γ-globulin by 1 order of magnitude depending on the conjugation site and the class of sugar moiety; (iv) methylation of hydroxyl groups on flavonoids weakened the affinities for γ-globulin by 1.20–38.0 times; (v) galloylated catechins and catechol-type catechins exhibited higher binding affinities for γ-globulin than non-galloylated and pyrogallol-type catechins, respectively; (vi) glycosylation of resveratrol decreased its affinity for γ-globulin.

Polyphenol-protein interaction in blood is a complicated issue. There are many small molecules, such as metal ions, glucose, fatty acids, and metabolites in blood. Recently, it was found that the metal ions such as $Al3^+$, $Zn2^+$, $Cu2^+$, and $Fe3^+$ significantly affect the interaction of several falvonoids for albumin *in vitro* [23]. The concentrations of metal ions in normal human blood and patient blood are different from each other. The detailed reports on difference of polyphenol-protein interaction in normal human blood and patient blood were few till now. The further work will focus on the effects of metal ions and glucose in serum on the interaction between polyphenols and normal human/patient plasma proteins *in vitro* and *in vivo*.

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