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Influence of B-Ring Hydroxylation on Interactions of Flavonols with Bovine Serum Albumin

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The B-ring substitution pattern of flavonols is a significant structural feature for their function as free radical scavengers and antioxidants. In this paper, four differently substituted B-ring hydroxylation flavonols (galangin, kaempferol, quercetin, and myricetin) and a flavonol glycoside (quercitrin) were studied for their ability to bind BSA by quenching the protein intrinsic fluorescence. From the spectra obtained, the biomolecular quenching constants, the apparent static binding constants, and the binding site values were calculated. The B-ring hydroxylation of flavonols significantly affected the binding/quenching process; in general, the binding affinity increased with the number of hydroxyl groups on the B-ring. The binding constants (K_a) were determined as myricetin (4.90×10^8 L/mol) > quercetin (3.65×10^7 L/mol) > kaempferol (2.57×10^6 L/mol) > galangin (6.43×10^5 L/mol). The glycoside substitute at the C-ring position decreased the binding affinity. The chromatographic retention factor (K') and logarithms of apparent partition coefficient (log K_{ow}) were linear to the logarithms of apparent binding constants (log K_a) for flavonols with increasing hydroxyl groups on the B-ring. These results showed that the hydrogen bond force play an important role in binding flavonols to BSA. These results are also in agreement with the generally accepted structure-dependent free radical scavenger and antioxidant abilities of flavonols.

KEYWORDS: Bovine serum albumin; flavonol; fluorescence quenching; partition coefficient; chromatographic retention factor; free radical scavenger activity; antioxidant activity

INTRODUCTION

Flavonoids are the important phytonutrient components present in a wide range of fruits, vegetables, nuts, and beverages, including wine and tea (*1, 2*). Flavonols are polyphenol compounds possessing two benzene rings joined by a linear three carbon chain (C2, C3, C4), represented as the C6−C3−C6 system. The flavonol moiety (2,3-double bond in conjugation with a 4-oxo group and a 3-hydroxyl group) and the 5,7-dihydroxylation at the A-ring are important structural features for significant antioxidant activity (*3*). In addition to –OH moieties in the structural arrangements of flavonols, the resonance of electrons between the A- and B-rings is very important for their antioxidant and biological activities (*4*). The B-ring –OH moiety is the most significant determinant factor in the scavenging of reactive oxygen species (*5*).

Galangin (**Figure 1**) is present in *Alpinia officinarum,* honey, and propolis. The latter two drugs were used in many countries to treat respiratory and viral infections. Galangin exerted antioxidative (*6*) and antibacterial activities (*7*) and an antiproliferative effect on HL-60 cells (*8*). Kaempferol (**Figure 1**) is widely present in broccoli, *Ginkgo biloba*, fruits, and vegetables (*2*). Several biological activities have been attributed to kaempferol, including antioxidant (*9*) and antiatherogenic activities (*10*) and inhibition of iron bioavailability (*11*). Quercetin (**Figure 1**) is the most abundant bioflavonoid found in vegetable and fruits, and this compound is mainly present in the glycoside form, for example, as quercitrin. Quercetin, in addition to having antibacterial and antioxidant activities (*12*), has been suggested to inhibit monoamine oxidases (*13*) and increase oxidative stress resistance and longevity in *Saccharomyces cerevisiae* cells (*14*). Myricetin (**Figure 1**) is present in a large number of plants, including tea, berries, fruits, vegetables, and medicinal herbs. It has shown strong antioxidative (*15*) and antiradical activities (*16*). Quercitrin, the 3-*O*-β-glucoside of quercetin (**Figure 1**), is a flavonol glycoside. Studies have demonstrated that when flavonols are present in the diet as aglycons, they could be partly

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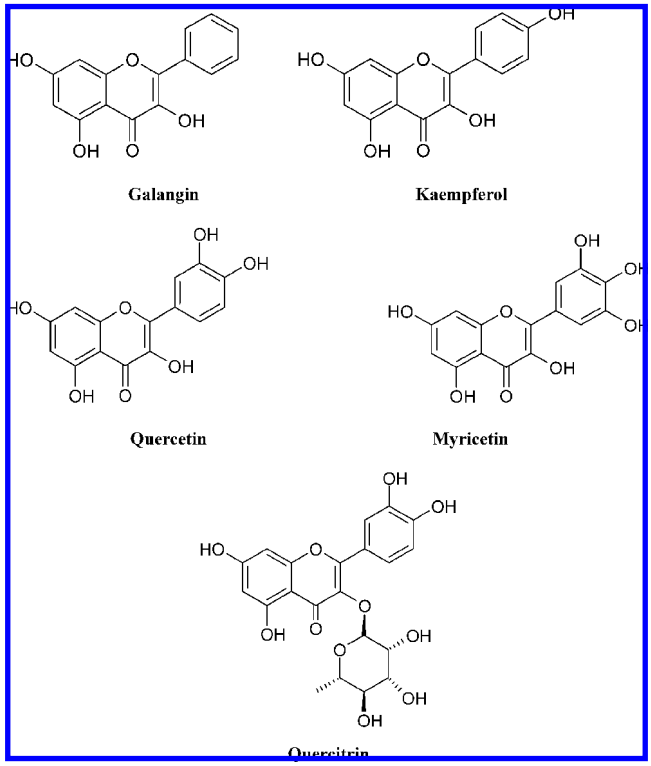

Figure 1. Structure of flavonoids.

absorbed in the stomach, but their glycosidic forms cannot be absorbed (*17*).

The interaction between biomacromolecules and drugs has attracted great interest among researchers for several decades (*18–25*). Among biomacromolecules, serum albumins are the major soluble protein constituents of the circulatory system and have many physiological functions (*18–20*). Bovine serum albumin (BSA) has been one of the most extensively studied of proteins particularly because of its structural homology with human serum albumin (HSA) (*18–24*). The interaction between protein and drug molecules results in formation of a stable protein−drug complex, which may be considered as a model for gaining general fundamental insights into drug–protein binding (*23–25*). Fluorescence spectroscopy is an appropriate method to determine the interaction between the small molecules and biomacromolecules (*18–25*). By analysis of the fluorescence parameters, much information concerning the structural changes in biomacromolecules could be obtained.

There have been several studies on fluorescence quenching of proteins induced by flavonoids (*18–25*). However, the influence of the B-ring hydroxylation pattern in flavonols on binding characteristics with BSA was not reported. In the present study, we evaluated the binding affinities of myricetin, quercetin, kaempferol, and galangin, which share the same substituents on the A and C rings but have different numbers of hydroxyl groups on the B-ring, with BSA.

MATERIALS AND METHODS

Apparatus and Reagents. Fluorescence spectra were recorded on a JASCO FP-6500 spectrofluorometer (Tokyo, Japan). UV–vis spectra were recorded on a Shimadzu UV-2450 spectrophotometer (Tokyo, Japan). The pH measurements were carried out on a PHS-3C Exact Digital pH meter (Cole-Parmer Instrument Co., Vernon Hills, IL). BSA (fraction V), quercitrin (≥98%), galangin (≥98%), and myricetin (≥98%) were purchased from Sigma Co. (St. Louis, MO). Kaempferol (≥98%) and quercetin (≥98%) were obtained commercially from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). A working solution of flavonol (1.0×10^{-4} mol/L) was prepared by dissolving flavonol in methanol–water solution (2:8, v/v). 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 solution of BSA (1.0×10^{-5} mol/L) was prepared with Tris-HCl buffer and stored in the refrigerator prior to use. All other reagents and solvents were of analytical reagent grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double-distilled water.

Fluorescence Spectra. Appropriate quantities of 1.0×10^{-4} mol/L flavonoids solution were transferred to a 10 mL flask, and then 1.0 mL of BSA solution was added, and the solution was diluted to 10 mL with Tris-HCl buffer. The resultant mixture was subsequently incubated at 310.15 K for 1 h. The solution was scanned on the spectrofluorometer with the range of 290–500 nm. The fluorescence intensity at 340 nm was determined under the excitation at wavelength of 280 nm. The dynamic quenching constant (K_{SV}) and the apparent binding constants (K_a) were attained according to refs *22–25*.

High-Performance Liquid Chromatography. The chromatographic retention factor (K') was determined by HPLC method according to Fang et al. (*26*). HPLC analysis was performed on a Shimadazu LC-2010A liquid chromatograph with a Shimadazu SPD-M10A diode array detector (Shimadazu, Kyoto, Japan). Flavonols were separated on a Kromasil RP-C_{18} column (250 mm × 4.6 mm ID, 5 μm; Hanbon Science & Technology Co., Ltd., Huaiyin, China).

The chromatographic retention factor (K') was expressed as

$$K' = (t_r - t_0)/t_0 \quad (1)$$

where t_r stands for retention time and t_0 stands for dead time.

RESULTS AND DISCUSSION

Effect of Flavonols on BSA Fluorescence Spectra. The fluorescence spectra of BSA with the addition of galangin (a), kaempferol (b), quercetin (c), myricetin (d), and quercitrin (e) are shown in **Figure 2**. In all cases, the fluorescence intensity of BSA decreased remarkably with the increasing concentration of flavonoids. Weak blue shifts of the maximum λ_{em} (1−2 nm) were observed for these five flavonoids. This suggested that there was some change in the environment of the tyrosine and tryptophan residues.

Fluorescence Quenching of BSA. The raw data for quenching of BSA fluorescence by addition of galangin (a), kaempferol (b), quercetin (c), myricetin (d), and quercitrin (e) are shown in **Figure 3**. These results indicated that the changes of the environment of tryptophan residues depended on the structure of flavonol. Galangin and myricetin were found to lead to 70–80% fluorescence quenching, while quercitrin quenched only 10% of BSA fluorescence. Kaempferol and quercetin quenched 40–60% of BSA fluorescence. **Figure 4** shows the Stern−Volmer plots for the BSA fluorescence quenching by galangin (a), kaempferol (b), quercetin (c), myricetin (d), and quercitrin (e). In the linear range of the Stern−Volmer regression curve, the average quenching constants (K_{SV}) for galangin, kaempferol, quercetin, myricetin, and quercitrin (having the lowest quenching effect, inset in **Figure 4**) at 310.15 K were 2.26

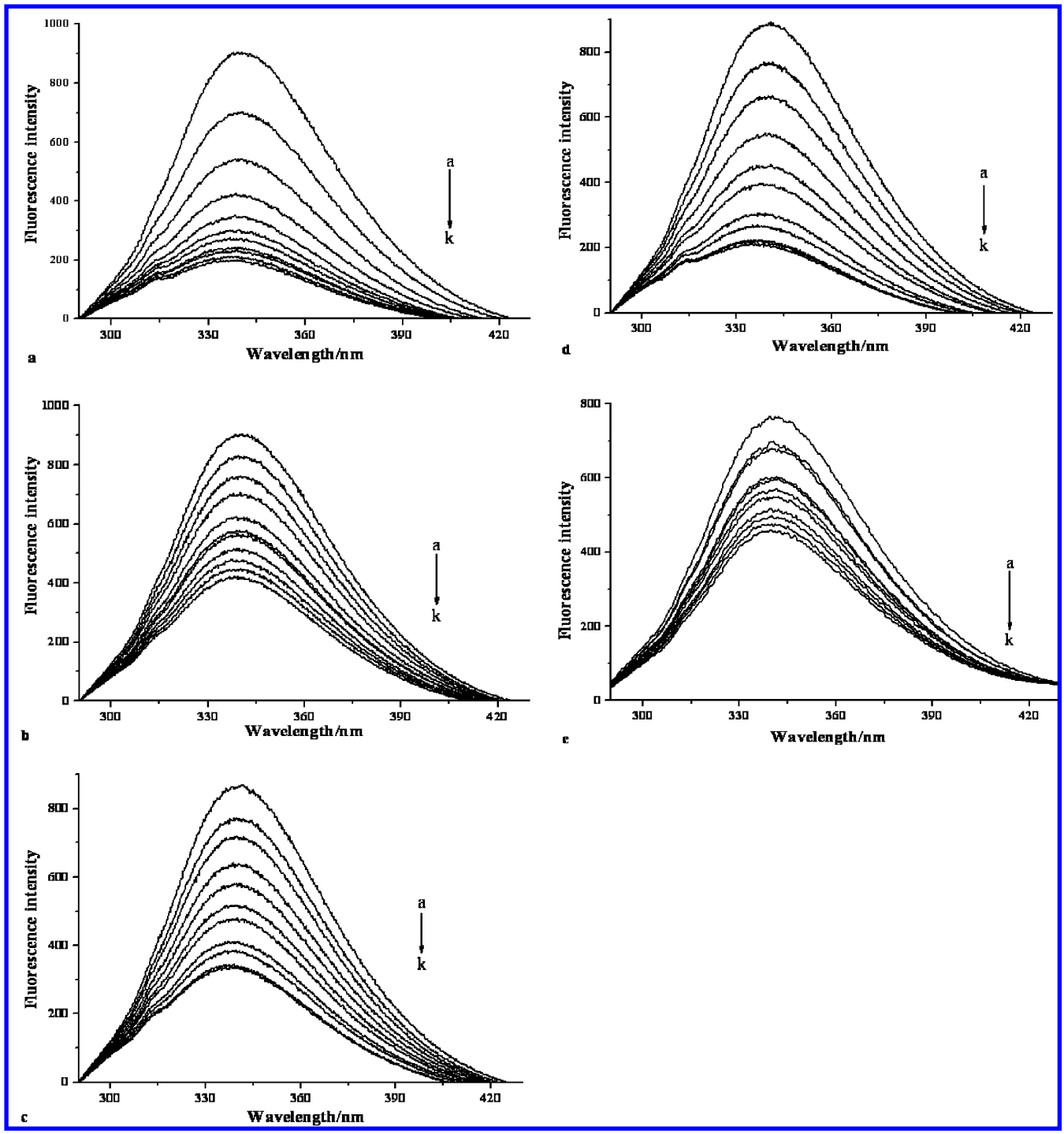

Figure 2. The quenching effect of flavonols on BSA fluorescence intensity, λ_{ex} =280 nm, [BSA] = 1.00 × 10^{-6} mol/L: (**a**−**d**) spectra a−k of solutions containing (0.00, 2.00, 4. 00, 6.00, 8.00, 10.00, 12.00, 14.00, 16.00, 18.00, or 20.00) × 10^{-7} mol/L of galangin (a), kaempferol (b), quercetin (c), and myricetin (d); (**e**) spectra a−k of solutions containing (1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, or 10.00) × 10^{-6} mol/L of quercitrin.

× 10^6 (R = 0.9984), 6.34 × 10^5 (R = 0.9935), 6.04 × 10^5 (R = 0.9945), 1.34 × 10^6 (R = 0.9893), and 4.86 × 10^4 L/mol (R = 0.9999), respectively.

Binding Constant and Binding Sites. Figure 5 shows the double-logarithm curve of flavonol quenching of BSA fluorescence at 310.15 K and **Table 1** gives the corresponding calculated results. The apparent binding constants (K_a) and the binding site values (n) between flavonols and BSA increased with increasing number of hydroxyl groups on the B-ring (inset in **Figure 5**) and decreased after glycoside substitution at the C-ring position. Dangles et al. (*27*) studied the binding constants of quercetin and quercetin glycosides to BSA by Scatchard analysis and also found a similar result. The native protein contains hydrophobic groups in the interior of the tertiary structure and polar groups at the surface. Hydrogen bonding may take place between the −OH groups in flavonols and the polar groups at the BSA surface. However, after glycoside substitution at the C-ring position, steric hindrance may take place, which weakens the binding affinity. Another mechanism may be that glycosylation of the flavonol increasing molecular weight and polarity weakens the capacity to penetrate into the tryptophan-rich hydrophobic regions of BSA, which are fre-

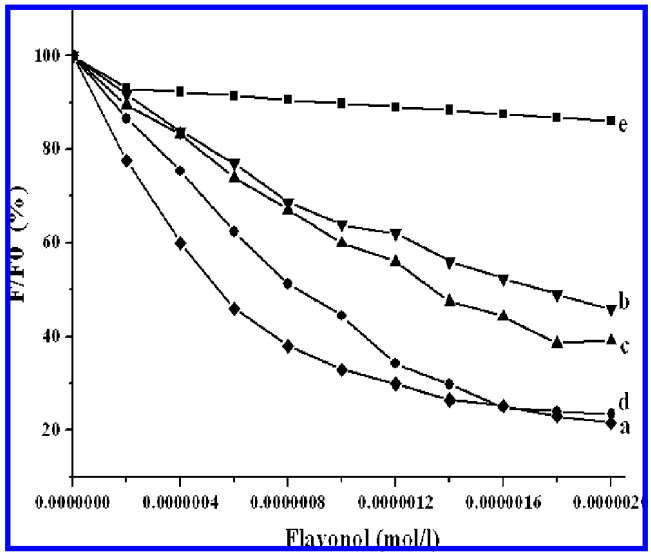

Figure 3. Tryptophan fluorescence quenching of BSA (1.00×10^{-6} mol/L) plotted as extinction of BSA tryptophans (F/F_0, %) against flavonol concentration for galangin (a), kaempferol (b), quercetin (c), myricetin (d), and quercitrin (e). The fluorescence emission intensity was recorded at λ_{ex} =280 nm and λ_{em}=340 nm.

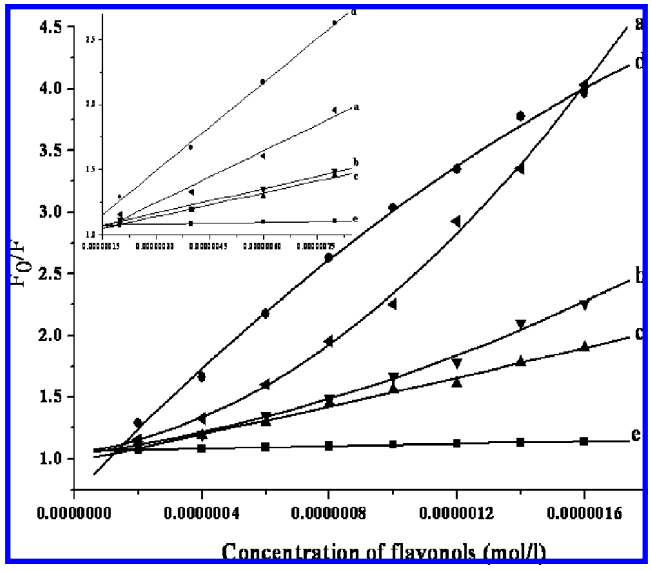

Figure 4. The Stern−Volmer curves of fluorescence quenching of BSA by galangin (a), kaempferol (b), quercetin (c), myricetin (d), and quercitrin (e) at 310.15 K. Inset shows the the Stern−Volmer curves with low concentrations of quenchers.

quently buried in the interior of the folded BSA. Also, the fact that BSA contains 21 tyrosines and 3 tryptophans that are all distributed over the amino acid sequence automatically suggests that BSA does not contain only one fluorophore. The different exposure and location of all these fluorophores may well result in a deviation from linearity of the Stern−Volmer plot.

UV Spectra of Flavonols Bound to BSA. Figure 6 shows the UV absorption spectra of galangin (a), kaempferol (b), quercetin (c), myricetin (d), and quercitrin (e) after interacting with BSA. It can be noted from **Figure 6** that the UV absorption band I (300–400 nm) of flavonols and quercitrin had an obvious bathochromic shift (4−12 nm), indicating that the structure of flavonoids was affected after interaction with BSA. The UV absorption band II (200–300 nm) of flavonols also had obvious bathochromic shifts

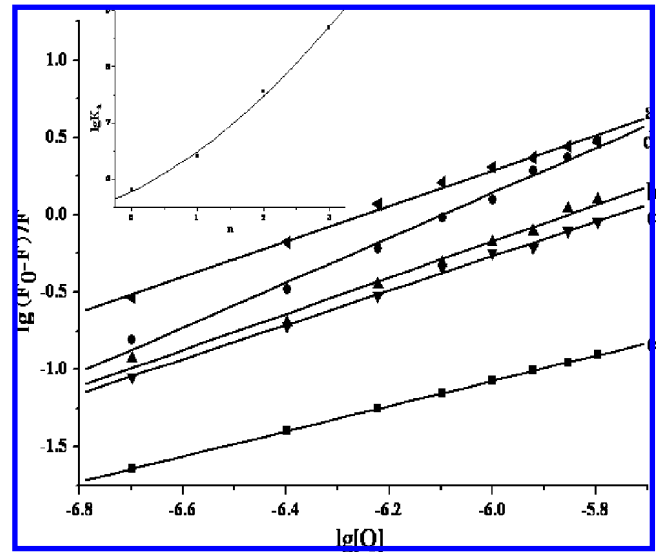

Figure 5. Double-log plot of galangin (a), kaempferol (b), quercetin (c), myricetin (d), and quercitrin (e) quenching effect on BSA fluorescence at 310.15 K. Inset shows the relationship of apparent binding constants (log K_a) and the binding site values (n).

Table 1. The Binding Parameters for Flavonoid−BSA Systems

flavonols	log K_a	n	R
quercitrin	3.8107	0.814	0.9835
galangin	5.8081	0.920	0.9973
kaempferol	6.4122	1.115	0.9988
quercetin	7.5626	1.291	0.9946
myricetin	8.6571	1.421	0.9951

(6−13nm). However, the UV absorption band II of quercitrin hardly changed, which revealed that the affinity of quercitrin for BSA was weaker than that of flavonols.

Relationship of Partition Coefficient and Binding Constants. The octanol–water partition coefficient (K_{ow}) (also referred to as P) is a measure of the propensity of a compound to differentially dissolve in these immiscible phases. The partition coefficient serves as a quantitative descriptor of lipophilicity and is one of the key determinants of pharmacokinetic properties. The log K_{ow} values are important to many industries and areas of research in determining how to deliver chemical substances to specific sites or eliminate chemicals from others, as well as limiting unwanted dispersal of chemicals through the environment.

The lipophilicity of the four flavonols was assessed by their partition coefficient values (K_{ow}) according to Moreira et al. (*28*). The log K_{ow} values for galangin, kaempferol, quercetin, and myricetin were 3.94, 3.507, 2.906, and 2.082, respectively. The log K_{ow} values were inversely proportional to log K_a for flavonols with different numbers of hydroxyl groups on the B-ring (**Figure 7**). The linear regression equation using the Origin 7.5 software was log K_{ow}= 7.59181 – 0.62986 log K_a (R = 0.9976). The galangin (0 -OH moieties on the B-ring) with highest partition coefficient (log K_{ow} = 3.940) had smallest affinity with BSA (log K_a = 5.8081) in four flavonols. From this point, the binding between these four flavonols and BSA was not caused by hydrophobic forces.

Relationship of Chromatographic Distribution Ratio and Binding Constants. To further investigate the hydrogen bonds force between these four flavonols and BSA. A reversed-phase C_{18} chromatography column was used to calculate the chromatographic distribution ratio of these four flavonols. The

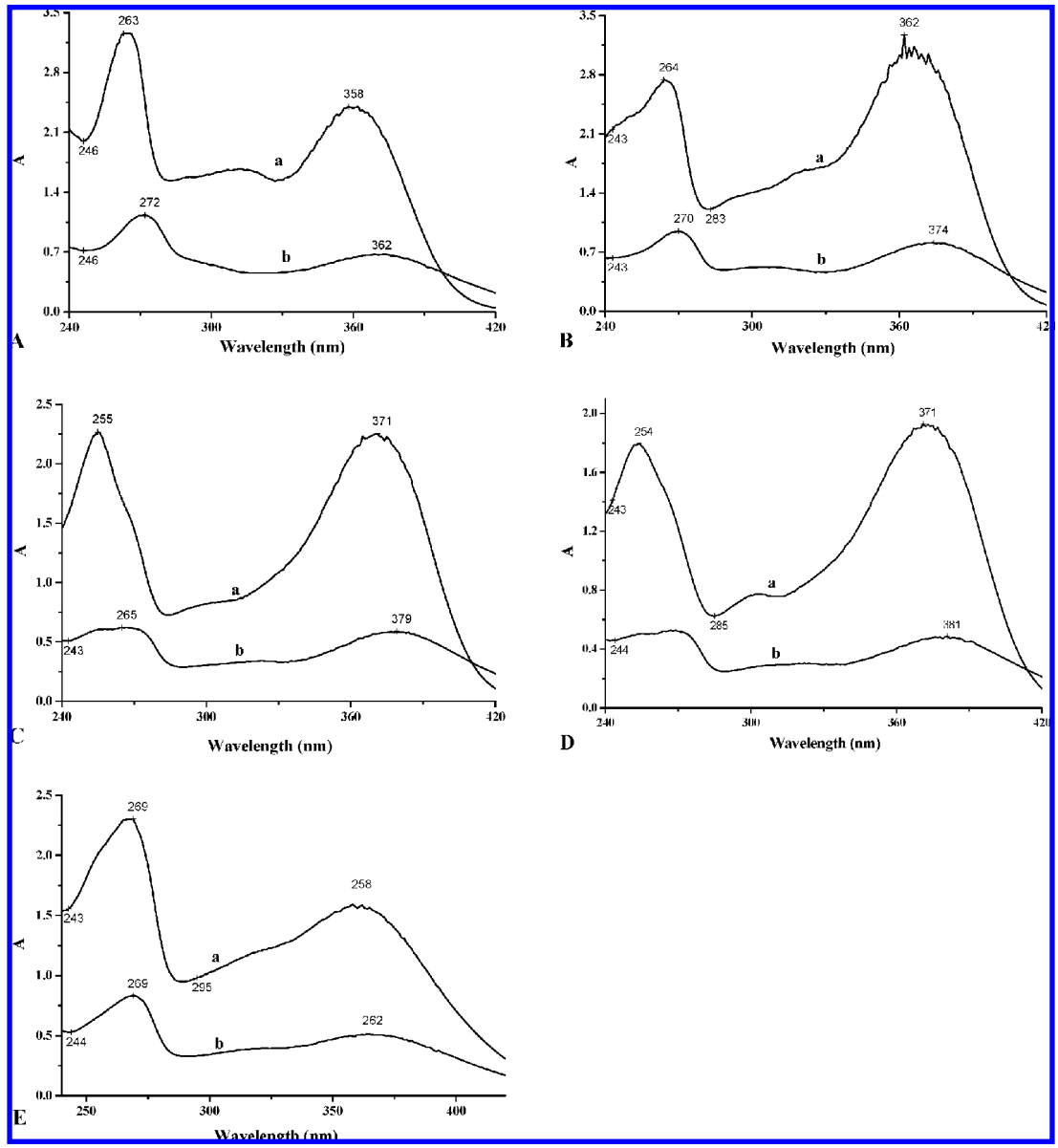

Figure 6. UV absorption spectra of galangin (**A**), kaempferol (**B**), quercetin (**C**), myricetin (**D**), and quercitrin (**E**) before (a) and after (b) interacting with BSA.

chromatographic retention factor (K') for galangin, kaempferol, quercetin, and myricetin were determined as 4.71, 3.87, 1.92, and 0.62, respectively. The chromatographic retention factor (K') were inversely proportional to the binding constants value (log K_a) for flavonols with different numbers of hydroxyl groups on the B-ring (**Figure 8**). The linear regression equation using the Origin 7.5 software was $K' = 13.09705 - 1.44953 \log K_a$ ($R = 0.9967$).

A reversed-phase C_{18} chromatography column consists of a monolayer of octadecyl silane (C_{18}) with very low polarity. The component with lower polarity has higher binding affinity with the C_{18} column, which causes longer retention time for chromatographic separation. However, being different from the octadecyl silane, BSA consists of large number of NH_2, OH, and SH groups, which indicates that BSA is high-polarity biomacromolecule.

Relationship of Binding Constants and Bioactivities of Flavonols. The presence of a dihydroxyl group (catechol-type) or three adjacent hydroxyl groups (pyrogallol-type) on the B ring of the flavonoid molecule, the number of free hydroxyl groups, a C_2-C_3 double bond in the C ring, or the presence of

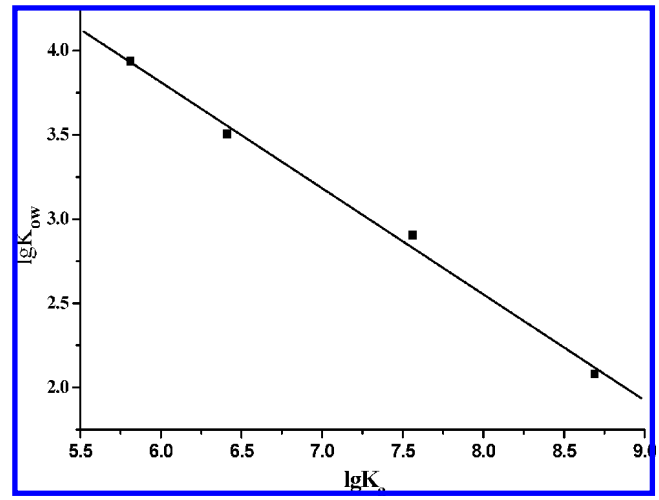

Figure 7. Relationship of binding constants (log K_a) with partition coefficient (K_{ow}) of flavonols. The partition coefficient values (K_{ow}) were from reference of Moreira et al. (*28*).

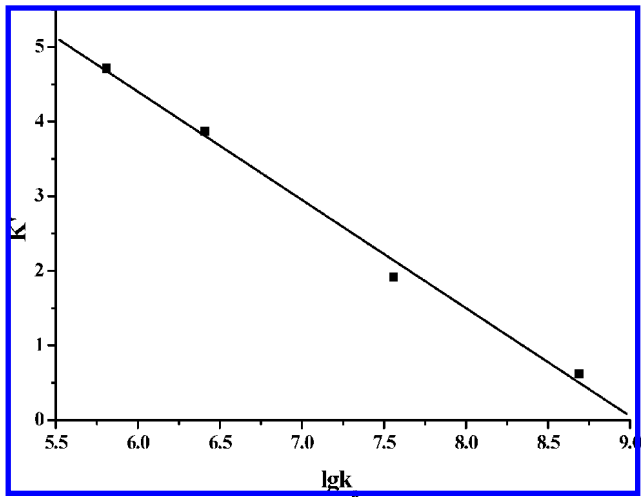

Figure 8. Relationship of binding constants (log K_a) with the chromatographic retention factor (K').

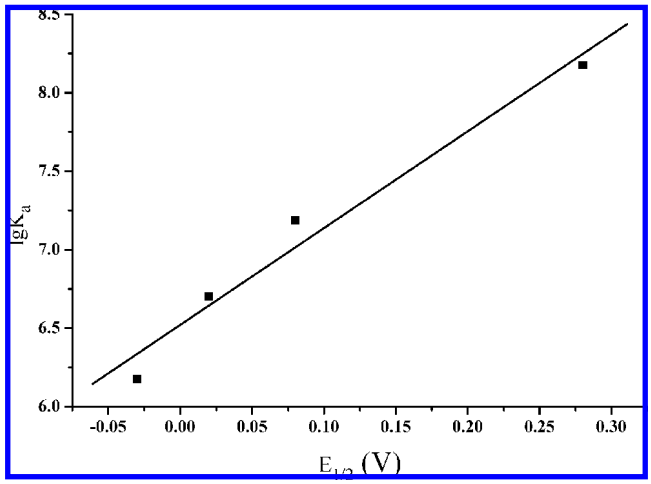

Figure 9. Relationship between binding constants and half-wave potential ($E_{1/2}$) of the four flavonols tested. The half-wave potential values ($E_{1/2}$) were from the work of Yang et al. (*33*).

C-3, C-5, and C-7 hydroxyl groups are usually listed as requirements for antioxidant and antiradical activity (*29, 30*). William et al. (*31*) indicated that OH substitutions were important in the antioxidant activities of flavonoids. The studies of structure−FPTase inhibitory activity relationships indicated that the number, position, and substitution of OH groups of the A and B rings of flavonoids are important factors affecting inhibition on FPTase (*32*). Yang et al. (*33*) used the half-wave potential values ($E_{1/2}$) to estimate the antioxidant activities of flavonols. A correlation ($r = 0.9853$) was found between binding constant values (log K_a) and the values of the half-wave potential ($E_{1/2}$) (*33*) of the four flavonols tested (**Figure 9**). The linear regression equation using the Origin 7.5 software was $\log K_a = 6.5215 + 6.1647E_{1/2}$.

McPhail et al. studied the free radical scavenger capacity of these four flavonols by electron spin resonance (ESR) spectroscopy (*15*). Reactivity is highly dependent on the configuration of the OH groups on the flavonol B ring, there being little contribution from the A ring to antioxidant effectiveness. The reaction stoichiometries of these compounds with the galvinoxyl free radical was determined; antioxidant capacities were determined as myricetin (4.08) > quercetin (3.27) > kaempferol (1.84) > galangin (1.01). A correlation ($r = 0.9900$) was also found between log K_a and reaction stoichiometries of these compounds with the galvinoxyl free radical (data not shown).

Our results are in agreement with the generally accepted structure-dependent free radical scavenger and antioxidant abilities of flavonols described above. Addition of a single 4′-hydroxyl group on the B-ring led to a significant increase in the interaction with the transport protein. It was likely due to the potential for conjugation between the 4′-hydroxyl group and the 3-hydroxyl group through the C-ring (*34*).

LITERATURE CITED

(1) Bravo, M. N.; Silva, S.; Coelho, A. V.; Boas, L. V.; Bronze, M. R. Analysis of phenolic compounds in Muscatel wines produced in Portugal. *Anal. Chim. Acta* **2006**, *563*, 84–92.

(2) Volpi, N.; Bergonzini, G. Analysis of flavonoids from propolis by on-line HPLC-electrospray mass spectrometry. *J. Pharm. Biomed.* **2006**, *42*, 354–361.

(3) Woodman, O. L.; Meeker, W. F.; Boujaoude, M. Vasorelaxant and antioxidant activity of flavonols and flavones: Structure-activity relationships. *J. Cardiovasc. Pharm.* **2005**, *46*, 302–309.

(4) Heijnen, C. G. M.; Haenen, G.R.M.M.; van Acker, F. A. A.; van der Vijgh, W. J. F.; Bast, A. Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups. *Toxicol. in Vitro* **2001**, *15*, 3–6.

(5) Chen, J. W.; Zhu, Z. Q.; Hu, T. X.; Zhu, D. Y. Structure-activity relationship of natural flavonoids in hydroxyl radical-scavenging effects. *Acta Pharmacol. Sin.* **2002**, *23*, 667–672.

(6) Miyake, T.; Shibamoto, T. Antioxidative activities of natural compounds found in plants. *J. Agric. Food Chem.* **1997**, *45*, 1819–1822.

(7) Cushnie, T. P. T.; Hamilton, V. E. S.; Chapman, D. G.; Taylor, P. W.; Lamb, A. J. Aggregation of Staphylococcus aureus following treatment with the antibacterial flavonol galangin. *J. Appl. Microbiol.* **2007**, *103*, 1562–1567.

(8) Bestwick, C. S.; Milne, L. Influence of galangin on HL-60 cell proliferation and survival. *Cancer Lett.* **2006**, *243*, 80–89.

(9) Park, J. S.; Rho, H. S.; Kim, D. H.; Chang, I. S. Enzymatic preparation of kaempferol from green tea seed and its antioxidant activity. *J. Agric. Food Chem.* **2006**, *54*, 2951–2956.

(10) Tu, Y.; Lian, T.; Yen, J.; Chen, Z.; Wu, M. Antiatherogenic effects of kaempferol and rhamnocitrin. *J. Agric. Food Chem.* **2007**, *55*, 9969–9976.

(11) Hu, Y.; Cheng, Z.; Heller, L. I.; Krasnoff, S. B.; Glahn, R. P.; Welch, R. M. Kaempferol in red and pinto bean seed (*Phaseolus vulgaris* L.) coats inhibits iron bioavailability using an in vitro digestion/human Caco-2 cell model. *J. Agric. Food Chem.* **2006**, *54*, 9254–9261.

(12) Ramos, F. A.; Takaishi, Y.; Shirotori, M.; Kawaguchi, Y.; Tsuchiya, K.; Shibata, H.; Higuti, T.; Tadokoro, T.; Takeuchi, M. Antibacterial and antioxidant activities of quercetin oxidation products from yellow onion (Allium cepa) skin. *J. Agric. Food Chem.* **2006**, *54*, 3551–3557.

(13) Chimenti, F.; Cottiglia, F.; Bonsignore, L.; Casu, L.; Casu, M.; Floris, C.; Secci, D.; Bolasco, A.; Chimenti, P.; Granese, A.; Befani, O.; Turini, P.; Alcaro, S.; Ortuso, F.; Trombetta, G.; Loizzo, A.; Guarino, I. Quercetin as the active principle of *Hypericum hircinum* exerts a selective inhibitory activity against MAO-A: Extraction, biological analysis, and computational study. *J. Nat. Prod.* **2006**, *69*, 945–949.

(14) Belinha, I.; Amorim, M. A.; Rodrigues, P.; de Freitas, V.; Moradas-Ferreira, P.; Mateus, N.; Costa, V. Quercetin increases oxidative stress resistance and longevity in Saccharomyces cerevisiae. *J. Agric. Food Chem.* **2007**, *55*, 2446–2451.

(15) McPhail, D. B.; Hartley, R. C.; Gardner, P. T.; Duthie, G. G. Kinetic and stoichiometric assessment of the antioxidant activity of flavonoids by electron spin resonance spectroscopy. *J. Agric. Food Chem.* **2003**, *51*, 1684–1690.

(16) Butkovic, V.; Klasinc, L.; Bors, W. Kinetic study of flavonoid reactions with stable radicals. *J. Agric. Food Chem.* **2004**, *52*, 2816–2820.

(17) Crespy, V.; Morand, C.; Besson, C.; Manach, C.; Demigne, C.; Remesy, C. Quercetin, but not its glycosides, is absorbed from the rat stomach. *J. Agric. Food Chem.* **2002**, *50*, 618–621.

(18) Papadopoulou, A; Green, R. J.; Frazier, R. A. Interaction of flavonoids with bovine serum albumin: A fluorescence quenching study. *J. Agric. Food Chem.* **2005**, *53*, 158–163.
(19) Rawel, H. A.; Meidtner, K.; Kroll, J. Binding of selected phenolic compounds to proteins. *J. Agric. Food Chem.* **2005**, *53*, 4228–4235.
(20) Kim, D.; Park, J.; Kim, J.; Han, C.; Yoon, J.; Kim, N.; Seo, J.; Lee, C. Flavonoids as mushroom tyrosinase inhibitors: A fluorescence quenching study. *J. Agric. Food Chem.* **2006**, *54*, 935–941.
(21) Soares, S.; Mateus, N.; Freitas, V. Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary α-amylase (HSA) by fluorescence quenching. *J. Agric. Food Chem.* **2007**, *55*, 6726–6735.
(22) Xiao, J. B.; Chen, J. W.; Cao, H.; Ren, F. L.; Yang, C. S.; Chen, Y.; Xu, M. Study of the interaction between baicalin and bovine serum albumin by multi-spectroscopic method. *J. Photochem. Photobiol. A* **2007**, *191*, 222–227.
(23) Rawel, H. M.; Frey, S. K.; Meidtner, K; Kroll, J; Schweigert, F. J. Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence. *Mol. Nutr. Food Res.* **2006**, *50*, 705–713.
(24) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Kluwer Academic/Plenum Publishers: New York, 1999.
(25) Xiao, J. B.; Shi, J.; Cao, H.; Wu, S. D.; Ren, F. L.; Xu, M. Analysis of binding interaction between puerarin and bovine serum albumin by multi-spectroscopic method. *J. Pharm. Biomed.* **2007**, *45*, 609–615.
(26) Fang, F.; Li, J. M.; Pan, Q. H.; Huang, W. D. Determination of red wine flavonoids by HPLC and effect of aging. *Food Chem.* **2007**, *101*, 428–433.
(27) Dangles, O.; Dufour, C.; Bret, S. Flavonol-serum albumin complexation. Two-electron oxidation of flavonols and their complexes with serum albumin. *J. Chem. Soc., Perkin Trans.* **1999**, *2*, 737–744.
(28) Moreira, M. R.; Kanashiro, A.; Kabeya, L. M.; Polizello, A. C. M.; Azzolini, A. E. C. S.; Curti, C.; Oliveira, C. A.; Amaral, A. T.-d.; Lucisano-Valim, Y. M. Neutrophil effector functions triggered by Fc-gamma and/or complement receptors are dependent on B-ring hydroxylation pattern and physicochemical properties of flavonols. *Life Sci.* **2007**, *81*, 317–326.
(29) Burda, S.; Oleszek, W. Antioxidant and antiradical activities of flavonoids. *J. Agric. Food Chem.* **2001**, *49*, 2774–2779.
(30) Pietta, P. G. Flavonoids as antioxidants. *J. Nat. Prod.* **2000**, *63*, 1035–1042.
(31) William, R. J.; Spencer, J. P.; Rice-Evans, C. Flavonoids: Antioxidants or signaling molecular. *Free Radical Biol. Med.* **2004**, *36*, 838–894.
(32) Kang, H. M.; Kim, J. H.; Lee, M. Y.; Son, K. H.; Yang, D. C.; Baek, N. I. Relationship between flavonoid structure and inhibition of farnesyl protein transferase. *Nat. Prod. Res.* **2004**, *18*, 349–356.
(33) Yang, B.; Kotani, A.; Arai, K.; Kusu, F. Estimation of the antioxidant activities of flavonoids from their antioxidant potentials. *Anal. Sci.* **2001**, *17*, 599–604.
(34) Pannala, A. S.; Chan, T. S.; O'brien, P. J.; Rice-Evans, C. A. Flavonoid B-ring chemistry and antioxidant activity: Fast reaction kinetics. *Biochem. Biophys. Res. Commun.* **2001**, *282*, 1161–1168.

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