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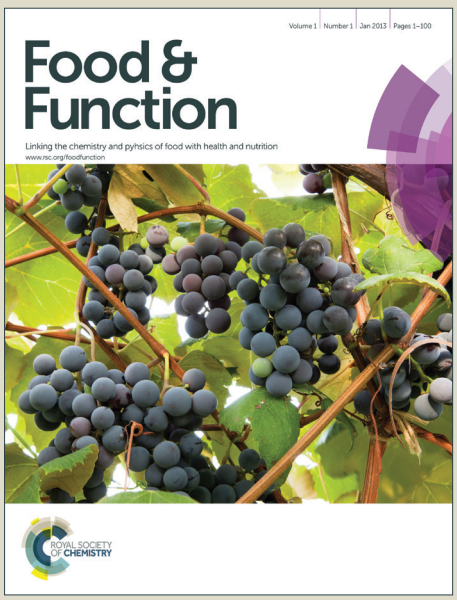

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Inhibiton of flavonoids on acetylcholine esterase: binding and structure-activity relationship

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Abstract

The inhibitory effects of flavonoids on acetylcholinesterase (AChE) have attracted great interest among researchers. However, few reports have focused on the structure-activity relationship for AChE inhibition of flavonoids. This work mainly concerns the structural aspects of inhibitory activities and binding affinities of flavonoids as AChE inhibitors. The results show hydroxyl groups in the A ring of flavonoids are favorable for inhibiting AChE, and the hydroxylation increases the affinities for AChE. However, methoxylation may decrease or increase the activities depending on the class of flavonoids. The glycosylation decreases the AChE inhibitory activities of flavonoids and lowers the affinities for AChE by 1 to 5 times depending on the conjunction site and the class of sugar moiety. The hydrogenation of C_2-C_3 double bond of apigenin decreases both the affinity for AChE and AChE inhibition. The molecular property–affinity relationship reveals that hydrogen bond force plays an important role in binding flavonoids to AChE. The AChE inhibitions generally increase with the increasing affinities of flavonoids within the class, especially for flavones and flavonols.

INTRODUCTION

Acetylcholinesterase is the key enzyme that terminates the nerve impulse at the cholinergic synapses by rapid hydrolysis of acetylcholine (ACh), a neurotransmitter found in the synaptic gap, thus keeping the normal transmission of nerve impulses.[1] It has been suggested that decreased concentration of the acetylcholine (ACh) appears to be critical element in the development of dementia of some progressive neurodegenerative disease, such as Alzheimer's disease (AD).[2] Hence, to restore acetylcholine levels by inhibiting AChE has become the primary treatment for the cognitive deficits of AD.[3] The inhibition of AChE is beneficial not only to the enhancement of cholinergic transmission in the brain, but also to reducing the aggregation of β-amyloid and the formation of the neurotoxic fibrils in AD.[4] So far, a few AChE inhibitors such as donepezil, rivastigmine, and galantamine, have been approved by US FDA to treat mild to moderate AD.[5] However, these drugs are not clinically satisfying for the low cost-effectiveness and the side effects including gastrointestinal tract reaction, insomnia, anorexia and hepatotoxicity.[6-8] In the recent decades, the researchers have been devoted to develop new AChE inhibitors, especially the so called “multifunctional AChE inhibitors” with additional efficacy in vascular dementia treatment.[9, 10] In addition to chemical synthesis, the potential for plants to yield new therapeutic agents has stimulated extensive research to screen novel AChE inhibitors from natural products.[11] There have been plenty of phytochemicals that found to be effective in inhibiting AChE, which mainly consist of alkaloids, cannabinoids, curcumins, stilbenes, and flavonoids.[12] Among them, flavonoids have attracted more and more interests for the high inhibitory activity and low toxicity[13]. Moreover, their diverse activities such as anti-oxidation, inhibition on advanced glycation products, and cardio-cerebrovascular protection give them extra advantages to be the potential multifunctional therapeutic agents for aging related diseases.[14-16]

Flavonoids are ubiquitously existed in plants, and entering into human diet via vegetable, fruits, beverage and other plant-derived food. The flavonoids are structurally characterized by a C_6-C_3-C_6 skeleton which are labeled A, B, and C (Table 1) and are divided into subclasses based on the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings.[17] The inhibitory activities of flavonoids against AChE have been broadly investigated by *in vitro*, *in vivo* and molecular docking studies[18-21]. Few studies, however, have concerned the relationship between the molecular structure of flavonoids and their inhibitory activities on AChE. Moreover, it has also rarely been discussed that relationship between the flavonoid-AChE interactions and the AChE inhibitory

activities of flavonoids.

In the present work, 20 flavonoids were tested for the inhibitory activities against AChE *in vitro*, and their binding affinities for AChE were obtained by fluorescence titration method. Both the structure-activity relationship for inhibitory effects of flavonoids on AChE and relationship between the binding affinities and inhibitory activities were discussed.

MATERIALS AND METHODS

Chemicals and Reagents

Acetylcholinesterase (AChE) from *Electrophorus electricus* (Type-VI-S, EC 3.1.1.7), acetylthiocholine iodide (≥99.0%), and 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, ≥98%) were purchased from Sigma-Aldrich (MO, USA). Sodium dodecyl sulfonate (SDS, ≥97%) was purchased from Sinopharm Group Co., Ltd. (Beijing, China). Baicalein, baicalin, chrysin, formononetin, 7,8-dihydroflavone, apigenin, puerarin, luteolin, rutin, fisetin, naringenin, daidzein and daidzin were obtained commercially from TCI Co., Ltd. (Tokyo, Japan). Myricetin, myricetrin, quercetin, quercetrin, kaemperferol, kaemperferide, and genistein were purchased from Aladdin Reagent Int. (Shanghai, China). All other reagents and solvents were analytical grade and used without further purification. All aqueous solutions were prepared using Millipore-pure water.

AChE Fluorescence Quenching Study

The fluorescence spectra were recorded on a Cray Eclipse fluorescence spectrophotometer (Varian, Mulgrave, Australia). The fluorescence quenching study was performed according to the method of Ryu and coworkers.[22] In a typical flurorescence measurement, 1mL AChE dissolved in 0.2mol/L phosphate buffered saline (PBS, pH 8.0) with the concentration of 5U/mL was added accurately in a 10mm quartz cell and then titrated by successive additions of flavonoids (1×10^{-3} mol/L of methanol solution) . The intrinsic fluorescence emission spectra of AChE were recorded from 300 to 450 nm, under an excitation wavelength of 276 nm. The fluorescence intensity was measured at 335 nm under the same excitation wavelength. Each fluorescence intensity determination was repeated 3 times. The band widths for excitation and emission were both 10 nm. All the investigated flavonoids showed no emission spectra in the scanned range under excitation at 276nm.

The fluorescence quenching of AChE by flavonoids was described by the Stern-Volmer equation:[23-25]

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

where F_0 and F represent the fluorescence intensities in the absence and presence of flavonoids,

[Q] is the concentration of flavonoids, K_q is the quenching rate constant, and τ_0 is the average lifetime, K_{sv} is the Sterm-Volmer quenching constant.

For static quenching, the relationship between fluorescence quenching intensity and the concentration of flavonoids can be described by the binding constant formula:[26]

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

where F_0 and F represent the fluorescence intensities in the absence and presence of flavonoid. [Q] is the concentration of flavonoids, K_a is the binding constant, and n is the number of binding sites per AChE molecular. After the fluorescence quenching intensities on AChE at 335 nm were measured, the double-logarithm algorithm was assessed by equation 2.

AChE Inhibition Assay

Inhibition of AChE was measured using the colorimetric method described by Ellman *et al.* with slight modifications.[27] Briefly, 775 μL of PBS (pH 8.0), 20 μL of AChE solution (0.5U/mL), 250 μL of DTNB (1.5×10^{-3}mol/L), and 30 μL of test flavonoid sample were mixed in a tube. 25 μL of acetylthiocholine iodide (1.5×10^{-2} mol/L) was then added to initiate the reaction. After 8 min of incubation at 37 ℃, the reaction was stopped by adding 400μL of SDS (4%) and the absorbance of the mixture was measured at 412 nm. A control mixture was prepared by using 30 μL of methanol instead of the flavonoid sample, with all other procedures similar to those used in the case of the sample mixture. The inhibitory percentage of AChE activity (In %) was calculated by using the equation:

$$In\ (\%) = (A_{control} - A_{sample})/A_{control}\ \} \times 100\% \quad (2)$$

where A_{sample} was the absorbance of the mixture containing flavonoid sample and $A_{control}$ was the absorbance of the control mixture. All assays were run in triplicate at minimum. The concentration of flavonoid in the mixture required for 50% inhibition of AChE activity (IC_{50}) was calculated by plotting the inhibition percentages against the corresponding flavonoids concentrations. For weak inhibitors, the activities are expressed by the In % at a dosage of 600 μ mol/L.

RESULTS AND DISCUSSION

Quenching effect of flavonoids on AChE fluorescence

As representative examples, the fluorescence spectra of AChE with addition of genistein (A), chrysin (B), bacailin (C), and apigenin (D) are shown in Figure 1 (The fluorescence spectra of AChE quenched by other flavonoids are not given here.) All flavonoids tested can quench the fluorescence of AChE markedly with increasing concentration of flavonoids. There are no obvious shifts of the maximum λ_{em} of AChE fluorescence for flavonoids tested, indicates no alteration of

the secondary and tertiary structure of AChE by the interaction between the flavonoids and AChE.[28]

The quenching ratios (F/F_0) of AChE fluorescence with addition of the other five representative flavonoids are shown in Figure 2. The intensities of AChE fluorescence decreased rapidly with the addition of quercetin and rutin. However, myricetin, myricetrin and naringeine showed a much slower quenching rates on AChE fluorescence. 10.0 μmol/L of quercetin was found to quench 50.24% of AchE fluorescence; however, the same amount of naringenine only quenched that by 22.48%. These results indicated that the quenching effects of flavonoids on AChE fluorescence depended on the structures of flavonoids.

Figure 3 shows the stern-volmer plots for AChE fluorescence quenching by quercetin, rutin myricetin, myricetrin and naringeine. As seen from Figure 3, the stern-volmer plots of the five flavonoids are quiet liner, which means the K_{sv} and K_q values can be calculated according to equation 2. The calculated K_q values of AChE fluorescence quenching by flavonoids were in the range of 3.88×10^{12} $Lmol^{-1}s^{-1}$ to 8.82×10^{13} $Lmol^{-1}s^{-1}$(data not shown here). For dynamic quenching, the maximum scatter collision quenching constant (K_q) of various quenchers with a biopolymer is reported to be 2.0×10^{10} L $mol^{-1}s^{-1}$, and if the Kq is much greater than that, then it can be concluded that the quenching is initiated by static quenching instead of dynamic quenching.[26] As the K_q values obtained here were much higher that 2.0×10^{10}, the fluorescent quenching of AChE by flavonoids was probably initiated by static quenching resulting from the formation of flavonoid-AChE complex. These results were in agreement with the investigation by Falé et al, which suggested that the static quenching of the fluorescence of AChE by rosmarinic acid, apigenin, luteolin and quercetin was originated by the formation of complex.[28]

The Binding Constants (K_a) and the Number of Binding Sites (n)

Since the quenching of AChE fluorescence by flavonoids are initiated by static quenching, the binding constant (K_a) and the number of binding sites per AChE molecular (n) can be calculated by equation 2. Table 1 summarizes the calculated results according to eq 1. The values of lg K_a are proportional to the number of binding sites (n) (Figure 4), which indicates that the eq. 2 used here is suitable for the study of the interaction between flavonoids and AChE.[29, 30] The magnitudes of apparent binding constants for AChE were almost all in the range of 10^4-10^6 L/mol, which were similar to the affinities of flavonoids for common human plasma proteins.[23] However, these data were much smaller than the affinities of flavonoids for human serum albumin (HSA) from our previous study (10^4-10^8 L/mol).[31]

Influence of structural alteration of flavonoids on their affinities for AChE and AChE inhibitory activities

Hydroxylation and methoxylation. The inhibitory activities of flavonoids against AChE were investigated *in vitro* and the results in the form of IC_{50} were listed in Table 1. The activities are found to be varied depending on the structures of flavonoids, and flavones and flavonols are generally stronger inhibitors than isoflavones. Previous studies have proven that the patterns of the hydroxyl and methoxyl groups on both of the A and B rings of flavonoids are closely related to the bioactivities and binding affinities for proteins.[32-35] The effects of hydroxylation and methoxylation of flavonoids on the affinities and AChE inhibitory activities were summed in table 2. It is noted that the hydroxylation on both ring A and ring B increased the inhibitory activities of flavonoids against AChE, while methoxylation may decrease or increase the activities depending on classes of flavonoids. As can be seen, hydroxylation on the position 5(ring A) of fisetin increases the activity by 20.3 fold, and baicalein with additional hydroxyl group on position 6(ring A) shows 6.8 fold higher activity than chrysin. However, both the hydroxylation on position 4' (ring B) of chrysin and position 5' (ring B) of quercetin hardly influence their inhibitory activities on AchE. Luteolin and quercetin with additional hydroxyl groups on position 3' (ring B) for both only show 4.7 and 6.3 higher activities than apigenin and kaempeferol, respectively. These results indicate that the hydroxyl groups in the A ring may be particularly important for flavonoids in inhibiting AChE. The results also support the docking study that shows hydroxyl groups of flavonoids form hydrogen bonds with active site residues of AChE.[20]

As for the affinities of flavonoids for AChE, the hydroxylation seems to enhance the AChE inhibitory activities of all the tested flavonoids except for quercetin and chrysin. Similar results were obtained in the literature which showed that luteolin had much greater binding constant than apigenin, and that luteolin was also a little bit stronger AChE inhibitor than apigenin.[28] Taking the affinities and inhibitory activities simultaneously into account, it is notable that the affinities of flavonoids changed consistently with the inhibitory activities, but not proportionally.

Glycosylation. The dietary flavonoids in nature occur mostly as β-glycosides. The flavones and flavonols are found mainly as the 3- and 7-O-glycoside, although the 8-C glycoside may also be found in isoflavones (Table 1). It has been reported that the glycosylation of dietary flavonoids decreased the affinities for plasma protein in vitro.[24] Herein, the effects of glycosylation of dietary flavonoids on the affinities for AChE and inhibitory activities on it were investigated. The sugar moieties are at 3, 7 and 8 position of the flavonoids. As shown in Figure 5, the glycosylation decreased both the affinities and inhibitory activities of flavonoids except for dadzein. For example, baicalein showed an 18.5 times higher inhibitory activity on AChE than its

glucuronoside (baicalin), and also a 1.5 time larger affinity than baicalin. The glycosylation of dietary flavonoids has been found to decrease the affinities for HSA by 1to 3 orders of magnitude depending on the conjunction site and the class of sugar moiety.[31] Here, the glycosylation only lowered the affinities for AChE by 1 to 5 times which also depended on the forms of glycosides. In addition, the present result that glycosylation decreased the inhibitory activities against AChE was similar to the conclusion we made in a recent review that glycosylation of flavonoids decreased the inhibitory activities both on α-amylases and α-glucosidases.[36]

Hydrogenation of the C_2-C_3 Double Bond. The C_2-C_3 double bond in conjugation with a 4-oxo group plays the key role for the flavonoids to maintain a plane molecular structure. When the double bond was saturated, the spatial structure of flavonoid changed consequently. As shown in table 1, apigenin showed both higher inhibitory activity and affinity than naringenin, which indicated hydrogenation of C_2-C_3 double bond of flavonoids might decrease both the affinities for AChE and inhibitory activities on it.

The Nature of flavonoid-AChE Noncovalent Interaction

The non-covalent interactions between flavonoids and proteins generally include four major forms, namely, hydrogen bonding, van der Waals interaction, hydrophobic interaction, and electrostatic interaction. The nature of the flavoniod–AChE interaction was studied by investigating the molecular property–affinity relationship. Here, the hydrogen bond acceptor/donor numbers and lipophilicity of flavonoids were used. The relationships between the hydrogen bond acceptor/donor numbers (Data were from PubChem Public Chemical Database [37]) of flavonoids and the affinities for AChE were shown in Figure 6. The affinities for AChE obviously increased with increasing hydrogen bond acceptor numbers of flavonoids, which indicated that hydrogen bond force plays an important role in binding flavonoids to AChE.

To further investigate whether or not the lipophilicity plays an important role in binding flavonoids to AChE, the relationship of affinities with partition coefficient values (XLogP3, data from reference [37]) was assessed. As shown in Figure 7, the binding affinities decreased with the increasing XLogP3 values, indicating that the hydrophobic force is not the main force to bind flavonoids to AChE. According to the above results, the noncovalent interaction between flavonoid and AChE seems to be similar to the previous investigated flavonoid-γ-globulin interaction[38], while different from the interactions in binding flavonoid to serum albumin, hemoglobin[39], common plasma proteins[23], and milk proteins[29].

Relationship between affinities and AChE inhibitory activities

AChE consists of a complex protein of the α/β hydrolase fold type having an overall ellipsoid shape containing a deep groove, usually called the gorge, and the active site of the enzyme is

believed to be located at the bottom of the gorge.[40, 41] A catalytic triad of Ser200-His 440-Glu327 in the esteratic subsite of gorge makes up the catalytic machinery of the enzyme, while some other aromatic residues at the entrance of the gorge, such as Tyr341, Tyr72 and Tyr124, comprises the so called "peripheral anionic site(PSA)" to modulate the entry of small molecules including substrates or inhibitors into the gorge.[42] To inhibit the catalytic activity of AChE, flavonoids may cross the PSA and enter the gorge to occupy the active site, or may just bind to the PSA and block the entrance for acetylcholine.[43] Herein, the relationship of the binding affinities of flavonoids with their AChE inhibition was investigated. As shown in figure 8, there is no obvious relationship between the affinities and inhibition. However, the inhibitory activities increased generally with the increasing affinities within the class, especially for flavones and flavonols. These results may be explained by that higher affinity increases the chance to enter the catalytic gorge, but the inhibition finally depends on the direct interaction between the flavonoid and the active site.

Relationship between TPSA and AChE inhibitory activities

The topological polar surface area (TPSA) is defined as the surface sum over all polar atoms. It is a descriptor showing the correlation with passive molecular transport through membranes, which has been successfully applied for the prediction of human intestinal absorption, Caco-2 monolayers permeability, and blood-brain barrier penetration.[44] It has been suggested that molecules with a TPSA value of greater than 140 angstrom squared showed low human intestinal absorption and tended to be poor at permeating cell membranes. [45, 46] It has also proven that lower TPSA values indicate higher blood –brain barrier (BBB) partition values which are necessary for a drug targeted at central nervous system.[47] To be potential AD drug candidates, flavonoids are preferable to passively cross the blood–brain barrier. Herein, the relationship between TPSA and the AChE inhibitory activites of flavonoids was investigated. As shown in Figure 9, there was no direct relationship between the two parameters, while three types of flavonoids might be roughly divided considering both the inhibitory activities and BBB penetration abilities. The type Ⅰ was the most favorable group with high activities (low IC_{50} value) and penetration abilities (low TPSA), and the type Ⅱ was the less preferred group with good penetration abilities but low activities. Although the favonoids in type Ⅲ group were of great inhibitory activities on AChE *in vitro*, few distributions in the brain may make them totally ineffective in treating AD.

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Legends:

Figure 1. The quenching effects of genistein (A), chrysin (B), bacailin (C), and apigenin (D) on AChE fluorescence spectra at 300.15 K. λex = 280 nm; AChE, 5U/mL; a→f: 0.00, 2.00, 4.00, ..., 10.00 (×10-6 mol/L) of flavonoids.

Figure 2. The quenching ratio (F/F_0) of AChE fluorescence spectra with addition of myricetin (My) ,myricetrin (Mt), naringenin (Na), quercetin(Q), rutin (Ru).

Figure 3. The stern-Volmer plots for AChE fluorescence quenching by flavonoids at 298 K

Figure 4. The relationship between the binding affinities (lgKa) and the numbers of binding sites (n) between flavonoids and AChE .

Figure 5. The effect of glycosylation of flavonoids on their affinities for AChE and AChE inhibitory activities.

Figure 6. Relationships of the hydrogen bond acceptor/donor number of flavonoids with the affinities (lgKa) for AChE. The hydrogen bond acceptor/donor numbers were taken from the PubChem Public Chemical Database

Figure 7. Relationship of apparent binding constants (lgKa) with partition coefficient (XLogP3) of flavonoids. The partition coefficient (XLogP3) were taken from the PubChem Public Chemical Database

Figure 8. Relationship of AChE inhibitory activities (IC_{50}) of flavonoids with affinities for AChE (semi solid points represent the values more than 600).

Figure 9. Relationship of TPSA with AChE inhibitory activities (IC_{50}) of flavonoids (semi solid points represent the values more than 600).

Figures and tables

Figure 1

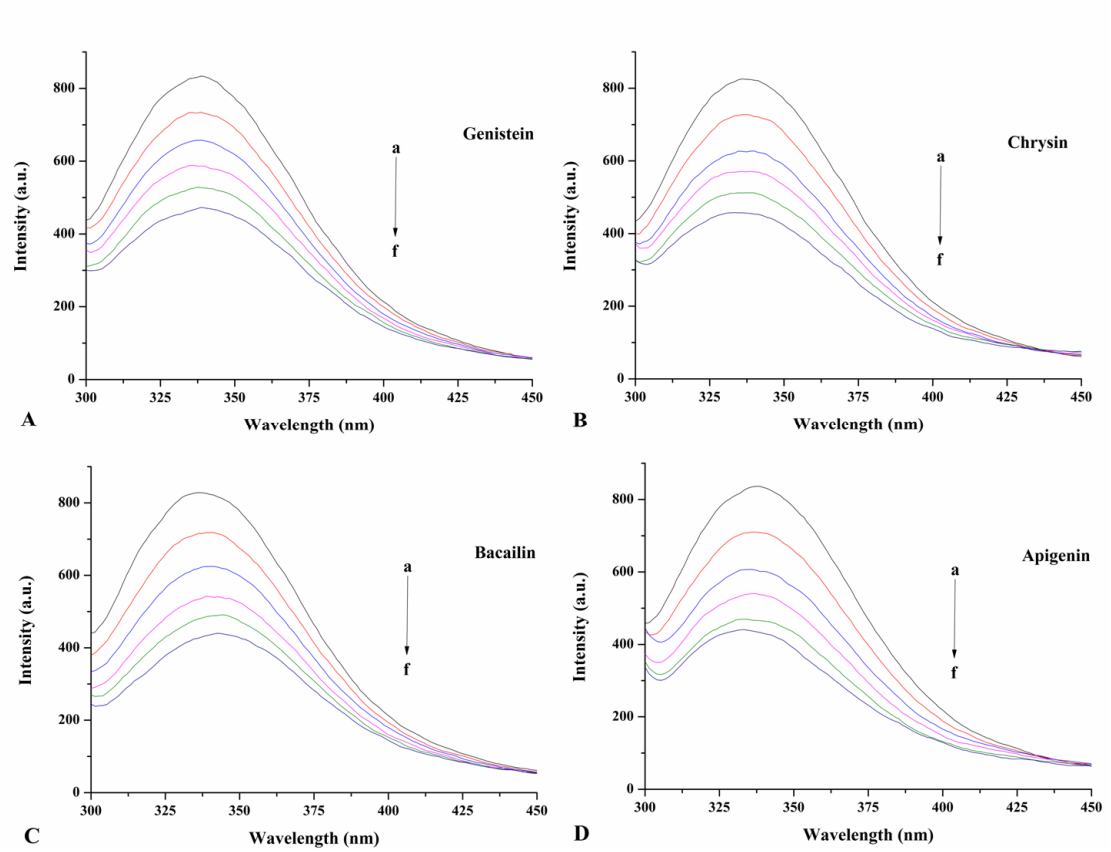


Figure2

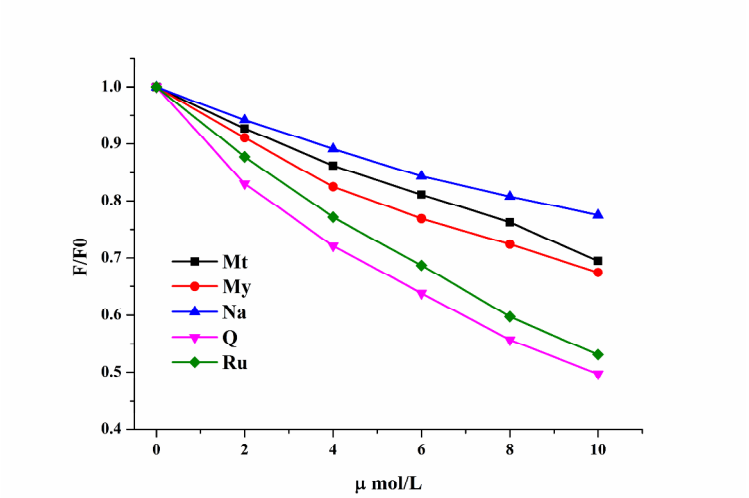


Figure 3

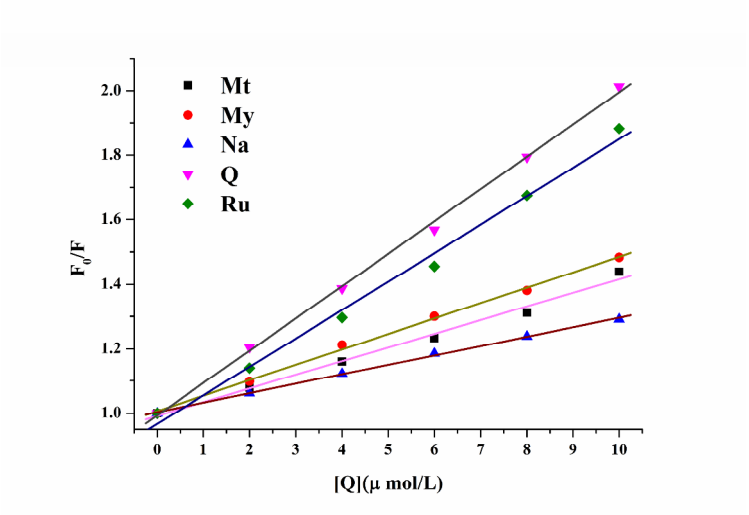


Figure4

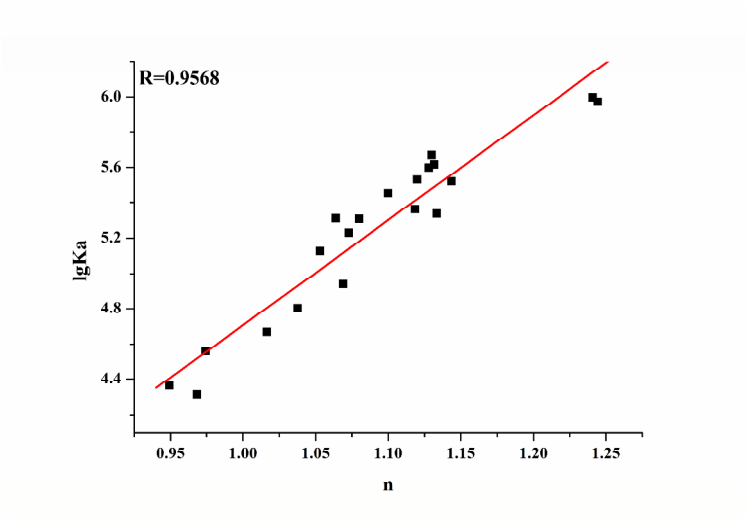


Figure 5.

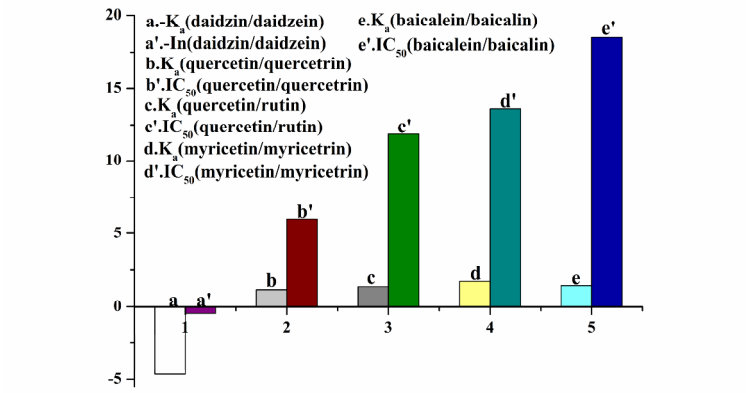


Figure 6

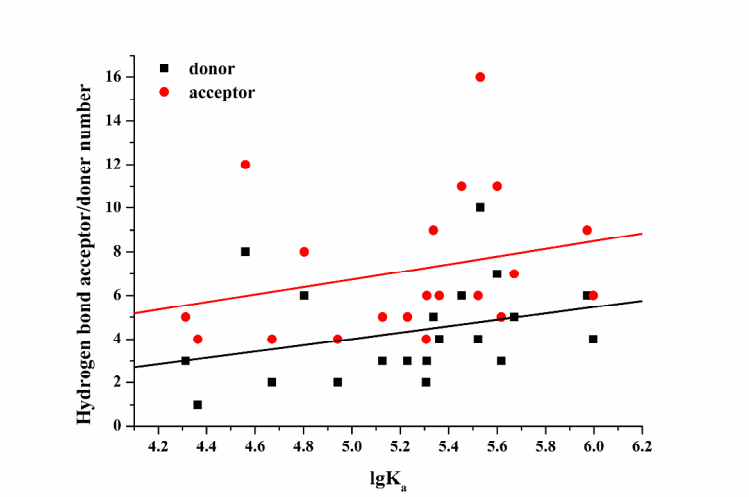


Figure 7

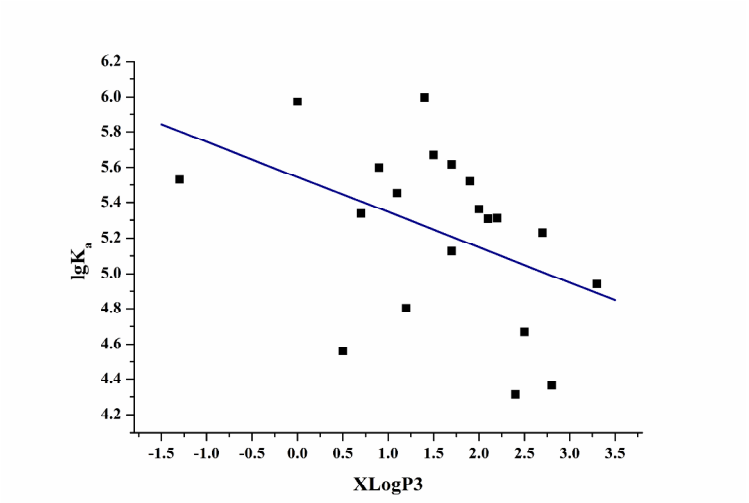


Figure 8

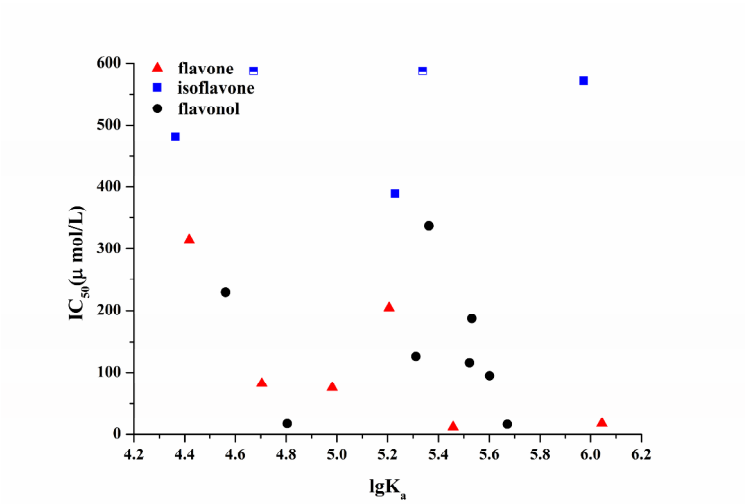


Figure 9

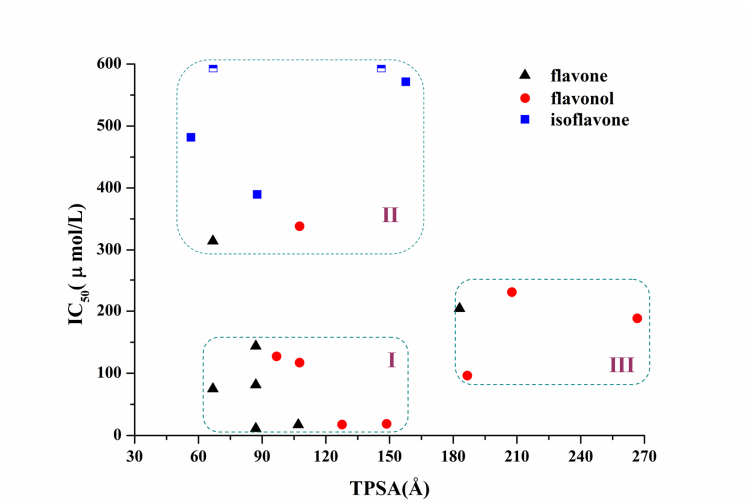
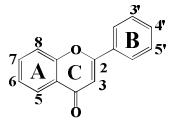
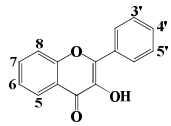
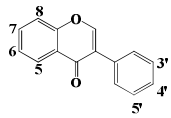


Table1. Chemical structures of dietary flavonoids and their affinities for AChE and AChE inhibitory activities

Subclass	Name	Subtitutions			IC_{50} /μ mol/L	lgK_a	n
		OH	OCH_3	others			
Flavones	Luteolin	5,7,3', 4'			17.3±2.0	5.99±0.11	1.24±0.02
	Chrysin	5,7			74.9±1.1	5.31±0.22	1.08±0.04
	Baicalein	5,6,7			11.0±1.4	5.62±0.12	1.13±0.02
	Baicalin	5,6		7-β-D-glucuronide	204.1±16.5	5.45±0.12	1.10±0.02
	Apigenin	5,7, 4'			81.5±8.6	5.13±0.70	1.05±0.13
	7,8-dihydroflavone	7,8			314.3±10.2	4.94±0.02	1.07±0.03
Flavanone	Naringenin#	5,7, 4'			143.6±16.2	4.31±0.08	0.97±0.01
Flavonols	Kaempferide	3,5,7	4'		125.4±10.2	5.31±0.19	1.06±0.04
	Kaempferol	3,5,7, 4'			115.3±8.9	5.52±0.06	1.14±0.01
	Quercetin	3,5,7,3', 4'			15.7±0.8	5.67±0.15	1.13±0.03
	Quercitrin	5,7,3', 4'		3-o- rhamnoside	94.5±3.8	5.60±0.29	1.13±0.11
	Myricetin	3,5,7,3',4', 5'			16.8±1.9	4.80±0.22	1.03±0.04
	Myricetrin	5,7,3',4', 5'		3-o-rhamnoside	228.6±12.3	4.56±0.19	0.97±0.03
	Fisetin	3,7,3', 4'			336.4±4.3	5.36±0.09	1.12±0.02
	Rutin	5,7,3', 4'		3-α-L-Rham-1,6-D-Glc	186.6±16.3	5.53±0.15	1.12±0.03
Isoflavones	Formononetin	7	4'		481.5±19.9	4.36±0.34	0.95±0.06
	Daidzin	4'		7-glucoside	8.8±1.5% *	4.66±0.12	1.01±0.02
	Daidzein	7,4'			18.4±2.9%*	5.33±0.30	1.13±0.06
	Genistein	5,7,4'			389.3±9.4	5.23±0.16	1.07±0.03
	Puerarin	7,4'		8-C-glucoside	571.2±34.9	5.97±0.27	1.24±0.05

* Inhibition percentage (In %) at 600 μ mol/L; # C_2-C_3 is saturated

Table 2. Effects of hydroxylation and methoxylation of flavonoids on their affinities for AChE and AChE inhibitory activities

Subclass	Structural Alteration	Example	Effects (times)	
			Af.	Ac.
Flavones	6 H→OH	Chrysin→Baicalein	2.1↑	6.8↑
	4' H→OH	Chrysin→Apigenin	≈	≈
	3' H→OH	Apigenin→Luteolin	5.3↑	4.7↑
Flavonols	3' H→OH	Kaempferol→Quercetin	1.4↑	6.3↑
	5' H→OH	Quercetin→Myricetin	7.4↓	≈
	5 H→OH	Fisetin→Quercetin	2.0↑	20.3↑
	4' H→OCH_3	Kaempferol→Kaemperide	1.4↓	↓

Isoflavones	5 H→OH	Daidzein→Genistein	2.6↑	↑
	4' H→OCH_3	Daidzein→Formononetin	2.0↑	↑