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Cholinestrase inhibitory effects of geranylated flavonoids from *Paulownia tomentosa* fruits

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ABSTRACT

Alzheimer's disease is rapidly becoming one of the most prevalent human diseases. Inhibition of human acetylcholinestrase (hAChE) and butyrylcholinestrase (BChE) has been linked to amelioration of Alzheimer's symptoms and research into inhibitors is of critical importance. Purification of the methanol extract of *Paulownia tomentosa* fruits yielded potent hAChE and BChE inhibitory flavonoids (**1**–**9**). A comparative activity screen indicated that a geranyl group at C6 is crucial for both hAChE and BChE. For example, diplacone (**8**) showed 250-fold higher efficacy than its parent eriodictyol (**12**). IC_{50}s of diplacone (**8**) were 7.2 μM for hAChE and 1.4 μM for BChE. Similar trends were also observed for 4′-*O*-methyldiplacone (**4**) (vs its parent, hesperetin **10**) and mimulone (**7**) (vs its parent, naringenin **11**). Representative inhibitors (**1**–**8**) showed mixed inhibition kinetics as well as time-dependent, reversible inhibition toward hAChE. The binding affinities of these compounds to hAChE were investigated by monitoring quenching of inherent enzyme fluorescence. The affinity constants (K_{SA}) increased in proportion to inhibitory potencies.

1. Introduction

Dementia is one of the most rapidly growing diseases. By 2050 an estimated 100 million people worldwide will suffer from dementia. This is a threefold increase based on the number of sufferers today.[1] Alzheimer's disease (AD) is the most common form of dementia in the elderly. Despite its prevalence, deaths from AD have risen by 66% in the USA from 2000–2008. On the other hand, deaths associated with stroke, heart disease and HIV have each fallen by >13% over the same period.[2]

It is thus no surprise that a huge research effort is being directed to understanding the causes of and potential treatments for AD. AD has been associated with amyloid-β-peptide (Aβ),[3] hyperphosphorylated tau protein,[4] and cholinesterases (ChEs) including acthylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). Given the complexity of the disease, other mechanisms are also involved in AD development. However, there is abundant evidence that defects in cholinergic synaptic transmission, in particular nicotinic acetylcholine receptor (nAChR)-mediated signaling, plays a major role in AD. It is generally agreed that the Aβ peptide plays an important role in the development of AD. For instance, Aβ expression is toxic to cultured neuronal cells.[5] Recent studies indicate that brain nAChRs can initiate signaling pathways that protect against Aβ toxicity.[6,7] Importantly, the ligand-gated ion channel, nAChR, is gated in response to the binding of the neurotransmitter ACh.[8] AChE and BChE, respectively catalyze the rapid hydrolysis of the neurotransmitters acetylcholine (ACh) and butyrylcholine (BCh) in the nerve synapse, and help terminate ACh signaling by hydrolyzing the transmitter, thereby inactivating nAChRs.[9] Thus an optimum level of acetylcholine should be maintained in the brain for its proper function. These findings have led to the cholinergic hypothesis of AD and the development of therapies targeting cholinergic molecular components.[10,11] Given the huge potential medical benefits, it is very attractive to explore leading medicinal plants or structures that may mediate AD through action on AChE-BChE.[12] *Paulownia tomentosa* steud. belonging to the family scrophulariaceae in deciduous tree distributed throughout Korea, China, and Japan. Iridoids, lignans and flavonoids have been reported as bioactive compounds of *P. tomentosa*.[13,14] Among them, geranylated flavonoids are known as its main bioactive constituents.[15] Some studies have reported that this species displays cytotoxic, antioxidant and antimicrobial activities,[16,17] but these studies have been restricted.

Herein we show that the MeOH (95%) extract from *P. tomentosa* fruits had a potent inhibitory activity against cholinesterase. Subsequent bioactivity-guided fractionation of MeOH extract led to

Abbreviations: IC_{50}, the inhibitor concentration leading to 50% activity loss; K_i, inhibition constant; K_m, Michaelis–Menten constant.

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the isolation of nine geranylated flavonoids (**1–9**). We thus report *P. tomentosa* is a rich source of cholinesterase inhibitors. This was proven by enzyme inhibition assays using purified compounds, kinetic mechanistic analysis of the mode of inhibition, and assessing the affinity between the inhibitors and hAChE by enzyme fluorescence quenching. In particular, we demonstrate that inhibition by these compounds requires a geranyl group at the flavonoid C6 position.

2. Results and discussion

The extracts from three different polar solvents (chloroform, methanol, and water) were tested for their enzymatic inhibitory activities against hAChE and BChE. The enzyme was assayed according to a standard literature procedure by following the hydrolysis of acetylthiocholine or butyrylthiocholine spectrophotometrically.[18–20] As shown Table 1, MeOH extracts exhibited a

Table 1
Inhibitory effects of extracts and compounds (**1**–**12**) on cholinesterase activities

Compound	Cholinesterase			
	IC_{50}[a] (μM) human erythrocytes AChE	Type of Inhibition K_i[b] (μM)	IC_{50}[a] (μM) equine serum BChE	Type of Inhibition K_i[b] (μM)
$CHCl_3$	3.68 mg/ml	NT[c]	2.12 mg/ml	NT
MeOH	1.44 mg/ml	NT	0.97 mg/ml	NT
Water	6.98 mg/ml	NT	5.22 mg/ml	NT
1	31.9 ± 1.2	Mixed (34.3)	12.7 ± 1.3	Mixed (18.7)
2	48.5 ± 2.1	Mixed (68.2)	11.2 ± 2.1	Mixed (18.2)
3	15.6 ± 0.8	Mixed (16.9)	3.8 ± 0.8	Mixed (4.5)
4	92.4 ± 4.1	Mixed (94.5)	25.6 ± 1.6	Mixed (23.5)
5	109.2 ± 8.4	Mixed (149.5)	24.5 ± 1.2	Mixed (23.6)
6	22.9 ± 1.6	Mixed (30.1)	6.4 ± 0.9	Mixed (7.6)
7	91.5 ± 5.3	Mixed (120.5)	20.6 ± 1.1	Mixed (22.2)
8	7.2 ± 0.6	Mixed (8.4)	1.4 ± 0.3	Mixed (1.2)
9	316.3 ± 12.5	NT	80.0 ± 2.6	Mixed (75.2)
10 (hesperetin)	2476.1	NT	1774.1	NT
11 (naringenin)	2045.0	NT	1493.7	NT
12 (eriodictyol)	1663.8	NT	1124.4	NT
Eserine	0.15 ± 0.03	NT	3.7 ± 0.6	NT

[a] All compounds were examined in a set of experiments repeated five times; IC_{50} values of compounds IC_{50} represent the concentration that caused 50% enzyme activity loss.
[b] Values of inhibition constant.
[c] NT is not tested.

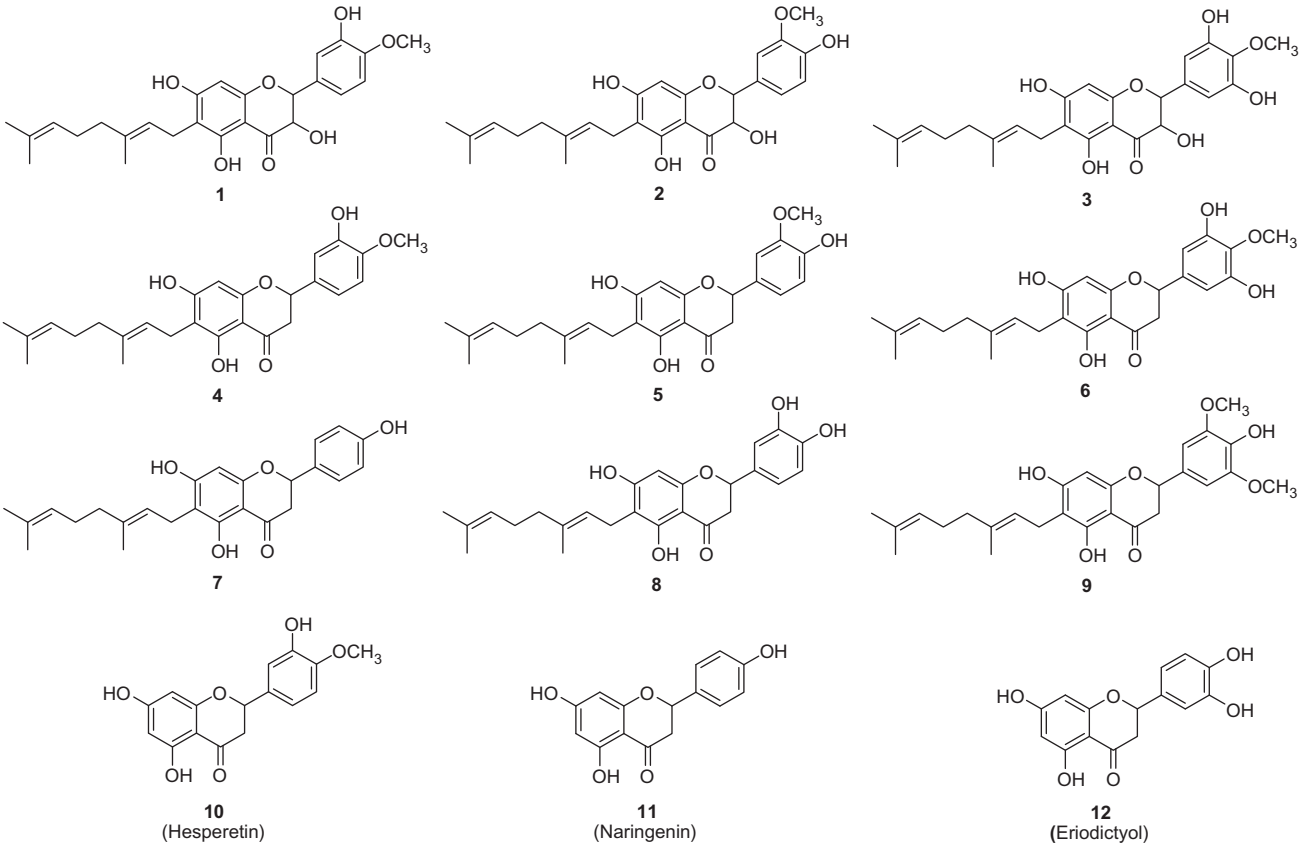

Figure 1. Chemical structures of geranylated flavonoids **1**–**9** from the *Paulownia tomentosa*, and their parent compounds (**10**–**12**).

significant degree of hAChE (1.44 mg/ml), and BChE (0.97 mg/ml) inhibition. The high potency of the MeOH extract encouraged us to identify compounds responsible for its cholinesterase inhibitory. Activity-guided fractionation of the MeOH extract gave geranylated flavonoids (**1–9**), which were purified over silicagel, sephadex LH 20, and octadecyl-functionalized silicagel as delineated above. The structures of isolated flavonoids (**1–9**) (Fig. 1) were elucidated using established spectroscopic analysis which was compared with previously reported data.[13–17] Isolated compounds **1–9** were identified as 4′-*O*-methyldiplacol (**1**), 3′-*O*-methyldiplacol (**2**), 6-geranyl-3,3′,5,5′,7-pentahydroxy-4′-methoxyflavane (**3**), 4′-*O*-methyldiplacone (**4**), 3′-*O*-methyldiplacone (**5**), 6-geranyl-3′,5,5′,7-tetrahydroxy-4′-methoxyflavanone (**6**), mimulone (**7**), diplacone (**8**), 6-geranyl-4′,5,7-trihydroxy-3′,5′-dimethoxyflavanone (**9**). Although the isolated flavonoids fall into two different classes, dihydroflavonols (**1–3**) and flavanones (**4–9**), all compounds share a common feature, a geranyl group at the C6 position. The most effective inhibitor **8** was obtained as a yellowish powder having molecular formula $C_{25}H_{28}O_6$ and 12 degrees of unsaturation, as established by HREIMS (*m*/*z* 424.1889 [M$^+$], calcd 424.1886). ^{1}H and ^{13}C NMR data in combination with molecular formula indicated a tricyclic skeleton with two aromatic rings, corresponding to a flavanone. The geranyl chemotype was confirmed by successive connectivities from H-1″ to H-10″ in the COSY spectrum. HMBC correlation of H-1″ with C-5, C-6, and C-7 unveiled that this geranyl chemotype was situated on C6. The absolute configuration (S-form) at C2 was assigned with negative Cotton effect ($\Delta\varepsilon$ nm −2.0) at 292 nm.[15] Thus, compound **8** was characterized as diplacone (Supplementary data). The biological activities of geranylated flavonoids (**1–9**), and their parent compounds (**10–12**) were assessed against hAChE, and BChE, and confirmed by the positive control with eserine. It is important to note that these compounds had never been evaluated for cholinesterase inhibitory activity before. Table 1, shows that all isolated compounds exhibited a significant degree of cholinesterase inhibi-

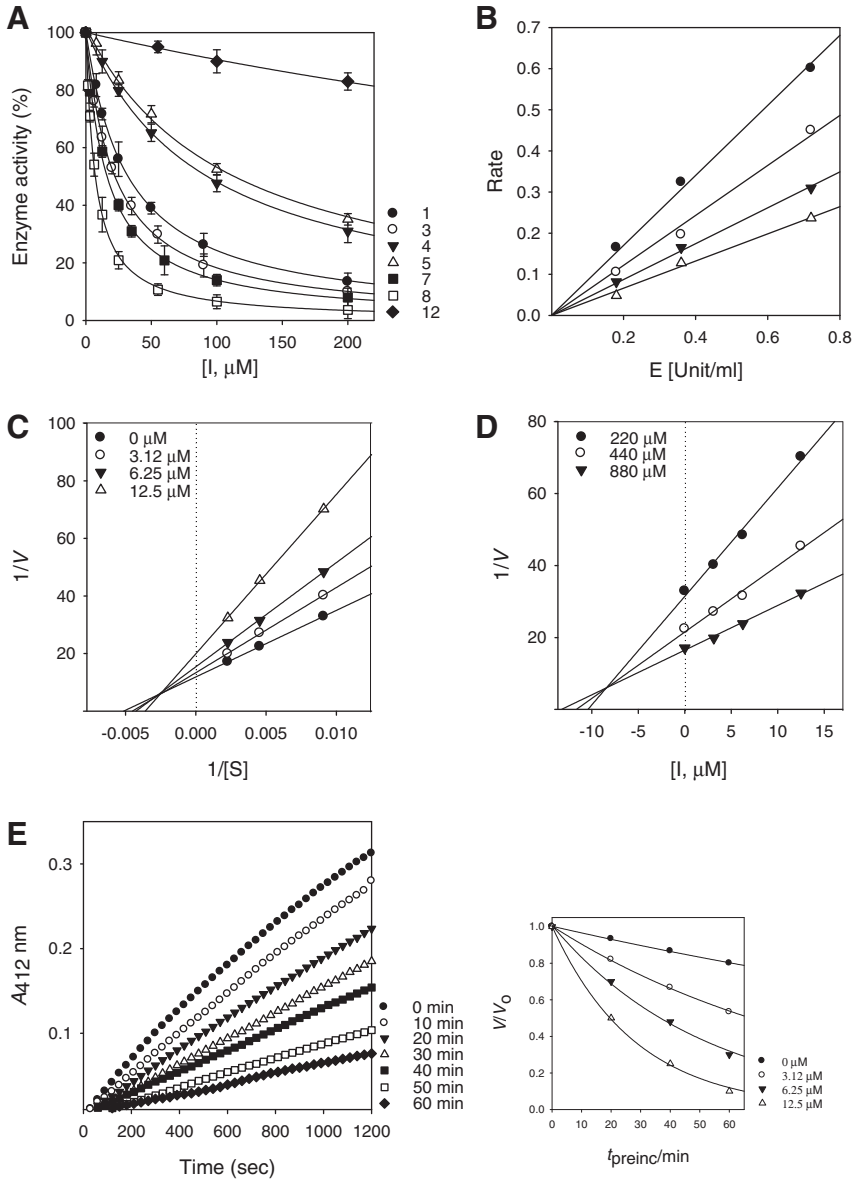

Figure 2. (A) Effect of isolated compounds on the acetylcholinestrase-catalyzed hydrolysis of acetylthiocholine iodide at 37 °C. (A) Activities represent production of thiocholine by the enzyme, The product then reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). (B) Relationship of the hydrolytic activity of hAChE with enzyme concentrations at different concentrations of **8** (●, 0 μM; ○, 3.12 μM; ▼, 6.25 μM; △, 12.5 μM). (C) Lineweaver–Burk plots for the effect of compound **8** on the hydrolytic activity of hAChE. (D) Dixon plot for compound **8** determining the inhibition constant K_i. (E) Time-dependent inhibition of hAChE in the presence of 6.25 μM compound **8**. (Inset) Decrease in slopes of the lines of panel E as a function of time (●, 0 μM; ○, 3.12 μM; ▼, 6.25 μM; △, 12.5 μM).

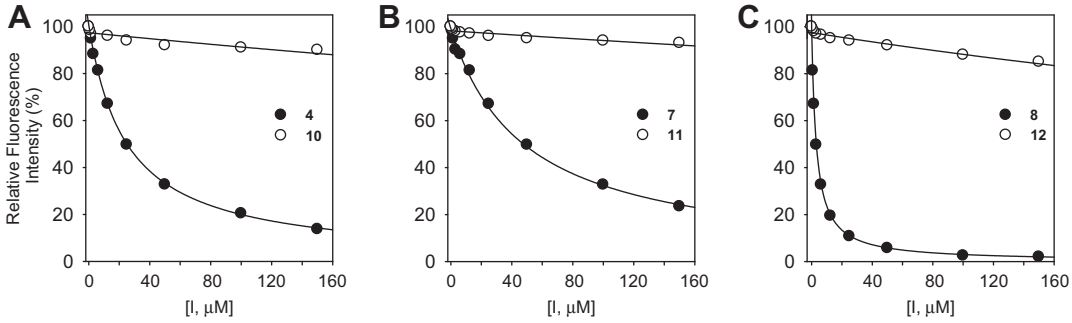

Figure 3. Relative fluorescence quenching effect of geranylated flavonoids and their corresponding parent compounds. (A) Compound **4** versus hesperetin **10**. (B) Compound **4** versus naringenin **11**. (C) Compound **8** versus eriodictyol **12**.

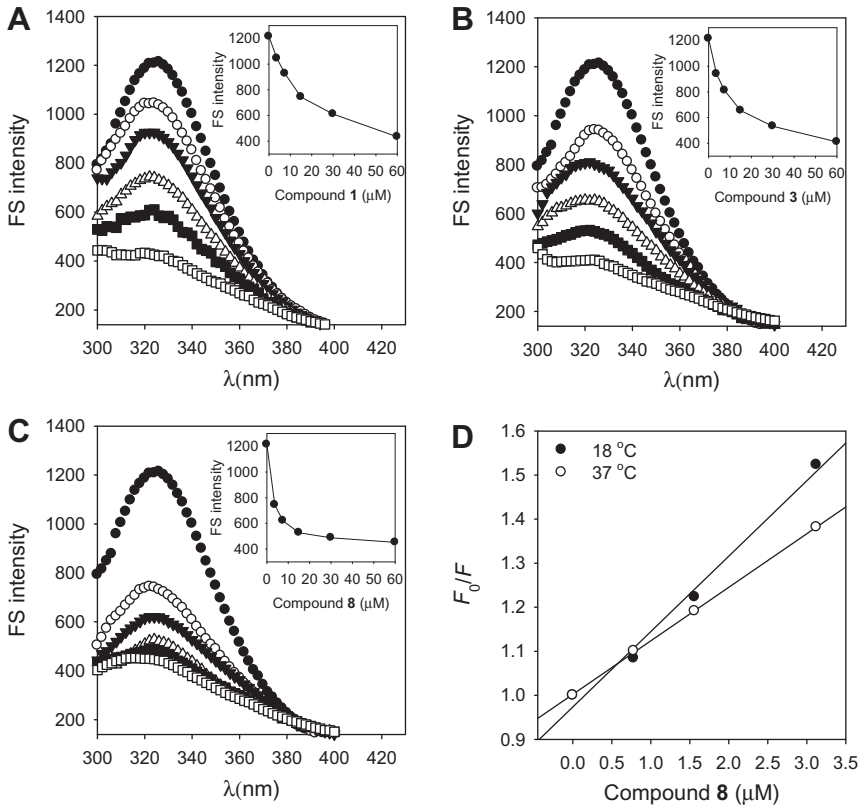

Figure 4. (A)–(C) The effect of compounds **1**, **3** and **8** on fluorescence spectra of hAChE after they were added into the enzyme solution. (Inset) normalized fluorescence intensity of hAChE with flavonoids **1**, **3**, **8**. Measurement condition: flavonoids (**1**, **3**, **8**) concentrations were at 0 (●), 3.7 (○), 7.5 (▼), 15 (△), 30 (■), and 60 μM (□), respectively. [hAChE] = 0.25 units, pH 8.0, at 37 °C, λ_{ex} = 276 nm, λ_{em} = 305 nm. (D) Stern–Volmer plots for flavonols **8** with hAChE at 18 and 37 °C (pH 8.0).

Table 2
Binding and quenching constants and binding sites for the tested compounds (**1**, **3**, **4**, and **8**) from *P. tomentosa*

Compound	K_{sv} ($\times 10^5$ LM^{-1})	R^2	K_A ($\times 10^3$ LM^{-1})	n	R^2
1	0.0285	0.9989	2.697	0.7548	0.9926
3	0.0369	0.9773	4.535	0.7724	0.9999
4	0.0103	0.9989	0.924	0.6824	0.9993
8	0.0577	0.9715	5.486	0.7258	0.9999

tion, with IC_{50}s ranging from 7.2–316 μM for hAChE and 1.4–80.0 μM for BChE. It is promising that the most potent flavonoids (**1–9**) could serve as inhibitors of both hAChE and BChE (Table 1). For example, compound **8** inhibited hAChE (IC_{50} = 7.2 μM) and BChE (IC_{50} = 1.4 μM), respectively. The potency of these inhibitors was affected by subtle changes in structure. It appears that better inhibition is observed when the flavanone B ring bears free hydroxyl groups. This is apparent from a comparison of compound **8** (IC_{50} = 7.6 μM) and compound **4** (IC_{50} = 92.4 μM), and **5** (IC_{50} = 109.2 μM) I this instance, compounds **4** and **5** have one of their two hydroxyl functions capped by a methyl group. In parallel comparison between homologous dihydroflavonols and flavanones (**1** vs **4**, **2** vs **5**, **3** vs **6**) established that dihydroflavonols were almost twofold as effective as flavones. The most striking aspect of these results, however is that geranylation at C6 is pivotal in the inhibition of both cholinesterases. Thus, compound **8** was 200-fold potent than unsubstituted analogue, eriodictyol **12** (IC_{50} = 1663 μM). A similar trend was observed for **4** vs hesperetin **10** (IC_{50} >2 mM) and **7** versus naringenin **11** (IC_{50} >2 mM). As expected from this trend, all geranylated flavonoids (**1–8**) apart from **9** inhibited hAChE dose-dependently (Fig. 2A).

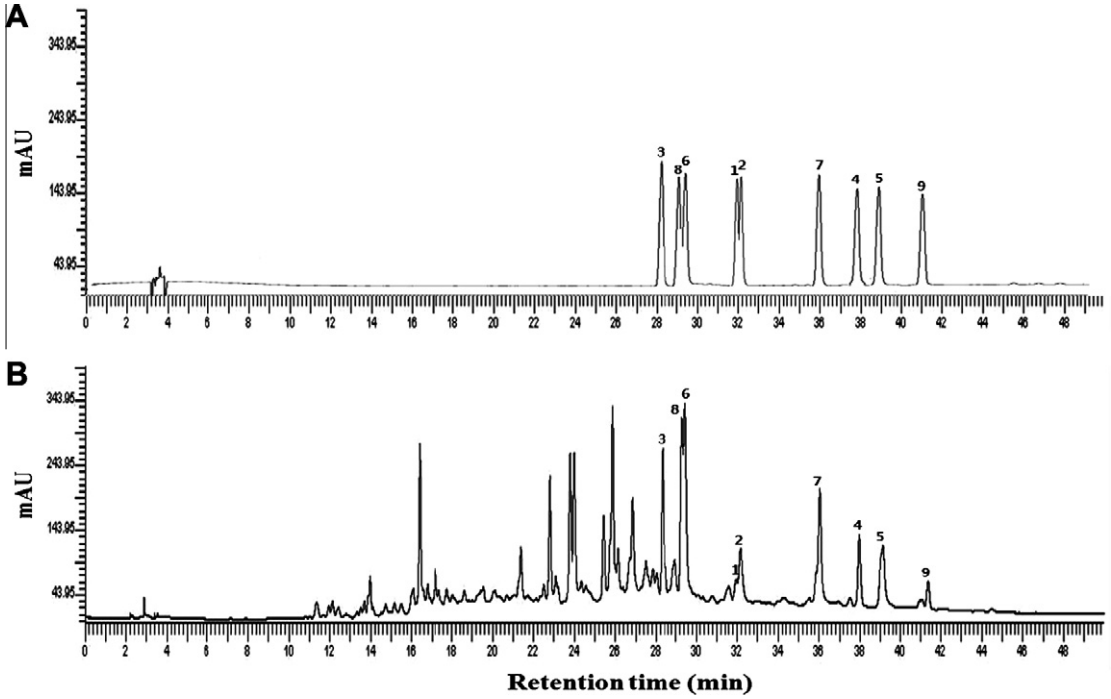

Figure 5. (A) Chromatogram of a mixture of all isolated polyphenols in this study; (B) chromatogram of MeOH extract.

The inhibition of hAChE by compound **8** is illustrated in Figure 2, representatively. Plots of the initial velocity versus concentration of **8** gave a family of straight lines, all of which passed through the origin. Increasing the inhibitor concentration resulted in a lowering of the line gradients, indicating that compound **8** was a reversible inhibitor (Fig. 2C). Further analysis showed that **8** exhibited mixed inhibition kinetics because increasing concentration of substrate resulted in a family of lines which declined with a common intercept on the left of vertical axis and above the horizontal axis. The K_i value of **8** was determined to be 8.4 μM by Dixon plot (Table 1 and Figure 2D).

The time dependence of the most potent inhibitor **8** on the hAChE-catalyzed hydrolysis of acetylcholine was subsequently investigated in more detail. This was achieved by measuring initial velocity of substrate hydrolysis as a function of preincubation time of the enzyme with **8**. In these experiments, hAChE was preincubated with inhibitor for a duration of between 0 and 60 min in 10 min intervals, and the residual velocity (v/v_0) was measured as function of incubation time. The enzyme was found to lose no more than 10% activity across the whole assay due to intrinsic decay (control). At fixed inhibitor concentrations, the residual velocity decayed exponentially with preincubation time. Such behavior is characteristic of a slow-binding inhibitor, as shown in Figure 2E.

We also studied the binding of inhibitors to hAChE by measuring quenching of intrinsic protein fluorescence. The fluorescence intensity of hAChE is attributed to its three intrinsic fluorophores, Trp 12, Tyr 22, and Phe 27. Under our measurement conditions (i.e., emission form 300 to 400 nm), there was no significant emission from any of the other components of the assay mixture. The experimental data were restricted to analysis of K_{SV} and K_A, since the τ_0 for tryptophan quenching in hAChE is not known. K_{SV} was analyzed using the Stern–Volmer equation:[21]

$$F_0 - F = 1 + K_{SV}[Q] \quad (1)$$

F and F_0 represent fluorescence intensity with and without quencher, respectively. K_{SV} is the Stern–Volmer constant, which can reflect the quenching extent, and [Q] is the quencher concentration. A decrease in the Fluorescence (FS) intensity, caused by quenching, was observed for all tested compounds in proportion to increasing concentration (Supplementary data). The FS quenching effects of compounds **1**, **3**, and **8** is displayed in Figure 4, representatively. The FS reductive tendencies accorded with their hAChE inhibitory potencies (Figure 4 and Table 1). In particular, a dramatic difference in fluorescence quenching was observed between geranylated flavonoids and their corresponding parent compounds (**4** vs **10**, **7** vs **11**, and **8** vs **12**) (Figure 3). This is in agreement with the sizeable difference in inhibitory potencies between these compound classes. It also suggests that the fluorescent chromophore of hAChE might be placed in a more hydrophobic environment. The fluorescence quenching spectra of hAChE by flavonoids were recorded at two temperatures (18 and 37 °C). As seen from Figure 4D, the values of K_{SV} decreased with the increase of temperature. For static quenching, the relationship between the change in the fluorescence intensity and the concentration of quencher for the set of reaction can be described by the following equation:

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

where F_0 and F are fluorescence intensities in the absence and the presence of quencher; Q_f is the concentration of free flavonoid; n is the number of binding sites; K_A is the binding constant.[22] From the plots of linear eq 2 obtained by $\log[(F_0\text{-}F)/F]$ versus $\log[Q]_f$, one can calculate the values of K_A and n as shown Table 2. The values of n approximates to 1 indicating that only a single binding site exists in hAChE for geranyl flavonoides. The binding constants of the inhibitors could be ranked in the following order **8** > **3** > **1** > **4**, which is in agreement with the order of their inhibition constants (K_i) as shown Tables 1 and 2.

To set the importance of these inhibitors in a practical context, we performed a comparative analysis of individual cholinesterase inhibitory flavonoids within the native fruits using HPLC. Relevant peaks in the HPLC trace were verified as one of the relevant compounds in this work not only by comparison with retention time of the pure compounds but also by LC/MS of each fraction. All compounds examined in this study were detected in the HPLC chromatogram, as shown in Figure 5. Importantly, LC/MS analysis of

each fraction shows characteristics consistent with our assigned structure. Moreover, the most active cholinesterase inhibitors **3**, **6**, and **8** were present in very high concentrations in the crude extract because they appeared as principal peaks in the chromatogram, such as at 28.32 min, 6-geranyl-3,3′,5,5′,7-pentahydroxy-4′-methoxyflavane (**3**), 29.08 min, diplacone (**8**), and 39.52 min, 6-geranyl-3′,5,5′,7-tetrahydroxy-4′-methoxyflavanone (**6**).

3. Conclusion

We have established *P. tomentosa* fruits as a rich source of cholinesterase inhibitors. Importantly, the inhibitors we characterized elicit a dual effect, in that they act against AChE as well as BChE. This is important because both enzymes have been implicated in AD progression, and thus drugs/compounds treating AD may be more effective should they inhibit both enzymes. Our work in this publication shows various aspects required for inhibitory activity, most importantly geranylation of C6, and this knowledge will certainly prove useful in further study. Furthermore, our thorough analysis demonstrates that our compounds are highly effective, slow-binding inhibitors, which renders these skeletons more attractive as lead compounds for SAR studies.

4. Materials and methods

4.1. General apparatus and chemicals

Melting points were measured on a Thomas Scientific capillary apparatus. Circular Dichroism (CD) spectra were measured in methanol (ca 0.1 mg/mL) using a Jasco J-715 CD spectropolarimeter (Gross-Umstadt, Germany). NMR spectra were recorded on a Bruker AM 500 spectrometer with TMS as an internal standard, and chemical shifts are expressed in δ values. EIMS and HREIMS were obtained on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan). Qualitative analyses were made using a Perkin–Elmer HPLC S200 (Perkin–Elmer, Bridgeport, USA). All purifications were monitored on commercially available glass-backed Merck precoated TLC plates and visualized under UV illumination at 254 nm and/or 366 nm or stained with 10% H_2SO_4 solution. Silica gel (230–400 mesh, Merck), RP-18 (ODS-A, 12 nm, S-150 μM, YMC), and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden) were used for column chromatography. All solvents used for extraction and isolation were of analytical grade.

4.2. Plant material

P. tomentosa fruits were collected at Jinju in Gyeongsang National University, Gyeongsangnam-do, Korea, in July 2010 and identified by Prof. Jae Hong Park. A voucher specimen (KHPark 071210) of this raw material is deposited at the Herbarium of Kyungpook National University (KNU). All chemicals used were of reagent grade and were purchased from Sigma Chemical Co. (St. Louis, Mo, USA), unless otherwise stated. All solvents were distilled before use.

4.3. Extraction and isolation of the inhibitors from *P. tomentosa* Fruits

The air-dried *P. tomentosa* Fruits (4.0 kg) were chopped, and extracted with MeOH (10 L × 3) at room temperature for 7 days. The combined filtrate was concentrated in vacuo to yield a dark brown gum (476 g, 11.9%). The MeOH extract was subjected to column chromatography (CC) on silica gel (10 × 40 cm, 230–400 mesh, 750 g) using a hexane to acetone gradient (50:1→1:1) to give 7 fractions (A–F). Pure compounds **1–9** were isolated by a range of chromatographic methods and characterized as described previously.[13–17] Fraction A (4.2 g) was fractionated by silica gel flash CC employing a gradient of hexane to acetone resulting in 9 subfractions (A1–A9). Subfractions A3 and A4, enriched with **5** and **9**, were combined (460 mg) and further purified by silica gel flash CC to yield compounds **5** (25 mg) and **9** (18 mg). Fraction B (8.9 g) was subjected to flash CC employing a gradient $CHCl_3$ to acetone giving 10 subfractions (B1–B10). Subfraction (B4–B6), enriched with compounds **4** and **7**, were combined (512 mg) and subjected to flash CC employing hexane/acetone gradient (30:1→5:1) to give compounds **4** (24 mg) and **7** (23.2 mg). Fraction C (1.8 g) was fractionated by silica gel flash CC employing a gradient of hexane to acetone, resulting in 9 subfractions. Subfractions (C6–C8), enriched with compounds **1** and **2**, were combined (362 mg) and further purified by reversed-phase CC (ODS-A, 12 nm, S-150 μM) eluting with CH_3OH/H_2O (4:1) to afford compounds **1** (42 mg) and **2** (33.2 mg). Fraction D (13.1 g) was fractionated by silica gel flash CC employing a gradient of hexane to acetone, resulting in 25 subfractions. Subfractions (D12–D15), enriched with compounds **6** and **8** were combined (308 mg) and further purified by Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden) with 80% CH_3OH as eluent, yielding compounds **6** (17 mg) and **8** (13 mg). Fraction E (4.6 g) was subjected to flash CC employing a gradient of $CHCl_3$ to EtOAc, giving 12 subfractions. Subfractions E2 and E3 were purified using Sephadex LH-20 column chromatography, eluting with 95% CH_3OH to afford compound **3** (18 mg).

4.3.1. 6-Geranyl-3,3′,5,7-tetrahydroxy-4′-methoxyflaane (1)

Pale yellow powder; mp >140 °C; EIMS, *m*/*z* 454 $[M]^+$; HREIMS, *m*/*z* 454.1993 (calcd for $C_{26}H_{30}O_7$ 454.1992); ^{1}H NMR (500 MHz, CH_3OH) δ 1.46 (3H, s, H-10″), 1.52 (3H, s, H-9″), 1.67 (3H, s, H-4″), 1.84 (2H, d, *J* = 7.0, 8.1 Hz, H-5″), 1.94 (2H, d, *J* = 7.2, 7.4 Hz, H-6″), 3.11 (2H, d, *J* = 7.1 Hz, H-1″), 3.78 (3H, s, H-4′OCH_3), 4.41 (1H, d, *J* = 10.8 Hz, H-3), 4.82 (1H, s, H-2), 5.10 (1H, t, *J* = 0.8, 6.17 Hz, H-7″), 5.12 (1H, d, *J* = 6.2 Hz, H-2″), 5.75 (1H, s, H-8), 6.73 (1H, d, *J* = 8.1 Hz, H-5′), 6.85 (1H, m, H-6′), 7.00 (1H, d, *J* = 1.8 Hz, H-2').[14]

4.3.2. 6-Geranyl-3,4′,5,7-tetrahydroxy-3′-methoxyflavane (2)

Pale yellow powder; mp >140 °C; EIMS, *m*/*z* 454 $[M]^+$; HREIMS, *m*/*z* 454.1990 (calcd for $C_{26}H_{30}O_7$ 454.1992); ^{1}H NMR (500 MHz, acetone–d_6) δ 1.43 (3H, s, H-10″), 1.49 (3H, s, H-9″), 1.64 (3H, s, H-4″), 1.82 (2H, t, *J* = 7.0, 8.0 Hz, H-5″), 1.94 (2H, t, *J* = 1.3, 2.1 Hz, H-6″), 3.15 (2H, d, *J* = 7.1 Hz, H-1″), 3.74 (3H, d, *J* = 2.4 Hz, H-3′OCH_3), 4.53 (1H, d, *J* = 11.6 Hz, H-3), 4.91 (1H, d, *J* = 11.6 Hz, H-2), 4.95 (1H, d, *J* = 7.0 Hz, H-7″), 5.12 (1H, d, *J* = 7.0 Hz, H-2″), 5.91 (1H, s, H-8), 6.74 (1H, d, *J* = 8.0 Hz, H-6′), 6.89 (1H, d, *J* = 6.3 Hz, H-2′), 7.07 (1H, d, *J* = 1.7 Hz, H-5′).[14]

4.3.3. 6-Geranyl-3,3′,5,5′,7-pentahydroxy-4′-methoxyflavane (3)

white needles; EIMS, *m*/*z* 470 $[M]^+$; HREIMS, *m*/*z* 470.1943 (calcd for $C_{26}H_{30}O_8$ 470.1941); ^{1}H NMR (500 MHz, acetone–d_6) δ 1.44 (3H, s, H-10″), 1.49 (3H, s, H-9″), 1.64 (3H, s, H-4″), 1.83 (2H, t, *J* = 7.0, 8.2 Hz, H-6″), 1.92 (2H, d, *J* = 2.2 Hz, H-5″), 3.15 (2H, d, *J* = 7.1 Hz, H-1″), 3.71 (2H, s, H-4′OCH_3), 4.49 (1H, d, *J* = 11.4 Hz, H-3), 4.84 (1H, d, *J* = 11.4 Hz, H-2), 4.96 (1H, dd, *J* = 5.6, 7.0 Hz, H-7″), 5.13 (1H, t, *J* = 1.1, 6.1 Hz, H-2″), 5.91 (2H, s, H-8), 6.62 (2H, d, *J* = 4.2 Hz, H-2′, 6′).[16]

4.3.4. 6-Geranyl-3′,5,7-trihydroxy-4′-methoxyflavanone (4)

Amorphous yellow powder; mp >102 °C; EIMS, *m*/*z* 438 $[M]^+$; HREIMS, *m*/*z* 438.2040 (calcd for $C_{26}H_{30}O_6$ 438.2042); ^{1}H NMR (500 MHz, acetone–d_6) δ 1.58 (3H, s, H-10″), 1.64 (3H, s, H-9″), 1.79 (3H, s, H-4″), 1.98 (2H, t, *J* = 7.0, 8.2 Hz, H-5″), 2.07 (2H, m, H-6″), 2.74 (1H, t, *J* = 3.0, 17.1 Hz, H-3b), 3.18 (1H, d, *J* = 13.0 Hz, H-3a), 3.30 (2H, d, *J* = 7.2 Hz, H-1″), 3.90 (3H, s, H-4′OCH_3), 5.10

(1H, d, J = 1.3 Hz, H-7″), 5.29 (1H, d, J = 1.2 Hz, H-2″), 5.29 (1H, d, J = 1.2 Hz, H-2″), 5.41 (1H, m, H-2), 6.07 (1H, d, J = 1.1 Hz, H-8), 6.89 (1H, d, J = 8.1 Hz, H-5′), 7.00 (1H, t, J = 2.0, 6.2 Hz, H-6′), 7.18 (1H, d, J = 1.9 Hz, H-2′), 12.48 (1H, d, J = 6.3 Hz, H-4).[14]

4.3.5. 6-Geranyl-4′,5,7-trihydroxy-3′-methoxyflavanone (5)

colorless powder; mp >103 °C; EIMS, m/z 438 $[M]^+$; HREIMS, m/z 438.2035 (calcd for $C_{26}H_{30}O_6$ 438.2042); ^{1}H NMR (500 MHz, CD_3OD) δ 1.47 (3H, s, H-10″), 1.53 (3H, s, H-9″), 1.65 (3H, s, H-4″), 1.84 (2H, t, J = 7.1, 8.2 Hz, H-5″), 1.95 (2H, t, J = 7.4, 7.5 Hz, H-6″), 2.45 (1H, t, J = 3.1, 17.0 Hz, H-3b), 2.85 (1H, d, J = 12.6 Hz, H-3a), 3.08 (2H, d, J = 6.9 Hz, H-1″), 3.77 (3H, s, H-3′OCH_3), 4.99 (1H, t, J = 1.2, 1.3 Hz, H-7″), 5.08 (1H, t, J = 3.3, 9.3 Hz, H-2), 5.14 (1H, t, J = 1.1, 5.7 Hz, H-2″), 5.62 (1H, s, H-8), 6.69 (1H, t, J = 3.5, 8.1 Hz, H-5′), 6.78 (1H, t, J = 1.8, 6.4 Hz, H-6′), 6.94 (1H, d, J = 1.8 Hz, H-2′).[15]

4.3.6. 6-Geranyl-3′,5,5′,7-tetrahydroxy-4′-methoxyflavanone (6)

Colorless powder; mp >108 °C; EIMS, m/z 454 $[M]^+$; HREIMS, m/z 454.1992 (calcd for $C_{26}H_{30}O_7$ 454.1992); ^{1}H NMR (500 MHz, acetone–d_6) δ 1.58 (3H, s, H-10″), 1.64 (3H, s, H-9″), 1.78 (3H, s, H-4″), 1.97 (2H, t, J = 7.0, 8.1 Hz, H-5″), 2.06 (2H, t, J = 4.4, 12.0 Hz, H-6″), 2.73 (1H, dd, J = 2.8, 17.1 Hz, H-3b), 3.16 (1H, dd, J = 13.0, 10.9 Hz, H-3a), 3.28 (2H, d, J = 7.1 Hz, H-1″), 3.86 (3H, s, H-4′OCH_3), 5.09 (1H, t, J = 6.2, 6.8 Hz, H-7″), 5.26 (1H, d, J = 10.9 Hz, H-2), 5.27 (1H, t, J = 7.0, 14.1 Hz, H-2″), 6.07 (1H, s, H-8), 6.72 (1H, s, H-6′), 6.73 (1H, s, H-2′).[14]

4.3.7. 6-Geranyl-4′,5,7-trihydroxyflavane (7)

Colorless powder; mp >116 °C; EIMS, m/z 408 $[M]^+$; HREIMS, m/z 408.1937 (calcd for $C_{25}H_{28}O_5$ 408.1937); ^{1}H NMR (500 MHz, CD_3OD) δ 1.52 (3H, s, H-10″), 1.60 (3H, s, H-9″), 1.73 (3H, s, H-4″), 1.99 (2H, d, J = 6.2 Hz, H-5″), 2.02 (2H, t, J = 6.5, 12.7 Hz, H-6″), 2.70 (1H, dd, J = 2.6, 17.1 Hz, H-3b), 3.00 (1H, dd, J = 13.0, 17.1 Hz, H-3a), 3.26 (2H, d, J = 7.0 Hz, H-1″), 5.18 (1H, t, J = 6.7, 13.63 Hz, H-2″), 5.24 (1H, dd, J = 2.5, 12.9 Hz, H-2), 5.92 (1H, s, H-8), 6.79 (1H, s, H-5′), 6.80 (1H, s, H-3′), 7.22 (1H, s, H-6′), 7.24 (1H, s, H-2′). [16]

4.3.8. 6-Geranyl-3′,4′,5,7-tetrahydroxy-flavanone (8)

Colorless powder; mp >124 °C; EIMS, m/z 424 $[M]^+$; HREIMS, m/z 424.1889 (calcd for $C_{25}H_{28}O_6$ 424.1886); ^{1}H NMR (500 MHz, CD_3OD) δ 1.45 (3H, s, H-10″), 1.51 (3H, s, H-9″), 1.64 (3H, s, H-4″), 1.84 (2H, t, J = 7.1, 7.9 Hz, H-5″), 1.94 (2H, m, H-6″), 2.57 (1H, dd, J = 2.8, 17.1 Hz, H-3b), 2.94 (1H, m, H-3a), 3.11 (2H, d, J = 7.1 Hz, H-1″), 4.95 (1H, t, J = 7.0, 7.1 Hz, H-7″), 5.09 (1H, t, J = 7.0, 7.1 Hz, H-2″), 5.13 (1H, dd, J = 2.7, 12.8 Hz, H-2), 5.83 (1H, s, H-8), 6.68 (2H, s, H-2′, 6′), 6.81 (1H, s, H-5′).[15]

4.3.9. 6-Geranyl-4′,5,7-trihydroxy-3′,5′-dimethoxyflavanone (9)

Colorless powder; mp >78 °C; EIMS, m/z 468 $[M]^+$; HREIMS, m/z 468.2151 (calcd for $C_{27}H_{32}O_7$ 468.2148); ^{1}H NMR (500 MHz, acetone–d_6) δ 1.43 (3H, s, H-10″), 1.49 (3H, s, H-9″), 1.64 (3H, s, H-4″), 1.83 (2H, t, J = 7.0, 8.2 Hz, H-5″), 1.93 (2H, t, J = 1.4, 2.0 Hz, H-6″), 2.59 (1H, dd, J = 2.8, 17.1 Hz, H-3b), 3.06 (1H, t, J = 3.6, 8.9 Hz, H-3a), 3.14 (2H, d, J = 7.1 Hz, H-1″), 3.72 (6H, s, H-3′OCH_3, 5′OCH_3), 4.95 (1H, t, J = 1.2, 1.3 Hz, H-7″), 5.13 (1H, t, J = 0.9, 6.2, H-2″), 5.26 (1H, t, J = 2.8, 13.0, H-2), 5.93 (1H, s, H-8), 6.74 (2H, d, J = 6.8, H-2′, 6′).[15]

4.4. Assay of cholinesterase inhibitory activity

Cholinesterase was assayed according to standard procedures by following the hydrolysis of acetylthiocholine iodide (AtCh) or butyrylthiocholine iodide (BtCh) by monitoring formation of 5-thio-2-nitrobenzoate spectrometrically at 412 nm from 5-5′-dithiobis (2-nitrobenzoic acid).[18–20] The reaction mixture contained 100 mM sodium phosphate buffer (pH 8.0), 20 μL of test sample solution and either 2 μL of human erythrocyte AChE (0.25 U/mL) or 2 μL of equine serum BChE (0.05 U/mL) solution, which were mixed and incubated for 10 min at room temperature. Assays were then initiated with the addition of 30 μL 5-5′-dithiobis(2-nitrobenzoic acid) (3.3 mM) and, respectively, 20 μL of AtCh (human erythrocyte, 1 mM), and 7 μL of BtCh (1 mM). All assays were performed in triplicate. Compounds showing the highest inhibitory activities were further characterized by determining the concentration required to inhibit 50% of the enzyme activity under the assay conditions (defined as the IC_{50} value). Kinetic parameters were determined using the Lineweaver–Burk double-reciprocal-plot method at increasing concentration of substrates and inhibitors. The inhibitors quercetin and eserine (Sigma–Aldrich, St. Louis, MO) were used in the assays for comparison.

4.5. Slow and time-dependent inhibitory activity

Slow and time-dependent assays and progress curves were carried out using 0.25 U/mL unit hAChE, and 1 mM AtCh in 100 mM sodium phosphate buffer (pH 8.0) at 37 °C. Enzyme activities were measured continuously for 300 s spectrophotometrically. To determine the kinetic parameters associated with time-dependent inhibition of acetylcholinesterase, progress curves for 300 s were obtained at one inhibitor concentration using fixed substrate concentrations.[23,24] The data were analyzed using the nonlinear regression program [Sigma Plot (SPCC Inc., Chicago, IL)].

4.6. Fluorescence quenching measurements

All fluorescence spectra were measured on a SpectraMax M2 Multi-Mode Microplate Reader (Molecular Devices, CA, USA) equipped with a 10.0 mm quartz cell and a thermostat bath. In a typical fluorescence measurement, 30 μL of hAChE solution (100 mM sodium phosphate buffer, pH 8.0) with a concentration of 5 U/mL was added to the quartz cell and then titrated by successive additions of inhibitors. Titrations were operated manually and mixed moderately. The fluorescence emission spectra were measured at 18 and 37 °C with the width of the excitation and emission slit both adjusted at 2.0 nm. The excitation wavelength was 276 nm, and the emission spectrum was recorded from 300 to 400 nm.[25] All experiments were performed in triplicate, and the mean values were calculated.

4.7. HPLC analysis

Quantification of the relative abundance of the compounds isolated and assayed in this manuscript within the crude fruit extract was carried out by HPLC (Perkin–Elmer 200 series, Perkin–Elmer Co., Bridgeport, USA) using a Zorbax SB-C 18 column (4.6 × 150 mm, 5 μM, Agilent, USA). Absorbances were measured at 280 nm. About 10 μL of extract was loaded onto the column. Gradient elution was carried out with water/0.1% acetic acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 1.0 mL/min. The linear gradient elution program was as follows: 0–10 min, 0–20% B; 10–20 min, 20–40% B; 20–30 min, 40–60% B; 30–40 min, 60–80% B; 40–50 min, 80–100% B.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.02.044.

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