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Isolation of Acetylcholinesterase Inhibitors from the Flowers of *Chrysanthemum indicum* Linne

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Abstract There is significant interest in finding new sources of acetylcholinesterase (AChE) inhibitors for use in treating Alzheimer's disease, since only a few AChE inhibitors are available for clinical use, such as galanthamine, physostigmine, and tacrine. The ethanol extract of *Chrysanthemum indicum* Linne flowers was analyzed and found to markedly decrease AChE activity. Acaciin and acacetin-7-*O*-β-D-galactopyranoside were identified as the active compounds responsible for the AChE inhibition by using an activity-guided fractionation strategy. The relationship between structure and activity for five flavonoids (acaciin, acacetin-7-*O*-β-D-galactopyranoside, luteolin, and two other commercially available flavonoids, i.e., apigenin and acacetin) was also investigated, revealing that the presence of methoxy groups at C-4' in the B ring and a sugar at O-7 in ring A appear to be essential for the inhibition of AChE.

Keywords: acetylcholinesterase inhibitor, *Chrysanthemum indicum*, Acaciin, acacetin-7-*O*-β-D-galactopyranoside, luteolin

Introduction

The identification of acetylcholinesterase (AChE) inhibitors has become an important area of pharmaceutical research due to the involvement of AChEs in Alzheimer's disease (AD) and other related dementias. AChE is a key component of cholinergic brain synapses and neuromuscular junctions. The major biological role of the enzyme is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine (1). Therefore, the inhibition of AChE is generally considered to be an effective treatment for AD (2, 3). Galanthamine has been widely used as an AChE inhibitor for the treatment of AD, and is claimed to have fewer side effects than other inhibitors such as physostigmine and tacrine (4).

Previous studies have reported that *Chrysanthemum indicum* contains antibacterial, antiviral, antioxidant, anti-inflammatory, and immunomodulatory properties (5). Phytochemical profiles of the plant have shown the presence of flavonoids, terpenoids, and phenolic compounds (6-9). Through a series of studies evaluating naturally occurring AChE inhibitors, we discovered that the methanol extract of *C. indicum* flowers, which has been used for the treatment of headache, insomnia, dizziness, influenza, eye diseases, etc. in various Chinese medicines (10), exhibited significant inhibitory activity toward AChE *in vitro*. In the present study, we analyzed the ethanol extract of *C. indicum* Linne flowers in order to characterize the active compound(s) responsible for its AChE inhibitory activity.

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Materials and Methods

Equipment The following instruments were used to obtain the indicated physical measurements: specific rotations, Horiba SEPA-300 digital polarimeter (*l*:55 cm; Horiba, Kyoto, Japan), UV spectra (UV-1600 spectrometer; Shimadzu, Kyoto, Japan), CD spectra (J-720WI spectropolarimeter; Jasco, Tokyo, Japan), IR spectra (FTIR-8100 spectrometer; Shimadzu), MS and high-resolution MS (JMS-GCMATE mass spectrometer; Jeol, Tokyo, Japan), ^{1}H-NMR spectra (at 400 MHz) and ^{13}C-NMR (at 100 MHz) spectra (DPX 400 spectrometer; Bruker BioSpin GmbH, Rheinstetten, Germany) with tetramethyl-silane as an internal standard.

The following experimental conditions were used for chromatography: ordinary-phase column chromatography; TLC, silica gel 60 F_{254} Al sheets (E. Merck Co., Darmstadt, Germany) using *n*-Hexane-EtOAc (1:1); reverse phase HPLC was carried out using the HP-1100 (Hewlett-Packard Inc., Palo Alto, CA, USA) HPLC system equipped with a binary pump, an Agilent Prep-C18 Scalar column (Agilent, 250 mm × 4.6 mm i.d.) and a guard column (ODS, 4 mm × 3.0 mm i.d.; Phenomenex Inc., Torrance, CA, USA). Semi-preparative HPLC was performed to fractionate the active fraction on a JAI-908-C60 HPLC (Japan Analytical Industry Co., Tokyo, Japan) equipped with a JAI RI and JAI UV detector operating at 280 nm. A commercially available prepacked ODS-BP column (JAI-GEL 50×2.1 cm i.d.) was employed. A Hewlett-Packard 1100 series on-line photodiode array detector (DAD) was used for detection. The instrumentation was controlled by HP Chemstation software. GLC was carried out with the following system: HP-6890 GC (Hewlett-Packard), with a Supelco STBTM-1 (30 m × 0.25 mm i.d.); and a capillary column with a 230°C injector temperature, 230°C detector temperature, and 240°C column temperature. The flow rate was 15 mL/min.

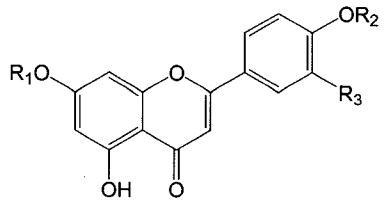
Plant material *Chrysanthemum indicum* Linne flowers were purchased from Dea-Gang Herb, Chuncheon, Gangwon, Korea in Sept. 2004. The botanical identity was confirmed by emeritus professor Heung Jun Chi, Department of Pharmacy, Seoul National University, Korea. The voucher specimens are kept at Regional Innovation Center, Hallym University Chuncheon, Gangwon, Korea.

AChE inhibition assay AChE activity was measured using a 96-well microplate reader based on Ellman's method (11, 12). The enzyme hydrolyzes the substrate acetylthiocholine, producing thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 405 nm. In the 96-well plates, 25 mL of 15 mM ATCI in water, 125 mL of 3 mM DTNB in buffer C (50 mM Tris–HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2 \cdot 6H_2O$), 50 mL of buffer B (50 mM Tris-HCl, pH 8, containing 0.1% BSA), 25 mL of sample (10 mg/ mL in MeOH diluted ten times with buffer A (50 mM Tris-HCl, pH 8), to give a concentration of 1 mg/ mL) were added and the absorbance was measured at 405 nm every 13 sec a total of five times. After 25 mL of 0.22 U/ mL of enzyme was added, the absorbance was again read every 13 sec a total of eight times. The reaction rate was calculated by the Microplate Manager Software version 4.0 (Bio-Rad Laboratories, Hercules, CA, USA). Any increase in absorbance due to the spontaneous hydrolysis of substrate was corrected by subtracting the rate of the reaction before adding the enzyme from the rate after adding the enzyme. Inhibition rates were calculated by comparing the samples with the control (10% MeOH in buffer).

Extraction and isolation of compounds Dried flowers (2,000 g) were extracted with hot ethanol three times, concentrated under reduced pressure to dryness (350 g), and then partitioned into methylene chloride (32 g) and ethylacetate (11 g) soluble fractions with residue (105 g). The ethylacetate soluble fraction, which had the most potent AChE inhibitory activity, was subjected to silica gel column (100×5 cm i.d.) chromatography and eluted with ethylacetate-MeOH (gradient, 1-20% MeOH), resulting in the production of 9 fractions (A to I). All fractions were tested using the AChE assay, which showed fractions B (1.22 g) and D (0.85 g) to have inhibitory activity. Fraction B was further fractionated using silica gel column (40×3 cm i.d.) chromatography and elution with MeOH-chloroform (gradient, 5-9% methanol), giving fractions B-1 to B-12 each of which was tested by the AChE assay. Purification of the remainder of fraction B-5 (250 mg) was performed using a semi-preparative HPLC system with a MeCN-H_2O (5:1) mixture and UV detection at 280 nm to yield compound I (63 mg). Gel filtration of fraction B-8 on Sephadex LH-20 (120×2 cm i.d.) with methanol yielded compound III (25 mg). Finally, 25 mg of compound II was obtained after separation of fraction D by semi-preparative HPLC with MeCN-H_2O (8:1) and UV detection at 280 nm.

Compound I (acaciin): Yellow powder (recrystallized from MeOH); m.p. 269-271; $C_{28}H_{32}O_{14}$, molecular weight: 592.55, $[\alpha]_D^{24}$ = -90.3° (Py) UV(λ_{max}, nm) (MeOH): 286, 326; (+NaOMe): 280, 368; (+$AlCl_3$): 276, 301, 343, 384; (+ $AlCl_3$ + HCl): 277, 300, 339, 384; (MeOH + NaOAc): 271, 320; (+ NaOAc + H_3BO_3): 269, 328. IR ν (/cm, KBr): 3420 (OH), 1640 (α,β-unsaturated ketone), 1480, 1600 (aromatic C=C), 1070, 1030 (glucosidic C-O). EI-MS (70eV): $[M+H]^+$ = 593, *m/z* = 285 (100, aglycone H), 461, 307, HR-MS (FAB) $[M+H]^+$ Exact mass 593.1870, Found mass: 593.1875. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 12.91 (1H, s, 5-OH), 8.06 (2H, d, J=8.91Hz, H-2',6'), 7.15 (2H, d, J=8.93Hz, H-3',5'), 6.95 (1H, s, H-3), 6.79 (1H, d, J=2.1Hz, H-8), 6.45 (1H, d, J=2.1Hz, H-6), 5.06 (1H, d, J=7.26Hz, H-1"), 4.55 (1H, s, H-1'"), 3.92 (1H, m, H-6"), 3.86 (3H, s, 4'-OCH_3), 3.73 (1H, m, H-2'"), 3.65 (1H, m, H-5'"), 3.52 (1H, m, H-3'"), 3.49 (1H, m, H-6'"), 3.45 (1H, m, H-5'"), 3.38 (1H, m, H-2"), 3.35 (1H, m, H-3"), 3.19 (1H, m, H-4"), 3.18 (1H, m, H-4'"), 1.07 (3H, d, J=6.29Hz, 6'"-CH_3), 5.44 (1H, d, J=4.9Hz, 3"-OH), 5.21 (1H, dd, J=5.4, 4.9Hz, 4"-OH), 4.70 (1H, d, J=5.5Hz, 4'"-OH), 4.60 (1H, d, J=4.4Hz, 2"-OH), 4.45 (1H, d, J=5.5Hz, 3'"-OH). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 162.8 (C-2), 103.7 (C-3), 181.9 (C-4), 162.3 (C-5), 99.6 (C-6), 163.8 (C-7), 94.7 (C-8), 156.9 (C-9), 105.3 (C-10), 122.5 (C-1'), 128.3 (C-2'), 114.6 (C-3'), 161.0 (C-4'), 114.6 (C-5'), 128.3 (C-6'), 55.5 (C-4'-OCH_3), 99.8 (C-1"), 72.9 (C-2"), 76.2 (C-3"), 69.4 (C-4"), 75.6 (C-5"), 66.0 (C-6"), 100.4 (C-1'"), 70.6 (C-2'"), 70.2 (C-3'"), 70.2 (C-4'"), 68. 3(C-5'"), 17.7 (C-6'").

Compound II (acacetin-7-*O*-β-D-galactopyranoside): Yellow powder (recrystallized from MeOH); m.p. 259-261°C; $C_{22}H_{22}O_{10}$, molecular weight: 446.4, $[\alpha]_D^{24}$ = -60° (Py) UV (λ_{max}, nm) (MeOH): 268, 325; (+ $AlCl_3$): 277, 302, 350, 380; (+ $AlCl_3$/HCl): 278, 300, 340, 381; (+NaOMe): 244 (sh), 290, 355; (+ NaOAc): 268, 320; (+ NaOAc/H_3BO_3): 269, 328. IR ν (/cm, KBr): 3350 (br.,-OH), 1660 (flavone >CO), 1610, 1519 (aromatic), 820 (*p*-substituted phenyl ring), 1075 (glycosidic C=O). EI-MS (70eV): M^+ = 446, *m/z* = 284 (100, aglycone), 241, 152, 132. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 12.93 (1H, s, 5-OH), 8.06 (2H, d, J=8.26Hz, H-2',6'), 7.12 (2H, d, J=8.4Hz, H-3',5'), 6.94



Compound		R_1	R_2	R_3
	I	Glc $\overset{6}{\underset{1}{\rightarrow}}$ Rha	CH_3	H
	II	Gal	CH_3	H
	III	H	H	OH
	IV	H	H	H
	V	H	CH_3	H

* Glc,glucopyranoside; Rha, rhamnopyranoside; Gal, galactopyranoside

Fig. 1. Flavonoid Structures.

(1H, d, J=1.8Hz, H-8), 6.86 (1H, s, H-3), 6.46 (1H, d, J=1.6Hz, H-6), 5.44 (1H, J=4.1Hz, OH), 5.16 (1H, J=3.19 Hz, OH), 5.09 (1H, overlapped, OH), 5.09 (1H, J=7.22Hz, anomeric H), 3.87 (3H, s, 4'-OCH_3), 3.21-3.74 (sugar, 6H). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 162.9 (C-2), 103.6 (C-3), 181.9 (C-4), 162.3 (C-5), 99.4 (C-6), 163.7 (C-7), 94.8 (C-8), 156.8 (C-9), 105.3 (C-10), 122.0 (C-1'), 128.3 (C-2'), 114.5 (C-3'), 161.0 (C-4'), 114.5 (C-5'), 128.3 (C-6'), 55.8 (C-4'-OCH_3), 99.8 (C-1"), 73.0 (C-2"), 77.0 (C-3"), 69.4 (C-4"), 76.3 (C-5"), 60.5 (C-6").

Acid hydrolysis of compounds I and II A solution of compounds I and II (2 mg each) in 5% aqueous H_2SO_4-1,4-dioxane (0.5 mL, 1:1, v/v) was heated under reflux for 1 hr. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH_2 form) and the residue removed by filtration. After removal of the solvent *in vacuo*, the residue was transferred to a Sep-Pak C18 cartridge with H_2O and MeOH. The H_2O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (4 mg) in pyridine (0.5 mL) at 60°C for 1 hr. The solution was then treated with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (0.2 mL) at 60°C for 1 hr. The supernatant was then subjected to GLC analysis to identify the derivatives of D-glucose [i, *t*R: 24.8 min (from I)] and D-galactose [ii, *t*R: 26.5 min (from II)].

Compound III (luteolin): Yellow powder (recrystallized from MeOH); m.p. 328°C; $C_{15}H_{10}O_6$, molecular weight: 286.2, UV(λ_{max}, nm) (MeOH): 253, 267, 291 (sh), 349; (+NaOMe) 266 (sh), 329 (sh), 401, IR ν (/cm, KBr): 3200-3400 (br., -OH), 1660 (>C=O), 1605, 1498 (C=C), EI-MS (70 eV): M^+ = 286, *m/z* = 258, 229, 153 (100.0), 152, 137, 134; ^{1}H-NMR (DMSO-D_6, 400 MHz) δ: 6.18 (1H, d, J= 2.0Hz, H-6), 6.43 (1H, d, J=9.0Hz, H-5'), 7.38 (1H, s, H-2'), 7.45 (1H, dd, J=2.2, 9.0Hz, H-6'), 9.31, 9.81, 10.74 (br.s, 3×OH), 12.96 (1H, s, 5-OH). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 164.5 (C-2), 103.3 (C-3), 182.2 (C-4), 162.1 (C-5), 99.2 (C-6), 164.7 (C-7), 94.2 (C-8), 157.9 (C-9), 104.2 (C-10), 122.1 (C-1'), 113.8 (C-2'), 150.1 (C-4'), 116.4 (C-5'), 119.3 (C-6').

Results and Discussion

AChE inhibitors, such as tacrine (THA, Cognex®)(13), donepezil (Aricept®)(14), rivastigmine (Exelon®)(15), and galanthamine (Reminyl®)(16), are known to increase brain acetylcoline (ACh) levels by preventing the degradation of released neurotransmitter, thereby enhancing neurotransmission at cholinergic synapses (17). Tacrine, the first approved drug for AD treatment in the United States, is a potent AChE inhibitor, but its side effects, low selectivity and hepatotoxicity (18), are considered to be unfavorable.

In our ongoing screening of natural products to identify sources of nontoxic and potent AChE inhibition, the ethanol extract of *C. indicum* Linne flowers exhibited the highest potency. Subsequently, in order to isolate any compound(s) responsible for the AChE inhibition activity, an activity-guided fractionation strategy was followed throughout the separation procedure as described in Methods. As shown in Table 1, the ethylacetate soluble fraction was found to have the most potent inhibitory activity, its IC_{50} values being 2.02×10^{-7} g/mL. Further fractionation and isolation of the ethylacetate fraction by repeated column chromatography produced three significant compounds (I, II, and III). The structures of compounds I to III were characterized by ^{1}H, ^{13}C NMR spectroscopy combined with EI mass spectrometry and determined to be acaciin (I), acacetin-7-*O*-β-D-galatopyranoside (II), and luteolin (III). The structure of luteolin (III) was confirmed by HPLC/UV as well as ^{1}H and ^{13}C-NMR in comparison with commercially available substances. NMR data of acaciin (I) and acacetin-7-*O*-β-D-galactopyranoside (II) were in agreement with the values obtained by Wagner *et al.* (19) and Chatterjee *et al.* (20), respectively. The three isolated compounds and two commercially available substances, apigenin (IV) and acacetin (V), were subjected to tests for AChE inhibitory activity at three graded concentrations with the results shown in Table 2.

Compounds I and II possessed a sugar moiety at O-7 and showed stronger activity than the corresponding aglycone, compound V. Compound V, with a 4'-methoxyl group, was far more potent than compound IV which has a hydroxyl group at the 4'-position. Compounds III and IV exhibited much weaker activities. These results provide some insight into the structure-activity relationship regarding AChE enzyme inhibition of flavone derivatives having substitutions at C-4' in the B ring and a sugar at O-7 in ring A.

Table 1. Effect of various fractions on AChE activity *in vitro*

Extract and fractions	IC_{50} (μg/mL)[1)]			
Ethanol extract	25.5			
Methylene chloride fr.	72.1			
Ethylacetate fr.	20.2			
Sub. A	35.9			
Sub. B	10.1			
Re-sub. B-1,	48.3		Re-sub. B-7,	29.9
Re-sub. B-2,	58.6		Re-sub. B-8,	25.2
Re-sub. B-3,	22.1		Re-sub. B-9,	98.4
Re-sub. B-4,	69.5		Re-sub. B-10,	87.3
Re-sub. B-5,	5.6		Re-sub. B-11,	39.8
Re-sub. B-6,	26.2		Re-sub. B-12,	102.4
Sub. C	48.9			
Sub. D	15.2			
Sub. E	88.4			
Sub. F	76.3			
Sub. G	65.2			
Sub. H	92.9			
Sub. I	88.9			
H_2O fr.	120.6			

[1)] IC_{50} values representing the concentration that caused 50% inhibition of enzyme activity were calculated from least-square regression equations in the plot of the logarithm of three graded concentrations vs. % inhibition.

Table 2. Effect of flavonoids on AChE activity *in vitro*

Compounds	IC_{50} (μM)[1)]
Acaciin (I)	3.2
Acacetin-7-*O*-β-D-galactopyranoside (II)	6.7
Luteolin (III)	20.5 % (at 100 μM)
Apigenin (IV)	15.6 % (at 100 μM)
Acacetin (V)	65.3
Galanthamine[2)]	0.64

[1)]IC_{50} values representing the concentration that caused 50% inhibition of enzyme activity were calculated from least-square regression equations in the plot of the logarithm of three graded concentrations vs. % inhibition.
[2)]Galanthamine, a reference compound and typical AChE inhibitor.

Compounds I (IC_{50}= 3.2 μM) and II (6.7 μM) showed the most potent inhibition of AChE. However, the inhibitory activities of compounds I and II were weaker than that of galanthamine (0.62 μM), the reference standard (21).

Oxidative stress has been implicated as a contributing factor in the neurodegeneration characteristic of AD (22). Strong antioxidative activity in *Chrysanthemum* spp. has been reported (23-26), and the antioxidative activity of luteolin and apigenin is well known (27, 28). The present one-drug-one-target paradigm in new drug exploration is considered partially responsible for the more-funding-less-drug predicament in the modern pharmaceutical industry. Any drug that is effective on multiple targets, whether it contains one or more ingredients, would be preferable. The present study suggests that natural antioxidants, including flavonoids such as compounds I, II, or III from *C. indicum* Linne, may improve the efficacy of AChE inhibition.

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