

8-Hydroxydihydrochelerythrine and 8-Hydroxydihydrosanguinarine with a Potent Acetylcholinesterase Inhibitory Activity from *Chelidonium majus* L.

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Ethanol extract of the aerial portion of *Chelidonium majus* L. inhibited acetylcholinesterase (AChE) activity without a significant inhibition of butyrylcholinesterase (BuChE). Using mass spectrometry and NMR studies, three active constituents were isolated and identified: 8-hydroxydihydrochelerythrine (1), 8-hydroxydihydrosanguinarine (2), and berberine (3). Compounds 1–3 showed potent inhibitory activity against AChE, with IC₅₀ (μM) values of 0.61–1.85. Compound 1 exhibited competitive and selective inhibition for AChE.

Key words *Chelidonium majus* L.; acetylcholinesterase; 8-hydroxydihydrochelerythrine; 8-hydroxydihydrosanguinarine

Alzheimer's disease is a degenerative disorder of the central nervous system and is the most common cause of dementia among the elderly. Neuropathological evidence has demonstrated that cholinergic functions decline in the basal forebrain and cortex in senile dementia of the Alzheimer type.^{1,2)} Accordingly, enhancement of cholinergic neurotransmission has been considered as one potential therapeutic approaches against Alzheimer's disease. One treatment strategy to enhance cholinergic function is the use of acetylcholinesterase (AChE, EC 3.1.1.7) inhibitors to increase the amount of acetylcholine present in the synapses between cholinergic neurons.^{3,4)} AChE inhibitors like tacrine, one of the most extensively studied AChE inhibitors, have been shown to significantly improve cognitive function in Alzheimer's disease.^{5,6)} Tacrine, however, is also known to cause hepatotoxic side effects by inhibiting butyrylcholinesterase (BuChE, EC 3.1.1.8), which is found in plasma.⁷⁾

In the course of screening natural products for anti-AChE activity, we found that an ethanol extract of the aerial portion of *Chelidonium majus* L. contained inhibitory substances that were selective for AChE. Subsequent activity-guided fractionation led to the isolation of three anti-AChE constituents, 8-hydroxydihydrochelerythrine (1), 8-hydroxydihydrosanguinarine (2), and berberine (3), along with the inactive related compound, noroxyhydrastinine (4).

C. majus L. has been traditionally used as an herbal medicine for treatment of gastric ulcer, gastric cancer, oral infection, liver disease, and general pains in Asian and European countries.⁸⁾ Recently, the extracts of *C. majus* L. were proven to be safe as components of veterinary and human phytopreparations, and of oral-hygiene agents.⁹⁾ Previous chemical studies of *C. majus* reported the isolation of isoquinoline alkaloids such as chelidonine, chelerythrine, sanguinarine, berberine, coptisine, and *dl*-stylopine.⁸⁾ Among them, chelidonine and protopine exhibited anti-tumor activity, and protoberberine showed anti-viral activity, while sanguinarine and chelerythrine had anti-inflammatory activity.¹⁰⁾ The AChE inhibitory activity of berberine from *Coscinium blueneaman*¹¹⁾ and *Corydalis speciosa*¹²⁾ has been evaluated. However, there is no report regarding the anti-AChE activity from *C. majus* L. Additionally, the potent and selective AChE inhibitory activity of 8-hydroxydihydrochelerythrine and 8-hydroxydihydrosanguinarine as constituents was reported for the first time in this study. Here, we report the isolation and

identification of the anti-AChE constituents contained in *C. majus* L.

MATERIALS AND METHODS

General Experimental Methods NMR spectra were recorded on a Bruker Biospin DMX 600. ESI-MS data were recorded on a Jeol JMS-HX110/110A mass spectrometer. AChE (E.C. 3.1.1.7) from electric eel, BuChE (E.C. 3.1.1.8) from horse serum, acetylthiocholine iodide (ATCh), butyrylthiocholine iodide (BuTCh), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and tacrine were purchased from Sigma Chemicals Co., Ltd.

Plants Dried plants were purchased from a Chinese herbal drug store in Daejeon and identified by staff at the Korea Research Institute of Bioscience and Biotechnology, Republic of Korea. A voucher specimen (K-2000-5) has been deposited in the Laboratory of Antioxidants, Korea Research Institute of Bioscience and Biotechnology.

Extraction and Isolation Dried plant materials (1.2 kg) were extracted three times with ethanol. After the ethanol solution was concentrated *in vacuo*, the resulting extracts were suspended in water and successively partitioned using chloroform twice, and the chloroform layer was concentrated *in vacuo*. The resultant residue (15.1 g) was subjected to SiO₂ column chromatography, followed by stepwise elution with CHCl₃–MeOH. The two active fractions (fraction I and II) were eluted with CHCl₃–MeOH (30 : 1) and CHCl₃–MeOH (5 : 1), respectively. Fraction I was pooled and concentrated *in vacuo* to yield an oily residue. The residue, which was subsequently redissolved in MeOH, was applied again to a Sephadex LH-20 column and then eluted with MeOH to yield two active fractions (Fr I-1 and I-2). After being dissolved in MeOH, the first active fraction (Fr I-1) was further purified by reverse-phase HPLC column chromatography. The column was eluted with CH₃OH–H₂O (90 : 10) at a flow rate of 6 ml/min to yield compound 2 (5.4 mg) and compound 4 (6.2 mg) at retention times (min) of 17.6 and 8.7, respectively. After being dissolved in MeOH, the second active fraction (Fr I-2) was also purified by reverse-phase HPLC column chromatography. The column was eluted with CH₃OH–H₂O (90 : 10) at a flow rate of 6 ml/min to yield compound 1 (2.1 mg) at a retention time (min) of 17.6. After it was pooled and concentrated *in vacuo*, fraction II was ap-

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plied to a Sephadex LH-20 column and then eluted with MeOH. For further purification, the active fraction was purified by reverse-phase HPLC column chromatography *via* elution with CH₃OH–H₂O (90:10) at a flow rate of 6 ml/min, which yielded compound **3** (4.4 mg) at a retention time of 22.7 min.

Compound 1: C₂₁H₁₉NO₅, a yellow powder. $[\alpha]_D^{25} +8.5^\circ$ ($c=0.1$, MeOH), UV (MeOH) λ_{max} nm (log ϵ): 204 (4.43), 232 (4.37), 283 (4.42), 323 (3.97), 351 sh (3.44). IR (KBr) ν_{max} cm⁻¹: 3432, 2922, 2852, 1646, 1494, 1463, 1273, 1238, 1037. ¹H-NMR (600 MHz, CDCl₃) δ : 7.80 (1H, d, $J=8.6$, H-6), 7.70 (1H, s, H-5), 7.67 (1H, d, $J=8.6$, H-12), 7.50 (1H, d, $J=8.6$, H-5), 7.14 (1H, s, H-4), 7.10 (1H, d, $J=8.6$, H-11), 6.08 (2H, d, $J=1.6$, –OCH₂O–), 5.57 (1H, s, H-8), 3.97 (3H, s, –OMe), 3.96 (3H, s, –OMe), 2.78 (3H, s, –NCH₃), ¹³C-NMR (125 MHz, CDCl₃) δ : 152.8 (C-10), 148.4 (C-2), 147.8 (C-3), 146.8 (C-9), 138.5 (C-14), 131.4 (C-4a), 127.0 (C-14a), 125.5 (C-8a), 125.2 (C-12a), 123.9 (C-5), 122.8 (C-13), 120.2 (C-6), 119.4 (C-12), 113.5 (C-11), 104.9 (C-4), 101.4 (–OCH₂O–), 100.8 (C-1), 86.4 (C-8), 61.9 (–OMe), 56.2 (–OMe), 40.8 (–NCH₃), ESI-MS: 366 [M+H]⁺.

Compound 2: C₂₀H₁₅NO₅, a yellow powder. $[\alpha]_D^{25} -2.5^\circ$ ($c=0.16$, MeOH), UV (MeOH) λ_{max} nm (log ϵ): 210 (4.22), 235 (4.30), 284 (4.35), 321 (3.96), 337 (3.90), 352 sh (3.43). ¹H-NMR (600 MHz, CDCl₃) δ : 7.76 (1H, d, $J=8.6$, H-6), 7.69 (1H, s, H-1), 7.48 (1H, d, $J=8.6$, H-5), 7.41 (1H, d, $J=8.2$, H-12), 7.13 (1H, s, H-4), 6.95 (1H, d, $J=8.2$, H-11), 6.12 (1H, d, $J=1.6$, –OCH₂O–), 6.06 (1H, d, $J=1.6$, –OCH₂O–), 6.07 (2H, d, $J=1.5$, –OCH₂O–), 5.38 (1H, s, H-8), 2.79 (3H, s, –NCH₃), ¹³C-NMR (125 MHz, CDCl₃) δ : 148.2 (C-2), 147.5 (C-3), 147.3 (C-10), 145.4 (C-9), 138.2 (C-14), 131.2 (C-4a), 126.9 (C-14a), 125.8 (C-12a), 123.8 (C-5), 122.8 (C-13), 120.2 (C-6), 116.4 (C-12), 113.1 (C-8a), 109.0 (C-11), 104.7 (C-4), 101.8 (–OCH₂O–), 101.2 (–OCH₂O–), 100.6 (C-1), 85.9 (C-8), 40.9 (–NCH₃), ESI-MS: 350 [M+H]⁺.

Compound 3: C₂₀H₁₈NO₄, ¹H-NMR (600 MHz, CDCl₃) δ : 9.90 (1H, s, H-12), 8.72 (1H, s, H-7), 8.00 (1H, m, H-9), 7.99 (1H, m, H-8), 7.64 (1H, s, H-1), 6.92 (1H, s, H-4), 6.14 (2H, s, –OCH₂O–), 4.99 (2H, t, $J=5.6$, H₂-6), 4.21 (3H, s, OMe), 4.12 (3H, s, OMe), 3.25 (2H, t, $J=5.6$, H₂-5), ¹³C-NMR (125 MHz, CDCl₃) δ : 150.6 (C-2), 150.4 (C-10), 148.3 (C-3), 145.1 (C-12), 144.2 (C-11), 137.9 (C-14), 133.5 (C-7a), 129.9 (C-4a), 126.7 (C-9), 123.4 (C-8), 121.8 (C-11a), 120.6 (C-7), 120.2 (C-14a), 108.4 (C-4), 105.5 (C-1), 102.2 (–OCH₂O–), 62.0 (OMe), 57.0 (OMe), 55.7 (C-6), 27.2 (C-5), ESI-MS: 337 [M+H]⁺.

Compound 4: C₁₀H₉NO₃, ¹H-NMR (600 MHz, CDCl₃) δ : 7.51 (1H, s, H-8), 6.65 (1H, s, H-1), 6.04 (1H, br s, NH), 5.99 (2H, s, –OCH₂O–), 3.51 (2H, dt, $J=6.6, 2.8$, H₂-3), 2.90 (2H, t, $J=6.6$, H₂-2), ¹³C-NMR (125 MHz, CDCl₃) δ : 166.0 (C-1), 150.9 (C-6), 146.9 (C-7), 134.5 (C-4a), 122.8 (C-8a), 108.0 (C-8), 107.2 (C-5), 101.5 (–OCH₂O–), 40.1 (C-3), 28.5 (C-4), ESI-MS: 192 [M+H]⁺.

Assay of AChE and BuChE The inhibitory activities against AChE were evaluated according to Ellman's coupled enzyme assay¹³ with the following modifications; 0.08 units AChE dissolved in 0.1 M potassium phosphate buffer (pH 7.4) and 5 μ l of ethanol extracts of plants or methanol solution of purified compounds were added to each well of a 96-well plate. After incubation for 3 min, ATCh and DTNB dis-

solved in 0.1 M potassium phosphate buffer (pH 7.4) were added to final concentrations of 20 μ M and 30 μ M, respectively, in each well. The reaction was carried out at room temperature for 5 min, and the initial rate of the enzyme was analyzed by measuring the formation of 5-thio-2-nitrobenzoate, yellow anion at 412 nm of UV wavelength with a microplate reader (Molecular Devices Co., Ltd.). Values for percentages of inhibition were calculated relative to a control sample. The inhibitory activities against BuChE were measured as described above for AChE, using 0.16 unit BuChE and 20 μ M BuTCh instead of AChE and ATCh as the enzyme and substrate, respectively.

RESULTS AND DISCUSSION

The ethanol extract of *C. majus* L. inhibited AChE by 98% at a concentration of 200 μ g/ml without significant inhibition (13% inhibition) of BuChE. To isolate the AChE inhibitory constituents of *C. majus* L., the methanolic extract was suspended in H₂O and partitioned with chloroform. Therefore, the activity was found in the chloroform fraction. Using several chromatographic methods, compounds **1–3**, along with the inactive compound **4**, were isolated as active constituents.

The structures of compounds **1–4** were independently determined by mass and NMR studies, since the structures of compounds **1–3** were quite complex. Compounds **1–4** were identified to be 8-hydroxydihydrochelerythrine,¹⁴ 8-hydroxydihydrosanguinarine,¹⁵ noroxyhydrastinine,¹⁶ and berberine,¹⁷ respectively, which mass and NMR spectral data

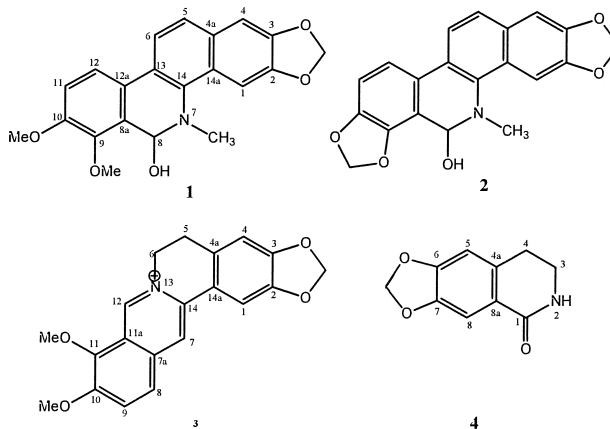


Fig. 1. Structures of Compounds **1–4**

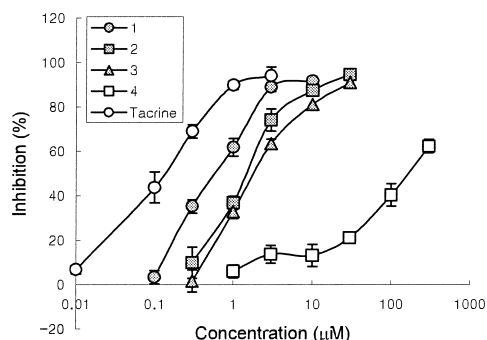
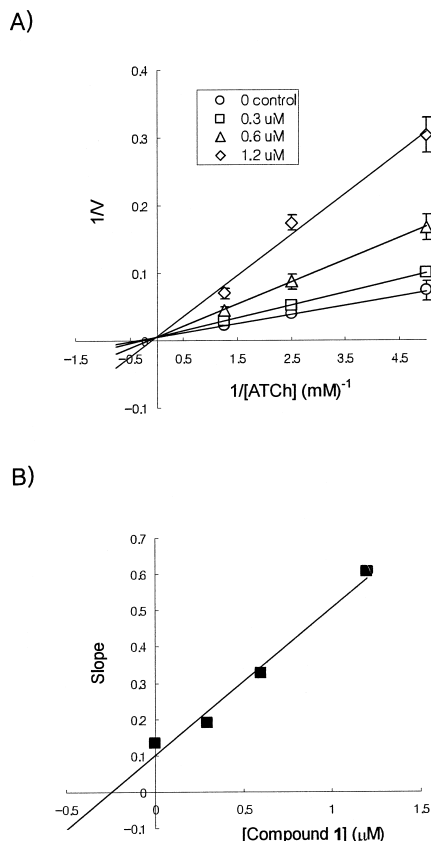


Fig. 2. Inhibitory Effects of Compounds **1–4** on AChE

The values were represented as the mean \pm S.D. of experiments performed in triplicate.

Table 1. Inhibitory Activity of Compounds 1–4 against AChE and BuChE

	IC ₅₀ (μM)		Selectivity (BuChE/AChE)
	AChE	BuChE	
1	0.61	34.6	56.7
2	1.37	12.8	9.3
3	1.85	78.9	42.6
4	>100	>100	—
Tacrine	0.12	0.006	0.05

Fig. 3. The Mechanism of AChE Inhibition by Compound 1 Respective to ATCh (A), and K_i Determination of Compound 1 (B)

(A) The reciprocals of the initial reaction and substrate concentration are plotted. (B) The slope values of the lines from graph A are plotted versus the inhibitor concentration, affording a line obtained by linear regression. The intercept point of this line with the x-axis gives an approximate K_i value of 0.25 μM for compound 1. The values were represented as the mean ± S.D. of experiments performed in triplicate.

were identical with those of published values. The physico-chemical properties of compound 1 and the ¹³C-NMR assignments of compounds 1 and 4 were reported for the first time in this study. Chelerythrine and sanguinarine were known to be quite susceptible to nucleophilic attack at C-8,¹⁸ and sanguinarine can be converted to either the 8-hydroxy-sanguinarine or the pseudomethanolate sanguinarine depending on the environment.¹⁹ The optical rotation of compounds 1 [+8.5° (*c*=0.1, MeOH)] and 2 [−2.5° (*c*=0.16, MeOH)] suggested that they are probably unequal mixtures of enantiomers like the reported 8-hydroxydihydrosanguinarine [−9° (*c*=0.4, MeOH)].¹⁵

The AChE inhibitory activities of compounds 1–4 were determined using Ellman's coupled enzyme assay. As shown

in Fig. 2, compounds 1–3 strongly inhibited AChE in a dose-dependent manner, with IC₅₀ (μM) values of 0.61, 1.37, and 1.85, respectively, while tacrine, an anti-dementia drug that is currently used, inhibited AChE activity with an IC₅₀ (μM) value of 0.12. To determine the selectivity of compounds 1–4 for AChE, their inhibitory activity on BuChE was assayed (Table 1). Compounds 1–3 exhibited selective inhibition for AChE compared with BuChE, with selectivities of 56.7, 9.3, and 42.6, respectively, while tacrine had low selectivity with a stronger inhibitory activity on BuChE than AChE in this assay system. The kinetic analysis of AChE inhibition of compound 1 is shown in Fig. 3. Compound 1 inhibited AChE in a competitive manner with the substrate ATCh. The K_i and K_m values for AChE were 2.5×10^{-7} M and 2.3×10^{-3} M, respectively.

The inhibitory activity selective for AChE of the ethanol extract of *C. majus* L., was reported in this study for the first time. 8-Hydroxydihydrochelerythrine and 8-hydroxydihydrosanguinarine, which were isolated from *Toddalia asiatica*¹⁴) and *Dactylicapnos torulosa*,¹⁵) respectively, were isolated from *C. majus* L. and reported for the first time in this study. Additionally, the strong AChE inhibitory activity of 8-hydroxydihydrochelerythrine and 8-hydroxydihydrosanguinarine was also reported for the first time in this study. Berberine, however, had been known as one of the AChE inhibitors isolated from *C. blueneman*¹¹) and *C. speciosa*.¹²) Since *C. majus* L. has been traditionally used as an herbal medicine in Asian and European countries, the extracts of the aerial portions of *C. majus* L., as well as its active components, 8-hydroxydihydrochelerythrine and 8-hydroxydihydrosanguinarine, may be useful as alternatives for anti-dementia nutraceuticals or currently used agents.

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