http://dx.doi.org/10.5012/bkcs.2014.35.2.655

Cholinesterase Inhibitors from the Aerial Part of *Piper hymenophyllum*

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Received November 14, 2013, Accepted November 27, 2013

Key Words : *Piper hymenophyllum*, Piperaceae, AChE, BChE, Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease and the predominant cause of dementia among the elderly. AD provokes progressive cognitive decline, psychobehavioral disturbances, and memory loss, and is characterized by the presence of senile plaque, neurofibrillary tangles, and reduced cholinergic transmission.[1,2] Although the pathogenesis of AD has not been fully elucidated, it is believed to be due to a deficiency of the neuromediator acetylcholine (ACh), which is referred to as the cholinergic hypothesis. Therefore, the inhibition of acetylcholinesterase (AChE; EC 3.1.1.7) (the key enzyme responsible for the hydrolysis of ACh to choline and acetic acid) has become a widely used treatment strategy.[3] Because reactive oxygen species have been reported to contribute to cellular aging and neuronal damage,[4] it is advantageous for an anti-AD drug candidate to possess antioxidant activity as well as an anti-cholinesterase effect. On the other hand, oxidative injury caused by free radical formation and iron accumulation also has been shown to contribute to the pathogenesis of AD.[5] Furthermore, AChE inhibitors, such as berberine which is reported to ameliorate spatial memory impairment by activating microglia and senile plaque clearance.[6] Moreover, berberine is also reported to inhibit AChE enzyme activity and play an important role in metabolic syndrome.[7] In addition, Peng *et al.* showed that anti-amnesic effect of berberin is related to increase peripheral and central cholinergic neuronal system activity.[8] Butyrylcholinesterase (BChE) may also play a role in Alzheimer's disease since inhibitors of this enzyme improve learning performance in rats and reduce β-amyloid protein levels.[9] Since ACh is also a physiological substrate for BChE, inhibition of BChE elevates the levels of this neurotransmitter in the brain.[9,10]

The *Piper* genus is the largest in Piperaceae family, and consists of approximately 1300 species in the Neotropics and an estimated 700 species in the tropics of the World.[11] Throughout the tropics, numbers of *Piper* sp. are used for many purposes, such as, foods, spices, perfumes, oils, fish poisons, insecticides, hallucinogens, and medicines.[12,13] In market, the pepper of Piperaceae is the world's most traded spice. The ripened fruit of *P. nigrum* are the source of white pepper, while its unripe fruit are the source of black pepper. Furthermore, a narcotic beverage is produced in Oceania from the roots of *P. methysticum*.[14] The chemistry of *Piper* species has been widely investigated and phytochemical investigations conducted in all parts of the World have led to the isolation of a number of physiologically active compounds, which include alkaloids/amides, propenylphenols, lignans, neolignans, terpenes, steroids, kawapyrones, piperolides, chalcones, dihydrochalcones, flavones and flavanones.[14] In Latin America, *Piper* species are used to treat a variety of gynecological ailments and to teat gastrointestinal problems, depression, anxiety, pain and inflammation, and bacterial and fungal infections.[11] However, no report has been issued on the constituents of *P. hymenophyllum* and their anti-cholinesterase activity. Therefore, to identify inhibitors of cholinesterase, we fractionated the $CHCl_3$- and EtOAc-soluble fractions of *P. hymenophyllum* and isolated a new compound (**1**) and six known compounds (**2**-**7**), and the results of investigation of their AChE and BChE inhibitory activities.

Repeated column chromatography (silica gel, RP-C18, and semi-preparative HPLC) of the $CHCl_3$- and EtOAc-soluble fractions of the aerial part of *P. hymenophyllum* resulted in the isolation of a new compound (**1**) and six known ones (**2**-**7**). The six known compounds were identified as neotaiwanensol B (**2**),[15] neotaiwanensol A (**3**),[15] caffeoylaldehyde (**4**),[16] hydroxychavicol (**5**),[17] guaiol (**6**),[18] and *N*-acetylanonaine (**7**)[19] by comparing physicochemical and spectroscopic data (IR, UV, MS, 1D and 2D NMR) with previously reported data.

Compound **1** was obtained as yellow amorphous solid, and was positive by the Dragendorff's reagent test. The molecular formula of **1** was $C_{15}H_{15}NO_4$ from the molecular ion peak at *m*/*z* 273.1003 based on a $[M]^+$ ion (calcd. 273.1001) by high resolution electron impact ionization mass spectrometry (HREIMS). Its UV spectrum showed maximum absorptions at 252 and 353 nm, and its IR spectrum indicated the presence of hydroxyl (3490 cm^{-1}), carbon-

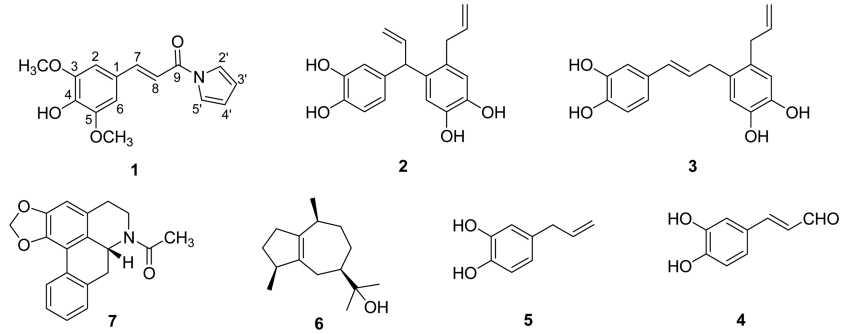

Figure 1. Chemical structure of isolated compounds (**1-7**) from *P. hymenophyllum.*

Table 1. ^{1}H NMR and ^{13}C NMR spectroscopic data for Compound **1**

Position	**1**		
	δ_H (ppm)[a]	δ_C (ppm)[b]	HMBC
1		126.2	
2	7.21 (1H, s)	107.7	C-1, C-3, C-4, C-6, C-7
3		149.1	
4		140.3	
5		149.1	
6	7.21 (1H, s)	107.7	C-1, C-2, C-4, C-5, C-7
7	7.89 (1H, d, 15.6)	149.0	C-1, C-2, C-6, C-8, C-9
8	7.45 (1H, d, 15.6)	113.9	C-1, C-7, C-9
9		163.9	
2′	7.61 (1H, t, 2.4)	120.2	C-9, C-3′, C-4′, C-5′
3′	6.32 (1H, t, 2.4)	113.5	C-2′, C-4′, C-5′
4′	6.32 (1H, t, 2.4)	113.5	C-2′, C-3′, C-5′
5′	7.61 (1H, t, 2.4)	120.2	C-9, C-2′, C-3′, C-4′
3-OCH_3	3.90 (3H, s)	56.8	C-3
5-OCH_3	3.90 (3H, s)	56.8	C-5

[a] ^{1}H NMR (400 MHz in acetone-d_6, *d* values) spectroscopic data. [b] ^{13}C NMR (100 MHz in acetone-d_6, *d* values) spectroscopic data

yl (1710 cm^{-1}), olefinic (1620 cm^{-1}), pyrrole (1460 cm^{-1}) groups, and an aromatic ring (1615 and 1519 cm^{-1}). The ^{1}H NMR spectra of **1** displayed signals indicating a 1,3,4,5-symmetrically tetrasubstituted phenyl [δ_H 7.21 (2H, s, H-2, H-6)], a pyrrole [δ_H 7.61 (2H, t, H-2, H-5) and 6.32 (2H, t, H-3, H-4)], olefinic [δ_H 7.89 (1H, d, H-7) and 7.45 (1H, d, H-8)] and two methoxy [δ_H 3.90 (6H, s, 3-OCH_3, 5-OCH_3)] groups (Table 1). The ^{13}C NMR spectra of **1** displayed 10 carbon signals. The signal at δ_C 56.8 (3-O$\underline{C}H_3$, 5-O$\underline{C}H_3$) was assigned to two methoxy groups, and the other 6 signals [δ_C 107.7 (C-2, 6), 140.4 (C-4) and 149.1 (C-3, 5) were consistent with an aromatic ring. Chemical shifts at δ_C 113.5 (C-3, 4) and 120.2 (C-2, 5) indicated a pyrrole ring. In addition, an amide carbonyl signal was observed at δ_C 163.9 (C-9), and two olefinic carbon signals were present at δ_C 149.0 (C-7) and 113.9 (C-8) (Table 1). In the HMBC spectrum, the olefinic proton at δ_H 7.89 (H-7) demonstrated correlations with carbon signals at δ_C 126.2 (C-1), 107.7 (C-2, 6), and 163.9 (C-9), and the olefinic proton at δ_H 7.45 (H-8) was coupled with the carbon signals at δ_C 126.2 (C-1), and 163.9 (C-9). In addition, protons at δ_H 7.61 (H-2', 5') also showed

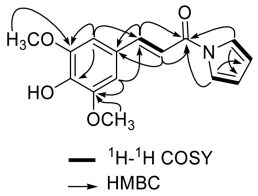

Figure 2. Selected HMBC correlations (H → C) and COSY spectra of Compound **1**.

correlations with the amide carbonyl at δ_C 163.9 (C-9) (Fig. 2). A comparison of compound **1** and *N*-cinnamoylpyrrole (isolated from *Piper argyrophyllum*)[20] showed that their ^{1}H and ^{13}C NMR spectra were similar, but that three aromatic protons at C-3, C-4, and C-5 in *N*-cinnamoylpyrrole are replaced by two methoxy and one hydroxyl group in **1**. These assignments were confirmed by HMBC correlations between methoxy proton signals at δ_H 3.90 (6H) and carbon signals at δ_C 149.1 (C-3, 5), and aromatic signals at δ_H 7.21 and δ_C [140.3 (C-4), 149.1 (C-3, 5)] (Fig. 2). Based on the above analysis, the structure of compound **1** was determined as *N*-3,5-dimethoxy-4-hydroxycinnamoylpyrrole.

In the assay study, the effects of compounds **1-7** on the activities of AChE and BChE were examined using a modification of Ellman's method.[21] As shown in Table 2, compounds **2**-**3** and **5** exhibited AChE inhibitory activities with IC_{50} values ranging from 14.46 to 51.07 μM, respectively, as compared with the berberine positive control (IC_{50} = 0.54 μM). Compound **2** was most effective with IC_{50}

Table 2. Inhibitory activities of compounds **1-7** from *P. hymenophyllum* against AChE and BChE

Compounds	AChE[a]	BChE[b]
1	> 100	> 100
2	14.46 ± 2.31	11.87 ± 0.03
3	28.31 ± 0.02	40.29 ± 0.03
4	> 100	> 100
5	51.07 ± 0.07	38.57 ± 0.80
6	> 100	> 100
7	> 100	> 100
Berberine[c]	0.54 ± 0.01	9.39 ± 0.05

[a,b] IC_{50}, μM, 50% inhibition concentrations, are expressed as the mean ± S.E.M. of triple experiments. [c] Positive control

values of 14.46 μM, respectively. In the BChE inhibitory assay, compounds **2**-**3** and **5** also exhibited potent activity with IC_{50} values ranging from 11.87 to 40.29 μM as compared with IC_{50} value of 9.39 μM for the berberine positive control. Compound **2** was most potent with IC_{50} values of 11.87 μM.

Experimental

General Experimental Procedure. Optical rotations were measured using a JASCO DIP 370 digital polarimeter. UV spectra were recorded in MeOH using a Thermo spectrometer. IR spectra were obtained using a Fourier Transform infrared spectrometer (Brucker Instrument, Inc., German). 1D- and 2D-NMR spectra were obtained using a Varian Unity Inova 400 MHz spectrometer with tetramethylsilanne (TMS) as the internal standard, and the chemical shifts were recorded in δ values (ppm). HREIMS was recorded using JEOL JMS-700 MStation^{TM} spectrometer (Japan). Silica gel (Merck, 63-200 μm particle size) and RP-18 (Merck, 75 μm particle size) were used for column chromatography. TLC was carried out using Merck silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was conducted using a Waters system (515 pump), a UV detector (486 Tunable Absorbance), and an YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size, YMC Co., Ltd., Japan). HPLC solvents were purchased from Burdick & Jackson (USA).

Plant Material. The aerial parts of *P. hymenophyllum* were harvested at the Cuc Phuong National Park, Ninh Binh province, Vietnam in April 2011. Botanical identification was performed by Associate Professor Vu Xuan Phuong, Department of Herbal Specimen, Vietnam Institute of Ecology and Biological resources. A voucher specimen (HVD 004-11) was deposited at the Department of Specimen, Vietnam Institute of Ecology and Biological Resources.

Extraction and Isolation. The aerial parts of *P. hymenophyllum* (15.0 kg) were extracted three times (3 h × 3 L) with MeOH under reflux at 60 °C. After removing the solvent under reduced pressure, the residue was suspended in H_2O and then partitioned successively with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. An activity-guided fractionation study resulted in the $CHCl_3$ and EtOAc fractions being chosen for further study.

The $CHCl_3$ soluble fraction (110.0 g) was chromatographed on a silica gel column using a stepwise gradient of *n*-hexane: acetone (15:1 to 0:1, each 4 L) to yield ten fractions (Fr.1-Fr.10, determined according to their TLC profiles). Fraction 3 (1.4 g) was subjected to silica gel column chromatography (CC) using *n*-hexane:acetone (30:1 to 5:1, 2 L for each step) as the eluent, to afford four sub-fractions (Fr.3-1 to Fr.3-4). Further purification of sub-Fr.3-2 (350.0 mg) using a ODS silica gel column eluted with MeOH-H_2O (4:1 to 1:0, 1 L for each step) resulted in the isolation of compound **6** (27.0 mg). Fraction 6 (43.9 g) was subjected to silica gel CC using CH_2Cl_2-acetone (100:1 to 0:1, 2 L for each step) as the eluent, to afford ten fractions (Fr.6-1 to Fr.6-10). Fraction 6-1 (1.1 g) was subjected to ODS silica gel CC, using MeOH-H_2O (1:2 to 3:1, 1.5 L for each step) gradient, to obtain two sub-fractions (Fr.6-1-1 to Fr.6-1-2). Sub-Fr.6-1-1 (525.0 mg) was purified by silica gel CC using *n*-hexane-acetone (5:1 to 2:1, 1 L for each step), which resulted in the isolation of compounds **5** (27.0 mg) and **7** (18.0 mg). Fraction 7 (18.3 g) was also subjected to a silica gel CC using *n*-hexane-acetone (10:1 to 1:1, 1.0 L for each step) gradient, to afford eight sub-fractions (Fr.7-1 to Fr.7-8). Fraction 7-4 (2.1 g) was subjected to silica gel CC using CH_2Cl_2-acetone (100:1 to 10:1, 1.0 L for each step), to obtain eight sub-fractions (Fr.7-4-1 to Fr.7-4-8). Further purification of sub-Fr.7-4-2 (86.0 mg) using a semi-preparative Waters HPLC system [using a gradient solvent system of MeOH-H_2O = 55:45 to 85:15; flow rate 5 mL/min; for 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 mm particle size] resulted in the isolation of compound **1** (6.5 mg; t_R = 38.2 min).

The EtOAc (19.3 g) soluble fraction was also subjected to silica gel CC using $CHCl_3$-MeOH (80:1 to 0:1, 2.0 L for each step) gradient, to obtain seven fractions (Fr.E-1 to Fr.E-7). Fraction E-2 (0.43 g) was subjected to silica gel CC using $CHCl_3$-MeOH (30:1 to 5:1, 0.5 L for each step) gradient, to obtain two sub-fractions (Fr.E-2-1 to Fr.E-2-2). Further purification of sub-Fr.E-2-1 (65.0 mg) using a semi-preparative Waters HPLC system [using a gradient solvent system consisting of MeOH-H_2O = 40:60 to 70:30; flow rate 5 mL/min; for 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 mm particle size] resulted in the isolation of compound **4** (11.0 mg; t_R = 40.2 min). Fraction E-4 (0. 87 g) was subjected to a silica gel CC using $CHCl_3$-MeOH (20:1 to 10:1, 1.0 L for each step) gradient, to afford three sub-fractions (Fr.E-4-1 to Fr.E-4-3). Sub-Fr.E-4-2 (565.0 mg) was further subjected to ODS CC using MeOH-H_2O (1:3 to 1:1, 1.0 L for each step) gradient, to obtain two sub-fractions (Fr.E-4-2-1 to Fr.E-4-2-2). Compound **2** (24.0 mg; t_R = 35.6 min) was obtained from sub-Fr.E-4-2-1 (154.0 mg) by preparative HPLC [using MeOH-H_2O = 50:50 to 85:15 gradient; flow rate 5 mL/min; for 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 mm particle size]. Sub-Fr.E-4-2-2 (258.0 mg) was purified by silica gel CC using with $CHCl_3$-MeOH (15:1 to 5:1, 0.5 L for each step) to afford compound **3** (42.0 mg).

***N*-3,5-Dimethoxy-4-hydroxycinnamoylpyrrole (1):** yellow amorphous solid; UV λ_{max} (MeOH): 252, 353 nm; IR (KBr) ν_{max} 3490, 3124, 1710, 1620, 1615, 1519, 1460 cm^{-1}; HREIMS *m*/*z* 273.1003 $[M]^+$ (calcd for $C_{15}H_{15}NO_4$, 273.1001). ^1H NMR (400 MHz, acetone-d_6) and ^{13}C NMR (100 MHz, acetone-d_6) spectroscopic data, see Table 1.

***In vitro* AChE Inhibitory Activity Assay.** The inhibitory activities of AChE and BChE were measured using the spectrophotometric method developed by Ellman with a slight modification.[6] Essentially, ACh and BCh were used as substrates to detect the inhibitions of AChE and BChE, respectively. The reaction mixture contained: 140 μL of sodium phosphate buffer (pH 8.0); 20 μL of tested sample solution [final concentration (f.c.) 100 μM for either compound]; and 20 μL of AChE or BChE solution, which

were mixed and incubated for 15 min at room temperature. All tested samples and the positive control (berberine) were dissolved in 10% analytical grade dimethyl sulfoxide (DMSO). Reactions were started by adding 10 μL of 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) and 10 μL of ACh or BCh. The hydrolysis of ACh or BCh was monitored by following the formation of the yellow 5-thio-2-nitrobenzoate anion (formed by the reaction between DTNB and thiocholine, released by the enzymatic hydrolysis of ACh or BCh) at 412 nm for 15 min. All reactions were performed in triplicate and recorded in 96-well microplates using a VERSA max ELISA Microplate Reader (Molecular Devices, Sunnyvale, CA, U.S.A.). Percent inhibition was calculated using (1–S/E) × 100, where E and S are enzyme activities with or without the tested sample, respectively. The ChEs inhibitory activity of each sample was expressed as IC_{50} values (μM, defined as the concentration required inhibiting the hydrolysis of substrate by ACh or BCh by 50%, as calculated using log-dose inhibition curves).

Acknowledgments. This study was financially supported by the National Foundation for Science and Technology Development, Vietnam (Project No: 104.01-2010-16), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (BK21 Plus program).

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