Cholinesterase Inhibitors from *Cleistocalyx operculatus* Buds

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(Received July 22, 2010/Revised August 17, 2010/Accepted August 18, 2010)

Five flavonoids, myricetin-3'-methylether 3-O-β-D-galactopyranoside (1), myricetin-3',5'-dimethylether 3-O-β-D-galactopyranoside (2), quercetin (3), kaempferol (4), and tamarixetin (5) were isolated from the buds of *Cleistocalyx operculatus* (Myrtaceae). The chemical structures of these compounds were determined on the basis of spectroscopic analyses, including 2D NMR. Their anti-Alzheimer effects were evaluated via acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity assays. All five compounds 1-5 showed potential inhibitory activities against AChE with IC<sub>50</sub> values of 19.9, 37.8, 25.9, 30.4 and 22.3 µM, respectively, while compounds 1, 3, 4 and 5 also possessed BChE inhibitory activity with IC<sub>50</sub> values of 152.5, 177.8, 62.5, and 160.6 µM, respectively.

**Key words:** *Cleistocalyx operculatus*, Myrtaceae, Flavonoids, Anti-Alzheimer, Acetylcholinesterase, Butyrylcholinesterase

**INTRODUCTION**

Alzheimer’s disease (AD) is a neurodegenerative disease and the most frequent and predominant cause of dementia in the elderly, provoking progressive cognitive decline, psychobehavior disturbances, memory loss, the presence of senile plaques, neurofibrillary tangles, and a decrease in cholinergic transmission (Scarpini et al., 2003; Parihar and Hemnani, 2004). Although the pathogenesis of AD is complicated and involved in numerous pathways, two major hypotheses are currently under consideration regarding the molecular mechanism, the cholinergic hypothesis and the amyloid cascade hypothesis. Thus, the focus herein is upon inhibitors of select cholinesterases (ChEs) to alleviate cholinergic deficits and improve neurotransmission and β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1; aspartyl protease, β-secretase, and memapsin 2) inhibitors to preclude formation and accumulation of amyloid β peptide (Aβ). Pursuant to this, both could then be established as viable therapeutic targets for AD (Yan et al., 1999; Vassar, 2002; Parihar and Hemnani, 2004; Rao et al., 2007).

In our screening program searching for cholinesterase inhibitors from medicinal plants, a water-soluble extract of the buds of *Cleistocalyx operculatus* (Roxb.) Merr and Perry (Myrtaceae) exhibited an acetylcholinesterase (AChE) inhibitory activity of >70% at 100 µg/mL. *C. operculatus* is a well-known medicinal plant, widely distributed and propagated in China, Vietnam and some other tropical countries. In Vietnam, *C. operculatus* is commonly called “Voi”. It is a well known medicinal plant whose buds are commonly used as an ingredient for tonic drinks in Southern China (Ye et al., 2004). It also showed strong protective effects on lipid peroxidation in rat liver microsomes, and on H<sub>2</sub>O<sub>2</sub>-induced trauma in PC12 cells (Lu et al., 2003). Recently, the anti-inflammatory activities of the essential oil of the *C. operculatus* buds were investigated in *in vitro* and *in vivo*. These results suggested that its essential oil might exert an anti-inflammatory effect by suppressing the expression of pro-inflammatory cytokines, and this suppression is partially mediated by blocking NF-κB activation (Dung et al., 2009). Previous phytochemical attention has led to the characterization of oleanane-type triterpenes (Zhang et al., 1990; Nomura et al., 1993), and flavonoids (Ye et al., 2004). Chalcone compounds from this plant possess antioxidant and anticancer activities (Ye et al., 2005a, 2005b, 2007).
Despite a number of biological studies on this plant, no paper has been published regarding the active principles and cholinesterase effects. In the present study, therefore, further phytochemical investigation of the water layer of this plant led to the isolation of five flavonoids (1-5). Details of the isolation, structural determination, and AChE and butyrylcholinesterase (BChE) inhibitors are described herein.

**MATERIALS AND METHODS**

**General experimental procedures**
Optical rotations were measured with a JASCO DIP 1000 digital polarimeter. UV spectra were recorded on a JASCO V-530 spectrophotometer. IR spectra were obtained on a JASCO FT/IR 300-E spectrometer. NMR experiments were conducted on a Varian Unity INOVA 400 spectrometer. 1H and 13C NMR spectra were recorded at 400 and 100 MHz, respectively, and tetramethylsilane was used as the internal standard. ESI-MS analyses were performed on a Micromass INOVA 400 spectrometer. 1H NMR experiments were conducted on a Varian Unity INOVA 400 spectrometer. 1H and 13C NMR spectra were recorded at 400 and 100 MHz, respectively, and tetramethylsilane was used as the internal standard. ESI-MS analyses were performed on a Micromass QTQF2 mass spectrometer. TLC was carried out on Merck silica gel F254-precoated glass plates and RP-18 F3548 plates. HPLC was performed on a Waters 600E multisolvant delivery system connected to a UV detector using YMC-Pack ODS-A (5 µm, 20 × 250 mm) semipreparative columns.

**Plant material**
The buds of *C. operculatus* were purchased in Dong Xuan herbarium market, Hanoi, Vietnam, in July 2007 and identified by Professor Pham Thanh Ky, Department of Pharmacognosy, Hanoi University of Pharmacy. A voucher specimen (0160) was deposited in the herbarium of the College of Pharmacy, Catholic University of Daegu, Korea.

**Extraction and isolation**
The buds (1.8 kg) were extracted with 3 L of MeOH, three times at room temperature. The MeOH extract (178.6 g) was combined and concentrated to yield a residue which was suspended in water and then successively partitioned with n-hexane, EtOAc, and BuOH. The H2O layer (18.0 g) was separated by Sephadex LH-20 column chromatography using a gradient of MeOH - H2O (from 40 : 60 to 100 : 0), to yield seventeen subfractions (W1 ~ W17) according to their TLC profiles. Subfraction W5 (0.5 g) was purified by semipreparative HPLC systems [using YMC-Pack ODS-A column (20 × 250 mm, 5 µm); mobile phase (10 to 75% MeOH in water for 60 min), flow rate 5 mL/min; UV-detection at 254 nm] resulted in the isolation of compound 3 [12.5 mg (7.0 × 10^{-6} %); tR = 38.5 min], compound 4 [10.0 mg (5.6 × 10^{-6} %); tR = 41.3 min], and compound 5 [11.0 mg (6.1 × 10^{-6} %); tR = 45.0 min], respectively. The subfraction W14 (0.68 g) was further purified by semi preparative HPLC [YMC-Pack ODS-A column (20 × 250 mm, 5 µm); mobile phase (15 to 65% MeOH in water + 0.1% Trifluoroacetic acid for 60 min), flow rate 5mL/min; UV-detection at 254 nm] to obtained compound 1 [6.0 mg (3.3 × 10^{-6} %); tR = 40.1 min], and compound 2 [5.4 mg (3.0 × 10^{-6} %); tR = 43.2 min], respectively.

**Myricetin-3'-methylether 3-O-β-D-galactopyranoside (1)**
Yellow amorphous powder; [α]D^25 = 16.1° (c 0.26, MeOH); UV λ_{max} (MeOH) nm: 256, 362; IR (KBr) cm^{-1}: 3234, 1627; negative ESI-MS (C_{23}H_{24}O_{13}) m/z: 493 [M - H]−; 1H NMR (400 MHz, C_{5}D_{5}N): δ 3.96 - 4.80 (6H, m, sugar protons), 4.10 (3H, s, OCH_{3}), 6.41 (1H, d, J = 6.8 Hz, H-1'), 6.66 (1H, s, H-6), 6.72 (1H, s, H-8), 7.86 (1H, s, H-6'), 8.40 (1H, s, H-2'); 13C NMR (100 MHz, C_{5}D_{5}N): δ 57.4 (3'-OCH_{3}), 62.4 (C-6''), 70.3 (C-4''), 73.9 (C-2'), 75.7 (C-3'), 78.1 (C-5'), 95.0 (C-8), 100.2 (C-6), 104.6 (C-1''), 104.9 (C-10), 107.5 (C-2'), 111.3 (C-6'), 121.6 (C-1'), 135.5 (C-3), 141.0 (C-5'), 147.8 (C-4'), 149.4 (C-3'), 158.0 (C-9), 158.1 (C-2), 163.2 (C-5), 166.3 (C-7), 179.2 (C-4).

**Myricetin-3',5'-dimethyl ether 3-O-β-D-galactopyranoside (2)**
Yellow amorphous powder; [α]D^25 = 31.4° (c 0.21, MeOH); UV λ_{max} (MeOH) nm: 256, 360; IR (KBr) cm^{-1}: 3340, 1628; negative ESI-MS (C_{22}H_{22}O_{13}) m/z: 493 [M - H]−; 1H NMR (400 MHz, C_{5}D_{5}N): δ 4.21 - 4.79 (6H, m, sugar protons), 4.03 (3H, s, OCH_{3}), 6.53 (1H, d, J = 8.0 Hz, H-1'), 6.73 (1H, d, J = 2.0 Hz, H-6), 6.77 (1H, d, J = 2.0 Hz, H-8), 8.07 (2H, s, H-2'), 13C NMR (100 MHz, C_{5}D_{5}N): δ 57.4 (3'-OCH_{3}), 62.4 (C-6''), 70.3 (C-4''), 73.8 (C-2'), 75.6 (C-3'), 78.1 (C-5'), 95.0 (C-8), 100.2 (C-6), 104.6 (C-1''), 105.7 (C-10), 107.5 (C-2'), 111.3 (C-6'), 121.6 (C-1'), 135.5 (C-3), 140.1 (C-5'), 147.8 (C-4'), 149.4 (C-3'), 158.0 (C-9), 158.1 (C-2), 163.2 (C-5), 166.3 (C-7), 179.2 (C-4).

**Quercetin (3)**
Yellow amorphous powder; UV λ_{max} (MeOH) nm: 256, 370; IR (KBr) cm^{-1}: 3382, 1706, 1634; ESI-MS (C_{15}H_{10}O_{7}) m/z: 301 [M - H]−; 1H NMR (400 MHz, C_{5}D_{5}N): δ 6.74 (1H, d, J = 2.0 Hz, H-6), 6.78 (1H, d, J = 2.0 Hz, H-8), 7.41 (1H, d, J = 8.4 Hz, H-5), 8.13 (1H, dd, J = 2.0, 8.4 Hz, H-6'), 8.64 (1H, d, J = 2.0 Hz, H-2'); 13C NMR (100 MHz, C_{5}D_{5}N): δ 94.7 (C-8), 99.6 (C-6), 104.9 (C-10), 117.1 (C-5'), 121.5 (C-2'), 123.4 (C-6'), 124.5 (C-1'), 138.3 (C-3), 147.5 (C-3'), 148.2 (C-4'), 150.2 (C-2'), 157.9 (C-4).