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Acetylcholinesterase Inhibitors from the Twigs of *Vaccinium oldhami* Miquel

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In the course of finding Korean natural products with acetylcholinesterase (AChE) inhibitory activity, we found that a methanolic extract of the twigs of *Vaccinium oldhami* significantly inhibited AChE. Bioassay-guided fractionation of the methanolic extract resulted in the isolation of two compounds, taraxerol (**1**) and scopoletin (**2**), as active constituents. These compounds inhibited AChE activity in a dose-dependent manner, and the IC_{50} values of compounds **1** and **2** were 33.6 (79 μM) and 10.0 (52 μM) μg/mL, respectively.

Key words: *Vaccinium oldhami*, Acetylcholinesterase, Taraxerol, Scopoletin

INTRODUCTION

Alzheimer's disease (AD), the most common cause of senile dementia in later life, is a major healthcare challenge due to the increasing longevity of the population. AD patients exhibit marked decline in cognitive ability and severe behavioral abnormalities such as irritability, anxiety, depression, disorientation, and restlessness (Sugimoto *et al.*, 2002). According to the cholinergic hypothesis of the pathogenesis of AD, memory impairments in AD patients result from a deficit of cholinergic functions in the brain. An important therapeutic strategy for activating central cholinergic functions has been the use of inhibitors of AChE, the enzyme responsible for the metabolic hydrolysis of the neurotransmitter acetylcholine (Kalauni *et al.*, 2002; Bartus *et al.*, 1982; Perry, 1986; Bartus, 2000). Some AChE inhibitors like physostigmine, tacrine, alkylpyridinium polymers, and carbamates have been identified and reported. But because of bioavailability problems and possible side effects like hepatotoxicity, the search for better AChE inhibitors still draws much attention (Park *et al.*, 1996; Rhee *et al.*, 2001).

As we screened natural resources for AChE inhibitors, we found that a methanolic extract of the twigs of *Vaccinium oldhami* (Ericaceae) inhibited AChE in a dose-dependent manner. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of taraxerol (**1**) and scopoletin (**2**).

The fruits of *V. oldhami* have been used in Korea and China as a folk medicine to treat inflammation, gonorrhea, vomiting, diarrhea, and eruption (Song *et al.*, 1989; Kim *et al.*, 1996). A survey of the related literature revealed that no phytochemical and pharmacological work has been carried out on *V. oldhami*.

This paper describes the isolation of taraxerol and scopoletin from *V. oldhami* and the inhibitory effects of these compounds on AChE.

MATERIALS AND METHODS

General procedure

^1H- and ^{13}C-NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. TLC was carried out on Merck precoated silica gel F_{254} plates, and the silica gel for the open column chromatography was Kiesel gel 60 (230-400 mesh, Merck). Sephadex LH-20 was used for the column chromatography (Pharmacia, 25-100 μm), and the column for LPLC was Lobar A (Merck Lichroprep Si 60, 240-10 mm). All solvents were routinely distilled prior to use. Other chemicals were commercial grade without further purification. Acetylthiocholine iodide (ASCh), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), and AChE (Type V-S, used for comparing with the prepared enzyme from the mouse brain) were purchased from Sigma Chemical Co.

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Plant materials

The twigs of *V. oldhami* were collected and air-dried in November 2001 at Wanju, Chonbuk, Korea. A voucher specimen was deposited in the herbarium of the college of pharmacy, Woosuk University (WSU-01-031).

Extraction and isolation

The shade-dried plant material (1 kg) was extracted (three times with MeOH for 5 hours at below 50°C) and filtered. The filtrate was evaporated *in vacuo* to give a dark brownish residue. The resultant methanolic extract (53 g) was followed by the successive solvent partition to give $CHCl_3$ (21 g), *n*-BuOH (15 g), and H_2O soluble fractions. Each fraction was tested for inhibitory effects on AChE. Among these fractions, the $CHCl_3$ soluble fraction showed the most significant inhibition of AChE. Silica gel column chromatography of the $CHCl_3$ soluble fraction with $CHCl_3$-EtOAc-MeOH (20:1:1) gave four fractions (fr.1-fr.4). The fraction fr.1 was rechromatographed on silica gel with $CHCl_3$-MeOH (5:1) to yield compound **1** (22 mg). The fraction fr.2 was applied with Sephadex LH-20 (MeOH) and purified by the Lobar-A column (MeOH: H_2O, 3:7) to yield **2** (12 mg).

Taraxerol (1)

Amorphous powder (MeOH), mp 283-284°C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 5.53 (1H, dd, *J*=7.6, 3.6 Hz, H-15), 3.19 (1H, dd, *J*=11.2, 4.4 Hz, H-3), 1.09 (3H, s, CH_3), 0.98 (3H, s, CH_3), 0.91 (6H, s, 2×CH_3), 0.95 (3H, s, CH_3), 0.93 (3H, s, CH_3), 0.82 (3H, s, CH_3), 0.80 (3H, s, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 158.1 (C-14), 116.9 (C-15), 79.1 (C-3), 55.6 (C-5), 49.3 (C-18), 48.8 (C-9), 41.4 (C-19), 39.0 (C-4), 38.8 (C-8), 38.0 (C-17), 37.8 (C-1), 37.8 (C-13), 37.6 (C-10), 36.7 (C-16), 35.8 (C-12), 35.2 (C-7), 33.7 (C-21), 33.4 (C-29), 33.1 (C-22), 29.9 (C-28), 29.9 (C-26), 29.8 (C-20), 28.0 (C-23), 27.2 (C-2), 25.9 (C-27), 21.3 (C-30), 18.8 (C-6), 17.5 (C-11), 15.5 (C-24), 15.4 (C-25).

Scopoletin (2)

Colorless prism (MeOH), mp 206-207°C; ^{1}H-NMR (400 MHz, $CDCl_3$+CD_3OD, 1:1) δ: 7.82 (1H, d, *J*=9.6 Hz, H-4), 7.03 (1H, s, H-5), 6.81 (1H, s, H-8), 6.22 (1H, d, *J*=9.6 Hz, H-3), 3.93 (3H, s, OCH_3). ^{13}C-NMR (100 MHz, $CDCl_3$+CD_3OD, 1:1) δ: 163.9 (C-2), 152.6 (C-9), 150.9 (C-7), 146.7 (C-6), 145.7 (C-3), 112.2 (C-4), 112.0 (C-10), 109.3 (C-5), 103.8 (C-8), 56.6 (OCH_3).

Enzyme extraction

Male ICR mice were used in this experiment, and the procedures were performed in accordance with the animal care guidelines of the NIH. In order to extract the AChE enzyme, the animals were put to death after total anesthesia by ether. Their brains were dissected. The forebrains were separated and homogenated with 5 volumes of a homogenation buffer [10 mM Tris-HCl (pH 7.2), containing 1 M NaCl, 50 mM $MgCl_2$, and 1% triton X-100] (Rieger *et al.*, 1980), then centrifuged at 10,000×g for 30 min. The resulting supernatant was used as an enzyme source. All extracting steps were carried out at 4°C. Protein concentration was determined using the BCA kit (biocinchoninic acid, Sigma Co., USA) with bovine serum albumin (BSA) as the protein standard.

Acetylcholinesterase inhibition assay

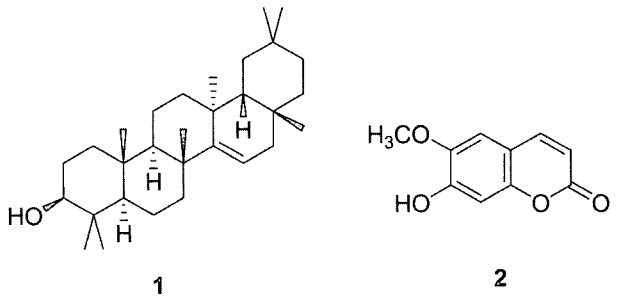
The AChE assay was performed by the method of Ellman *et al.*, with minor modifications, using acetylthiocholine iodide as a substrate (Ellman *et al.*, 1961). 0.5 M phosphate buffer was diluted with distilled water to make 50 mM phosphate buffer. Ellmans reaction mixture was made from a combination of 0.5 mM acetylthiocholine iodide and 1 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) in a 50 mM sodium phosphate buffer (pH 8.0). The rates of hydrolysis by AChE were monitored spectrophotomatically using a 96-well microtiter plate reader (Mortensen *et al.*, 1996). Each extract (or compound, 10 μL) and 50 mM sodium phosphate buffer (30 μL) were mixed with the enzyme solution (10 μL). An Ellmans reaction mixture (50 μL) was further added to give a final volume of 100 μL, and the mixture was incubated at 37°C for 30 min. Absorbance at 450 nm was read immediately after adding the Ellmans reaction mixture. Reading were repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. Blank reaction was measured by substituting saline for the enzyme (Chung *et al.*, 2001; Park *et al.*, 1996).

Statistical analysis

The IC_{50} values (the inhibitory dose that reduced the 50% of AChE activity) were calculated with the SPSS program (Version 8.0). The Michaelis constant (*K*m) was determined by means of Lineweaver-Burk plots, using initial velocities obtained over three concentrations of the substance (0.25, 0.125 and 0.063 mM, respectively).

RESULTS AND DISCUSSION

In the course of our search for AChE inhibitors from natural resources, we found that the methanolic extract of the twigs of *V. oldhami* exhibited anti-AChE activity (51 μg/mL). To isolate the AChE inhibitory constituents from *V. oldhami*, the total methanolic extract was suspended in water and partitioned successively with $CHCl_3$ and *n*-BuOH. As a result, the inhibitory activity was found in the $CHCl_3$ soluble fraction. Using several chromatographic techniques, compounds **1** and **2** were isolated as active constituents and identified as taraxerol (Ogihara *et al.*,



1 2

Fig. 1. Structures of compounds **1-2**

1987; Sakurai *et al.*, 1987) and scopoletin (Gunasekera *et al.*, 1980; Kim *et al.*, 1986), respectively, by comparing physicochemical and spectral data with those of published literatures.

Compounds **1** and **2** inhibited AChE activity in a dose-dependent manner (Fig. 2). The concentrations of **1** and **2** required for IC_{50} were determined to be 33.6 and 10.0 μg/mL, respectively (Table I), while the IC_{50} value of a positive control, berberine (Hwang *et al.*, 1996), was 1.2 μg/mL. Between them, the mechanism of compound **2** was studied in depth *in vitro*. The kinetic analysis of AChE inhibition of compound **2** is shown in Fig. 3. The K_m and V_{max} values were calculated from the Lineweaver-Burk plot. The V_{max} value of AChE, as plotted against [ASCh],

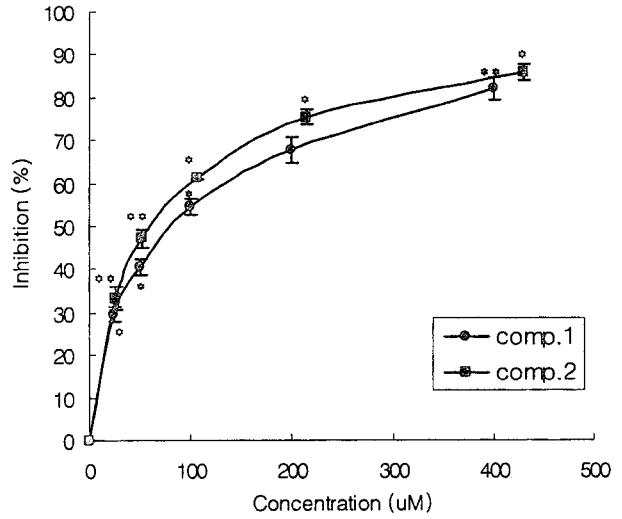


Fig. 2. The inhibitory activity of compounds **1** and **2** on AChE. Differs significantly from the control, effective *p<0.05, **p<0.01

Table I. The inhibitory acitivities of copounds **1-2** on AChE

Compound	IC_{50} (μg/mL)
1	33.6
2	10.0
berberine	1.2

The values indicate 50% AChE inhibitory effect and are the means of triplicate data.

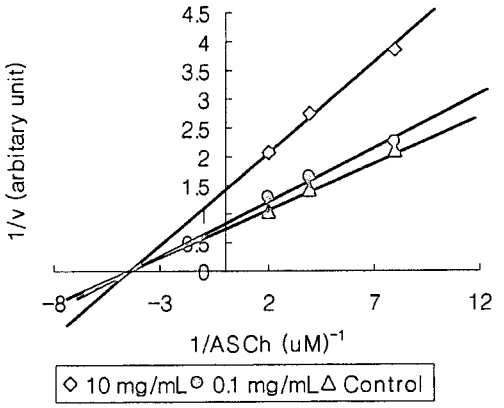


Fig. 3. Lineweaver-Burk plot of 1/v vs. 1/ASCh in the presence and absence of compound **2**

decreased significantly with the addition of compound **2**. However, the K_m values did not change. This result indicated that compound **2** inhibited AChE in a noncompetitive manner.

This study showed that compounds **1** and **2** isolated from *V. oldhamin*, taraxerol and scopoletin, inhibit AChE activity-although less effectively than tacrine derivatives. These compounds were purified from a natural plant, that has been used for folk medicine in Korea and China. On oral or transdermal administration, these low molecular substances can easily reach the site of action (brain) by crossing the blood-brain barrier, which is the tight junction controlling the transport of material into the brain (Broadwell *et al.*, 1993). In conclusion, the present study suggested the useful of the methanolic extract of *V. oldhami* and its isolated components, taraxerol and scopoletin, for the treatment of Alzheimers disease.

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