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Acetylcholinesterase Inhibitors from the Roots of *Angelica dahurica*

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In the course of finding Korean natural products for acetylcholinesterase (AChE) inhibitory activity, we found that a methanolic extract of the roots of *Angelica dahurica* showed significant inhibitory effects on AChE. Bioassay-guided fractionation of the methanolic extract resulted in the isolation of three furanocoumarins, isoimperatorin (**1**), imperatorin (**2**) and oxypeucedanin (**3**), as active principles. These compounds inhibited AChE activity in a dose-dependent manner, and the IC_{50} values of compounds **1-3** were 74.6, 63.7 and 89.1 uM, respectively.

Key words: *Angelica dahurica*, Anticholinesterase activity, Isoimperatorin, Imperatorin, Oxypeucedanin

INTRODUCTION

Alzheimers disease (AD) is the most common cause of senile dementia in later life. 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 acetylcholine (Bartus *et al.*, 1982; Perry, 1986; Bartus, 2000). Some AChE inhibitors like physostigmine or tacrine are known to have limitations such as short half-life or side-effects like hepatotoxicity, and alkylpyridinium polymers, dehydroevodiamine and carbamate type AChE inhibitors have been reported, but because of bioavailability problems and possible side-effects, there still is great interest in finding better AChE inhibitors (Park *et al.*, 1996; Rhee *et al.*, 2001).

During screening for AChE inhibitors from natural resources, we found that a total methanolic extract of the root of *Angelica dahurica* (Umbelliferae) showed significant inhibition toward AChE. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of three furanocoumarins, isoimperatorin (**1**), imperatorin (**2**) and oxypeucedanin (**3**).

The root of *A. dahurica* have been used in Korea, Japan and China as a folk medicine to treat menstrual disorder, abdominal pain, hysteria, bleeding, headache and excessive leukorrhea (Kimura *et al.*, 1996). Earlier investigations on the chemical constituents of *A. dahurica* dealt with the isolation of over twenty coumarins (Kwon *et al.*, 2002; Wang *et al.*, 2001; Baek *et al.*, 2000; Kim *et al.*, 1992; Qiao *et al.*, 1996).

This paper describes the isolation of three furanocoumarins from *A. dahurica* and their anti- AChE activity.

MATERIALS AND METHODS

General procedure

^{1}H- 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 silica gel for column chromatography was Kiesel gel 60 (230-400 mesh, Merck). And Sephadex LH-20 was used for column chromatography (Pharmacia, 25-100 μm). Column for LPLC was Lobar A (Merck Lichroprep Si 60, 240-10 mm). All other chemicals and solvents were analyticlal grade and used 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 roots of *A. dahurica* were collected in October 2000 at Sunchang, Chonbuk, Korea. A voucher specimen is deposited in the herbarium of college of pharmacy, Woosuk Jniversity (WSU-00-010).

Extraction and isolation

The air-dried plant materials (500 g) were finely ground and extracted twice with hot MeOH under reflux. The resultant methanolic extract (120 g) was chromatographed on a silica gel column using a mixture of *n*-hexane-EtOAc-MeOH with increasing polarity and yielded fifteen subfractions (fr.1-fr.15). Among these subfractions, fr.3 showed the most significant AChE inhibitory activity. Silica gel column chromatography of fr.3 with a solvent gradient of EtOAc in *n*-hexane gave five subfractions (fr.31-fr.35). subfraction fr.31 was rechromatographed on silica gel column (*n*-hexane-EtOAc, 6:1) and crystallized with MeOH to yield **1** (250 mg). Subfraction fr.32 was rechromatographed on silica gel column with *n*-hexane-EtOAc (2:1) to give two subfractions (fr.321-fr.322). Subfraction fr.321 was purified with sephadex LH 20 (MeOH) to give **2** (95 mg). Subfraction fr.35 was applied over silica gel eluting with *n*-hexane-EtOAc-MeOH (5:1:1) and purified by Lobar-A column (n-hexane:EtOAc, 1:1) to yield **3** (25 mg).

Compound 1 (isoimperatorin): colorless prisms (MeOH), mp 109-111°C; ^{1}H-NMR and ^{13}C-NMR data were in good agreement with those of literature values (Baek *et al.*, 2000, Cho *et al.*, 1998; Kwon *et al*; 1991; Kim *et al.*, 1992 and Woo *et al.*, 1982).

Compound 2 (imperatorin): colorless prisms (MeOH), mp 101-102°C; ^{1}H-NMR and ^{13}C-NMR data were in good agreement with those of literature values (Baek *et al.*, 2000, Cho *et al.*, 1998; Kwon *et al*; 1991; Kim *et al.*, 1992 and Woo *et al.*, 1982).

Compound 3 (oxypeucedanin): colorless prisms (MeOH), mp 140-141°C; ^{1}H-NMR and ^{13}C-NMR data were in good agreement with those of literature values (Baek *et al.*, 2000. Cho *et al.*, 1998; Kwon *et al*; 1991; Kim *et al.*, 1992 and Woo *et al.*, 1982).

Acetylcholinesterase inhibition assay

The AChE assay was measured by the modified method of Ellman *et al.* using acetylthiocholine iodide as a substrate (Ellman *et al.*, 1961). For the enzyme source, mouse brain was homogenized 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 extraction steps were carried out at 4°C. Protein concentration was determined using the BCA kit (biocinchoninic acid, Sigma Co., JSA) with bovine serum albumin (BSA) as the protein standard. The rates of hydrolysis by AChE were monitored spectrophotomatically using a 96-well microtiter plate format. Each extract (10 μl) was mixed with an enzyme solution (10 μl) and incubated at 37 for 30 min. Absorbance at 450 nm was read immediately after adding an Ellmans reaction mixture [70 μl, 0.5 mM acetylthiocholine, 1 mM 5,5-dithil-bis-(2-nitrobenzoic acid)] in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. Reading was 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).

RESULTS AND DISCUSSION

The methanolic extract of the root of *A. dahurica* was found to exhibit significant anti-AChE activity. To isolate the AChE inhibitory constituents of *A. dahurica*, the total methanolic extract was chromatographed by silica gel column. As a result, the activity was found in the subfraction fr.3. Using several chromatographic techniques, compounds **1-3** were isolated as active constituents and identified as isoimperatorin, imperatorin and oxypeucedanin, respectively, from physicochemical and spectral data in comparison with those of published literatures (Baek *et al.*, 2000, Cho *et al.*, 1998; Kwon *et al*; 1991; Kim *et al.*, 1989 and Woo *et al.*, 1982).

Compounds **1-3** inhibited AChE activity in a dose-dependent manner (Fig. 2). The concentrations of **1-3** required for IC_{50} (50% AChE inhibitory effect) determined to be 74.6, 63.7 and 89.1 μM, respectively (Table I), while the IC_{50} value of a positive control, berberine (Hwang *et al.*, 1996), was 2.9 μM. Among them, the mechanism of compound **2** was more studed *in vitro*. Inhibition of AChE by compound **2** was independent of incubation time (up to 60

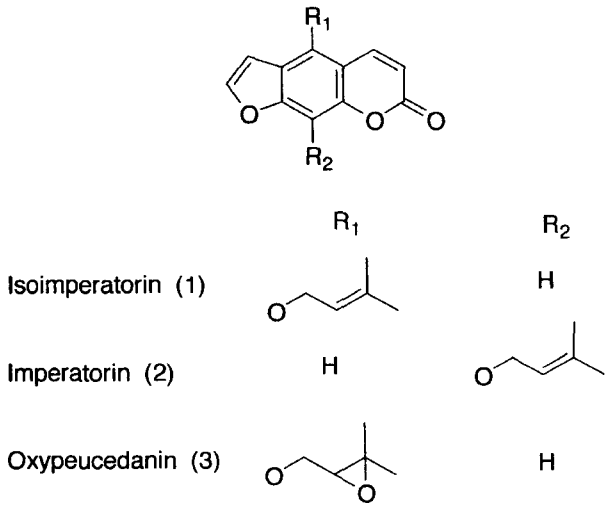

Fig. 1. Structures of compounds **1-3.**

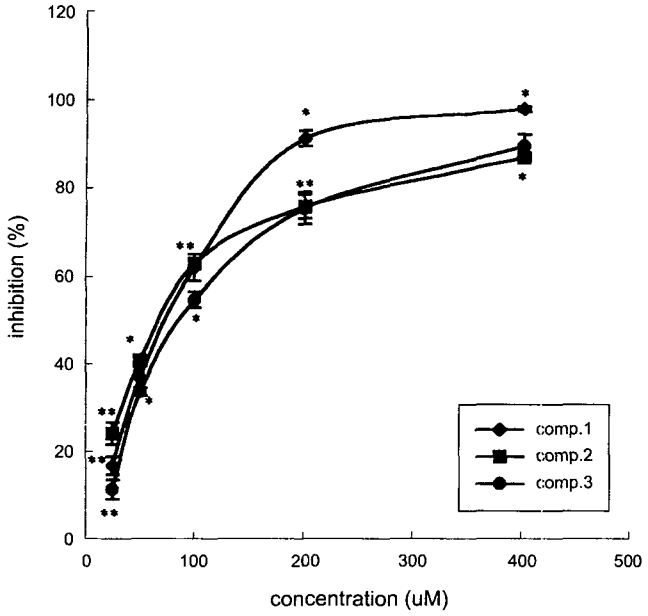

Fig. 2. The inhibitory activities of compounds **1-3** on AChE. Differs significantly from the control, effective *p < 0.05, **p < 0.01.

Table I. The inhibitory acitivities of copounds **1-3** on AChE.

Compound	IC_{50} (μg/ml)*
1	74.6
2	63.7
3	89.1
berberine	2.9

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

min, data not shown). This result suggests that compound **2** inhibited AChE reversibly. The kinetic analysis of AChE inhibition of **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] was decreased significantly by the addition of compound **2**. However, the K_m value was not changed. These results indicate that compound **2** inhibited AChE in a noncompetitive manner.

In this study, we have shown that the three furanocoumarins isolated from *A. dahurica* isoimperatorin, imperatorin and oxypeucedanin inhibits AChE activity. These are less effective than that of tacrine derivatives. However, these compounds were purified from a natural plant, which have been used a folk medicine from long time ago in Korea, Japan and China. And those low molecular materials could easily reach the site of action (brain) following oral or transdermal administration, since the molecules could cross the blood-brain barrier, the tight junction controlling the transport of material into the brain (Broadwell *et al.*, 1993). In conclusion, the present study suggests that the methanolic extract of *A. dahurica*, and its isolated furanocoumarin

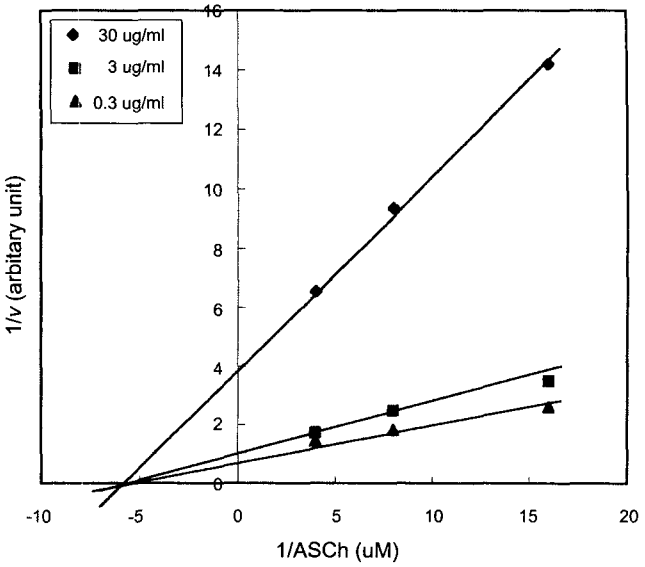

Fig. 3. Lineweaver-Burk plot of 1/v vs. 1/ASCh in the presence of compound **2**

components, isoimperatorin, imperatorin and oxypeucedanin may be useful for the treatment of AD.

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