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Inhibitory Effect of *Trans-N-p*-Coumaroyl Tryamine from the Twigs of *Celtis chinensis* on the Acetylcholinesterase

Dae Keun Kim and Kieseung Lee[1]

College of Pharmacy, Woosuk University, Samrye 565-701, Korea and [1]Department of Chemistry, Woosuk University, Samrye 565-701, Korea

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The methanolic extract of the twigs of *Celtis chinensis* was found to show inhibitory activity on acetylcholinesterase (AChE), an enzyme that plays a role in the metabolic hydrolysis of ACh. Bioassay-guided fractionation of the methanolic extract resulted in the isolation of *N-p*-coumaroyl tyramine, as an inhibitor on AChE. This compound inhibited AChE activity in a dose-dependent manner, and the IC_{50} value of *trans-N-p*-coumaroyl tyramine was 34.5 μg/mL (122 μM).

Key words: *Celtis chinensis*, Acetylcholinesterase, *Trans-N-p*-coumaroyl tyramine

INTRODUCTION

An important therapeutic strategy for activating central cholinergic functions has been the use of inhibitors of acetylcholinesterase (AChE), the enzyme responsible for the metabolic hydrolysis of acetylcholine (Bartus *et al.*, 1982; Perry, 1986; Bartus, 2000). In Alzheimers disease (AD) the brain is characterized by selective neuronal loss, neurofibrillary tangles, and extracellular deposits of insoluble amyloid that form senile plaques (Dennis *et al.*, 1998). 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. Some AChE inhibitors like physostigmine, tacrine, alkylpyridinium polymers, dehydroevodiamine, and carbamates have been 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).

In the course of screening Korean natural resources for AChE inhibitory activity, a total methanolic extract of the twigs of *Celtis chinensis* Persoon (Ulmaceae) was found to show an inhibitiory effect on AChE. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of *trans-N-p*-coumaroyl tyramine, as an active component.

Correspondence to: Dae Keun Kim, College of Pharmacy, Woosuk University, Samrye 565-701, Korea
E-mail: dkkim@woosuk.ac.kr

The bark of *C. chinensis* have been used in Korea, Japan and China as a folk medicine to treat lumbago, irregular menstruation, gastric disease, and abdominal pain (But *et al.*, 1997). The literature survey revealed that very little pharmacological work has been carried out on *C. chinensis*, and only its antifeedant effect on the larvae of the yellow butterfly has been reported (Numata *et al.*, 1984).

This paper describes the isolation of *trans-N-p*-coumaroyl tyramine from *C. chinensis* and the inhibitory effect of this compound on AChE.

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, with Kiesel gel 60 (230-400 mesh, Merck) used as the silica gel. Sephadex LH-20 was used for the column chromatography (Pharmacia, 25-100 μm). The column used for LPLC was Lobar-A (Merck Lichroprep Si 60, 240-10 mm). All other chemicals and solvents were analytical grade and used without further purification. Acetylthiocholine iodide (ASCh), 5,5'-dithio-bis-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.

Plant materials

The twigs of *C. chinensis* were collected and air-dried in

June 2001 at Bongdong, Chonbuk, Korea. A voucher specimen was deposited in the herbarium of the college of pharmacy, Woosuk University (WSU-01-020).

Extraction and isolation

The shade dried plant material (500 g) was extracted (three times with MeOH at room temperature) and filtered. The filtrate was evaporated *in vacuo* to give a dark brownish residue. The resultant methanolic extract (95 g) was followed by successive solvent partitioning to give *n*-hexane (7 g), $CHCl_3$ (15 g), EtOAc (5 g), *n*-BuOH (30 g) and H_2O soluble fractions. Each fraction was tested for inhibitory effects on AChE. Among these fractions, the EtOAc soluble fraction showed the most significant AChE inhibitory activity. Silica gel column chromatography of the EtOAc soluble fraction with $CHCl_3$-EtOAc-MeOH (10:1:1) gave five fractions (fr.1-fr.5). The major fraction fr.2 was rechromatographed on the Sephadex LH-20 column (MeOH) and purified by Lobar-A column chromatography ($CHCl_3$-EtOAc, 7:1) to yield compound **1** (40 mg).

Compound 1 (*Trans-N-p*-coumaroyl tyramine): Amorphous powder (MeOH), mp 234-236°C; EIMS (m/z): 283[M^+], 176, 164, 147 (100), 120, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.34 (1H, d, *J* = 15.6 Hz, H-7), 7.30 (2H, d, *J* = 8.8 Hz, H-2, 6), 6.95 (2H, d, *J* = 8.4 Hz, H-2', 6'), 6.68 (2H, d, *J* = 8.8 Hz, H-3, 5), 6.62 (2H, d, *J* = 8.4 Hz, H-3', 5'), 6.28 (1H, d, *J* = 15.6 Hz, H-8), 3.36 (2H, t, *J* = 7.5 Hz, H-7), 2.65 (2H, t, *J* = 7.5 Hz, H-8), ^{13}C-NMR (100 MHz, CD_3OD): 169.2 (C-9), 160.6 (C-4), 156.9 (C-4), 141.8 (C-7), 131.3 (C-1'), 130.7 (C-2', 6'), 130.6 (C-2, 6), 127.6 (C-1), 118.3 (C-8), 116.7 (C-3', 5'), 116.3 (C-3, 5), 42.6 (C-8'), 35.8 (C-7).

Animal care and 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). We diluted 0.5 M phosphate buffer with distilled water to make 50 mM phosphate buffer. Ellman's 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 Ellman's 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 Ellman's reaction mixture. Readings 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 50% of AChE activity) were calculated by the use of 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

The methanolic extract of the twigs of *C. chinensis* was found to exhibit anti-AChE activity (IC_{50} = 41 μg/mL). To isolate the AChE inhibitory constituents from *C. chinensis*, the total methanolic extract was suspended in water and partitioned successively with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. As a result, the inhibitory activity was found in the EtOAc soluble fraction. Using several chromatographic techniques, compound **1** was isolated as an active constituent and identified as an amide compound, *trans-N-p*-coumaroyl tyramine, by comparing physicochemical and spectral data with that of published literatures (Yosihra *et al.*, 1978; Okuyama *et al.*, 1986; Zhao *et al.*, 1992).

Trans-N-p-coumaroyl tyramine inhibited AChE activity in a dose-dependent manner (Fig. 2). The IC_{50} of compound **1** was determined to be 34.5 μg/mL (122 μM), while the IC_{50} value of a positive control, berberine (Hwang *et al.*, 1996), was 1.21 μg/mL (3.6 μM). The inhibition mechanism of compound **1** was studied in depth *in vitro*. Inhibition of

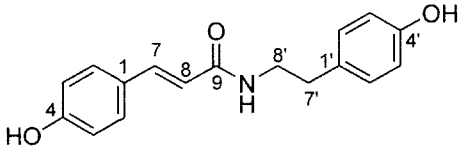

Fig. 1. Structure of compound **1**

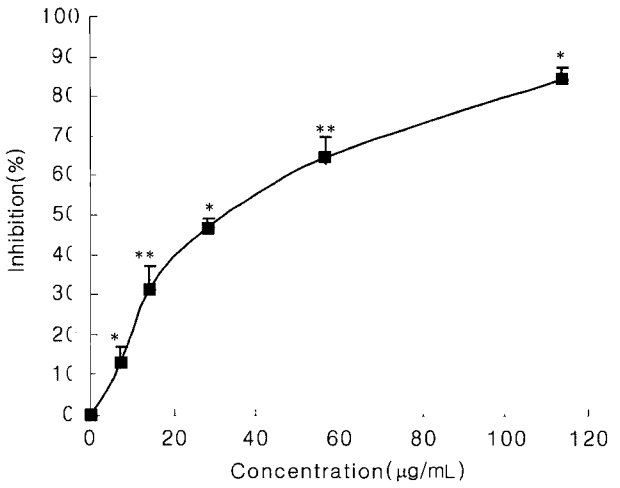

Fig. 2. The inhibitory activity of compound **1** on AChE. Differs significantly from the control, effective *p<0.05, **p<0.01.

AChE by compound **1** was independent of incubation time (up to 60 min, data not shown), suggesting that compound **1** inhibited AChE reversibly. The kinetic analysis of AChE inhibition by compound **1** is shown in Fig. 3. As the intersection of the lines occurred in the second quadrant, the Lineweaver-Burk plot for compound **1** indicated that with increased concentration of the active component, the K_m value of AChE against the active component increased and the V_{max} value decreased. This result indicated that this active component inhibited AChE in a mixed (competitive/noncompetitive) manner.

This study showed that an amide compound isolated from *C. chinensis, trans-N-p*-coumaroyl tyramine, inhibits AChE activity, although less effective than that of tacrine derivatives. The compound was purified from a natural plant that has been used as a folk medicine for a long time in Korea, Japan and China. On oral or transdermal administration, this low molecular material 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 indicated that the methanolic extract of *C. chinensis*, and its isolated amide component, *trans-N-p*-coumaroyl tyramine, may be useful for the treatment of Alzheimers disease.

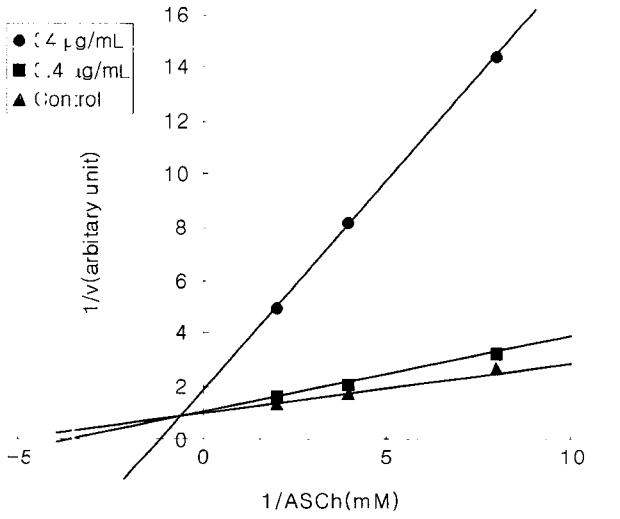

Fig. 3. Lineweaver-Burk plot of 1/v vs. 1/ASCh in the presence and absence of *trans-N-p*-coumaroyl tyramine

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