Inhibitory Effect of *Poncirus trifoliate* on Acetylcholinesterase and Attenuating Activity against Trimethyltin-Induced Learning and Memory Impairment

Jae Kyeom Kim,¹ Heyri Baé,¹ Mi-Jeong Kim,² Soo Jung Cho,³ Hong Yon Cho,¹ Han-Joon Wang,¹ Young Jun Kim,¹ Seung Taik Lim,² Eun Ki Kim,⁴ Hye Kyung Kim,⁵ Bok Yong Kim,⁶ and Dong-Hoon Shin¹

¹Department of Food and Biotechnology, Korea University, Seoul 136-701, Republic of Korea
²Brain Korea 21 Project for Medicine, Yonsei University, Seoul 120-752, Republic of Korea
³Graduate School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea
⁴Department of Biological Engineering, Inha University, Incheon 402-751, Republic of Korea
⁵Department of Food and Biotechnology, Hanseo University, Seosan 356-706, Republic of Korea
⁶Han-il Ginseng Industry Company, Chuncheon 200-170, Republic of Korea

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Various native Korean plants were screened to find an effective acetylcholinesterase (AChE) inhibitor for the treatment of Alzheimer’s disease (AD). Among these plants, the ethanol extract of *Poncirus trifoliate* was selected for isolating the AChE inhibitor because it exhibited the highest inhibitory activity (47.31%). To separate the active compound from *Poncirus trifoliate*, solvent partition, open column chromatography, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) were utilized. The putative chemical structure of the AChE inhibitor was identified as methoxsalen by successive analysis with electron ionization mass spectrometry (EI-MS) and $^{13}$C/$^1$H-nuclear magnetic resonance (NMR). To confirm the attenuating effect of the *Poncirus trifoliate* extract on trimethyltin (TMT)-induced neurotoxicity, *in vivo* behavior tests were carried out. Our findings suggest that the *Poncirus trifoliate* extract significantly reversed TMT-induced learning and memory impairment. These results demonstrate that the *Poncirus trifoliate* extract could possess a wide range of beneficial activities for neurodegenerative disorders, notably AD.

Key words: methoxsalen (8-methoxy-2',3',6,7-furocoumarin); *Poncirus trifoliate*; Alzheimer’s disease; acetylcholinesterase; amyloid beta peptide

Alzheimer’s disease (AD) was discovered by Dr. Alois Alzheimer in 1907, and is described as a degenerative disease of the central nervous system with many cognitive and neuropsychiatric manifestations that result in progressive disability and eventual incapacitation. AD is not just among the leading causes of senile dementia, but it is also the most prevalent neurodegenerative disease affecting populations worldwide, and thus is an increasingly threatening international health problem. According to a report from the Alzheimer’s Association, one in eight Americans over the age of 65 and about half of Americans over the age 85 currently suffer from this disease.¹ Moreover, the annual economic cost of AD health care and lost wages for both patients and caregivers is estimated to be approximately 80–100 billion dollars. With the present rate of growth among the elderly population, it is predicted that around 16 million seniors will be affected by 2050.²⁻⁴

AD patients present a progressive loss of cholinergic synapses in the brain regions associated with higher mental functions, mainly the hippocampus and neocortex. In these AD patients, a decrease in acetylcholine (ACh), a neurotransmitter, appears to be a critical element in the development of dementia; this finding has led to research on ways to enhance the diminished level of the cholinergic neurotransmitter. Hence, AD and other forms of dementia could be treated by the use of agents that restore the level of acetylcholine through the inhibition of both major forms of cholinesterase: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Moreover, the inhibition of AChE plays a key role not only in enhancing cholinergic transmission in the brain, but also in reducing the aggregation of amyloid beta peptide (Aβ) and the formation of the neurotoxic fibrils in AD. The AChE inhibitors so far approved by the U.S. Food and Drug Administration for the treatment of symptomatic patients with mild to moderate AD are tacrine, donepezil, and rivastigmine.⁵⁻⁶

Despite intensive advances in research, effective therapeutic options against AD are limited, and have thus increased the demand for new drugs. Polyphenolic compounds from fruits and vegetables are currently gaining interest for their beneficial effects, including...
antioxidative, antiviral, anticancer, and anti-inflammatory actions, as well as the prevention of cardiovascular diseases. However, previous interest in these phytochemicals, with respect to their health-promoting effects, has primarily been focused on examining their roles in the protection against cancer and ischemic heart disease. Few studies have investigated their effects on brain functions and neurodegenerative diseases.

In this study, various Korean plants were screened in a search for effective AChE inhibitors. Among these plants, the ethanol extract of *Poncirus trifoliata* was selected for the isolation and purification of an AChE inhibitor, because it showed the highest inhibitory activity. To separate the active compound from the extract of *Poncirus trifoliata*, solvent partition, open column chromatography, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) were utilized. The isolated compound was then analyzed by EI-MS and 1H-NMR to elucidate its putative chemical structure. To confirm the attenuating effect of the extract of *Poncirus trifoliata* against trimethyltin (TMT)-induced learning and memory impairment, *in vivo* behavior tests, i.e., the Y-maze test and passive avoidance test, were performed.

### Materials and Methods

**Materials.** Dimethyl sulfoxide (DMSO), trimethyl chloride (TMT), tacrine (9-amino-1,2,3,4 tetra-hydroacridine), acetylthiocholine iodide, and 5,5′-dithio-bis(2-nitro) benzoic acid (DTNB) were purchased from Sigma Co. (St. Louis, MO, USA). Silica gel and analytical thin-layer chromatographic plates were obtained from Merck Co. (Darmstadt, Germany). The PC12 cell line was from the American Type Culture Collection (Manassas, VA, USA). The Roswell Park Memorial Institute (RPMI) 1640 medium, donor horse serum, fetal bovine serum, and antioxidant-antimycotic were purchased from Gibco-BRL™ (Grand Island, NY, USA). All other chemicals used were of analytical grade.

**Methods.**

Preparation of the plant extracts for screening. Various Korean plants were obtained from a local market or oriental medicine store in Seoul, Republic of Korea. Each sample was dried in a drying oven (Vision Scientific, Seoul, Korea). The mice were housed nine per cage in a room maintained at a 12-h light-dark cycle, 55% humidity, and 23–25 °C temperature. The *Poncirus trifoliata* extract was mixed in a commercial diet (Purina Korea, Seoul, Republic of Korea) at concentrations of 400, 800, and 1,200 mg/kg of body weight (0.25%, 0.50% and 0.75%, respectively).

**Animals.** Institute of Cancer Research (ICR) mice (male, 5 weeks old) were obtained from Daehan Biolink (Chungnam, Republic of Korea). The mice were placed in the lighted compartment; as soon as it entered the dark compartment, an inescapable electric shock was provided (0.5 mA, 1 s). The testing trial, which was given 1 d after the training trial, the mouse was again placed in the lighted compartment, and the time until it re-entered the dark compartment was measured (the step-through latency maximum testing limit was 300 s).

**Y-maze test.** Recording spontaneous alternation behavior in the Y-maze test was used to assess the immediate working memory performance. The Y-maze test was performed 2 d after the TMT injection. The maze was made of black-painted plastic, and each arm of the maze was 33 cm long, 15 cm high, and 10 cm wide, and was positioned at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze for 8 min. The series of arm entries was recorded visually, and arm entry was considered to have been completed only when the hind paws of the mouse were placed completely in the arm of the maze. Alternation is defined as successive entries into the three arms in overlapping triplet sets. The percentage alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus two), multiplied by 100.

**Measurement of AChE activity from the brain homogenate.** Once the Y-maze and passive avoidance tests had been completed, the whole...
brain tissues were immediately collected from the mice and homogenized with 5 vol. of a homogenization buffer [10 mM Tris–HCl (pH 7.2) containing 1 M NaCl, 50 mM MgCl2, and 1% Triton X-100] to measure the AChE activity by using the modified method of Ellman et al.12) The homogenate was centrifuged at 10,000 g for 30 min, and the resulting supernatant was used as an enzyme source. All the enzyme preparation steps were carried out at 4°C. The rate of hydrolysis by AChE was spectrophotometrically monitored in the 96-well plates as already described.

Statistical analyses. Each result is expressed as the mean ± S.D. Data were analyzed by Duncan’s multiple-range test in the Statistical Analysis System (SAS) software package (NC, USA). The statistical significance of differences among groups was calculated by a one-way analysis of variance (ANOVA).

Results and Discussion

ACh, a neurotransmitter, is widely distributed in the nervous system and has been implicated to play an important role in cerebral cortical development, cortical activity, cerebral blood flow control, modulation of cognitive performance, and signal transfer in the synapses. Loss of cholinergic innervation, as demonstrated by reduced choline acetyltransferase (ChAT) and increased AChE activity, is correlated with the degree of dementia and the severity of the neuropathological hallmarks of AD. AChE inhibitors decrease the hydrolysis of ACh to elevate the endogenous level of ACh in the brain, and to boost cholinergic neurotransmission. Therefore, it was considered that elevating the level of ACh might be helpful in attenuating the symptoms of neuronal deficits and treating cognitive dysfunction in mild to moderate cases of AD. In some cases, the patients showed a dramatic improvement in cognitive scores, and these effects were readily observable in terms of their daily functions.17–20)

In order to search for an effective AChE inhibitor from natural sources, various native edible plants were screened by using Ellman’s method. The inhibitory activities of these plant extracts on AChE are summarized in Table 1. The ethanol extract of Poncirus trifoliata exhibited the highest inhibitory activity against AChE when compared with that of the other extracts (Poncirus trifoliata; 47.31%, Allium monanthum; 28.26%, Paeonia suffruticosa Andr.; 28.21%). The ethanol extract of the selected sample was subsequently partitioned three times with n-hexane, chloroform, and ethyl acetate, respectively. As depicted in Fig. 1, the second fraction of chloroform exhibited the highest inhibitory activity on AChE (28.95 ± 1.66%), and was then chromatographed in a silica gel column, which divided it into 33 subfractions. Among them, the second of the 33 fractions was found to inhibit AChE activity by more than 50% (54.35 ± 1.21%, Fig. 2). To purify the active compound, the AChE inhibitor, the selected

![Fig. 1. Inhibitory Effect against AChE Activity of the Poncirus trifoliata Extract Fractionated by Solvent Partition.](image)

The value (%) of AChE activity for each fraction was calculated as compared with the control activity (100%). The ethanol extract of the Poncirus trifoliata extract was dissolved in deionized water and respectively partitioned with n-hexane (H1, H2, H3), chloroform (C1, C2, C3), and ethyl acetate (E1, E2, E3) three times. The sample concentration was 1 mg/ml. Three hundred μM of tacrine, a specific AChE inhibitor, was utilized as a positive control. Data are represented as the mean inhibition (n = 3) ± S.D. Duncan’s multiple-range test of SAS indicates a significant difference (p < 0.05 vs. the sample fraction).
Inhibitory Effect of the Poncirus trifoliate Extract against the AChE Activity Fractionated by Silica Gel Open Column Chromatography. The value (%) of AChE activity for each fraction was calculated as compared with the control activity (100%). The second fraction of chloroform from the solvent partition (C2) was eluted by a mixture of CHCl₃ and EtOH (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100; v/v). Column elution of each mixture was individually performed three times. The sample concentration was 1 mg/ml. Three hundred μl of tacrine, a specific AChE inhibitor, was utilized as a positive control. Data are represented as the mean inhibition (n = 3) ± S.D. Duncan’s multiple-range test of SAS indicates a significant difference (*p < 0.01 vs. tacrine and the sample fraction).

Table 2. Inhibitory Effect of the Poncirus trifoliate Extract at Different R_f Values Separated by Preparative TLC

<table>
<thead>
<tr>
<th>R_f value</th>
<th>Inhibitory effect against AChE (%)</th>
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<tbody>
<tr>
<td>0.08</td>
<td>23.70 ± 0.81</td>
</tr>
<tr>
<td>0.12</td>
<td>31.34 ± 1.04</td>
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<tr>
<td>0.15</td>
<td>29.90 ± 2.01</td>
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<tr>
<td>0.17</td>
<td>31.48 ± 1.27</td>
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<tr>
<td>0.24</td>
<td>23.44 ± 2.07</td>
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<tr>
<td>0.28</td>
<td>33.95 ± 1.02</td>
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<tr>
<td>0.34</td>
<td>49.55 ± 0.42</td>
</tr>
<tr>
<td>0.41</td>
<td>38.67 ± 0.82</td>
</tr>
<tr>
<td><strong>0.44</strong></td>
<td><strong>54.01 ± 0.51</strong> *</td>
</tr>
<tr>
<td>0.51</td>
<td>31.52 ± 1.04</td>
</tr>
<tr>
<td>0.63</td>
<td>26.82 ± 2.50</td>
</tr>
<tr>
<td>0.77</td>
<td>18.99 ± 2.51</td>
</tr>
</tbody>
</table>

The mobile phase was a mixture of CHCl₃, EtOH, and NH₃ (99.5:0.5 ml and 16 μl). The sample concentration was 1 mg/ml. Data are presented as the mean inhibition (n = 3) ± S.D. Duncan’s multiple-range test of SAS indicates a significant difference (*p < 0.01).

Fraction was subjected to successive preparative TLC and HPLC. TLC plates were visualized under visible and ultraviolet light (at 254 and 365 nm), and R_f values were measured (Table 2). The separated band from TLC that represented the best inhibition was scratched together and extracted with chloroform or EtOH. The sample was then applied to a μ-Bondapak™ C₄8 column (Waters Co., MA, USA; reverse phase, 3.9 mm × 300 mm) with an HPLC system (Fig. 3). The final yield of methoxsalen from dried Poncirus trifoliate (3 kg) was 1.5 mg. According to the resulting ¹³C/¹H-NMR and EI-MS data (Figs. 4 and 5), the putative AChE inhibitor was finally identified as methoxsalen; this compound has already been reported to possess various bioactive properties,¹²⁻¹³ and belongs to a group of compounds known as the psoralens or furocoumarins (Fig. 6).

TMT, a potent neurotoxicant, has been shown to cause damage to the limbic system. The neurodegenerative effect of TMT is the most evident due to pyramidal cell loss in the hippocampus, leading to various behavioral and biochemical deficits.¹⁴⁻¹⁵ To confirm the attenuating effect of the extract of Poncirus trifoliate against TMT-induced learning and memory impairment, the Y-maze test and passive avoidance test were utilized. The ICR mice were treated with or without a Poncirus trifoliate extract mixed diet for up to 3 weeks, and were then injected with a single dose of TMT (0.0025 mg/g of body weight). All mice treated with the extract of Poncirus trifoliate gained body weight normally (38.33 ± 1.78 g for the control group versus 38.28 ± 0.4 g for the sample groups treated with the Poncirus trifoliate extract) and did not exhibit any acute toxicity during the experimental period (data not shown). The neuroprotective effects of the Poncirus trifoliate extract on learning and memory in vivo were then assessed by using a TMT-induced amnesia model.

Memory impairment in AD patients is a condition that makes them dependent upon their caregivers. In our results, the Poncirus trifoliate extract exhibited a learning memory enhancing effect in the passive avoidance test. The TMT treatment significantly shortened the latency time (a 38.06% decrease in step-through latency) in the retention trial compared with that of the control group. However, treating the mice with the Poncirus trifoliate extract for 3 weeks significantly reversed this TMT-induced amnesia in all sample groups (400 mg/kg, 95.07 ± 10.22%; 800 mg/kg, 102.26 ± 0.89%; 1,200 mg/kg, 102.53 ± 0.30%; Table 3). No significant difference between the sample groups was apparent.

Recording spontaneous alternation behavior in a Y-maze test was used to assess the immediate working memory performance. The Y-maze test was carried out two days after the TMT injection. As depicted in Fig. 7, the administration of TMT significantly impaired the spatial working memory (78.93 ± 6.26%) compared with that of the control group (100.00 ± 7.15%). Groups pretreated with the sample extract showed a significantly
ameliorated TMT-induced decrease in alteration behavior, and protective effects were clearly shown in all of the sample groups (400 mg/kg, 96.03 ± 11.31%; 800 mg/kg, 99.96 ± 4.41%; 1,200 mg/kg, 103.49 ± 5.69%). More importantly, the numbers of arm entries were not significantly different among the experimental groups. These results indicate that the general locomotor activity of the mice was not affected by either the sample extract or TMT administration, but that the impaired spatial working memory was attenuated by the administration of the *Poncirus trifoliate* extract. There were no significant differences in memory enhancement among any of the sample groups.

To assess the inhibitory activity of the *Poncirus trifoliate* extract against AChE, brain tissues were dissected from the mice immediately after the behavior tests. As shown in Table 4, the group with TMT injection demonstrated higher AChE activity compared with the control group, whereas all sample groups exhibited significant inhibitory activity against AChE (control group, 100.00 ± 0.47%; TMT group, 77.97 ± 0.41%; 400 mg/kg, 84.32 ± 1.91%; 800 mg/kg, 84.99 ± 2.02%; 1,200 mg/kg, 99.68 ± 5.79%). This result is in agreement with a previous study that TMT decreased the level of acetylcholine in the hippocampus.26 Natural dietary phytochemicals, including flavo-

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**Fig. 3.** Isolation of Active Compound from the *Poncirus trifoliate* Extract by HPLC.

The analytical column used was a μ-Bondapak™ C18 (Waters Co., MA, USA; reverse phase, 3.9 mm × 300 mm). Separation was carried out at a 0.5 ml/min flow rate using a linear gradient of 0–100% of analytical grade ethanol. The injection volume was 10μl, and a distinct peak was monitored at 248 nm with a PDA detector.

**Fig. 4.** EI-MS Data for the AChE Inhibitor Isolated from the *Poncirus trifoliate* Extract.

The spectrum was recorded by a positive ion EI mass spectrometer (JMS-AXS505WA, Jeol, Tokyo, Japan). The sample (0.3 mg) was dissolved in MeOH. EI-MS enabled a peak of high abundance to be observed, showing the molecular weight of the active component to be 216 m/z.
noids, are substrates for several enzymes located in the small intestine, colon, and liver. Contrarily, we should not exclude the possibility of metabolic modification of certain phytochemicals altering their lipophilicity to enhance the permeability against the blood brain barrier (BBB). Therefore, it is unreasonable to draw the conclusion that certain phytochemicals inhibited AChE either directly or indirectly. However, our present results indicate that the administration of the *Poncirus trifoliate* extract effectively inhibited AChE in the brains of the

**Fig. 5.** $^{13}$C/$^1$H-NMR Spectrum of the AChE Inhibitor Isolated from the *Poncirus trifoliate* Extract. $^{13}$C/$^1$H-NMR was operated at 600 MHz and 25 °C (Avance-600, Bruker, Karlsruhe, Germany). $^1$H-NMR spectrum (A), $^{13}$C-NMR spectrum (B). The sample (1.5 mg) was dissolved in methyl-d3 alcohol-dl (MeOD).

**Fig. 6.** Chemical Structure of Methoxsalen. Molecular formula, C$_{12}$H$_8$O$_4$; molecular weight, 216.18.
mice, so that it may have contributed to increasing the ACh level in the synaptic clefts.

Poncirus trifoliate fruits are green and then ripen to yellow, with a 3–4 cm diameter, resembling a small orange. The anti-cancer, anti-inflammatory, anti-Helicobacter pylori, and anti-anaphylactic activities of this fruit have recently been reported. In this study, methoxsalen was purified from the Poncirus trifoliate extract and presented inhibitory activity against AChE. Methoxsalen has been reported to be isolated from medicinal herbs including Treculia obovoidea (Moraceae) and Angelica archangelica. Methoxsalen has also shown anti-microbial, anti-oxidative, and anti-AChE activities in vitro. However, this may be the first report to indicate that the Poncirus trifoliate extract had anti-amnesic activity by inhibiting AChE.

AD is accompanied by several pathological changes in the brain, including diffuse loss of neurons, intracellular proteins, and extracellular proteins such as senile plaques. The most abundant constituent of senile plaques that can be observed in AD is Aβ peptide, a cleaved form of the amyloid precursor protein (APP) by β-, γ-secretase. Although its pathological mechanism and cellular pathway have not been fully elucidated, several lines of evidence have indicated that the Aβ peptide induced the intracellular accumulation of reactive oxygen species (ROS), eventually resulting in peroxidation of the cell membrane, modification of protein, DNA/RNA damage, and cell death. On the other hand, α-secretase cleaves APP within the Aβ peptide sequence and releases soluble N-terminal non-aggregating fragments (sAPP). Many studies have shown that the activation of sAPP release was related to the reduced formation of amyloidogenic peptide. Interestingly, based on the results obtained from superfused brain cortical slices of the rat, AChE inhibitors significantly increased the release of sAPP, thus slowing the deterioration of the AD patient. Moreover, several lines of evidence have also indicated AChE to be a potent amyloid-promoting factor when compared with other Aβ-associated proteins, and this might be suppressed by the action of another AChE inhibitor, the binding peripheral anionic site ligand. Therefore, we cannot dismiss the possibility that methoxsalen may exert a beneficial effect not merely to increase the level of the neurotransmitter between the synapse clefts by inhibiting AChE, but also to stimulate the release of sAPP in the AD brain or to bind with a peripheral anionic site, thereby decreasing the formation of neurofibrillary tangles (NFT) and senile plaques.

We found in the present work that the extract of Poncirus trifoliate exerted an inhibitory activity against AChE, and provided substantial evidence for the therapeutic potential of methoxsalen, thus inviting...
further clinical investigation. It will be more interesting to study how this active compound affects inhibitory activity at the molecular level and changes the cellular machinery of RNA and proteins. The results of this study indicate that methoxsalen from Poncirus trifoliate might perform an inhibitory action against AChE, and clearly show an improvement of both memory and cognitive ability against TMT-induced neuronal deficits. Therefore, the extract of the possible AChE inhibitor, methoxsalen from Poncirus trifoliate, could possess a wide range of beneficial pharmacological activities for neurodegenerative disorders, most notably AD.

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References