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# Effects of *Chrysanthemum indicum* Linne Flowers on Acetylcholinesterase Activity and Learning Performance in Mice

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**Abstract** Alzheimer's disease (AD) is the most common neurodegenerative disorder and is responsible for more than 50% of all dementia cases. There is significant interest in finding new sources of compounds that inhibit acetylcholinesterase (AChE) to be used in the treatment of AD, since only a few AChE inhibitors, such as galanthamine, physostigmine, and tacrine, are available for clinical use. In the present study, ICR mice were treated with a 1 mg/kg scopolamine, which caused impaired cognitive ability. The steady consumption of a water extract of *Chrysanthemum indicum* Linne flowers for 3 months significantly prevented the scopolamine induced deficit of the spatial cognitive capability of mice. It also improved long-term memory in mice with amnesia induced by scopolamine, as assessed by the Morris water maze and passive avoidance tests. In addition, water extract consumption significantly decreased AChE activity in mouse brain, leading to inhibition of acetylcholine hydrolysis.

**Key words:** *Chrysanthemum indicum* Linne, acetylcholinesterase inhibitor, Morris water maze, passive avoidance test

## Introduction

Alzheimer's disease (AD) is one of the most common forms of the dementia that affects many older people and its primary symptom is loss of memory. Besides the neuropathological hallmarks of this disease, namely neurofibrillar tangles and neuritic plaques, it is characterized neurochemically by a consistent deficit in cholinergic neurons in the basal forebrain (1,2). The cholinergic neural system plays an important role in learning and memory in humans and animals. Evidence for this stems from the data of several authors who investigated the effects of a reduction in the activity of the enzyme involved in the synthesis of acetylcholine (ACh), choline acetyl transferase, or of excess degradation of ACh by acetylcholinesterase (AChE) (3-5).

The principal role of AChE is the termination of nerve impulse transmission at cholinergic synapses by rapid hydrolysis of ACh. Inhibition of AChE serves as a strategy for the treatment of AD, senile dementia, ataxia, and Parkinson's disease (6-8). There are a few synthetic medicines, such as tacrine and donepezil, and a natural product-based medicine, rivastigmine, that are used in the treatment of the cognitive dysfunction and memory loss associated with AD (9). These compounds have been reported to have adverse effects, including gastrointestinal disturbances, and also have problems associated with bioavailability (10). These reason necessitate the search for better AChE inhibitors from natural resources (11).

Previous studies have reported that *Chrysanthemum indicum* has antibacterial, antiviral, antioxidant, anti-inflammatory, and immunomodulatory properties. Phytochemical profiles of the plant have shown the presence of flavonoids, terpenoids, and phenolic compounds (12-14). Through a series of studies evaluating naturally occurring AChE inhibitors, the ethanol extract of *C. indicum* flowers was shown to significantly inhibit AChE *in vitro*, and 3 compounds (acaciin, acacetin-7-*O*-β-D-galactopyranoside, and luteolin) were recently identified to be responsible for this AChE inhibition (15). In this study, the effects of the water extract of *C. indicum* Linne flowers on learning and memory functions of scopolamine-treated mice were investigated in the Morris water maze and the passive avoidance test, and AChE activity in mouse brain was also assessed.

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## Materials and Methods

**Extraction** The dried flowers of *C. indicum* Linne were purchased from Dea-Gang Herb, Chuncheon, Gangwon, Korea in Sept. 2005. The plant materials were authenticated by Dr. Kim YD, professor of the Department of Biology, Hallym University. Dried flowers (3,000 g) were extracted with 10 L of distilled water for 1 hr using a no pressure extractor (CMB-K2-151L; Kyung Seo Machines Co., Incheon, Korea), concentrated under reduced pressure using a rotary vacuum evaporator (Rotavapor R-220; BÜCHI Labortechnik AG, Flawil, Switzerland), and finally lyophilized (Bondiro; Ilshin Lab Co., Ltd., Yangju, Korea) to obtain a powder (600 g, 20%) that was used as the test sample.

**Animals** ICR mice, weighing 37-45 g, were purchased from the Orient Co., Ltd. (Seongnam, Korea) and acclimated for 7 days prior to the commencement of the experiment. Mice were randomly distributed into 4 groups (10 mice per group): control (C); scopolamine treated control (CS); water extract of *C. indicum* Linne flower treated (K); and scopolamine & water extract of *C. indicum* Linne flower

treated (KS). The mice were placed in polypropylene cages (5/cage) with rice husk as bedding. Mice were allowed access to pelleted animal feed (Agribrand Purina; Cargill Agri Purina, Inc., Seongnam, Korea) and water *ad libitum*, and maintained under a 12-hr light/dark cycle (light on 07.30-19.30 hr) in an air-regulated and soundproof laboratory (23±1°C, 55±5% humidity). The lyophilized water extract powder of *C. indicum* Linne flower dissolved in distilled water (0.05 g/100 mL) was put into the water bottles of the water extract and the scopolamine & water extract treated groups for 3 months, while distilled water alone was provided for the 2 other groups. All experiments were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of Hallym University.

**Scopolamine treatment** An animal model in which cholinergic hypofunction has been caused pharmacologically by the administration of scopolamine, is a useful tool in the evaluation of new cholinergic agonists (16). Scopolamine (1 mg/kg) dissolved in 0.1% dimethyl sulfoxide (DMSO) was administered intraperitioneally 30 min prior to the test, in order to induce learning and memory impairment in the mice.

**Passive avoidance test** The passive avoidance test was carried out in identical part illuminated and part non-illuminated boxes (Gemini Avoidance System; San Diego, CA, USA). The illuminated compartment (20×20×20 cm) contained a 50 W bulb. The non-illuminated compartment (20×20×20 cm) had a floor composed of 2 mm stainless steel rods spaced 1 cm apart. These 2 compartments were separated by a guillotine door (5×5 cm). For the acquisition trial, mice were initially placed in the illuminated compartment and the door between the 2 compartments was opened 10 sec later. When mice entered the dark compartment, the door automatically closed and an electrical foot shock (0.5 mA) of 3 sec duration was delivered through the stainless steel rods. Twenty-four hr after the acquisition trial had been performed 3 times a day for 2 days, each mouse was again placed in the illuminated compartment to test retention. The time taken for a mouse to enter the dark compartment after the door opened was defined as the avoidance latency. Ten mice were used per treatment and scopolamine treated mice were administered with scopolamine (1 mg/kg) before 30 min prior to the test.

**Morris water maze test** The spatial memory of the mice was assessed using a Morris water maze (17). This is a circular stainless steel pool (150 cm in diameter and 45 cm in height) with a featureless inner surface. The pool was filled to a depth of 30 cm with water (20±1°C) which was made opaque with the addition of milk powder. A small platform (10 $cm^2$) was placed in 1 of 4 quadrants. The top of the platform was 1 cm below the surface of the water. During acquisition trials for 4 days, mice were trained to swim from variable starting points around the tank to the platform. A total of 16 training trials were given (4 trials per day for 4 days with an interval of 5-7 min). Each mouse was placed facing the wall at 1 quadrant of the pool. The mouse was allowed to remain in it for 30 sec, and then was gently removed from the pool to its home cage. On the 5$^{th}$ day, the animals were tested for spatial memory retention in the water maze. The platform was removed, and each mouse was again placed at the starting point and observed. The stop-through latency to reach the original platform location was recorded in seconds, and the frequency to pass the original platform location for 120 sec was recorded. Ten mice were used per treatment and scopolamine treated mice were administered scopolamine (1 mg/kg) before 30 min prior to the test.

**AChE activity** AChE activity in the mice brains was measured using an acetylthiocholine iodide substrate based colorimetric method, as described by Ellman *et al.* (18). Ten animals from each group were decapitated and whole mouse brain was obtained, and homogenized with 0.1 mM sodium phosphate buffer (pH 7.4). Each homogenate was preincubated for 5 min at 37°C with 0.1 mM tetraisopropyl pyrophosphoramide (TPPA), a selective inhibitor of butyrylcholinesterase. For the assay of AChE activity, a reaction mixture that contained 470 μL sodium phosphate buffer (0.1 mM; pH 8.0), 167 μL 4% dithiobisnitrobenzoic acid (DTNB) and 33 μL homogenate was incubated for 5 min at 37°C. After the addition of 280 μL acetylthiocholine iodide (1 mM) as the substrate, the absorbance was measured for 5 min at 410 nm. Results were given as unit/min/mg protein for specific activities. Protein concentrations were determined using Lowry's method (19) using bovine serum albumin as a standard.

**ACh content measurement** The quantification of ACh was done according to the method by Galgani *et al.* (20). Five animals from each group were decapitated and whole mouse brain was obtained, and homogenized (10 mg/mL) in 0.1 M phosphate buffer, pH 7.2. An aliquot of 50 μL 1% hydroxylamine was added to a 50 μL aliquot of the homogenized sample, and the pH was adjusted to 1.2±0.2 by HCl addition. An aliquot of 500 μL 10% $FeCl_3$ in 0.1 N HCl was added and mixed, and the absorbance was measured at 530 nm (UV-1600 spectrometer; Shimadzu, Kyoto, Japan), with the results expressed as acethylcholine ng/mg protein.

**Statistical analysis** Analysis of variance (ANOVA) and Duncan's multiple-range test were used to determine the significance of differences among means, and $p<0.05$ was considered to be statistically significant.

## Results and Discussion

**Passive avoidance test and Morris water maze test** In the present study, the effects of water extract of *C. indicum* Linne flowers on scopolamine-induced memory impairments were investigated in the passive avoidance and Morris water maze tasks in mice. Scopolamine, a nonselective muscarinic antagonist, blocks cholinergic signaling without changing the ACh concentration, and produces memory deficits that are similar to those found in age-related senile dysfunction involving cholinergic neurons in the central nervous system (CNS) (21). Thus, the scopolamine-induced amnestic murine model is useful for investigating age-related senile CNS dysfunction.

The ameliorative effects of water extract of *C. indicum* Linne flowers were investigated in the passive avoidance task. As shown in Fig. 1, K group did not enter the dark

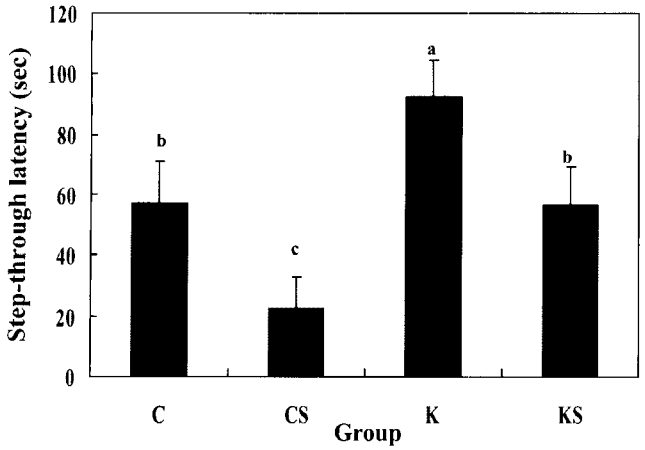


**Fig. 1. Effect of water extract from *Chrysanthemum indicum* Linne on the memory deficit in mice induced by scopolamine in the passive avoidance performance test.** C, control group; CS, scopolamine treated control group; K, water extract of *C. indicum* Linne flowers treated group; KS, scopolamine & water extract treated group. Bars with the same small letters are not significantly different by ANOVA and Duncan's multiple range test, $p<0.05$ was considered significant.

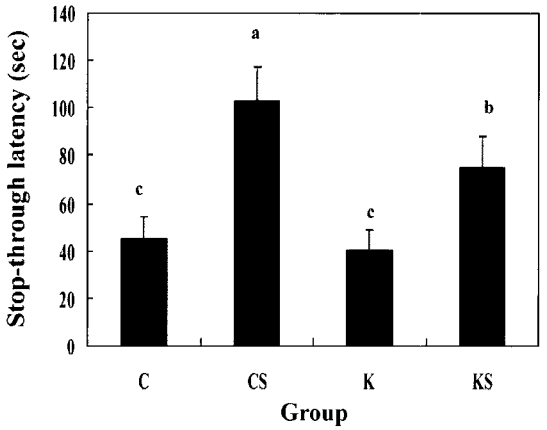


**Fig. 2-1. Effect of water extract from *Chrysanthemum indicum* Linne on the stop-through latency in Morris water maze test.** C, control group; CS, scopolamine treated control group; K, water extract of *C. indicum* Linne flowers treated group; KS, scopolamine & water extract treated group.

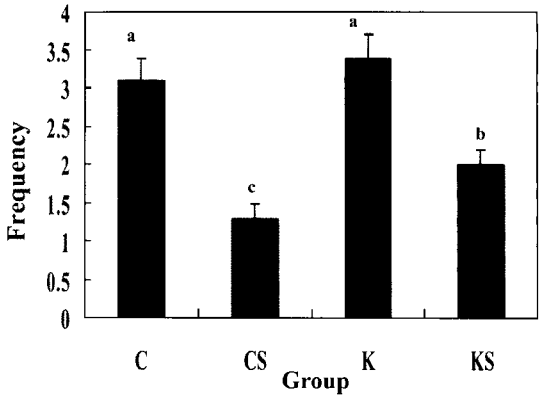


**Fig. 2-2. Effect of water extract from *Chrysanthemum indicum* Linne on frequency in the Morris water maze test.** C: control group; CS, scopolamine treated control group; K, water extract of *C. indicum* Linne flowers treated group; KS, scopolamine & water extract treated group.

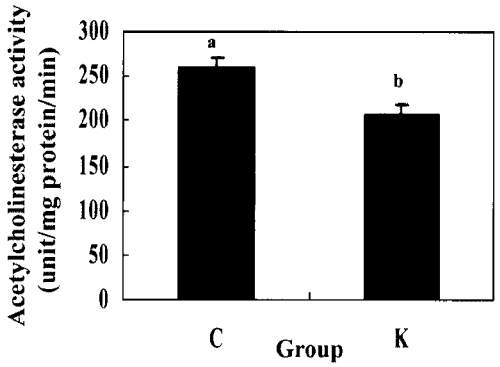


**Fig. 3. Effect of water extract from *Chrysanthemum indicum* Linne on acetylcholinesterase activity in brain of mouse.** C, control group; K, water extract of *C. indicum* Linne flowers treated group.

compartment for 92.6 sec, showing a significant improvement in working memory compared to the control group. The step-through latency to enter the dark compartment was significantly decreased by scopolamine administration; the control group had a latency of 57.4 sec compared to the CS group whose latency was 22.7 sec. The shorter step-through latency induced by scopolamine was significantly reversed by the administration of water extract of *C. indicum* Linne flowers. The KS group had a significantly longer step-through latency of 56.6 sec, showing the water extract had ameliorated the scopolamine induced deficit, so that the latency was the same as that of the control group. The Morris water maze task is used to assess hippocampal-dependent spatial learning ability. Escape latency reductions from day to day reflect learning with respect to reference or long-time memory (22). ICR mice trained in spatial navigation showed a marked reduction in stop-through latencies (the time period to pass the platform location) from the first to the second trials and day by day. As shown in Fig. 2-1, the trained control group had a stop-through latency of 45.1 sec, and the K group had a latency of 40.7 sec, with no significant difference between the two groups. The CS group exhibited a marked impairment in long-term memory with a stop-through latency of 102.8 sec. However, latency in the KS group was 75.2 sec, showing a significant preventive effect of the water extract on the impairment of memory capability, compared to scopolamine alone. As shown in Fig 2-2, the control group had a frequency of 3.1 to pass the original platform location over 120 sec, and the K group had a 3.4 frequency, with no significant difference between the 2 groups. The frequency was far lower in the CS group at 1.3. The KS group had a significantly improved frequency of 2.0, compared to the CS group, but this was still lower than the control group. The patterns of the stop-through latency and frequency results were similar to each other.

These results demonstrate that the water extract of *C. indicum* Linne flowers significantly prevented the scopolamine induced deficit of the spatial cognitive capability of the mice, and improved long-term memory in mice with scopolamine-induced amnesia.

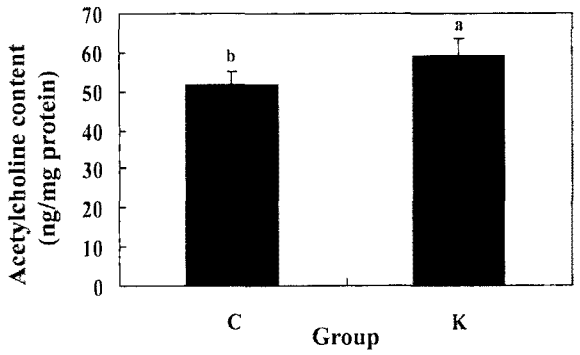


**Fig. 4. Effect of water extract from *Chrysanthemum indicum* Linne on acetylcholinesterase content in brain of mouse.** C, control group; K, water extract of *C. indicum* Linne flowers treated group.

**AChE inhibition and ACh content** To investigate the mode of action of water extract of *C. indicum* Linne flowers, its AChE inhibitory activity and its effect on ACh content were assessed using mouse brain homogenates. As shown in Fig. 3, there was a significant difference between the mouse brain AChE activity in the K group (205.6±14.7 unit/mg protein/min) compared to the control group (259.7±13.5 unit/mg protein/min). In addition, as shown in Fig. 4, the brain ACh contents of the 2 groups were 59.28 ±4.09 ng/mg protein and 51.64±3.46 ng/mg, respectively. The results demonstrate that the steady consumption of the water extract of *C. indicum* Linne flowers significantly inhibited AChE activity, leading to reduced AChE hydrolysis in the mouse brain.

AChE inhibitors, such as tacrine, donepezil, rivastigmine, and galanthamine, are known to increase the brain ACh level by preventing the degradation of released neurotransmitter, thereby enhancing neurotransmission at cholinergic synapses (23). Tacrine, the first approved drug for AD treatment in the United States, is a potent AChE inhibitor, but its side effects, low selectivity, and hepatotoxicity (23) make use of this drug problematic.

Rubioa *et al.* (24) showed that scopolamine administration increased the AChE activity of control mice by 1.5 fold. Black Maca extracts were used to inhibit AChE and resulted in a 45% decrease in AChE activity compared to scopolamine-treated mice. This decrease was associated with an improvement in learning and memory in male mice, showing the relationship between the inhibitory effect of the extracts on AChE activity and learning and memory improvement. In addition, according to Saxenaa *et al.* (25), gugulipid (12.5, 25, and 50 mg/kg) caused a dose dependent improvement in scopolamine-induced deficits in the passive avoidance test, with simultaneous inhibition of AChE activity in the brain of streptozotocin-treated mice, indicating that chronic treatment of gugulipid may also enhance cholinergic activity by inhibiting AChE so causing cognitive improvement.

In our ongoing screening of natural products to identify sources of nontoxic and potent AChE inhibitors, the ethanol extract of *C. indicum* Linne flowers was shown to be the most potent AChE inhibitor. Subsequently, in order to isolate any compound(s) responsible for the AChE inhibition activity, an activity-guided fractionation strategy was followed throughout the separation procedure (15). It was recently reported that three compounds (acaciin, acacetin-7-*O*-β-D-galactopyranoside, and luteolin) in the ethanol extract of *C. indicum* Linne flowers were identified to be responsible for the improvement in the efficacy of AChE inhibition (15).

In conclusion, the present study suggests that the water extract of *C. indicum* Linne flowers can improve or ameliorate spatial long-term and working memory dysfunction, in part, by by inhibiting AChE activity, so enhancing the cholinergic nervous system and this extract may have anti-amnestic activity *in vivo*.

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