Biomolecules & Therapeutics, **17(3)**, 249-255 (2009)
www.biomolther.org

ISSN: 1976-9148(print)/2005-4483(online)
DOI: 10.4062/biomolther.2009.17.3.249

Forsythiaside, a Constituent of the Fruits of *Forsythia suspense*, Ameliorates Scopolamine-Induced Memory Impairment in Mice

Sunho KIM[1,3], Dong Hyun KIM[1,3], Ji Joung CHOI[4], Jong Gu LEE[4], Choong Ho LEE[4], Se Jin PARK[1,3], Won Yong JUNG[1,3], Dong Hyun PARK[1,3], Kwang Ho KO[5], Seung Ho LEE[4], and Jong-Hoon RYU[1,2,3,*]

[1]Department of Life and Nanopharmaceutical Sciences , [2]Department of Oriental Pharmaceutical Science and, [3]Kyung Hee East-West Pharmaceutical Research Institute, College of Pharmacy, Kyung Hee University, Seoul 130-701, [4]College of Pharmacy, Yeungnam University, Gyeongsan 712-749, [5]Department of Pharmacology, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

(Received February 7, 2009; Revised May 8, 2009; Accepted May 11, 2009)

Abstract − Forsythiaside is a polyphenolic constituent of the fruits of *Forsythia suspense* Vahl which are widely used as anti-inflammatory herbal raw materials in traditional Chinese medicine. In the present study, the authors assessed the effects of forsythiaside on learning and memory impairments induced by scopolamine using a passive avoidance and the Morris water maze tests in mice. Drug-induced amnesia was induced by scopolamine treatment (1 mg/kg, i.p.). Forsythiaside (10 mg/kg, p.o) administration significantly prevented scopolamine-induced step-through latency reduction in the passive avoidance test and scopolamine-induced increased escape latency in the Morris water maze test ($p < 0.05$). Moreover, in an *ex-vivo* study, forsythiaside treatment (10 mg/kg, p.o) significantly reduced the increase of thiobarbituric acid reactive substance levels induced by scopolamine ($p < 0.05$). Taken together, the present study suggests that forsythiaside could be useful for the treatment of cognitive impairment, and that its beneficial effects are mediated, in part, by its antioxidative properties.

Keywords: Forsythiaside, Memory, Passive avoidance task, Morris water maze task, Antioxidant

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder and the most common form of senile dementia, and predominantly appears to affect the cholinergic neurotransmitter system (Kristensen, 1990; Perry *et al*., 1999). The impairment of learning and memory is the most characteristic manifestation of dementia and can be chemically induced in experimental animals by the administration of scopolamine, a muscarinic receptor antagonist (Misane *et al*., 2003). In addition, scopolamine treatment also causes oxidative stress, which suggests the scopolamine-induced learning and memory impairment model is also useful for investigating anti-amnesic agents with anti-oxidative properties (El-Sherbiny *et al*., 2003). Lipid peroxidation is thought to be a prominent and especially deleterious form of neuronal oxidative injury (Weinstock *et al*., 2004). Furthermore, the findings of several clinical researchers also suggest that oxidative stress is implicated in the pathophysiology of dementia and other age-related neurodegenerative disorders (Cruz-Aguado *et al*., 1998; Cruz *et al*., 2003). In addition, it has also been reported that antioxidants decrease the risk of memory deficits in AD (Bassett *et al*., 2003).

The fruits of *Forsythia suspense* Vahl. (Oleaceae) are widely used in traditional Chinese medicine to treat inflammation, pyrexia, or emesis (Zhu, 1998). Recently, it was reported that some compounds isolated from the fruits of *F. suspense* have antioxidant and antibacterial effects (Qu *et al*., 2008). However, no attempt has been previously made to determine whether *F. suspense* or its constituents have anti-amnesic activity. We isolated forsythiaside, a phenylethanoid glycoside, from the fruits of *F. suspense*, and considered that if forsythiaside ameliorates scopolamine-induced memory impairment, it might be useful for treating amnesia because forsythiaside also exhibits anti-oxidative activity (Qu *et al*., 2008). To test these possibilities, we undertook to determine whether forsythiaside ameliorates memory impairments and lipid peroxidation in-

*Corresponding author
Tel: +82-2-961-9230 Fax: +82-2-966-3885
E-mail: jhryu63@khu.ac.kr

duced by scopolamine in mice. Behavioral parameters were evaluated using the Morris water maze and the passive avoidance tasks in mice. The antioxidant activities of forsythiaside were measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and thiobarbituric acid reactive substance (TBARS) assay *in vitro* and *ex vivo*; the TBARS assay provides a reliable index of *ex vivo* lipid peroxidation (Ilic *et al*., 1999).

MATERIALS AND METHODS

Materials

The fruits of *F. suspense* were purchased from a folk medicine market "Yak-ryong-si" in Daegu (Korea), and authenticated by Prof. Je Hyun Lee (College of Oriental Medicine, Dongguk University, Gyeongju, Korea). A voucher specimen has been deposited at the College of Pharmacy, Yeungnam University (Korea). Forsythiaside (Fig. 1) was isolated from the fruits of *F. suspense* as follows. The dried fruits of *F. suspense* (10 kg) were extracted three times with MeOH at room temperature for 7 days. The MeOH solution obtained was concentrated under reduced pressure to yield a residue (750 g), which was partitioned with EtOAc and *n*-BuOH, respectively. The *n*-BuOH extract (142 g) was then loaded onto a silica gel column (98×9.0 cm) and eluted with a CH_2Cl_2/MeOH gradient (100:0, 98:2, 95:5, 92:8, 90:10, 85:15, 80:20, 75:25, 70:30, 60:40, 50:50, 20:80, 0:100) to give 15 fractions. Fraction 10 (6.5 g) was chromatographed on a silica gel column (40×4.0 cm) eluted with CH_2Cl_2/MeOH (from 100:0 to 50:50) to give 5 fractions. Of these, fraction 10-4 (3.8 g) was chromatographed on a silica gel column (35×4.0) and eluted with CH_2Cl_2/acetone using a step gradient (3-60%) to afford forsythiaside (2,200 mg, purity 98.5%). The structure of forsythiaside was confirmed by comparing with literature values (Nishibe *et al*., 1982). Tacrine (9-amino-1, 2, 3, 4-tetrahydroacridine hydrochloride), (−)-scopolamine hydrobromide, DPPH, DTNB (5, 5'-dithiobis [2-nitrobenzoic acid]), and ascorbic acid were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). All other materials were obtained from normal commercial sources and were of the highest grade available.

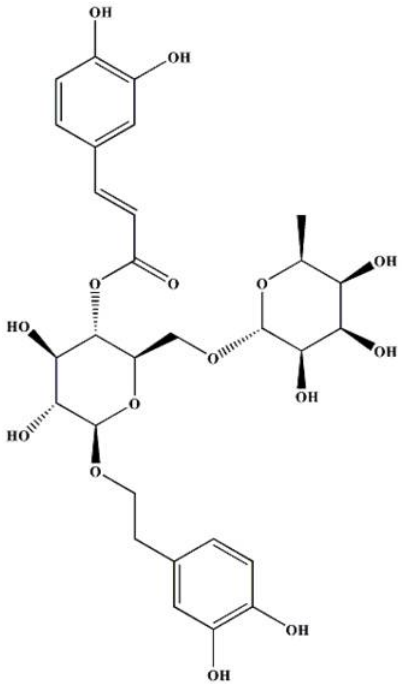

Fig. 1. Structure of forsythiaside.

Animals

Animal maintenance and treatment were carried out in accordance with Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines issued by Kyung Hee University, Korea. Male ICR mice (25-30 g) were purchased from the Orient Co., Ltd, a branch of Charles River Laboratories (Seoul). Animals were housed 4 or 5 per cage, allowed access to water and food ad libitum, and maintained under a constant temperature (23 ± 1°C) and humidity (60 ± 10%) under a 12-h light/ dark cycle (light on 07.30-19.30 h). We used a total 125 mice in all the experiments; different mice were used in each experiment. All efforts were made to minimize the number of animals as well as their suffering.

The passive avoidance task

Testing was carried out in identical illuminated and non-illuminated boxes (20×20×20 cm), separated by a guillotine door (5×5 cm) (Gemini Avoidance System, San Diego, CA). The illuminated compartment contained a 50 W bulb, and the floor of the non-illuminated compartment (20×20×20 cm) was composed of 2 mm stainless steel rods spaced 1 cm apart. For acquisition trials, mice were initially placed individually in the illuminated compartment and the door between the two compartments was opened 10 s later. When a mouse entered the dark compartment, the door automatically closed and an electrical foot shock (0.5 mA) of 3 s duration was delivered through the stainless steel rods. Twenty-four hours after the acquisition trial, mice were again individually placed in the illuminated compartment for the retention trial. The time taken for a mouse to enter the dark compartment after door opening was defined as latency for both acquisition and retention trials. Latencies to enter the dark compartment were re-

corded for up to 300 s. To investigate the anti-amnesic effects of forsythiaside, mice were treated with forsythiaside (2.5, 5, 10 or 20 mg/kg, p.o) or tacrine (10 mg/kg, p.o) as a positive control 1 h before acquisition trial, and scopolamine (1 mg/kg, i.p.) was administered 30 min after forsythiaside. Control group received 10% Tween 80 solution only.

The Morris water maze task

The Morris water maze is a circular pool with a featureless inner surface and a diameter and height of 90 and 45 cm, respectively. The pool was filled to a depth of 30 cm with water containing 500 ml of milk (20 ± 1°C). The tank was placed in a dimly lit, soundproof test room containing various visual cues. A white platform (6 cm in diameter, 1 cm below the surface of the water) was then placed in one of the pool quadrants. The first experimental day was dedicated to swimming training for 60 s in the absence of the platform. During the five subsequent days, the mice were given two training trials per day with the platform in place (Kim *et al*., 2006). The time interval between each training trials was 30 min. For two training trials, mice were placed in the water facing the pool wall in one of the pool quadrants. The entry point was changed for each trial. Forsythiaside (10 mg/kg, p.o), tacrine (10 mg/kg, p.o) as a positive control, or vehicle was administered 1 h before the first training trial, scopolamine (1 mg/kg, i.p.) was administered 30 min after forsythiaside treatment on each of the 5 trial days. Control group received 10% Tween 80 solution only. When a mouse located the platform, it was permitted to remain on it for 10 s. If a mouse did not locate on the platform within 60 s, it was placed on the platform for 10 s. The animal was returned to its home cage and dried under an infrared lamp after each trial. During each trial session, the time taken to find the hidden platform (latency) was recorded using a video camera-based Ethovision System (Nodulus, Wageningen, The Netherlands). One day after final training trial sessions, mice were subjected to a probe trial session, during which the platform was removed from the pool. Mice were then allowed to swim for 60 s to search for the platform. A record was kept of the swimming time in the pool quadrant where the platform had been previously placed.

Free radical scavenging assay

The free radical scavenging activity of forsythiaside was determined using DPPH dissolved in aqueous methanol. Various concentrations (5, 10, 50, or 100 μM) of forsythiaside solution were added to this DPPH solution (0.1 mM). After incubation at 25°C for 30 min, absorbance was measured at 515 nm. Percentages of DPPH remaining were calculated by comparing absorbance versus a control. The control solution consisted of 0.1 ml of aqueous methanol and 2.0 ml of DPPH radical solution. All tests were run in triplicate and averaged. Ascorbic acid was used as positive control.

Measurement of lipid peroxidation levels

Lipid peroxidation in brain tissue was determined by measuring levels of malondialdehyde (MDA) which reacts with thiobarbituric acid. Mice were administered forsythiaside (10 mg/kg p.o) and 30 min later scopolamine (1 mg/kg, i.p.) and sacrificed 60 min post-forsythiaside administration, and whole brains were removed. Isolated whole brain was homogenized in ice-chilled [20 mM Tris-HCl (pH 7.4)] buffer containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, 15 μg/ml leupeptin, 10 μg/ml bacitracin, 10 μg/ml pepstatin, 15 μg/ml trypsin inhibitor, 50 mM NaF, and 1 mM sodium orthovanadate. Samples of homogenates (100 μg/protein) were the mixed with trichloroacetic acid (1 ml of a 10% w/v solution). Thiobarbituric acid solution (1 ml of 0.67% (w/v)) was added and the mixture was heated at 100°C for 30 min, cooled, and centrifuged at 3,000 g for 10 min. TBARS levels were determined by measuring absorbance at 535 nm (OPTIZEN 2120UV, Mecasys Co. Ltd., Korea).

Statistical analysis

Values are expressed as means ± SEM. For the passive avoidance task, data were analyzed using a Kruskal-Wallis non-parametric test, and when results were significant, treatment groups were compared using Tukey's *post hoc* test. Data from the probe trial of the Morris water maze task and TBARS levels were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. In particular, group differences in escape latency of training trial session in the Morris water maze task were analyzed using two-way ANOVA with repeated measures. Statistical significance was accepted for p values of < 0.05.

RESULTS

Effect of forsythiaside on memory impairment induced by scopolamine in the passive avoidance test

We assessed whether forsythiaside ameliorates memory dysfunction induced by scopolamine using the passive avoidance task. When placed into the bright side of a step-through box, mice quickly entered the dark compartment. Mice were conditioned using a mild foot shock

after entering the dark compartment and hesitated to re-enter the dark compartment when tested 24 h later (Fig. 2). In the passive avoidance task, there was a significant group effect in terms of latency time [H (6)=36.158, $p<$ 0.001] (Fig. 2). Step-through latencies of scopolamine-treated mice were significantly shorter than those of vehicle-treated control mice ($p<0.05$, Fig. 2). Furthermore, when mice were treated with tacrine (a positive control) 30 min before scopolamine, step-through latencies were greater than for scopolamine-treated mice, and when treated with forsythiaside (10 mg/kg, p.o) step-through latencies were also greater than for scopolamine-treated mice ($p<0.05$). However, at other doses, forsythiaside did not exhibit a significant effect.

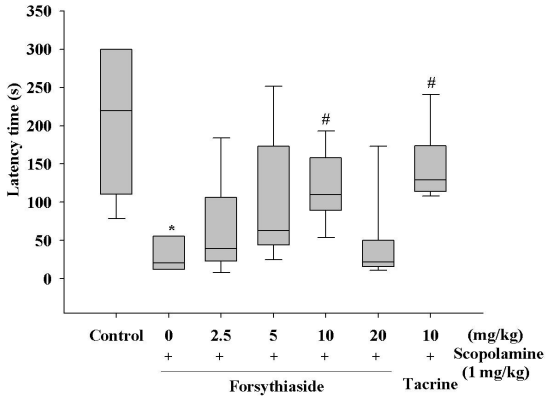

Fig. 2. Effect of forsythiaside on scopolamine-induced memory impairment by the passive avoidance task. Mice were administered forsythiaside (2.5, 5, 10 or 20 mg/kg, p.o) or tacrine (10 mg/kg, p.o, as a positive control) 1 h before the acquisition trial. Memory impairment was induced by scopolamine (1 mg/kg, i.p.), and acquisition trials were conducted at 30 min after scopolamine treatment. Data represent means ± SEM (n=10/group) ($^{*}p<0.05$ versus the vehicle control group; $^{\#}p<0.05$ versus the scopolamine-treated group).

Effect of forsythiaside on memory impairment induced by scopolamine in the water maze task

The effect of forsythiaside (10 mg/kg, p.o) on spatial learning was evaluated using the Morris water maze test. As shown in Fig. 3A, the scopolamine-treated group exhibited longer escape latencies throughout the training

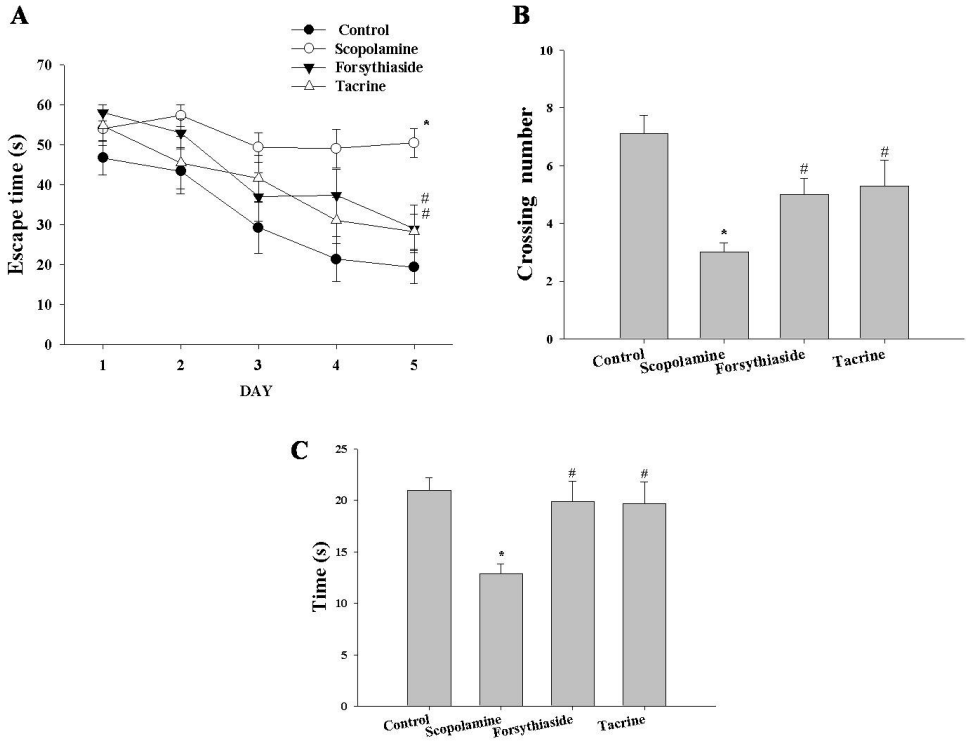

Fig. 3. Effects of forsythiaside on latency times (A) during the Morris water maze trial sessions, and on crossing numbers (B) and swimming times (C) during probe trial sessions on scopolamine-induced memory deficits in mice. At 60 min before trial sessions, forsythiaside (10 mg/kg, p.o), tacrine (10 mg/kg, p.o) or vehicle (same volume of 10% Tween 80) solutions were administered to mice. Memory impairment was induced by scopolamine treatment (1 mg/kg, i.p.). The training trial and probe trial sessions were performed as described in the Materials and Methods. Data represent means ± SEM (n=10/group) ($^{*}p<0.05$ versus vehicle controls; $^{\#}p<0.05$ versus scopolamine-treated mice).

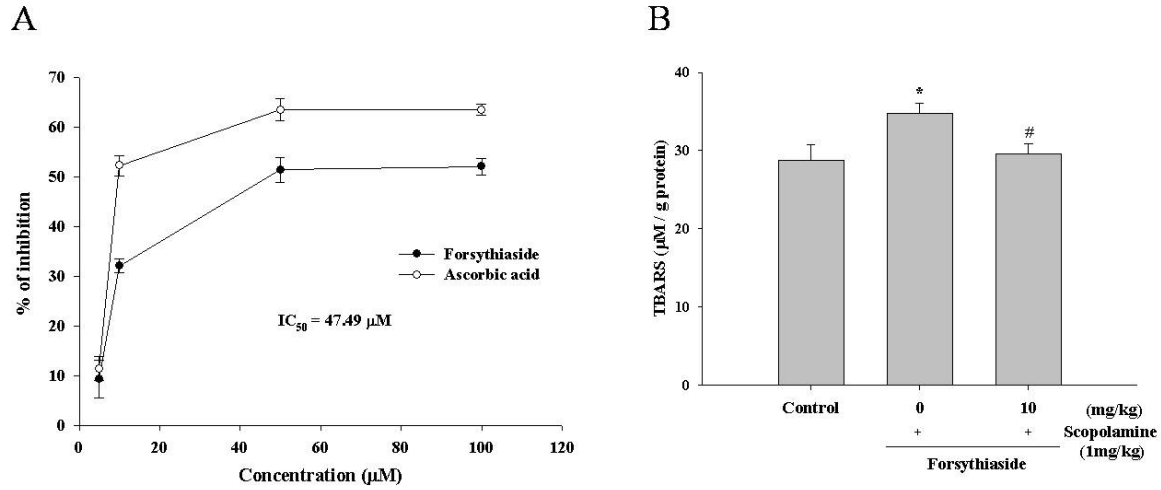

Fig. 4. (A) Effect of forsythiaside on DPPH free radical scavenging activity *in vitro*. The concentration of forsythiaside required to inhibit 50% (IC_{50}) was 47.49 μM *in vitro* (ascorbic acid; 9.384 μM). (B) Effect of forsythiaside on thiobarbituric acid reactive substance (TBARS) levels in the whole brains of mice treated with scopolamine. In this *ex-vivo* study, animals (n=5/group) were treated with forsythiaside (10 mg/kg, p.o) or the same volume of 10% Tween 80 solution at 1 h before sacrifice, and scopolamine (1 mg/kg, i.p.) at 30 min before sacrifice. TBARS levels were determined as described in Materials and Methods. Data represent means ± SEM (*$p < 0.05$ versus vehicle controls; $^{\#}p < 0.05$ versus scopolamine-treated group).

days than did the control group [F (1, 90)=54.918, $p < 0.001$]. Forsythiaside (10 mg/kg) significantly shortened the escape latencies prolonged by scopolamine treatment [F (1, 90)=10.268, p=0.002]. Moreover, tacrine also significantly reduced escape latencies [F (1, 90)=16.155, $p < 0.001$], compared with scopolamine-treated group. On the day following the final trial day, probe testing revealed a significant group effect in terms of crossing numbers [F (3, 36)=6.999, $p < 0.001$] (Fig. 3B) in the zone platform previously existed and swimming times [F (3, 36)=3.853, p=0.017] (Fig. 3C) in the target quadrant. During the probe trial session, swimming times and crossing numbers in the scopolamine-treated group were significantly lower than in the vehicle-treated control group ($p < 0.05$, Fig. 3B, C). Moreover, the shorter swimming times and crossing numbers induced by scopolamine were significantly reversed by by forsythiaside (10 mg/kg, p.o) or tacrine treatment (10 mg/kg, p.o.) ($p < 0.05$, Fig. 3B, C).

Anti-oxidant effects of forsythiaside on lipid peroxidation

Forsythiaside showed an antioxidant effect in the DPPH free radical scavenging test (IC_{50}=47.49 μM, Fig. 4A). In addition, scopolamine was found to increase lipid peroxide levels by TBARS assays in whole brain, and forsythiaside significantly reduced these lipid peroxide levels in scopolamine-treated mice [F (2, 12)=14.881, $p < 0.001$, Fig. 4B].

DISCUSSION

In the present study, it was found that forsythiaside pretreatment ameliorates memory impairments induced by scopolamine in the passive avoidance test and in the Morris water-maze test in mice. Furthermore, forsythiaside was also found to inhibit scopolamine-induced MDA generation, which suggests that the ameliorating effects of forsythiaside on memory dysfunction induced by scopolamine are associated with its anti-oxidative effects.

The ameliorative effects of forsythiaside on learning and memory deficit were investigated by the passive avoidance testing. The step through latency reduced by scopolamine treatment was recovered to approximately 58% of the vehicle-treated control group by forsythiaside administration (10 mg/kg). In addition, step through latencies induced by tacrine (10 mg/kg; the positive control) plus scopolamine were 70% of those of vehicle-treated controls, which concurs with previously published data (Bejar *et al*., 1999). However, during acquisition trial, no differences in latencies were observed between mice in any treatment or control group.

The Morris water maze task was used to assess hippocampal-dependent spatial learning ability (Morris, 1984; Barnes *et al*., 1996). Furthermore, it has been reported that escape latencies observed on a day-to-day basis reflect long-term memory (Morris, 1984). In the present study, long-term memory impairment was observed in sco-

polamine-treated mice, and forsythiaside pretreatment significantly shortened escape latencies as compared with scopolamine alone during training trial sessions. Furthermore, during probe trial sessions, forsythiaside improved swimming times and crossing numbers within the target zone to approximately 95% and 71% of the control level, respectively, and tacrine was found to have similar effects. Blokland *et al*. (2004) has shown that animals which spend more time and swim greater distances in the target pool quadrant during probe trials have better this spatial memories. Accordingly, our findings suggest that forsythiaside improves long-term and spatial memory in our scopolamine-induced amnesic mouse model. Thus, forsythiaside showed very similar memory ameliorating activities with the tacrine, as a positive control, on the passive avoidance and the Morris water maze tasks. However, although forsythiaside showed a memory ameliorating effect on scopolamine-induced mice, its IC_{50} on the acetylcholinesterase activity determined by the method of Ellman *et al*. (1961) using the mouse whole brain was >500 μM (data not shown). Accordingly, forsythiaside might have other effects, such as, a cholinergic agonist or modulatory effect on cholinergic transmission.

An elevated brain oxidative status in amnesic rats resembles the clinical situation in demented patients, which have been reported to have elevated oxidative stress and membrane lipid peroxidation levels (Palmer, 1999). In addition, overall peroxidation activity in the brains of AD patients has been reported to be significantly elevated (Marcus *et al*., 1998). More specifically, Balazs *et al*. (1994) showed that the entire AD brain is subjected to oxidative challenges. Peroxidation process and the overproduction of free radicals in brain could lead to consumption of detoxifying endogenous antioxidants, such as, glutathione. Our data also show that scopolamine treatment caused substantial lipid peroxidation in brain. Forsythiaside is considered to be an effective free radical scavenger and antioxidant (Qu *et al*., 2008). Furthermore, consistent with previous findings, our DPPH analysis results show that forsythiaside scavenges free radicals (IC_{50}=47.49 μM) and prevents scopolamine-induced lipid peroxidation in whole brain. Scopolamine is known to trigger the inductions of reactive oxygen species (ROS) and to cause free radical injuries (Annunziato *et al*., 2003; Fan *et al*., 2005), and El-Sherbiny *et al*. (2003) reported that memory impairment induced by scopolamine is associated with brain oxidative stress status. We also observed that scopolamine increased proinflammatory cytokines such as tumor necrosis factor-α both *in vivo* (hippocampal tissue) and *in vitro* (BV-2 cell) (Jung *et al*., 2009). Furthermore, several natural anti-oxidants have been found to attenuate memory impairment (El-Sherbiny *et al*., 2003; Singh *et al*., 2003; Dhingra *et al*., 2004; Vitor *et al*., 2004; Fan *et al*., 2005). Acteoside, a phenylethanoid glycosides distributed in *C. dichotoma* and other medicinal plants, has been reported to exhibit anti-oxidative, anti-inflammatory, anti-nephritic and anti-hepatotoxic activities (Hayashi *et al*., 1994; Xiong *et al*., 1998; He *et al*., 2000; Sahpaz *et al*., 2002), and to have anti-amnesic activity in a scopolamine-induced memory impairment model, as confirmed by the passive avoidance and the Morris water maze tasks (Lee *et al*., 2006). They suggested that anti-amnesic effects of acteoside result from its anti-oxidative effect, which implies that an anti-oxidative characteristic protects memory deficits. Taken together, forsythiaside may be a useful treatment for the memory impairment states caused by oxidative or neuroinflammatory insults, such as, ischemia and AD. Indeed, we observed that memory impairment induced by transient hypoperfusion by occlusion of common carotid artery in gerbil was ameliorated by the forsythiaside administration tested by the Y-maze task (data not shown). However, further researches will be needed to clarify these issues.

In conclusion, the present study shows that forsythiaside ameliorates the memory impairments caused by central cholinergic dysfunction, and suggests that these activities are mediated by its anti-oxidative effect. Furthermore, our findings suggest that forsythiaside has therapeutic potential for the treatment of AD.

ACKNOWLEDGMENTS

This research was supported by a grant funded by Korean Food and Drug Administration (2007).

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