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Biflorin Ameliorates Memory Impairments Induced by Cholinergic Blockade in Mice

Se Jin Jeon[1,2,†], Boseong Kim[1,2,†], Byeol Ryu[1,2], Eunji Kim[1,2], Sunhee Lee[1,2], Dae Sik Jang[1,2,*] and Jong Hoon Ryu[1,2,*]

[1]Department of Life and Nanopharmaceutical Science, [2]Kyung Hee East-West Pharmaceutical Research Institute, College of Pharmacy, Kyung Hee University, Seoul 02447, Republic of Korea

Abstract

To examine the effect of biflorin, a component of *Syzygium aromaticum*, on memory deficit, we introduced a scopolamine-induced cognitive deficit mouse model. A single administration of biflorin increased latency time in the passive avoidance task, ameliorated alternation behavior in the Y-maze, and increased exploration time in the Morris water maze task, indicating the improvement of cognitive behaviors against cholinergic dysfunction. The biflorin-induced reverse of latency in the scopolamine-treated group was attenuated by MK-801, an NMDA receptor antagonist. Biflorin also enhanced cognitive function in a naïve mouse model. To understand the mechanism of biflorin for memory amelioration, we performed Western blot. Biflorin increased the activation of protein kinase C-ζ and its downstream signaling molecules in the hippocampus. These results suggest that biflorin ameliorates drug-induced memory impairment by modulation of protein kinase C-ζ signaling in mice, implying that biflorin could function as a possible therapeutic agent for the treatment of cognitive problems.

Key Words: Biflorin, N-methyl D-aspartate receptor, Cognition, Protein kinase C-ζ

INTRODUCTION

Dementia is a neurodegenerative disease characterized by the loss of intellectual ability, and the dementia patient suffers severe interference with occupational and social performance. With increasing age, the prevalence of dementia increases, and the overall number is increasing worldwide. According to the causes and symptoms, dementia is classified into Alzheimer's disease (AD), vascular dementia or dementia with Lewy's bodies (Burns and Iliffe, 2009). In AD, the most prevalent dementia, one of the main pathological hallmarks is severe cholinergic dysfunction, such as decreased choline acetyltransferase (ChAT) and increased acetylcholinesterase (AChE) activities, in the basal forebrain cholinergic neurons (Schliebs and Arendt, 2011). Hence, numerous researchers have investigated ways to increase the cholinergic neurotransmitter system. As a result, AChE inhibitors (AChEI) were developed for AD therapy. Donepezil, an approved drug targeting AChE inhibition, prevents the decomposition of acetylcholine in the synapse and is prescribed clinically (Tune and Sunderland, 1998; Schneider, 2000). However, these AChEIs have some adverse effects such as diarrhea, insomnia or vomiting. Therefore, it would be necessary to explore new therapeutic agents that increase cognitive function with reduced side effects.

Meanwhile, the N-methyl-D-aspartate (NMDA) receptor system in the brain has been implicated in many fundamental functions, such as neuronal plasticity, neurotoxicity, learning, and memory. In particular, NMDA receptors have been demonstrated to be involved in spatial learning (Tsien *et al*., 1996; Morris *et al*., 2013; Yamada *et al*., 2015), working and reference memory (May-Simera and Levin, 2003; Levin *et al*., 2005), place preference (Swain *et al*., 2004), passive-avoidance learning (Danysz *et al*., 1988), and reversal learning (Dong *et al*., 2013). Pharmacological manipulations or lesion studies in experimental animals suggest that the NMDA receptor system may be important in cognitive function (Izquierdo and Medina, 1997; Scholtzova *et al*., 2008; van Zundert *et al*., 2010; Collingridge *et al*., 2013). These findings suggest a possibility that NMDA receptor would be a new therapeutic target for AD. If an NMDA receptor agonist or modulator

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***Corresponding Authors**
E-mail: jhryu63@khu.ac.kr (Ryu JH), dsjang@khu.ac.kr (Jang DS)
Tel: +82-2-961-9230 (Ryu JH), +82-2-961-0719 (Jang DS)
Fax: +82-2-966-3885 (Ryu JH), +82-2-966-3885 (Jang DS)
[†]The first two authors contributed equally to this work.

does not exert neurotoxicity, it would be a promising candidate for AD therapy. Recently, we observed that biflorin, a quinine compound, exhibits binding affinity to NMDA receptor.

Biflorin is an o-naphthoquinone, contained in various herbal materials including *Syzygium aromaticum*. According to the American Herbal Products Association (AHPA), *Syzygium aromaticum* is classified as one of Class I herb, which means safe and able to eat (McGuffin *et al*., 1997). Several reports revealed that biflorin has antioxidant and protective effects against cytotoxicity, genotoxicity, mutagenicity, and intracellular lipid peroxidation (Cai and Wu, 1996; Vasconcellos *et al*., 2005; Wisintainer *et al*., 2014). However, the effects of biflorin on cognitive functions remain unknown. Here, we investigated whether biflorin has ameliorating effects on scopolamine-induced memory impairment using the passive avoidance, the Y-maze or the Morris water maze tasks. In addition, we employed an antagonism study and Western blot analysis to investigate the changes in memory-related signaling molecules.

MATERIALS AND METHODS

Animals

ICR male mice (6 weeks old, 25-30 g) were purchased from the Orient Co (a branch of the Charles River Laboratories, Gyeonggi, Korea). Mice were housed 5 per cage, provided with food and water ad libitum, and kept under a 12 h light/dark cycle (light on 07:30-19:30 h) at a constant temperature (23 ± 1°C) and relative humidity (60 ± 10%). Animal treatment and maintenance were conducted in accordance with the Animal Care and Use Guidelines issued by Kyung Hee University (Seoul, Korea). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (approval number: KHP-2013-01-04).

Materials

Biflorin was provided by one of the authors (D.S. Jang) and suspended in 10% Tween 80 solution. Donepezil hydrochloride monohydrate, scopolamine hydrobromide, and dizocilpine (MK-801) were purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies against calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C-ζ (PKC-ζ), phosphorylated PKC-ζ at Thr 410, cAMP response element-binding protein (CREB) at Ser 133, extracellular signal-regulated kinase (ERK), and phosphorylated ERK at Thr202/Tyr204 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody against phosphorylated CaMKII at Thr 286 (pCaMKII) was purchased from Abcam (Cambridge, UK). All other materials were obtained from normal commercial sources and were of the highest grade available. All drugs were freshly made on the day of testing. Donepezil, a positive control, and scopolamine were dissolved in a 0.9% saline solution.

Passive avoidance task

For the assessment of the passive avoidance task, male ICR mice were trained (acquisition trial) 24 h prior to the retention trial. The acquisition trial was performed in a box consisting of two identical chambers (20×20×20 cm), one illuminated with a 50 W bulb and another non-illuminated chamber, separated by a guillotine door (5×5 cm), as described elsewhere (Lee *et al*., 2013). Mice were administered either biflorin (0, 0.1, 0.3, 1, or 3 mg/kg, p.o.), or donepezil (5 mg/kg, p.o.) 1 h before the acquisition trial. The control group received the vehicle solution (10% Tween 80 solution).

Mice were initially placed in the illuminated compartment during the acquisition trial. The door between the two compartments was opened 10 s later. After the mice entered the non-illuminated compartment, the door automatically closed, and a 3-s electrical foot shock (0.5 mA) was delivered through the stainless steel rods. Mice that did not enter the non-illuminated compartment within 60 s after the opening of the door were excluded from the retention trial. The retention trial was conducted 24 h after the acquisition trial by returning individual mice to the illuminated compartment. Scopolamine (1 mg/kg, i.p.) or MK-801 (0.1 mg/kg, i.p.) was administered 30 min after the treatment of biflorin. The time for the mouse to enter the dark compartment after the door opening was defined as latency in both trials. Latencies of up to 300 s were recorded.

In the memory enhancing study, mice were administered only biflorin (0, 0.3, 1, or 3 mg/kg, p.o.) or the same volume of 10% Tween 80 solution 1 h before the acquisition trial. When the mice entered the non-illuminated compartment, a 3-s electrical foot shock (0.25 mA) was delivered through the stainless steel rods to avoid ceiling effects. Latencies of up to 600 s were recorded. Other procedures were the same as described above.

For an antagonism study, biflorin (1 mg/kg) was administered 1 h before the acquisition trial, and a sub-effective dose of MK-801 (0.1 mg/kg, i.p.) (Kim *et al*., 2009), an NMDA receptor antagonist, was administered 30 min after the administration of biflorin. Scopolamine (1 mg/kg) was administered 5 min after the treatment with MK-801. The acquisition trial was conducted 25 min after the administration of scopolamine. The dose of MK-801 in this study did not impair passive avoidance task performance when administered alone. Other procedures were the same as described above.

Y-maze

The Y-maze is a horizontal maze with three arms (40 cm-long×3 cm-wide×12-cm-high) symmetrically disposed at 120° angles from each other. The maze was constructed of dark opaque polyvinyl plastic, as described elsewhere (Jung *et al*., 2014). Mice were initially placed within one arm, and the sequence (e.g., ABC, CAB) and number of arm entries were recorded manually for each mouse over an 8 min period. An actual alternation was defined as entry into all three arms on consecutive choices (i.e., ABC, CAB, or BCA but not BAB). One hour before the test, mice were orally administered either biflorin (0, 0.1, 0.3, 1, or 3 mg/kg) or donepezil (5 mg/kg). The control group received 10% Tween 80 solution rather than biflorin or donepezil. Scopolamine (1 mg/kg) was introduced to induce memory impairment 30 min before the test. Maze arms were thoroughly cleaned with water spray between each test to remove residual odors and residues. The alternation score (%) for each mouse was defined as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries) minus two multiplied by 100, as shown by the following equation: % Alternation=[(Number of alternations)/(Total arm entries−2)]×100.

Morris water maze

The Morris water maze consists of a circular pool (90 cm in diameter and 45 cm in height) with a featureless inner sur-

face. The pool was filled to a depth of 30 cm with water containing 500 mL of black pigment (24 ± 1°C). The tank was placed in a dimly lit, soundproof test room with visual cues. A black platform (6 cm in diameter and 29 cm high) 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 four subsequent days, the mice were given two trials per session per day with the platform in place. The time interval between each trial per session was 30 min. When a mouse located the platform, it was permitted to remain on it for 10 s. If the mouse did not locate the platform within 60 s, it was forced into the platform and placed on it for an additional 10 s. During each trial session, the time taken and distance moved to find the hidden platform (latency time) were recorded using a video camera-based EthoVision System (Noldus, Wageningen, Netherlands). One day after the last training trial session, the mice were subjected to a probe trial session in which the platform was removed from the pool, allowing the mice to swim for 60 s to search for it. A record was kept of the swimming time in the pool quadrant where the platform had previously been placed. Biflorin (1 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) was given 1 h before the first trial at each session on every consecutive day. Memory impairment in mice was induced by scopolamine administration (1 mg/kg, i.p.) 30 min before the first trial in each session. The control group received 10% Tween 80 solution only.

Open field test

The open field test was conducted in a clear black Plexiglas box (40×40×40 cm) equipped with a video-based EthoVision System (Noldus), as described previously (Jung *et al*., 2006). Mice were administered biflorin (0, 0.3, 1 or 3 mg/kg, p.o.) or 10% Tween 80 solution 1 h before the test. Mice were initially placed in the center of the apparatus, and the total distance moved was recorded for 25 min. The horizontal locomotor activity is expressed in terms of the total ambulatory distance.

Western blot analysis

Mice were sacrificed 1 h after biflorin administration for Western blotting. The vehicle group received 10% Tween 80 solution. The isolated hippocampal tissues were homogenized in ice-chilled Tris-HCl buffer (20 mM, pH 7.4) containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM sodium orthovanadate, and one protease inhibitor tablet per 50 ml buffer. The homogenates (15 μg total protein) were then subjected to SDS-PAGE (10% gel) under reducing conditions. The proteins were transferred to PVDF membranes in the transfer buffer [25 mM Tris-HCl (pH 7.4) containing 192 mM glycine and 20% v/v methanol] at 400 mA for 2 h at 4°C. Then, the membranes were blocked with 5% skim milk and incubated with primary antibodies: anti-CaMKII or anti-pCaMKII antibodies and anti-CREB or anti-pCREB antibodies (1:3000 dilution in 2% skim milk); anti-PKC-ζ or anti-pPKC-ζ antibodies, and anti-ERK or anti-pERK antibodies (1:5000 dilution in 2% skim milk) overnight at 4°C and washed with Tris-buffered saline/Tween 20 (TBST). The membranes were then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h and finally developed with enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA). The membrane was analyzed using the LAS-4000 mini bio-imaging program (Fujifilm Lifescience USA, Stamford, CT, USA).

Acetylcholinesterase inhibition assay

In our *ex-vivo* study, mice were administered biflorin (1 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) and sacrificed 1 h after each administration (Kim *et al*., 2007b). Donepezil (5 mg/kg) was used as a positive control. An analysis of AChE activity was conducted using acetylthiocholine iodide substrate in a colorimetric method (Ellman *et al*., 1961). Whole mouse brain except cerebellum was homogenized in a glass Teflon homogenizer (Eyela, Tokyo, Japan) including 3.5 ml (10 times of each tissue weight) volumes of homogenizer buffer (0.1 M phosphate buffer, pH 8.0), and then centrifuged at 3000 g for 10 min at 4°C. The supernatant was used as enzyme source for the assay. The supernatant solution was mixed with 144 μl of Buffer A (0.1 M phosphate buffer, pH 8.0), 22 μl of buffered Ellman's reagent (10 mM 5, 5′-dithiobis [2-nitrobenzoic acid] and 15 mM sodium bicarbonate) and 1.1 μl of acetylthiocholine iodide solution, and then mixed with 4.4 μl of neostigmine solution in 96 well after then incubated at room temperature for 10 min. Absorbance was measured at 412 nm using a UV spectrophotometer (OPTIZEN 2120UV, Mecasys Co., Ltd., Daejeon, Korea).

Radioligand binding assay

In the receptor-binding assay, we found that biflorin only showed binding affinity to NMDA receptors among 27 receptors (AB23234) that are known to be involved in learning and memory processes. NMDA receptor binding studies using [^{3}H]TCP, an antagonist radioligand for NMDA receptor (Cat no. 233000), were performed (Custom Screen by Eurofins Panlab, formerly Riscerca Biosciences, LCC, Seattle, WA, USA). Briefly, CHO cells stably transfected with a plasmid encoding the Wistar rat NMDA receptor were used to prepare membranes in modified 10 mM Tris-HCl, pH 7.4 using standard techniques. A 10 μg aliquot of membrane was incubated with 4 nM [^{3}H]TCP for 45 min at 25°C. Non-specific binding was estimated in the presence of 1.0 μM MK-801. Membranes were filtered and washed 3 times, and the filters were counted to determine specifically bound [^{3}H]TCP. Biflorin was screened at 100 μM.

Statistics

Data are expressed as the mean ± standard error of mean (SEM). Data from behavioral tests were analyzed by one-way analysis of variance (ANOVA) with Tukey's post hoc comparison or by two-way ANOVA with Bonferroni's post hoc test. Western blot data were analyzed by one-way ANOVA followed by Tukey's post-hoc analysis for multiple comparisons. Statistical significance was set at $p<0.05$. All statistical analyses were performed using the Prism 5.0 software (GraphPad, La Jolla, CA, USA).

RESULTS

The effects of biflorin on the cognitive dysfunction induced by scopolamine or cognitive enhancement in the passive avoidance task

The passive avoidance task was performed to evaluate the ameliorating effects of biflorin on the cognitive dysfunction. Significant step-through latency effects were observed between the groups in the retention trial [$F(6, 68)=12.53$, $p<0.05$] (Fig. 1A). The reduction in the step-through latency

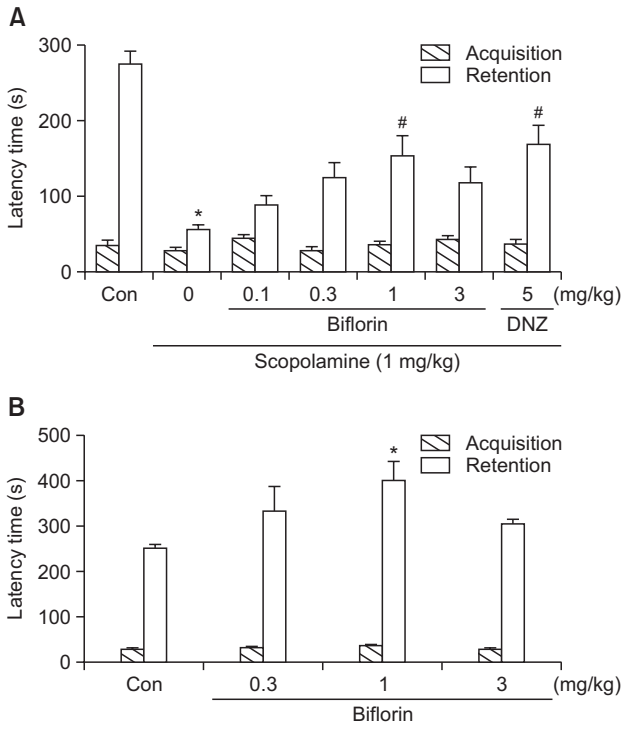

Fig. 1. Ameliorating effects of biflorin on the cognitive dysfunction induced by scopolamine or cognitive enhancement in the passive avoidance task. (A) The effects of biflorin on scopolamine-induced memory impairment. Biflorin (0, 0.1, 0.3, 1 or 3 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.), or the same volume of vehicle (10% Tween solution) was administered to mice 1 h before an acquisition trial. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before the acquisition trial. Twenty-four hours after the acquisition trial, a retention trial was conducted for 300 s. (B) The effects of biflorin on the cognitive performance of naïve mice. Biflorin (0, 0.3, 1 or 3 mg/kg, p.o.) or the same volume of vehicle (10% Tween solution) was administered to mice 1 h before the acquisition trial. In the acquisition trial, a mild electrical shock (0.25 mA) was given to the mice. A 10-min retention trial was performed 24 h after the acquisition trial. Data represent means ± SEM (n=8-10/group) (*p<0.05, versus the vehicle-treated controls; $^{\#}p$<0.05, versus the scopolamine-treated group). Con, control.

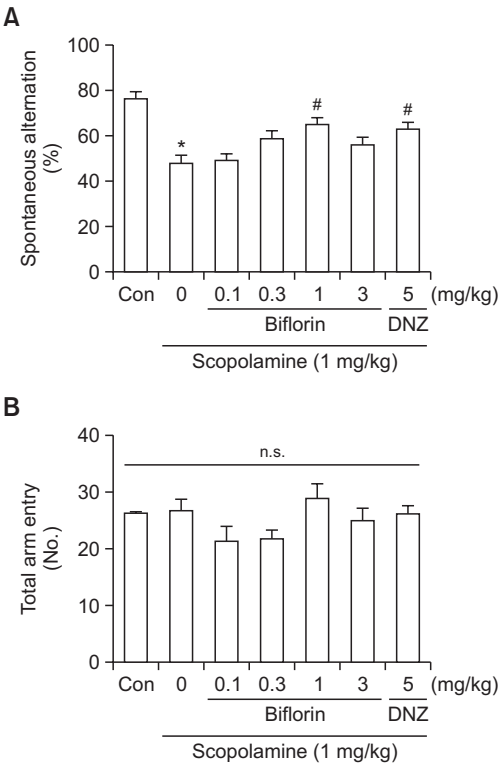

Fig. 2. Effects of biflorin on scopolamine-induced memory impairment in the Y-maze task. Biflorin (0, 0.1, 0.3, 1 or 3 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.), or the same volume of vehicle (10% Tween solution) was administered to mice 1 h before the Y-maze tests. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before the Y-maze tests. Spontaneous alternation behavior (A) and the number of arm entries (B) during an 8 min session were recorded. Data represent means ± SEM (n=8-9/group) (*p<0.05, versus the vehicle-treated controls; $^{\#}p$<0.05, versus the scopolamine-treated group). Con, control.

in the scopolamine-injected group was significantly reversed by the administration of biflorin (1 mg/kg, p<0.05; Fig. 1A), as observed in the donepezil-treated group (Fig. 1A). Additionally, the memory-enhancing effect of biflorin in the normal naïve mice was evaluated using the passive avoidance task (Fig. 1B). An increase in step-through latency was observed between the groups in the retention trial performed 24 h after the acquisition trial [F (3, 36)=3.132, p<0.05] (Fig. 1B). Biflorin (1 mg/kg) significantly increased the step-through latency compared with the vehicle-treated control (p<0.05). During the acquisition trial, there were no significant differences in the step-through latencies between the groups of unimpaired or scopolamine-treated mice.

The effect of biflorin on scopolamine-induced cognitive dysfunction in the Y-maze task

The Y-maze task was performed to examine the effect of biflorin on spontaneous alternation behavior. A significant group effect was observed in spontaneous alternation behavior upon the administration of biflorin [F (6, 69)=8.821, p<0.05]. The percentage of spontaneous alternations in the scopolamine-treated group was significantly lower than in the vehicle-treated control group (p<0.05, Fig. 2A), and the reduction in spontaneous alternation was significantly ameliorated by biflorin (1 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) (p<0.05, Fig. 2A). However, the mean numbers of arm entries were similar across all experimental groups (Fig. 2B), suggesting that general locomotor activity was not affected by biflorin.

The ameliorating effect of biflorin on scopolamine-induced cognitive impairment in the Morris water maze task

The Morris water maze task was performed to evaluate the effect of biflorin (1 mg/kg, p.o.) on spatial learning and memory. As shown in Fig. 3A, the scopolamine-treated group exhibited longer escape latencies than the vehicle-treated control group throughout the training days. However, the escape latencies of both the biflorin (1 mg/kg, p.o.) and donepezil-treated (5 mg/kg, p.o.) groups were significantly shortened compared with the scopolamine-treated group during training trial sessions 3 and 4 [trial session 3, F (3, 39)=8.348, p<0.05; trial session 4, F (3, 39)=11.65, p<0.05]. On the day after the final day of the training sessions (5th day), the effects on swimming time within

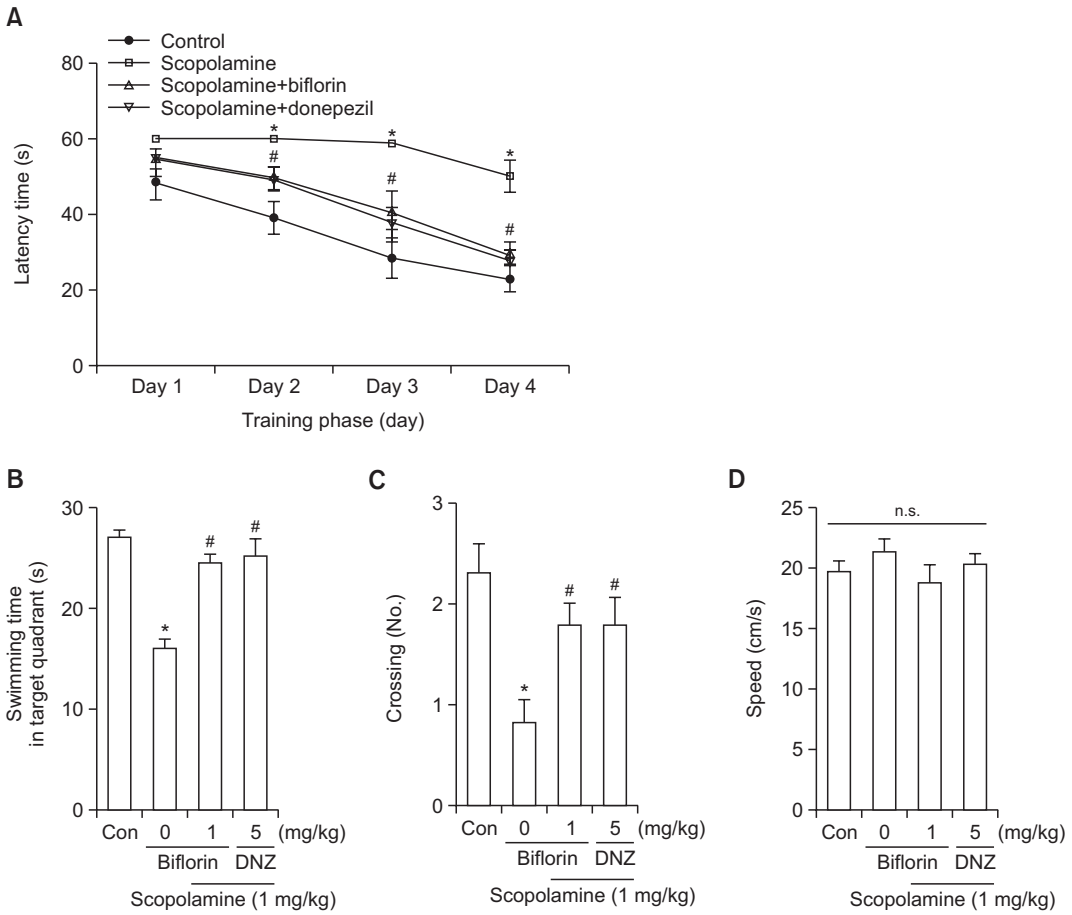

Fig. 3. Effects of biflorin on scopolamine-induced memory dysfunction in the Morris water maze task. The escape latency time throughout training trial sessions for 4 days (A), the swimming time in the target quadrant (B), the number of crossings (C) in the area where the hidden platform was located, and the swimming speed (D) during the probe trial section on day 5 in the Morris water maze task were measured. The training sessions were conducted for 4 days, and biflorin (1 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.), or the same volume of vehicle (10% Tween solution) was administered to the mice 60 min before the first training trial of each session. Memory impairment was induced by the administration of scopolamine (1 mg/kg, i.p.) 30 min before the first training trial. The training trial and probe trial sessions were performed over 60 s, as described in Section 2.5. Data represent means ± SEM (n=10/ group) (*p<0.05, versus the vehicle-treated controls; #p<0.05, versus the scopolamine-treated group). Con, control.

the target quadrant and the number of crossings of the target zone in the scopolamine-treated group were significantly reversed by biflorin (1 mg/kg, p.o.) or donepezil administration [F (3, 39)=5.834, p<0.05] (Fig. 3B, 3C). However, there were no significant differences in swimming speed across all groups (Fig. 3D).

The effect of biflorin on general locomotor activity in the open field test

Because the stimulatory effect of biflorin on exploratory behavior also affects cognitive behavior, the open field test was performed, and spontaneous locomotor activity was observed. There were no significant changes in total ambulatory distances across all groups (p>0.05, Fig. 4).

The ameliorating effects of biflorin against MK-801-induced cognitive impairment in the passive avoidance task

We conducted a receptor-binding assay to investigate the receptor signaling(s) involved in the cognitive function effects

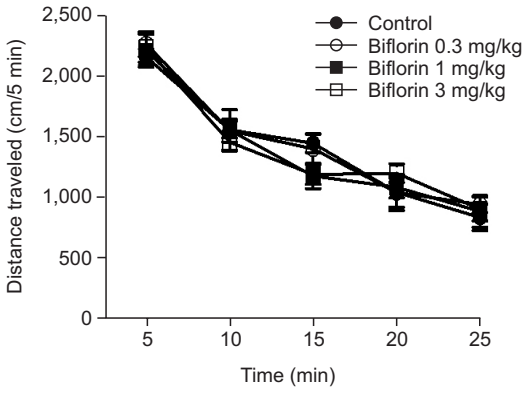

Fig. 4. Effects of biflorin on locomotor activity in the open field test. The exploratory behaviors of mice in the open field test were observed for 25 min. The mice were administered biflorin (0.3, 1 or 3 mg/kg, p.o.), donepezil (5 mg/kg) or the same volume of vehicle (10% Tween 80 solution) 1 h before the test. Data are expressed as means ± SEM (n=10/group).

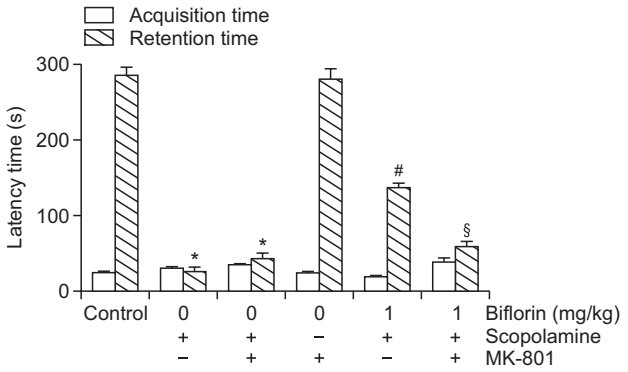

Fig. 5. The role of NMDA receptor signaling in biflorin-induced cognitive function. To test the antagonistic effect of a sub-effective dose of MK-801 on the effect of biflorin on scopolamine-induced memory impairment, biflorin (1 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution) was administered 60 min before the acquisition trial, and a sub-effective dose of MK-801 (0.1 mg/kg, i.p.) was given to the mice 30 min later. Scopolamine (1 mg/kg, i.p.) was administered 5 min after MK-801 injections. The retention trial was conducted 24 h after the acquisition trial. Data represent means ± SEM (n=8-10/group) (*p<0.05, versus the vehicle-treated controls; $^{\#}p$<0.05, versus scopolamine-treated groups; $^{\S}p$<0.05, versus biflorin plus scopolamine-treated group). Con, control.

of biflorin. Among the 27 targeted receptors associated with learning and memory, biflorin significantly inhibited the binding activity of [^{3}H]TCP, a radioligand for NMDA receptor, by approximately 60% at 100 μM. Based on the result that biflorin acts as an NMDA receptor ligand, we had a clue that the effect of biflorin on learning and memory was related to NMDA receptor.

To confirm whether the memory-ameliorating effect of biflorin is mediated via NMDA receptor signaling, a sub-effective dosage of MK-801 (0.1 mg/kg, i.p.) was co-administered with scopolamine in biflorin-treated mice (1 mg/kg), and the passive avoidance task was conducted. The reduced step-through latency induced by scopolamine was significantly reversed by biflorin administration (1 mg/kg) [F (5, 55)=178.2, p<0.05] (Fig. 5). The ameliorating effect of biflorin on scopolamine-induced memory impairment was inhibited by the administration of MK-801, and the step-through latency was similar to the results in the group treated with scopolamine alone. In the acquisition trial, there were no significant differences in step-through latency between the groups.

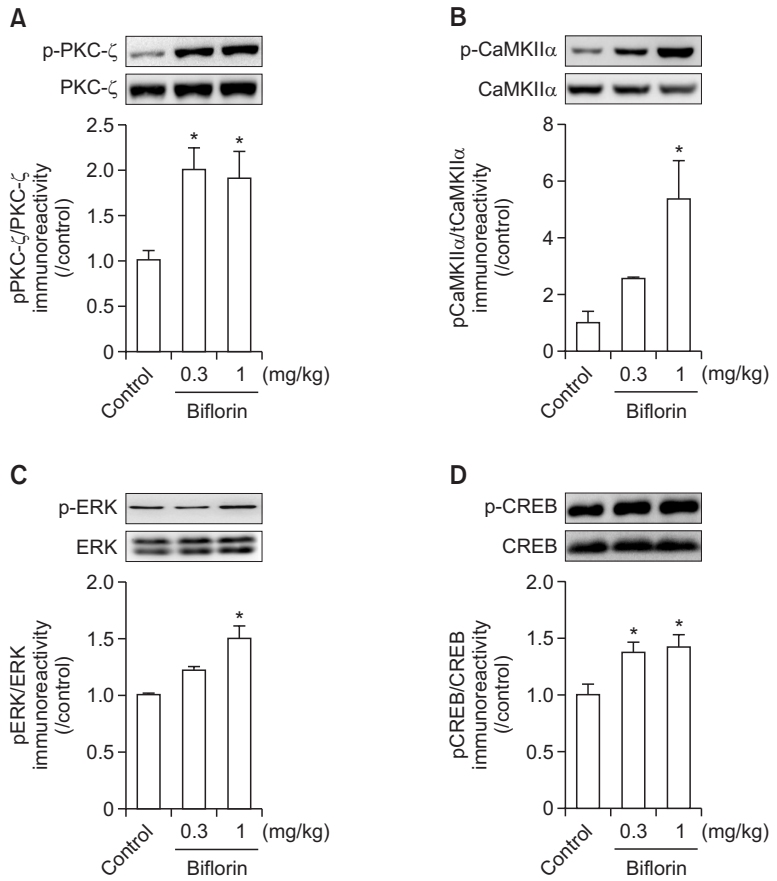

Fig. 6. Effects of biflorin on memory-related proteins in the hippocampus. The mice were administered biflorin (0.3 or 1 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution) and sacrificed 1 h after drug administration. The immunoreactivity and quantitative analysis of PKC-ζ, phosphorylated PKC (pPKC-ζ) (A), CaMKII, phosphorylated CaMKII (pCaMKII) (B), ERK, phosphorylated ERK (pERK) (C), CREB, and phosphorylated CREB (pCREB) (D) were measured in the hippocampal tissue. Data represent the means ± SEM (n=3-4/group) (*p<0.05, versus the vehicle-treated controls).

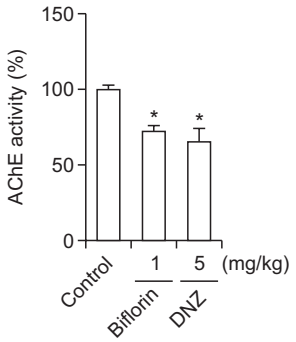

Fig. 7. Inhibitory effects of biflorin on acetylcholinesterase (AChE) activity in an *ex vivo* assay. In mice, biflorin (1 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) was administered 1 h before, and then, the mice were sacrificed. Whole brains were removed and homogenized using Buffer A. AChE activities were determined as described in Materials and Methods. Data represent means ± SEM (n=4/group) (*p<0.05, versus the vehicle-administered control group).

The effects of biflorin on the signaling molecules in the hippocampus

Based on the observation that the glutamate neurotransmitter pathway might be involved in the effects of biflorin on learning and memory, we investigated the molecular changes in the hippocampus regarding the NMDA receptor-activated signaling molecules, such as PKC, ERK, CREB or CaMKII. In the Western blot analysis, the administration of biflorin (0.3 or 1 mg/kg) significantly increased the phosphorylation levels of hippocampal PKC-ζ, ERK, CREB and CaMKII (n=5-6 per group, p<0.05, Fig. 6).

Effect of biflorin on AChE activity in *ex vivo* assay

Since biflorin reversed the cholinergic blockade-mediated cognitive dysfunctions in the several learning and memory tasks, we performed the AChE inhibitory activity assay under the *ex vivo* condition. As a result, there were significant group effects on AChE inhibition (p=0.0047). Biflorin (1 mg/kg) treatment showed significant AChE inhibitory activity as observed in the donepezil-treated group [F (2, 9)=10.30, p<0.05, Fig. 7]. Our results imply that the positive function of biflorin on the memory function is resulted from its AChE inhibitory activity, in part.

DISCUSSION

In this study, we found that biflorin exerted beneficial effects on cognitive function measured by several behavioral tasks, such as the passive avoidance, Y-maze, and the Morris water maze tasks. In addition, PKC-ζ, CaMKII, ERK and CREB, which are relevant to the activation of NMDA receptor and cognitive function, were phosphorylated upon the administration of biflorin.

During the detailed phytochemical investigations of *S. aromaticum* in relation to cognitive function, we isolated biflorin by activity-guided fractionation methods. Biflorin, as an O-naphthoquinone, is known to have anti-tumor activity and potent anti-oxidative activity (Cai and Wu, 1996; Vasconcellos *et al*., 2005). In structure, biflorin is similar to oroxylin A without the B ring (phenyl ring) and has a C-glycoside group at the 6 position instead of the methoxyl group in oroxylin A. Oroxylin A has been investigated with regard to its cognitive function and anti-psychotic activity (Kim *et al*., 2007a; Liu *et al*., 2013). Similarly, we reported that spinosin, a flavonoid with C-glucoside at the 6 position, exhibited cognitive enhancing activity, suggesting that a functional group at the 6 position of the A ring in flavonoids is essential for its cognitive enhancing activity. Therefore, it is reasonable to examine the pharmacological properties of biflorin in relation to cognitive function. In the passive avoidance task, which can evaluate the hippocampus-dependent long-term or contextual memory (Camp and Johnson, 2015), biflorin significantly prolonged step-through latency compared to the scopolamine-treated groups. In the Y-maze task, which evaluates the short-term or working memory (Sanderson *et al*., 2009), biflorin showed an ameliorating effect on the decreased spontaneous alternation induced by scopolamine in mice. In the Morris water maze task, evaluating hippocampus-dependent long-term and spatial memory (Dubue *et al*., 2015), decreased swimming time in the target quadrant induced by scopolamine was significantly recovered by biflorin administration. Thus, biflorin is a promising therapeutic candidate for cognitive dysfunction, such as AD.

As mentioned before, biflorin is very similar in structure to oroxylin A, which is a $GABA_A$ receptor antagonist (Liu *et al*., 2013), so we investigated whether biflorin exhibits $GABA_A$ receptor antagonistic properties. However, we did not obtain any significant information in behavioral study (data not shown). Therefore, we conducted receptor binding assay against several receptors including $GABA_A$ receptor, NMDA receptor, dopamine D1 receptor, or histamine H3 receptor, which are known to be related to learning and memory functions. Unexpectedly, biflorin exhibited binding affinity to NMDA receptor.

Both animal and human studies clearly indicate that the NMDA receptor system within the brain is involved in the processes of learning and memory formation. The administration of NMDA receptor antagonists has been shown to impair performance in tasks that seem to depend upon hippocampal or amygdaloid functions (Morris *et al*., 1986; Izquierdo and Medina, 1993; Jentsch and Roth, 1999; Liu *et al*., 2015). The noncompetitive, highly specific NMDA-receptor antagonist (MK-801) has been shown to induce the dose-dependent impairment of learning and memory (Benvenga and Spaulding, 1988; Butelman, 1990; de Lima *et al*., 2005; Liu *et al*., 2015). We also observed that MK-801 impaired cognitive function in the step-through passive avoidance task (Kim *et al*., 2009). Moreover, the biflorin-induced increase in latency in the scopolamine-treated group was reversed by MK-801. In addition, cognitive dysfunction induced by MK-801 was reversed by the administration of biflorin (data not shown). These results suggest that biflorin exerts its pharmacological activities through NMDA receptor signaling.

It is well known that the NMDA receptor-mediated calcium increase significantly enhances PKC and CaMKII activation, which stimulates synaptic LTP and learning and memory in mice (Moriguchi *et al*., 2011; Lisman *et al*., 2012). For example, PKC modulates the hippocampal long-term potentiation (LTP) (Sweatt *et al*., 1998), which is also related to learning and memory (Bliss and Collingridge, 1993; Lynch, 2004). Among the various subunits of PKC, PKC-ζ has recently been reported to affect the generation and maintenance of LTP in

the hippocampus-dependent memory process (Sacktor *et al*., 1993; Volk *et al*., 2013). In addition, CaMKII is highly expressed in the brain and is further enriched at excitatory synapses and their post-synaptic densities (Coultrap and Bayer, 2012). Thus, CaMKII is essential for mediating the induction and maintenance of the synaptic plasticity underlying learning and memory (Lisman *et al*., 2012). Furthermore, knockout mice of CaMKIIα, the major isoform in the brain, were the first transgenic animals with a behavioral phenotype in learning and memory (Silva *et al*., 1992). This evidence suggests that PKC-ζ and CaMKII activation play an important role in learning and memory. The administration of biflorin increased the phosphorylation level of PKC-ζ as well as of CaMKII in the hippocampus. Additionally, biflorin slightly facilitated LTP without affecting basal synaptic transmission (data not shown). In this work, we could not fully examine the exact mechanism by which biflorin activates PKC-ζ and CaMKII signaling; however, we assumed that enhancing the NMDA receptor agonistic property of biflorin would induce those signaling cascades.

Several studies have suggested that the activity of ERK1/2, a mitogen-activated protein kinase, is required for the establishment of synaptic activity and the development of several forms of memory. When a neuron is exposed to synaptic activation, the intracellular calcium level is elevated, and several kinases, including PKC, phosphoinositide-3 kinase (PI3K) and ERK1/2 are activated (Sutton and Chandler, 2002; Cohen-Matsliah *et al*., 2007; Zheng *et al*., 2009). In addition, CREB, which is located downstream of the signal transduction pathways of cAMP and calcium (Silva *et al*., 1998), is well established to act as a positive regulator during the cognitive process. The activation of CREB is mediated by several kinases such as CaMKII, ERK or cAMP-dependent PKA (Deisseroth and Tsien, 2002) and depends on its phosphorylation at serine 133 (Gonzalez and Montminy, 1989; Gonzalez *et al*., 1989). Activated CREB targets the transcription of memory-related genes, such as c-fos, activity-regulated cytoskeleton-associated protein or brain-derived neurotrophin factor, and is considered to enhance memory consolidation or LTP by regulating the expression of these genes (Sheng *et al*., 1991; Finkbeiner *et al*., 1997; Kawashima *et al*., 2009). Biflorin increased the phosphorylation levels of both ERK and CREB. As observed in the receptor binding study and signaling molecules, biflorin exhibited NMDA receptor agonist characteristics. However, biflorin did not exhibit cytotoxic activity in the primary hippocampal culture system, and the IC_{50} value was over 300 μM (data not shown), which means that biflorin is not a strong and selective NMDA receptor agonist but may be a modulator. Therefore, further study is in progress to determine the exact mechanism of action of biflorin on cognitive function. Moreover, biflorin showed an AChE inhibitory activity, which may affect cholinergic-blockade induced cognitive dysfunction in this study.

Through the activation of PKC, CaMKII and ERK-CREB signaling, biflorin significantly ameliorates the memory impairment induced by scopolamine or MK-801 treatment in several behavioral tasks. Due to its action as an NMDA receptor agonist, we expect biflorin to be effective in ameliorating cognitive deficits observed in AD in part by modulating NMDA receptor properties. Overall, these results strongly support the possibility of biflorin as a therapeutic agent in cognitive dysfunctions such as AD.

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