Ameliorating effect of *Citrus aurantium* extracts and nobiletin on β-amyloid (1-42)-induced memory impairment in mice

HAE JIN LEE[1], SUNG-KWON LEE[2], DONG-RYUNG LEE[2], BONG-KEUN CHOI[2], BAO LE[1] and SEUNG HWAN YANG[1]

[1]Department of Biotechnology, Chonnam National University, Yeosu, South Jeolla 59626; [2]Nutrapharm Tech, Jungwon-gu, Seongnam, Gyunggi 13201, Republic of Korea

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Abstract. The aim of the present study was to evaluate the neuroprotective effect of *Citrus aurantium* extract (CAE) and nobiletin against amyloid β 1-42 (Aβ 1-42)-induced spatial learning and memory impairment in mice. After injecting Aβ 1-42 (5 μl/2.5 min, intracerebroventricular injection), amnesic mice were orally administered CAE and nobiletin for 28 days. Memory, spatial and cognitive ability were measured using passive avoidance and a Morris water maze task. Acetylcholinesterase (AchE) activity was investigated in the hippocampus and cortex using commercial kits and the analysis of Bax, Bcl-2, and cleaved caspase-3 protein expression by western blot assays was used to confirm the anti-apoptotic mechanism of CAE and nobiletin. The present study confirmed impairments in learning and memory in the Aβ-induced neurodegenerative mice with increased AchE activity in the brain. However, the daily administration of CAE and nobiletin reduced the spatial learning deficits and increased the AchE activity in the cortex and hippocampus. Furthermore, CAE and nobiletin significantly downregulated the Bax and cleaved caspase-3 protein expression and upregulated the Bcl-2 and Bcl-2/Bax expression in the cortex and hippocampus of Aβ-treated mice. These results suggest that CAE and nobiletin exert a neuroprotective effect by regulating anti-apoptotic mechanisms, including reduced AchE activity in the cortex and hippocampus of the cognitive deficit mouse model.

Introduction

Dementia is a brain disorder in which learning and memory are compromised by various complex factors (1). Alzheimer's disease (AD), the most common form of dementia, is a typical age-related degenerative brain disease characterized by loss of neurons in the hippocampus and cortex (2-4). According to a previous study, approximately 34 million people suffer from AD, among them more than 5 million were diagnosed with AD patients (5). In the current aging society, neurodegenerative diseases such as AD have become critical medical issue worldwide (6). The pathogenesis of AD involves amyloid plaque accumulation, tau protein aggregation, and cholinergic dysfunction that induces neurotoxicity, accompanied by the impairment of cognition, behavior, and emotion (3,5,7).

Amyloid beta (Aβ) is generated from the cleavage of amyloid precursor protein (APP) by the combination of enzymes, β-secretase, and γ-secretase. Aβ 1-42 protein is a fragment of the full-length Aβ that can cause inflammation and synaptic toxicity by initiating different biochemical cascades (8,9). In addition to Aβ accumulation, hyperphosphorylation of the tau protein is a pathological feature observed in AD that triggers the neurodegenerative process (10). Furthermore, memory dysfunction is also triggered by cholinergic system damage that includes cholinergic neurons, neurotransmitters, and their receptors (5,11). Therefore, it is important to maintain the acetylcholine (Ach) level by inhibiting the acetylcholinesterase (AchE) activity. Various AchE inhibitors such as tacrine, donepezil, rivastigmine, and galantamine have been used to treat cognitive impairment by inhibiting AchE activity at the cholinergic synapses and thus, increasing the Ach content. However, the clinical efficacy remains unexplored; moreover, various side effects have been reported (12).

Citrus fruits have been traditionally used as medicine to increase immunity, alleviate indigestion, and reduce inflammation in Asia (13). The peels of *Citrus aurantium* contain a variety of active components, including naringin, hesperidin, narirutin, and polymethoxylated flavone (PMF) such as nobiletin and tangeretin. Previous studies, reported that nobiletin has various biological properties such as anti-cancer activity, anti-oxidant capacity, anti-inflammatory effects, and anti-obesity activity (13-15) as well as anti-dementia activity (16). Nobiletin is known to increase the levels of neprilysin protein, an enzyme that demonstrated Aβ degradation activity in SK-N-SH cells and activation of the protein kinase A (PKA)/extracellular signal-regulated kinase (ERK)/cAMP response element (CRE)-binding protein (CREB) intracellular

Correspondence to: Professor Seung Hwan Yang, Department of Biotechnology, Chonnam National University, Daehak-ro 50, Yeosu, South Jeolla 59626, Republic of Korea
E-mail: ymichigan@jnu.ac.kr

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signaling pathway in PC12D cells (17,18). However, this has not yet been tested on memory loss in an Aβ 1-42-induced normal mouse model.

In the present study, we administered 50 or 100 mg/kg *Citrus aurantium* extract (CAE) and 30 mg/kg nobiletin based on various references (3,13,19). The nobiletin concentration was determined by calculating the amount of nobiletin contained in the CAE in this experiment. We hypothesized whether CAE and nobiletin treatments have similar anti-dementia effects and underlying anti-apoptotic activity against β-amyloid-induced memory impairments in mice.

Materials and methods

Sample preparation. The CAE was supplied by KPLC Group (Paris, France) and prepared as described previously. The main component of CAE, nobiletin, was analyzed by high-performance liquid chromatography according to followed method: The solvent was a mixture of water (A) and Methanol (B) and delivered at 0.7 ml/min in a gradient flow as follows: 50-25% (20 min), 25-10% (24 min), and 10-50% (35 min) A. The nobiletin compound (purity; >98) used in the experiment was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals. Eight week-old 40 male C57BL/6J mice (weight, 20-24 g) were purchased from Orient-bio Co. (Seongnam, South Korea). The mice were individually housed in stainless steel cages under controlled conditions with a 12-h light-dark cycle at 23±3˚C and 55 ± 15% humidity and given free access to water and food. After a 7-day acclimation period, they were used in the experiment. All experiments were approved by an Ethics Committee (no. 2018-07-008) of ChemOn Inc. (Yongin, Korea) and performed in accordance with the national guidelines for the care and use of laboratory animals and the mice were maintained according to their guidelines. We monitored changes in body weight once a week, and observed changes in feed and water intake. In additions, appearance changes such as hair coat, activity, and posture were monitored once a day after β-amyloid injection over the course of the experiments. To improve animal well-being, we provided a sanitary environment to prevent disease and proper breeding and management and used appropriate painkillers and anesthetics to reduce pain. The humane endpoints were set by observing body weight change, hair coat, movement and posture of mice and applied in consultation with experimental committee. After the experiment, the mice were anaesthetized by the 100% carbon dioxide inhalation for 2-3 min and a fill rate of about 10-30% of the chamber volume per minute with carbon dioxide. When both sings as lack of respiration and faded eye color were observed, the mice were removed from the CO_2 chamber.

Aβ 1-42 injection and drug administration. Aβ 1-42 was dissolved in sterile 0.1 M phosphate-buffered saline (PBS) and incubated for 7 days at 37˚C to disrupt the aggregates. The mice were anesthetized with 1 ml/kg body weight Zoletil (40 mg/kg) and Rompun (5 mg/kg) (4:1, v/v) without any adverse events and the aggregates of Aβ 1-42 protein or vehicle (sterile 0.9% saline) was injected (5 μl/2.5 min, i.c.v.) using stereotaxic apparatus coordinates [anteroposterior (AP), -1.0 mm; mediolateral (ML), +1.0 mm; dorsoventral (DV), -2.5 mm]. After the injection of Aβ 1-42 peptide, the mice were divided into five groups: 1) control (vehicle), 2) Aβ 1-42 alone, 3) Aβ 1-42 + CAE (50 mg/kg), 4) Aβ 1-42 + CAE (100 mg/kg), and 5) Aβ 1-42 + nobiletin (30 mg/kg). The respective drugs were given orally (p.o.) for 4 weeks (Day 28). Based on the results of previous studies, the concentrations of CAE and nobiletin were set (3,13,19). The passive avoidance test was conducted in all groups for 3 days (Days 15-17) and Morris water maze (MWM) task for 7 days (Days 22-28). The mice were performed by cardiac puncture for exsanguination after carbon dioxide anesthesia at Day 28 and were confirmed the death by observing stop breathing and cardiac dysfunction through CO_2 anesthesia and exsanguination. Their cortex and hippocampus were stored at -80˚C for further analysis. The entire experimental schedule is shown in Fig. 1.

Step-through passive avoidance test. To determine learning and memory ability, the passive avoidance test was conducted in an acrylic shuttle box with two compartments and a guillotine door in the middle. The box consists of illuminated and non-illuminated compartments with an electric grid floor that allows for electronic shock stimuli. The test was performed on 3 consecutive days at 24-h intervals. On the first day (Day 15), the animals were allowed to explore the non-illuminated compartment for 2 min and then transferred back to the illuminated compartment. They were then allowed to access the illuminated and non-illuminated compartment freely for 60 sec. If the mice moved to the non-illuminated compartment, they were immediately removed and trained to adapt to the illuminated compartment. After the end of adaptation the time, on the second day, the mice moved between the two compartments for 120 sec, but when they entered the non-illuminated compartment, the guillotine door automatically closed and a scrambled shock was given for 2 sec. On the last day of the test, Day 17, the mice were placed in the illuminated compartment and the time taken for them to move to the non-illuminated compartment after the door opened was measured.

MWM task. On Day 22, the mice were tested using the MWM task. A circular water pool was divided into quadrants; the platform was randomly located within one quadrant. The mice were trained to find the platform for 60 sec. When the mice reached the platform, they were allowed to remain on the platform for 30 sec; if they could not find the platform within 60 sec, they were guided and placed on it for 30 sec to learn the extra maze cues. All animals performed two trials per day and the position of the platform in the circular pool was randomly changed. The task consisted of 2 days of training; 4 days of acquisition, during which the time to find the platform was recorded; and 1 day of probe trial. For the probe trial, the platform was removed and the animals were allowed to search it for 1 min. The number of crossings by the mice in the quadrant with the removed platform was measured on Day 28.

AchE activity. At the end of the experiment, the mice were sacrificed, and the cortex and hippocampus were dissected from the brain. Both tissues were rapidly homogenized and sonicated in 0.1 M phosphate buffer (pH 7.5), followed by centrifugation at

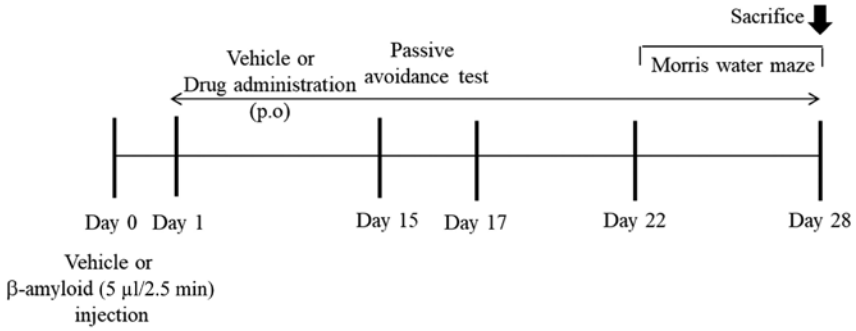

Figure 1. Experimental protocol of the present study.

14,000 rpm for 5 min. The supernatant was collected and stored at -80˚C until further analysis. AchE activity was determined using commercial assay kits (Abnova; cat. no. KA-1607, Taiwan) according to the manufacturer's instructions. The activity was calculated as the optical density (OD) at 412 nm and represented as OD values per milligram of protein.

Western blotting. The dissected cortex and hippocampus tissues were lysed in radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride and 1% protease inhibitor cocktail (Roche, Germany), followed by centrifugation at 12,000 rpm for 15 min at 4˚C. The supernatant was collected, and the protein concentration was calculated by bicinchoninic acid protein assay kit (Thermo, USA). Equal amount of proteins (20 μg/lane) was separated on a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Bio-Rad, USA). The membranes were blocked with commercial blocking buffer (Thermo, USA) for 1 h at room temperature and washed thrice with Tris-buffered saline containing 0.1% Tween 20 (TBS-T). After washing, the membrane was incubated at 4˚C overnight with the following appropriate antibodies: B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; 1:1,000; cat. no. 2772; Cell Signaling Technology, Inc., USA), B-cell lymphoma 2 (Bcl-2; 1:1,000; cat. no. 3498; Cell Signaling Technology, Inc., USA), Cleaved caspase-3 (1:1,000; cat. no. 9664; Cell Signaling Technology, Inc., USA), and β-actin (1:2,000; cat. no. 8457; Cell Signaling Technology, Inc., USA) The next day, the membranes were washed thrice with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were again washed thrice and enhanced using chemi-luminescence reagents. The protein bands on the membrane were detected by a chemi-luminometer (ATTO, Japan). Densitometry was performed using the Image-Pro Plus soft-ware (version 6.0; Media Cybernetics, Inc., USA).

Statistical analysis. Data are expressed as the mean ± standard error and were analyzed with SPSS Statistics 22.0. Different treatment groups were compared using one-way analysis of variance followed by multiple comparisons using Dunnett's post hoc test using Origin 7.0 software (Microcal, USA). $P<0.05$ was considered to indicate a statistically significant difference.

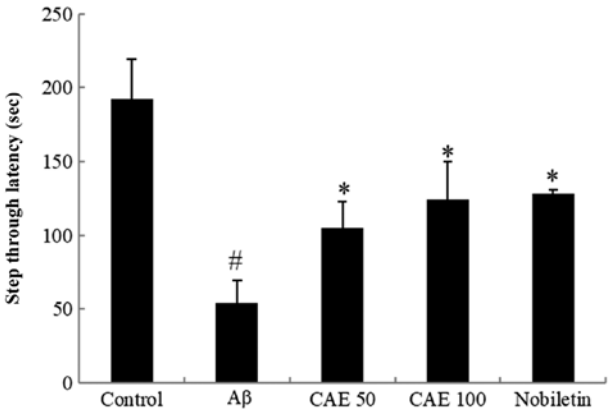

Figure 2. Effect of nobiletin and CAE on step-through latency in passive avoidance test in Aβ 1-42 induced memory impairment. The data are presented as mean ± standard error of the mean. *P<0.05 vs. Aβ 1-42 group; #P<0.05 vs. control group (n=8). Aβ, amyloid β; CAE, *Citrus aurantium* extract.

Results

Composition of CAE. The composition in CAE were investigated the chromatographic profiles of a standard on HPLC analysis. We confirmed the CAE contained 27% nobiletin, and 22% tangeretin respectively. The optimized CAE was used in all subsequent experiments.

Observation made regarding the human endpoints. The mice did not show any change in body weight during the experiment, but hair coat showed rough condition. Response to weak stimuli decreased and activity ability also decreased. Also, the mice was sitting on the floor with a curved posture was observed and through these symptoms, the humane endpoint was set. The animals were sacrificed by meeting the defined endpoint.

Effect of CAE and nobiletin on step-through passive avoidance task in Aβ 1-42-induced memory impairment. The effect of CAE and nobiletin on the Aβ 1-42-induced memory impairment was measured using a passive avoidance task. The step-through latency of the Aβ 1-42-only treated group was significantly shortened compared to the control group (Fig. 2). However, the reduced step latency with Aβ 1-42 was restored by the administration of CAE and nobiletin. CAE 50 and 100 mg/kg treatment increased the step-through

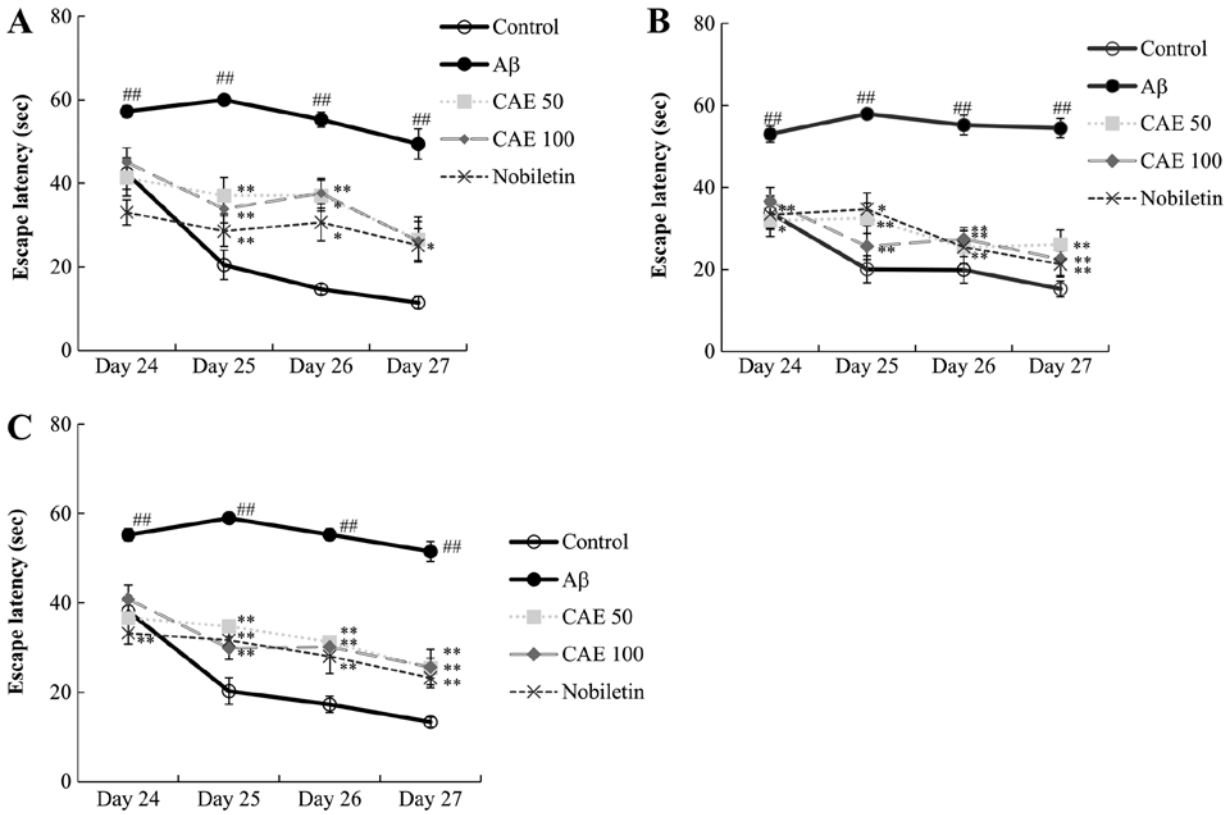

Figure 3. Effect of nobiletin and CAE on Aβ 1-42 induced memory impairment in the Morris water maze. The escape latency of (A) Trial 1 and (B) Trial 2, and the mean of (C) Trial 1 and Trial 2 during the training sessions for 4 days. The data are presented as mean ± standard error of the mean. *P<0.05 and **P<0.01 vs. Aβ 1-42 group; $^{\#\#}$P<0.01 vs. control group (n=8). Aβ, amyloid β; CAE, *Citrus aurantium* extract.

latency by 49.7% (105.06±17.75) and 57.3% (123.92±26.20), and nobiletin (30 mg/kg) recovered the induced memory impairment up to 58.8% (128.32±2.43) compared with Aβ 1-42-only treated mice.

Effect of CAE and nobiletin on the MWM task in Aβ 1-42-induced memory loss in mice. The efficacy of CAE and nobiletin in protection from the spatial memory impairment via Aβ 1-42 injection was further confirmed. The escape latency assessment was performed twice a day. During the test period, escape latency decreased slightly in the second trial compare to the first trial in all experimental groups (Fig. 3A and B). No difference was observed in the escape latency for 4 days in the amnesic mice, which were treated with Aβ 1-42. By contrast, the control group showed significantly decreased escape latency in two trials over 4 days (Fig. 3A). It is well-established that Aβ 1-42 induces memory loss and increases the escape latency. The CAE 50 and 100 mg/kg groups demonstrated a statistically significant (P<0.01) reduction in the escape latency from Day 25 to Day 27 compared to than the Aβ 1-42-only treated group (Fig. 3C). Similarly, the escape latency in mice administered nobiletin significantly decreased for 4 days compared to the Aβ 1-42-only injected group.

Effect of CAE and nobiletin on swim distance in the MWM task. The effect of CAE and nobiletin treatment on the swim distance to locate the platform in the MWM task is shown in Table I. The control group mice were able to swiftly locate the platform and reached the platform during the training session. However, the Aβ 1-42-treated group had difficulty learning to locate the platform. The swim distance was significantly increased compared to that of the control group. The swim distance of the CAE 50 and 100 mg/kg-treated groups was significantly reduced compared with those in the Aβ 1-42 group during the training session. The mice in the nobiletin-treated group were also able to find the platform easily with a short swim distance, especially on Day 24.

Effect of CAE and nobiletin on probe trial in the MWM task. To evaluate the spatial memory of the mice, the number of crossings to the platform was measured in the probe trial on Day 28. As shown in Fig. 4, a significantly increased number of crossings were observed in the control group than the mice treated with Aβ 1-42, which implies that the Aβ 1-42 group with memory impairment showed less learning than the control group. However, the administration of CAE 50 and 100 mg/kg enhanced the number of crossings by 5-fold and 5.33-fold and nobiletin treatment increased up to 5.17-fold compared with the Aβ 1-42-treated group. These results show that these drugs enhance spatial cognition, learning, and memory functions against Aβ 1-42-induced memory impairment.

Effect of CAE and nobiletin on AchE inhibitory activity in the cortex and hippocampus. To investigate the neuroprotective effect of CAE and nobiletin on brain tissue, AchE activity was measured in the cortex and hippocampus (Table II).

Table I. Effect of nobiletin and CAE on the distance swum by Aβ 1-42 treated mice to find the platform in the water maze task.

	Distance (mm)			
Treatment	Day 24	Day 25	Day 26	Day 27
Control	1,033.69±167.08	539.00±179.58	454.69±114.75	339.88±67.56
Aβ	1,297.44±152.80	1,570.13±83.02[c]	1,449.63±109.44[c]	1,232.38±116.74[c]
CAE 50	910.69±199.40	935.13±158.16[b]	869.88±230.11[a]	637.06±136.15[b]
CAE 100	1,056.81±172.04	663.75±105.84[b]	791.06±87.19[b]	610.38±142.27[b]
Nobiletin	923.19±131.32	847.94±121.70[b]	752.50±210.10[a]	512.81±187.10[b]

The data are presented as the mean ± standard error of the mean. [a]P<0.05 and [b]P<0.01 vs. Aβ 1-42 group; [c]P<0.05 vs. control group (n=8), Bonferroni corrected. Aβ, amyloid β; CAE, *Citrus aurantium* extract.

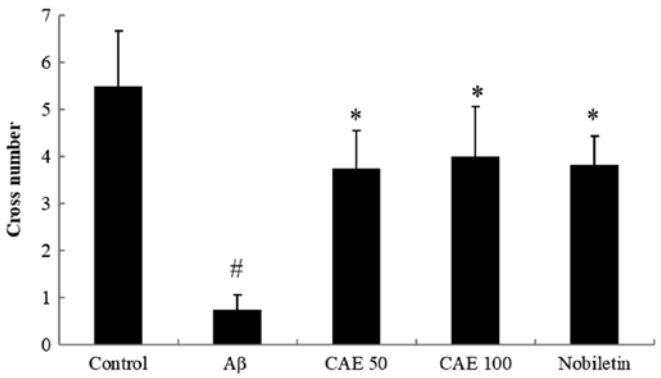

Figure 4. Effect of nobiletin and CAE on the number of crossing in a probe trail on Day 28. The data are presented as mean ± standard error of the mean. *P<0.05 vs. Aβ 1-42 group; #P<0.05 vs. control group (n=8). Aβ, amyloid β; CAE, *Citrus aurantium* extract.

Table II. Effect of nobiletin and CAE on AchE activity in the cortex and hippocampus of mice.

	U/mg protein	
Treatment	Cortex	Hippocampus
Control	38.2902±3.8548	24.7195±4.0319
Aβ	90.2940±10.2591[d]	45.6556±2.3807[c]
CAE 50	40.7787±3.9846[a]	41.5168±2.5505
CAE 100	38.0118±1.5175[a]	29.8937±2.7889[a]
Nobiletin	39.2531±2.1394[b]	12.7880±0.4159[b]

The data are presented as the mean ± standard error of the mean. [a]P<0.05 and [b]P<0.01 vs. Aβ 1-42 group; [c]P<0.05 and [d]P<0.01 vs. control group (n=8), Bonferroni corrected. Aβ, amyloid β; CAE, *Citrus aurantium* extract.

The AchE activity in the Aβ 1-42-treatment group was significantly increased compared to that in control group. CAE 50 and 100 mg/kg administration reduced the AchE activity in a dose-dependent manner by 56.6 and 58.8% in the cortex and 8.8 and 35.6% in the hippocampus, respectively compared to the Aβ 1-42-treated group. Similarly, the AchE activity in the nobiletin treatment group also decreased significantly by 56.5% in the cortex and 72.2% in the hippocampus.

Effect of CAE and nobiletin on expression levels of Bax, Bcl-2 and cleaved caspase-3 proteins in the cortex and hippocampus. The effect of CAE and nobiletin on the Bcl-2 family and caspase pathway was investigated in the cortex and hippocampus. As shown in Fig. 5, Bax and cleaved caspase-3 protein levels in the Aβ 1-42-treated group were decreased in the cortex and hippocampus compared to those in the control group. In contrast, Bcl-2 protein expression was higher in the control group than in the Aβ 1-42-only treated group in the cortex and hippocampus. In the hippocampus, the Bax protein levels in the CAE 100 mg/kg and nobiletin administration group was significantly reduced by 15 and 10%, while Bcl-2 expression was up-regulated in the CAE 100 mg/kg and nobiletin-treated group up to 1.4- and 2-fold compared to the Aβ 1-42-treated mice. In addition, the ratio of Bcl-2/Bax protein expression was significantly higher than in the Aβ 1-42-only treated group. The CAE 50 and CAE 100 mg/kg and nobiletin-treated groups showed down-regulation of the cleaved caspase-3 protein expression by 21, 22, and 20% respectively (Fig. 5A). A similar protein expression pattern was observed in the cortex. The Bax and cleaved caspase-3 protein level decreased with CAE 50 mg/kg, CAE 100 mg/kg, and nobiletin treatment, while the Bcl-2 and Bcl-2/Bax protein expression was upregulated compared to the levels in the mice treated with Aβ 1-42 only (Fig. 5B).

Discussion

The present study is the first report to evaluate the neuroprotective effects in Aβ 1-42-induced memory impairment animal model and not the transgenic or senescence accelerated mouse model. Our results showed that the Aβ 1-42-injection resulted in severe performance deficits in the passive avoidance and Morris water task as well as neurodegeneration in the mice brain that was evident from increased AchE activity in the hippocampus and cortex. In this study, we treated the amnesic mice with CAE and nobiletin and confirmed the anti-amnesic effect by regulating of apoptotic signaling.

Aβ plays a major role in the development of AD, particularly the neurotoxic Aβ 1-42 (10). The direct injection of Aβ 1-42 in

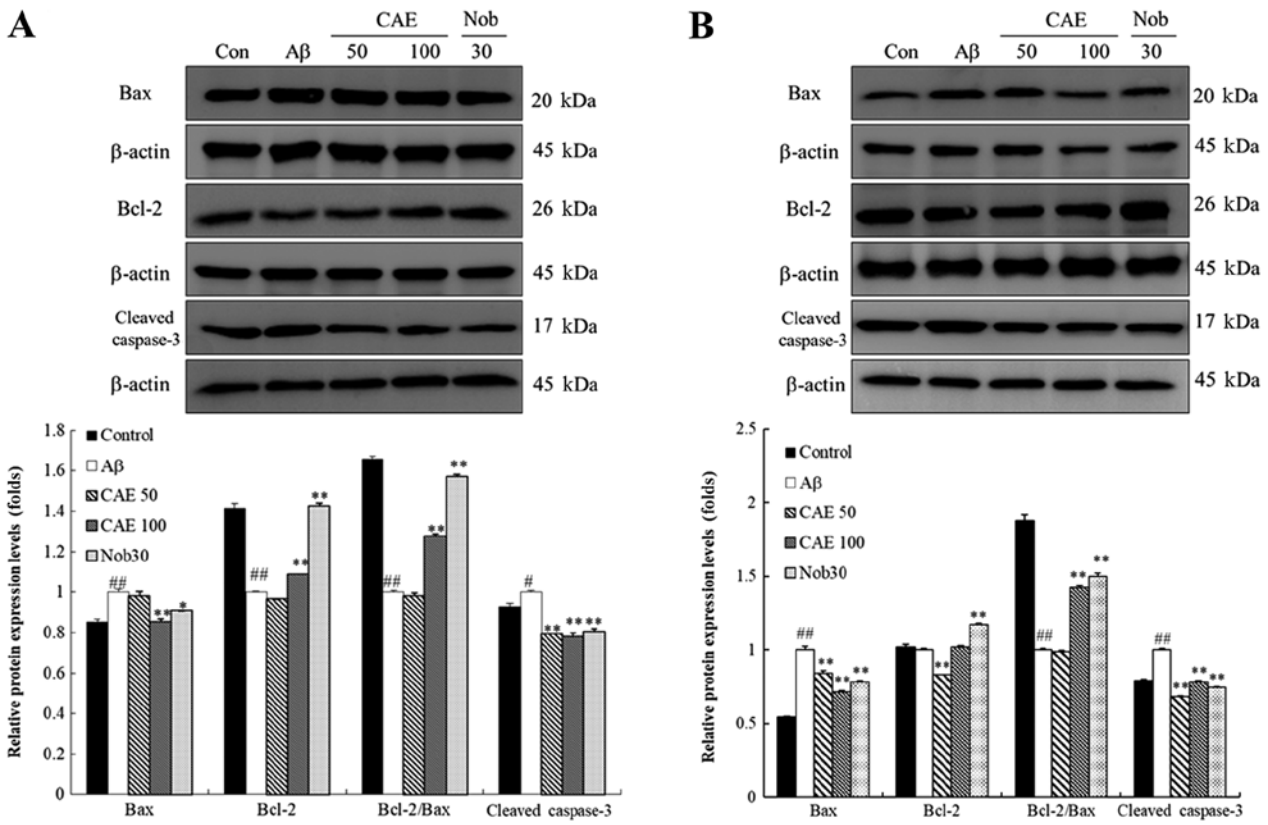

Figure 5. Effect of nobiletin and CAE on protein expression in (A) hippocampal and (B) cortex tissues. The expression was detected by western blot analysis. Bax, Bcl-2 and cleaved caspase-3 protein levels were normalized by separate control β-actin, respectively. *P<0.05 and **P<0.01 vs. Aβ 1-42 group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. control group (n=8). Aβ, amyloid β; CAE, *Citrus aurantium* extract; Nob, Nobiletin.

the rodent brain has been used to cause apparent memory deficits, and Aβ-exposed rats have shown hippocampus-dependent spatial learning dysfunction in long and short-term tasks (4). Also, the brains of AD patients demonstrated a high Aβ level compared with normal aged brain samples (20). The deposition of Aβ in the cortex and hippocampus, which are responsible for learning and memory performance, resulted in neuronal apoptosis (21,22).

To examine the protective effect of CAE and nobiletin, we performed the passive avoidance and MWM tasks to investigate learning and memory function. The passive avoidance task is a method that is used to measure the escape time from the space that induces pain and fear by electronic shock in rodents (23). It is commonly used to confirm the memory function, and we found in this study that CAE and nobiletin administration significantly increased the step-through latency to similar levels, a phenomenon that was reduced by the Aβ 1-42 injection. The MWM is an assessment method to evaluate hippocampal-dependent learning abilities and cognitive deficits in rodents. The animals were trained to learn spatial working information at the learning stage and assisted to build future memory (24). These results are consistent with those of previous studies that show Aβ-induced memory deficits in an MWM task than those in the saline group (25). As a result of two trials for 4 days on the MWM task, CAE treatment reduced escape latency in the second trial compared to the first trial, and escape time decreased over training days. The nobiletin administration group showed similar escape latency for 4 days in the first trial, and the escape latency decreased rapidly on Day 26 in the second trial. Although the pattern of escape latency of the CAE and nobiletin group was slightly different, the mean escape latency was decreased to a similar pattern. This means that CAE and nobiletin administration showed significant decreases in escape latency, improvement in cognitive performance, and amelioration of the memory deficits.

Furthermore, to investigate the neuroprotective effect of CAE and nobiletin, we examined the changes in the Ach system in the hippocampus and cortex. Ach is an essential enzyme that maintains the normal function of the nervous system and is hydrolyzed by AchE. In addition, Aβ deposition is increased in the presence of AchE, and AchE activity in AD is related to Aβ deposition (23). Therefore, it is important to reduce the level of AchE, which is used as a marker for the cholinergic nervous system. Here we found that AchE activity in the cortex was similar to that of CAE and nobiletin. However, nobiletin administration showed significantly lower AchE activity than CAE administration in the hippocampus. CAE and nobiletin administration benefits on the cholinergic neurotransmission by decreasing AchE activities in the cortex and hippocampus.

AchE can also be used as a marker of apoptosis. AchE expression or activity is increased when the cells undergo apoptosis, and enhanced AchE expression levels are detected in the brain of focal cerebral ischemic rats (26,27). AchE is usually present in the cytoplasm and moves to the nucleus before nuclear morphological changes occur. It then accelerates

chromatin condensation and fragmentation by modulating nuclear components Therefore, AchE can be detected on the fragmented nuclei of apoptotic cells, and increased AchE activity implies the occurrence of cell death (28).

Apoptosis is triggered via two major pathways: The mitochondrial (intrinsic) pathway and the death receptor-mediated (extrinsic) pathway. In this study, we focused on the mitochondrial pathway, which is regulated by Bcl-2 family and caspases (29). Bcl-2 is a known anti-apoptotic protein, while Bax is a pro-apoptotic protein that promotes apoptosis. These two proteins are the major factors responsible for cell death regulation. The Bcl-2 and Bax ratio determines whether a cell undergoes or escapes apoptosis (23,30). When the Bcl-2/Bax ratio is lower, the caspase pathway triggers apoptosis and results in the release of apoptosis-promoting factors such as cleaved caspase-3. Also, in the previous study, the decreased Bax/Bcl-2 ratio was seen in the AchE deficiency-mice model and inhibited the activation of cleaved caspase-3 when AchE was deficient and inhibited (31). In our study, the Aβ 1-42 injection group had increased Bax and cleaved caspase-3 protein expressions compared with the control group, while Bcl-2 protein levels were increased in the control group and reduced in the Aβ 1-42-treated group. CAE treatment significantly decreased the Bax and cleaved caspase-3 protein expression levels and simultaneously increased Bcl-2 protein expression in the hippocampus and cortex. Likewise, the treatments also increased the expression ratio of Bcl-2 to Bax in the cortex and hippocampus. The nobiletin treatment group displayed reduced expression levels of Bax and cleaved caspase-3 protein in the hippocampus to a level similar to that of the CAE 100 mg/kg group. On the other hand, Bcl-2 protein expression in the nobiletin administration group was increased to a level similar to that of the control group. Bax and cleaved caspase-3 protein expressions in the cortex were significantly inhibited by nobiletin treatment, while the Bcl-2 protein level was significantly enhanced compared the control group. Similar to this result, the Bcl-2/Bax ratio also increased significantly in the hippocampus and cortex.

In conclusion, our results indicate that the administration of CAE and nobiletin had a similar neuroprotective effect against Aβ-induced cognitive impairment through reduction of AchE activity and anti-apoptotic activity and regulating the Bcl-2 family and caspase pathway in the cortex and hippocampus. Our results provide evidence of the dietary intake of CAE or nobiletin as a valuable functional food since it has the ability to reduce cognitive impairment and memory dysfunction. However, to confirm the neuroprotective effects of CAE and nobiletin, we have to confirm the morphological change of brain tissues in further study. In addition, the effects of CAE and nobiletin on Aβ accumulation in cortex and hippocampus should be studied.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request with the permission of Nutrapharm Tech who granted the use of this data in the present study.

Authors' contributions

HJL carried out the experiments and wrote the original manuscript. SKL analyzed the experimental data. DRL and BL performed the data processing and quality control assessment. BKC and SHY designed the study, and proofread and finalized the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by an Ethics Committee (no. 2018-07-008) of ChemOn Inc. (Yongin, Korea) and performed in accordance with the national guidelines for the care and use of laboratory animals and the mice were maintained according to their guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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