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Effect of Antioxidant Flavanone, Naringenin, from *Citrus junos* on Neuroprotection

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Amyloid β protein (Aβ)-induced free radical-mediated neurotoxicity is known as a leading hypothesis for a cause of Alzheimer's disease. Aβ increased free radical production and lipid peroxidation in PC12 nerve cells, resulting in apoptosis and cell death. The protective effect of naringenin, a major flavanone constituent isolated from *Citrus junos*, against Aβ-induced neurotoxicity was investigated using PC12 cells. Pretreatment with isolated naringenin and vitamin C prevented the generation of the Aβ-induced reactive oxygen species. Naringenin resulted in the decrease of Aβ toxicity in a manner of concentration dependence, which was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. However, treatment with these antioxidants inhibited the Aβ-induced neurotoxic effect. The antiamnestic activity of naringenin in vivo was also evaluated using ICR mice with amnesia induced by scopolamine (1 mg/kg body weight). Naringenin, when administered to ICR mice at 4.5 mg/kg body weight, significantly ameliorated scopolamine-induced amnesia as measured in the passive avoidance test. Therefore, these results indicate that micromolecular Aβ-induced in vitro oxidative cell stress is reduced by naringenin and naringenin may be a useful chemopreventive agent against a neurodegenerative disease such as Alzheimer's disease.

KEYWORDS: Naringenin; oxidative stress; Alzheimer's disease; antiamnestic agent

INTRODUCTION

Knowledge of the molecular mechanisms of amyloid β peptide (Aβ) cytotoxicity is incomplete. However, the generation of ROS and oxidative damage are believed to be involved in the pathogenesis of neurodegenerative disorders (*1*). Evidence for increased oxidative stress (ROS, lipid peroxidation, protein modification, and oxidation of mitochondrial DNA) has been noted in the AD brain (*2*−*5*). There was also evidence suggesting that oxidative stress could contribute to the formation of amyloid plaques and neurofibrillary tangles (*6*, *7*). The Aβ peptide has been identified as a possible source of oxidative stress in the AD brain because it can acquire a free radical state contributing to its toxic effects (*8*−*10*). Some brain regions of AD patients showed increased sensitivity to oxygen free radicals; this could be due to a reduction in free radical defenses, an increase in free radical formation, or both (*11*). Aβ-induced cytotoxicity has been shown to be caused by the intracellular accumulation of H_2O_2, ultimately leading to the peroxidation of membrane lipids and to the cell death (*12*). Accumulation of ROS results in damage to major macromolecules in cells, including lipids, proteins, and nucleic acids (*2*, *4*, *5*). Free radical-induced oxidative alterations to neuronal lipids, proteins, and DNA are particularly extensive in AD brain areas where Aβ is abundant (*13*). The demonstration of ROS production by Aβ and direct evidence of oxidative damage in neurons by Aβ (*10*, *14*) has propelled the hypothesis of "Aβ-induced free radical-mediated neurotoxicity". We tested this hypothesis using the sympathetic nerve pheochromocytoma cell line that has been successfully used over years in neuronal function studies (*15*−*17*). It has been shown that addition of Aβ to these cells results in increased ROS production, apoptosis, and cell death (*14*−*16*). Neuronal apoptosis has been also implicated in AD (*18*, *19*) and is considered to be a result of the Aβ-induced ROS production. The cholinergic−neural system plays an important role in learning and memory in humans and animals (*20*). SCOP, a muscarinic antagonist, impairs learning and memory in rodents and humans, especially the processes of learning acquisition and short-term memory (*21*). As such, SCOP has been used as a model in screening antiamnestic drugs.

One of the reliable ways to prevent the cellular injuries induced by oxidative stress is to augment endogenous oxidative defense capacity through dietary or pharmacological intake of

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antioxidants (*22*, *23*). *Citrus junos* is cultivated in the southern part of Korea, whose fruit is processed into juice and is often preferred to vinegar as an ingredient in sauces and salad dressings for its characteristic flavor. In the present study, we have examined the neuronal protective effect of naringenin (4′,5,7-trihydroxyflavanone), which is found in *C. junos*, against oxidative cell death induced by Aβ peptide during the PC12 cells cultivation. Also, the antiamnestic effect of naringenin was evaluated using an in vivo mouse model.

MATERIALS AND METHODS

Materials. RPMI 1640 medium, fetal bovine serum, horse serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). Sigma Chemical Co. (St. Louis, MO) supplied Aβ_{25-35}. Aβ_{25-35} was dissolved in deionized distilled water at a concentration of 1 mM and stored at −20 °C. The stock solution was diluted to desirable concentrations immediately before use.

Preparation of *C. junos* Extracts and Naringenin. *C. junos* was purchased in a local market for Oriental medicines in Seoul, Korea, in 1999 and ground to pass through a fine screen (about 1 mm). The plant materials were authenticated by the Institute of Biotechnology, where voucher specimens were maintained, Korea University. The dried sarcocarp (2 kg) of *C. junos* was extracted with methanol in an agitating apparatus. This procedure was performed three times. Upon removal of solvent in a vacuum, the methanolic extract yielded 140 g. This methanolic extract was then suspended in water and partitioned successively with ethyl acetate (H_2O:EtOAc = 1:1). The ethyl acetate fraction of *C. junos* obtained was filtered through a 0.45 μm poly(tetrafluoroethylene) syringe tip filter. Using a μ-bondapack C_{18} reverse column (3.9 mm × 300 mm), the sample was analyzed by Waters 2690 HPLC analysis system (Waters Co., Milford, MA). Naringenin was detected at 248.5 nm with a Waters 996 PDA detector. The data were controlled and analyzed using the Millennium Manager System (Version 2.15; Waters Associates). Separations were carried out at 1 mL/min using a 90 min linear gradient of 0−100% methanol (data not shown). The structure of the resulting compound was analyzed by $^1H/^{13}C$ NMR and electron impact mass spectrometry (EI-MS) (data not shown).

Cells. Pheochromocytoma cells (PC12 cells) were cultured and maintained as previously described. The PC12 cell line was derived from a transplantable rat pheochromocytoma. The cells respond reversibly to nerve growth factor (NGF) by induction of the neuronal phenotype (*24*). Growth medium consisted of RPMI 1640, 10% heat-inactivated horse serum, 5% fetal bovine serum, 50 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained in the 37 °C incubator with water-saturated and 5% CO_2. PC12 cells were passaged when the culture was 80−90% confluent, dislodged from the surface of the culture dish (100 mm), and dispersed into single cell by repeated and forceful triturating of the culture medium directly onto the cells. The cells were subcultured once a week in the split ratio of 1:3 or 1:4.

Measurement of Oxidative Stress. Levels of cellular oxidative stress were measured using the fluorescent probe DCF (Molecular Probes) according to the method that Goodman and Mattson described (*25*). In brief, cells were cultured in 96 well plates in RPMI 1640 media supplemented with 10% horse serum and 5% fetal bovine serum. Cells were then treated for 48 h at the levels of various concentrations of the isolated naringenin or vitamin C. The cells were then treated with or without 100 μM H_2O_2 for 24 h. At the end of the treatment, cells were incubated in the presence of 50 μM DCF in PBS. Fluorescence was finally quantified using SER-NR 94572 fluorometer (TECAN, U.S.A.) equipped with 485 nm excitation and 530 nm emission filters.

MTT Assay. The MTT reduction assay was performed as described previously (*26*, *27*). Briefly, the cells were incubated with 0.25 mg MTT/mL for 0.5−6 h at 37 °C, and the reaction was stopped by adding a solution containing 50% dimethylformide and 20% sodium dodesyl sulfate (pH 4.8). The amount of MTT formazan product was determined by measuring absorbance using a microplate reader (Bio-Rad, Hercules, CA) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

LDH Assay. Damage of the plasma membrane was evaluated by measuring the amount of the intracellular enzyme, LDH, which is released into the medium. LDH activity was measured spectrophotometrically according to the method described by Cabaud and Wroblewski (*28*). The rate of reduction of pyruvate to lactate was measured at 450 nm. Supernatants of PC12 cells were collected from each well, and LDH activities were determined with a colorimetric LDH assay kit (Sigma). Lactic acid, NAD, and NADH were not absorbed significantly at this wavelength. The amount of pyruvate remaining after incubation was inversely proportional to that of LDH activity.

Trypan Blue Test for Cell Viability. Cell viability was assessed by trypan blue dye exclusion prior to all treatments. Viable cells maintained membrane integrity and did not allow trypan blue dye to pass through the cell membrane. Cells with compromised cell membranes appeared blue due to accumulation of dye and were counted as dead. At least 600 cells were counted in four different fields, and the number of viable cells was calculated as percent of the total cell population. PC12 cells with ≥98.0% viability were employed in all treatments (*29*, *30*).

Animals and Experimental Design. Male ICR mice (Samtaco BioKorea Co., Seoul, Korea), weighing 25−30 g, were used for the passive avoidance test following a 1 week adaptation period (20−23 °C; 12 h light cycle from 09:00 to 21:00; food, Agribrand Purina Korea, and water ad libitum). The experimental procedures were approved by the guidelines of the Animal Care and Use Committee of the Korea University. Mice were allowed free access to normal feed or feed mixed with naringenin for 3 weeks. Amnesia was induced in mice with SCOP (1 mg/kg body weight) given subcutaneously. Behavioral tests were started 30 min after injection.

Passive Avoidance Test. The passive avoidance box was divided into two compartments, one illuminated and one dark square box. The light compartment was illuminated by a 100 W bulb; the dark compartment was equipped with 2 mm stainless steel rods spaced 1 cm apart and extending the length of the compartment. A guillotine door separated those compartments. During the training trial, each mouse was placed in the lighted compartment; as soon as it entered the dark compartment, the door was closed and the mouse received an electrical foot shock (0.5 mA/mouse, 1 s). An inescapable shock was delivered through the stainless steel rods (one trial training run). In the testing trial, given 1 day after the training trial, the mouse was again placed in the lighted compartment and the time until it reentered the dark compartment was measured. If the mice did not enter the dark compartment within 300 s, we concluded that the mice had memorized the passive avoidance training after one training trial (*31*).

Statistical Analysis. All data were expressed as means ± SD of at least three independent experiments. Statistical analysis was performed by Student's *t*-test using Sigma Plot software (SPSS Inc., Chicago, IL). Statistical comparisons within the same group were performed for paired observations. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

One of the mechanisms responsible for Aβ-induced oxidative injury is the generation of hydrogen peroxide and induction of lipid peroxidation (*32*). Hydrogen peroxide was used to test Aβ-induced oxidative stress in PC12 cells in this work. Exposure of PC12 cells to H_2O_2 for 24 h resulted in ∼230% increase of ROS levels as compared to control (**Figure 1**). A 48 h pretreatment of the cells with increasing concentrations of naringenin from *C. junos* was followed up with 24 h exposure of H_2O_2. Vitamin C, a naturally occurring major nutrient, showed the reduction of oxidative stress ∼2.4 times higher than the H_2O_2 treatment group. The naringenin (25−100 μM) resulted in a 170−223% decrease of ROS levels as compared to the H_2O_2 exposure (**Figure 1**). Flavonoids contribute to the free radical scavenger effect (*33*). These results clearly demonstrate that naringenin, a potent antioxidant from *C. junos*, shows the antioxidative effect by reducing oxidative stress in cells at the biological activities to similar to vitamin C.

To evaluate Aβ neurotoxicity properly, it is important to employ an appropriate method for quantitating cell viability. Because the dye, MTT, is known to be converted into a purple

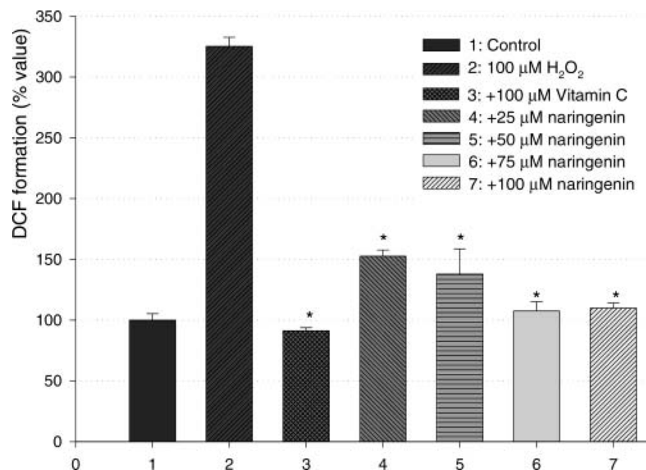

Figure 1. Effect of naringenin and vitamin C on free radical production determined in the presence and absence of Aβ in PC12 neuronal cell. PC12 cells were treated for 48 h with concentrations of the indicated compounds. The cells were then treated with 100 μM H_2O_2 for 24 h. Levels of cellular oxidative stress were measured using the fluorescent probe 2′,7′-dichlorofluorescein (DCF) as described. DCF value was not changed by vitamin C or naringenin (data not shown). Results shown are means ± SD ($n = 3$). Results differ significantly from the value in the H_2O_2-treated group: $^*P < 0.05$.

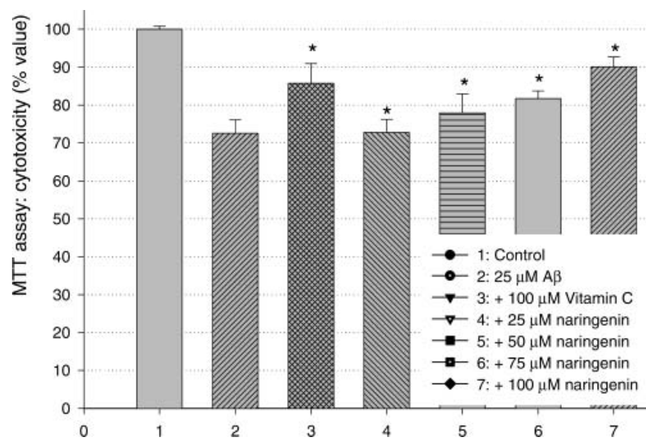

Figure 2. Effect of naringenin and vitamin C on PC12 cell viability determined in the presence and absence of Aβ. PC12 cells were treated for 48 h with various concentrations of the indicated compounds. The cells were then washed and treated with 25 μM Aβ_{25-35} for 24 h. Levels of cell viability were measured using the MTT assay as described in the Materials and Methods. Cell viability was not changed by vitamin C or naringenin (data not shown). Results shown are means ± SD ($n = 3$). A significant difference ($P < 0.05$) was observed on the Aβ-induced cell death.

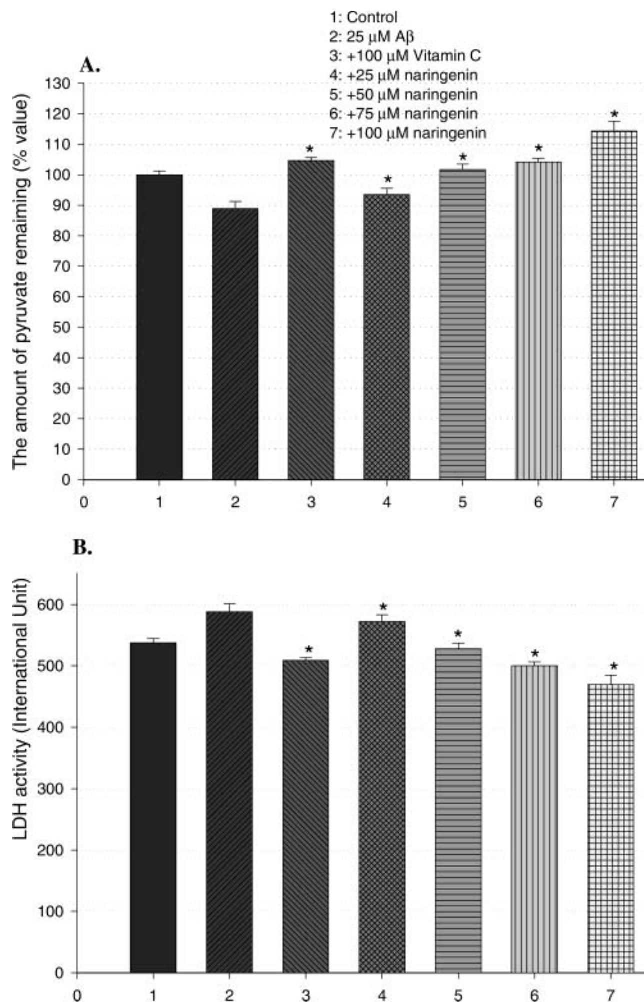

Figure 3. Preventive effects of naringenin and vitamin C against Aβ-induced lipid peroxidation in PC12 cells. PC12 cells were treated for 48 h with various concentrations of the indicated compounds. The cells were treated with Aβ_{25-35} (25 μM) for 24 h. LDH activity was measured spectrophotometrically at 450 nm. Supernatants of PC12 cells were collected from each well, and LDH activities were determined with a colorimetric LDH assay kit. Lactic acid, NAD, and NADH were not absorbed significantly at this wavelength. The amount of pyruvate remaining (**A**) after incubation was inversely proportional to that of LDH activity (**B**). One international unit (U) of an enzyme is defined as the amount of enzyme that will convert 1 μmol of substrate per minute. All data are represented as the mean ± SD. Values were obtained from three separate cultures. Statistical analysis indicated that the influence of the compounds used represented a significant effect on the Aβ-induced membrane toxicity (LDH release) ($P < 0.05$).

formazan by redox activity of living cells, the MTT reduction assay has been widely used for measuring cell viability (*26*, *27*). PC12 cells were treated with Aβ_{25-35} (25 μM) for 24 h, and the effect of Aβ-induced cytotoxicity was evaluated with the MTT assay. Aβ_{25-35} caused a decrease in cell viability (about 29%), but pretreatment cells with increasing concentrations of naringenin from *C. junos* inhibited Aβ-induced neurotoxicity (**Figure 2**). The cell protection effect of vitamin C against Aβ-induced neurotoxicity was less than that of 100 μM naringenin.

Because neuronal plasma membrane is sensitive to oxidative stress (*32*), the toxic effect of Aβ_{25-35} was measured by the LDH assay. LDH release measures the activity of this stable enzyme released into the medium from dead cells. A quantitative analysis of LDH activity can determine what percentage of cells is dead. Treatment with Aβ_{25-35} caused an increase in LDH release (**Figure 3**). The amount of pyruvate remaining (**Figure 3A**) after incubation was inversely proportional to that of released LDH activity (**Figure 3B**). One international unit (U) of an enzyme is defined as that amount of enzyme that will convert 1 μmol of substrate per minute. Pretreatment with naringenin inhibited LDH release in a dose-dependent manner (**Figure 3**).

To confirm whether naringenin from *C. junos* inhibits the Aβ-induced membrane damage, we checked the trypan blue exclusion assay. Viable cells maintained membrane integrity and did not allow trypan blue dye to pass through the cell membrane. As shown in **Table 1**, Aβ-induced oxidative stress increased plasma membrane damage. PC12 cell plasma membrane was damaged by Aβ-induced oxidative stress. The Aβ-

Table 1. Inhibited Aβ Toxicity in PC12 Cells after Pretreatment with Naringenin as Assessed by Trypan Blue Exclusion Staining Followed by Cell Counting

control cells[a]	100
25 μM Aβ alone	64.3 ± 3.2
+ 100 μM Vitamin C	81.0 ± 2.6[b]
+ 25 μM naringenin	60.3 ± 3.2
+ 50 μM naringenin	63.7 ± 1.5
+ 75 μM naringenin	71.0 ± 1.0
+ 100 μM naringenin	84.6 ± 2.1[b]

[a] PC12 cells were plated at low density in a 12 well plate. Cells were incubated with naringenin for 48 h before the addition of 25 μM Aβ protein. Cultures were observed after an additional 24 h, and trypan blue exclusion staining was performed. Data are presented as means ± SD for one representative triplicate determination and are expressed as the percent survival as compared to the corresponding controls. The viability of untreated control cells was defined as 100%. [b] $P < 0.01$ (cell survival after incubation with naringenin as compared with cell survival after incubation with Aβ protein) was considered significant.

induced membrane damage was reduced by naringenin. These results indicated that the effect of Aβ detected by the MTT assay corresponded with a decrease in cell viability and that Aβ-induced neurotoxicity was developed by plasma membrane damage. In this study, naringenin inhibited Aβ-induced membrane damage and neurotoxicity.

Evidence from animal and human studies indicates that learning and memory can be modified by drugs affecting central cholinergic function (*21*). For instance, such muscarinic antagonists as SCOP have been shown to impair memory, whereas such inhibitors of acetylcholinesterase (AChE) as physostigmine, tacrine, or velnacrine facilitate the cognitive process in animals and humans (*34*). To assess this efficacy, SCOP was used to induce memory impairment in mice, and this impairment was measured using a passive avoidance test with or without treatment with selective flavanone compounds. Intraperitoneal injection of SCOP hydrobromide, which is a muscarinic cholinergic receptor blocker, causes memory deficits and decreases cholinergic activity on behavioral performance (*35*); therefore, this method of SCOP exposure is a useful in vivo model for AD. In this respect, we just used the SCOP-induced in vivo method to investigate the in vivo effect of naringenin.

In this experiment, mice were treated with naringenin in the drinking water at various concentrations for up to 3 weeks prior to SCOP administration. Because the average water intake per mouse per day was approximately 8−10 mL, the amount of naringenin consumed by mice receiving 0.001−0.0045% naringenin in the drinking water ranged from 0.03 to 0.135 mg mouse^{-1} day^{-1}. All mice treated with naringenin gained body weight normally 37.2 ± 2.5 g for control mice vs 37.8 ± 1.7 g for mice treated with naringenin for 3 weeks) and did not show any signs of toxicity during the experiment (data not shown). Then, we tested the effects of naringenin on learning and memory in vivo using the SCOP-induced amnesia model (*36*). Treatment with SCOP (1 mg/kg) significantly shortened the latency time (44.9% decrease in step through latency) in the retention trial (**Figure 4B**). However, treatment of mice with naringenin for 3 weeks attenuated the SCOP-induced impairment in a dose-dependent manner (7.5% increase and 20−27% decreases in step through latency) with maximal effects observed at a concentration of 0.135 mg mouse^{-1} day^{-1} (0.0045%). In this study, our data showed that naringenin ameliorated SCOP-induced learning and memory deficit. Although we do not suggest the improving mechanism of naringenin on SCOP-induced deficit, naringenin can be considered as a useful therapeutic agent in AD.

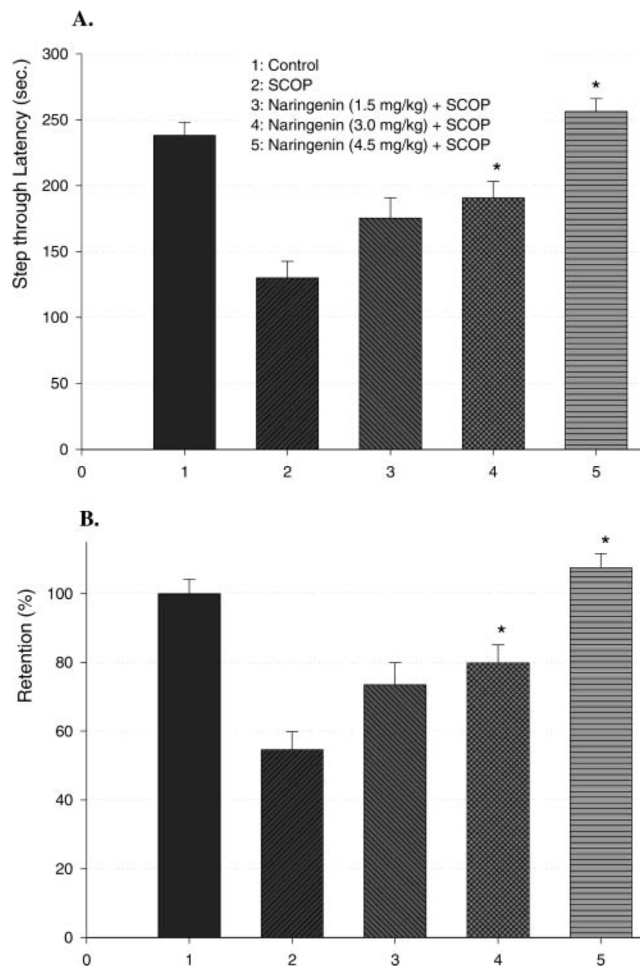

Figure 4. Protective effects of *C. junos* against the SCOP-induced impairment in learning and memory in mice. Effects of naringenin on step through latency (**A**) and retention percentage (**B**) in the retention trial of the passive avoidance task. After injection of SCOP (1 mg/kg), each behavioral test was done. Mice were trained on a one trial step through passive avoidance task. The testing trial was given 1 day after the training trial. The values shown are the mean latencies ± SD ($n = 10$). Control mice were injected with 0.85% (w/v) saline (100 μL). Results differ significantly from values in SCOP-treated group: $^*P < 0.01$.

Brain cells are at a particular risk of being damaged by free radicals because the brain has a high oxygen turnover, and CNS neuronal membranes are rich in polyunsaturated fatty acids, which are potential targets for lipid peroxidation (*12*). An imbalance of the equilibrium between free radical generation and various antioxidant defense systems leading to the accumulation of free radicals is called oxidative stress. Free radicals and oxidative stress-induced neuronal cell death have been implicated in various neurological disorders, such as AD (*37*). AD-associated Aβ accumulation in CNS plaques of AD patient's brains induces the generation of oxygen-free radicals, ultimately leading to the peroxidation of membrane lipids and cell lysis (*32*). The results in this study indicate that naringenin represents a higher protective effect against Aβ-induced neuronal damage than vitamin C and a less protective effect against Aβ-induced ROS increase than vitamin C. Considering the antioxidant effect of ginkgolides, these studies also indicate that the neuroprotective effect of the *Ginkgo biloba* extract Egb 761 is probably not only due to its antioxidant properties but to a separate yet unidentified activity (*38*, *39*). However, it should be noted that the absence of a role of ROS in Aβ-induced apoptosis and cell death, under the short-term conditions used,

does not exclude their involvement in the pathogenesis of AD, a long-term evolving process (*7*).

In this study, we have shown that naringenin (4′,5,7-trihydroxyflavanone) of *C. junos* was purified from natural edible plants. The effective dose, absorption rate, intake by regular diet, and metabolism of naringenin in humans is not yet known. Although it is likely that naringenin concentrations ingested by a regular diet are not high enough to trigger beneficial effects, naringenin from *C. junos* can be considered as a health-promoting food supplement. Naringenin has been reported that was isolated as topical photoprotective agents (*40*). Topical administration of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system and thus may be a successful strategy for diminishing ultraviolet radiation-mediated oxidative damage in the skin (*41*).

In conclusion, naringenin has a protective effect of Aβ-induced in vitro neuronal cytotoxicity. We used a SCOP-induced animal model as an experiment for Aβ-induced cholinergic impairment. Our data showed the significant antiamnesic effects in mice. Therefore, the neuronal cell protection by naringenin might explain the antiamnestic effects observed in the present study. Natural compounds that exert antiamnestic activity in vivo through blocking of Aβ-induced neuronal damage might offer a useful therapeutic agent in the treatment of AD.

ABBREVIATIONS USED

Aβ, amyloid β protein; AD, Alzheimer's disease; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCF, 2,7-dichlorofluorescein diacetate; LDH, lactate dehydrogenase; SCOP, scopolamine; CNS, central nervous system.

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