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Memory-Improving Activity of the Flower Extract from *Chrysanthemum boreale* (Makino) Maskino in Scopolamine-Treated Rodents

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Abstract: One of the factors related to the pathogenesis of Alzheimer's disease, a disease characterized by gradual cognitive and memory impairment, is an inflammatory process induced by the amyloid-β-mediated activation of microglia. In the present study, an extract of the *Chrysanthemum boreale* (Makino) Makino (CB) flower, which has inhibitory effects on inflammation and the production of phosphorylated tau in cells, was investigated for its ameliorative effect on memory dysfunction in scopolamine-treated Alzheimer's disease models. The CB-extract-diet-administered groups, which were treated chronically with scopolamine (intraperitoneal), showed increased spontaneous alterations (12.5–15.5% increase) in the Y-maze test and latency to escape (3.7–6.7-fold increase) in the passive avoidance test, compared to the negative control (NC) group. Rats administered the CB extract also showed a higher tendency (66–86% increase) of hippocampal brain-derived neurotrophic factor expression than NC rats. Moreover, the ratio of phosphorylated extracellular signal-regulated kinase/extracellular signal-regulated kinase in the CB-extract-administered group was lower (48.0–52.2%) than that (100%) in the NC group. In the Morris water maze test conducted on the fifth day, the free-swimming times of the CB-extract-administered mice that were also treated with scopolamine for a short time (5 d) increased (51.7–56.1%) significantly compared to those of the NC mice. Finally, high-performance liquid chromatography analysis confirmed that isochlorogenic acid A, linarin, and chlorogenic acid are the major phenolic components of the CB extract. These results suggest that the extract of CB flowers might be useful as a functional material with memory-enhancing effects.

Keywords: *Chrysanthemum boreale*; cognition; memory; brain-derived neurotrophic factor; isochlorogenic acid; Morris water maze

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1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder and the most common form of dementia, which possibly contributes to 60–70% of cases. According to the World Health Organization, AD is characterized by cognitive and functional decline associated with age [1–3]. Memory loss is a common symptom in most AD patients. Although genetic and environmental risk factors have been implicated in the pathogenesis of AD, it is currently suggested that age-related disease-promoting factors, including the neurotransmitter acetylcholine (ACh), inflammation, and amyloid beta (Aβ)/tau proteins, are also involved in the same [4–6].

In AD pathogenesis, inflammatory reactions are related to the accumulation of Aβ protein and activation of microglia and astrocytes [7,8]. Soluble Aβ and amyloid plaques induce secondary events such as inflammation, oxidative stress, hyperphosphorylation of

tau protein, and excitotoxicity, which in turn lead to cell death and neurotransmitter deficits, especially of ACh [3]. Thus, it is thought that certain compounds with anti-inflammatory and -Aβ actions might be able to slow or halt the progression of neurodegenerative diseases such as AD.

Many plants with traditional efficacies have been used to treat cognitive disorders, including AD. Components isolated from natural resources are known to improve cognition. For example, galantamine, a clinically used drug in AD treatment, is isolated from the plant *Galanthus nivalis* L. [9,10]. Moreover, it is suggested that microglia activated by pro-inflammatory Aβ could release neurotoxic factors such as NO and TNF-α [11]. Therefore, we conducted a preliminary experiment to screen the inhibitory effects of 210 plant extracts on nitric oxide (NO) production in lipopolysaccharide (LPS)-treated BV2 cells. From this preliminarily experiment, the flower extract from *Chrysanthemum boreale* (Makino) Makino (CB) was selected.

CB, which has yellow flowers, is a perennial plant widespread in East Asia that has been used for the treatment of disorders, including pneumonia, colitis, and stomatitis [12]. The various activities of different CB plant parts have been reported in the literature, as discussed below.

β-Caryophyllene isolated from the aerial part of CB exerts cytotoxic activity in lung cancer cells and induces apoptosis in human oral epidermoid carcinoma cells [12,13]. Whole plants have been shown to inhibit oxidative stress-induced neuronal damage in cells and MPTP-induced mice [14,15]. Sesquiterpene lactones from leaves and stems have been shown to inhibit ACAT and NO production [16,17]. The flowers have been shown to exhibit various activities, including cytotoxicity of carcinoma cells, life prolongation in sarcoma-implanted mice, anti-bacterial/inflammatory effects on RAW264.7 cells, antioxidant/melanogenic skin regeneration, remedy potential for vascular disorders, and the inhibition of aldose reductase, which is related to cataract formation [18–25]. The compounds isolated from the flowers include cumambrin A, which has a recovering effect on increasing blood pressure in spontaneously hypertensive rats; handelin (guaianolide dimer), which has suppressive activity in a carrageenan-induced paw edema model; 1-indolhexadecane, which has an alleviating effect on atopic dermatitis in mice; and flavone glycoside, which has sedative/anti-convulsant activity [11,26–30]. Although the flowers of CB have various physiological activities, as mentioned above, the potential effect of CB on memory/cognition improvement has not yet been studied.

In the present study, the extract of *C. boreale* flowers (CB extract), as a plant resource, showed anti-inflammatory activities in in vitro experiments carried out in LPS-treated BV2 microglial cells and inhibitory activity on phosphorylated tau (p-Tau) production in Aβ-treated SH-SY5Y cells. Furthermore, the memory-enhancing potential of the CB extract was investigated in scopolamine (Sco)-induced memory-impaired rats and mice, which served as AD models.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Resource and Extract Preparation

The samples for the study, i.e., flowers of CB, were collected and dried in October 2018 in Eumseong-gun, Chungcheongbuk-do, Korea. The plant was identified by comparing it with a specimen (voucher no. MPS005533) from the National Institute of Horticultural and Herbal Science (NIHHS), Eumseong, Korea. Powdered CB (450 g) was extracted with ethanol at room temperature and filtered twice. Solvents of the whole extract were eliminated by means of evaporation in a vacuum condition, at 50 °C. Finally, the crude CB extract (62.8 g) was obtained and stored at −20 °C until it was used.

2.1.2. Chemicals

Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum, and horse serum were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). In addi-

tion, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LPS, dimethyl sulfoxide, penicillin and streptomycin, sulfanilamide, N-1-napthyl-ethylenediamine dihydrochloride (NED), L-glutamate, Trizma base, ethylenediaminetetraacetic acid, all-trans retinoic acid (RA), Aβ protein fragment 1–42 ($A\beta_{1–42}$), Donepezil, and Sco were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against extracellular signal-regulated kinase 1 and 2 (ERK1/2), phosphorylated (p)-ERK1/2, and β-actin, as well as anti-rabbit IgG horseradish peroxidase-linked antibody, were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibody against brain-derived neurotrophic factor (BDNF) was provided by Abcam (Cambridge, UK). The Western blot ECL substrate was supplied by Bio-Rad (Hercules, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for analyzing choline acetyltransferase (ChAT), p-Tau, and ACh were supplied by Elabscience (Houston, TX, USA) and BioVision Inc. (Milpitas, CA, USA). ECL substrates, phosphate-buffered saline, and BDNF protein were provided by Bio-Rad (Hercules, CA, USA), WelGene (Gyeongsan, Korea), and Alomone Lab (Jerusalem, Israel), respectively.

2.2. Cell Lines and Cell Culture

The BV2 cell line, a microglial cell that originated from the mouse brain, was proliferated in complete (CM) RPMI-1640 media containing 10% fetal bovine serum and 1% penicillin and streptomycin. The SH-SY5Y cell line, which originated from human neuroblastoma (SK-N-SH), was proliferated in RPMI-1640 media containing 10% horse serum, 5% fetal bovine serum, and 1% penicillin and streptomycin.

2.3. In Vitro Assay

2.3.1. Analysis of NO Levels, IL-6 Levels, and Cell Proliferation in BV2

To measure the effect of the CB extract on NO production, BV2 cells (2×10^5 cells/mL) were allowed to grow in a 48-well plate for 24 h and then were treated with CB extract in serum-free media for 2 h. Thereafter, 0.5 μg/mL LPS in CM medium was added to the wells for 24 h. The supernatant (50 μL) from each well was transferred to a 96-well plate and allowed to react with 50 μL 1% sulfanilamide and 50 μL 0.1% NED. The NO level of the reactant was measured in terms of the nitrite concentration at a wavelength of 520 nm. Cell-free supernatant (100 μL) from each well was also used to measure the effect on IL-6 release using an ELISA kit at a wavelength of 450 nm. To measure the proliferation of BV2 cells, the cell remnants in each well after the procedure mentioned above were reacted with MTT reagent (0.6 mg/mL) for 1 h. After removing the MTT reagent, the formazan crystals dissolved in dimethyl sulfoxide were measured at a wavelength of 540 nm using a microplate reader. For these assays, the methods of Kwon et al. [31] were modified.

2.3.2. Analysis of p-Tau Production in SH-SY5Y Cells

To assess the inhibitory activity of the CB extract on p-Tau production, SH-SY5Y cells (1×10^5 cells/well) were seeded into a 6-well plate and incubated for 24 h. Following that, the cells were treated with 10 μM RA and 5 nM BDNF for 2 and 1 d, respectively. The CB extract was subsequently applied to the cells for 2 h, followed by the addition of 0.1 μM $A\beta_{1–42}$ in all wells except for the normal well. After 21 h, the supernatant in each well was removed by means of suction and the remaining cells were washed with ice-cold phosphate-buffered saline and lysed. The lysis buffer-treated cells were then collected and centrifuged at 13,201× *g* and 4 °C for 20 min. The p-Tau levels in the supernatant thus obtained were evaluated using an ELISA kit, according to the manufacturer's instructions. This experiment adopted the modified methods of Olivieri et al. [32].

2.4. In Vivo Experiments

2.4.1. Animal Experiments Using Rats Chronically Treated with Sco

Seven-week-old Sprague Dawley male rats (obtained from DBL Co., Eumseong, Republic of Korea) were acclimated to the environment for 1 week, and then distributed into five groups: normal, NC (Sco), CB extract-low (CBL), CB extract-high (CBH), and

Donepezil (positive control, PC) (n = 10 for each group). The diets for the CBL and CBH groups included a commercial diet (supplied by DBL Co.) containing 0.07% and 0.14% of CB extract, respectively. The normal, NC, and PC groups were fed a commercial diet. The diets and water for all groups were provided for 21 d *ad libitum* PC group was intraperitoneally (i.p.) administered 1 mg/kg Donepezil once per day, for 21 d. All the groups except the normal group were injected with 1 mg/kg Sco in 0.9% saline, while the normal group was treated with only 0.9% saline one time per day (i.p.). The passive avoidance test (PAT) was conducted from the 18th to 20th days, with the latency to escape from the light room to the dark room measured on the 20th day. The Y-maze test was executed on the 21st day. The rats were euthanized under CO_2 on the 22nd day. The hippocampal tissue and serum collected were stored at −80 °C before analysis. Doses of scopolamine and Donepezil were determined with reference to the methods of Bhuvanendran et al. [33]. The methods for the Y-maze test and PAT were adopted and modified from Sarter et al. [34] and Pury et al. [35], respectively. The result (alteration) of the Y-maze test was calculated as:

$$\text{Alteration (\%)} = (\text{spontaneous alteration/total entry} - 2) \times 100 \quad (1)$$

2.4.2. Analysis of Biomarkers in Rats Chronically Treated with Sco

Hippocampal ChAT activity and serum ACh levels were analyzed using ELISA kits. The expression of hippocampal proteins was measured using Western blot, which was conducted using anti-BDNF (1:500 dilution), anti-p-ERK, anti-ERK, and anti-β-actin (1:1000 dilution) as the primary antibodies, and horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (1:2000 dilution) as the secondary antibodies. Protein expression in the western ECL substrates was identified using the ChemiDoc™ Imaging System (Bio-Rad, Hercules, CA, USA).

2.4.3. Animal Experiments Using Mice Acutely Treated with Sco

Mice with memory dysfunction that were acutely treated with Sco were used for behavioral testing in the Morris water maze (MWM). Male ICR mice (five-week-old) were supplied by DBL Co. The mice were divided into normal (vehicle), NC (Sco), CBL (112.7 mg/kg CB extract in saline + Sco), CBH (328.0 mg/kg CB extract in saline + Sco), and Donepezil (1 mg/kg Donepezil in saline + Sco) (n = 10) groups. The doses of the CB extract and the administration period were determined according to a previous preliminary chronic Sco administration experiment. The mice were orally administered with the CB extract and Donepezil for 21 d. Additionally, from the 17th to 21st day, the mice were administered Sco (1 mg/kg/day, i.p.), following which the MWM test was performed. On the 5th test day, a cognition test was conducted using EthoVision® software (Noldus, Wageningen, The Netherlands) to measure the swimming times in the target area. The MWM test was conducted via the modified method of Weizner et al. [36].

All animal experiments followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and were approved by the Institutional Animal Care and Use Committee of NIHHS (approval number: NIHHS-2021-001, 21 January 2021) for the rat experiment and the Animal Experiment Ethics Committee of Daegu Haany University (Approval No.: DHU2021-075, 7 July 2021) for the mouse experiment.

2.5. Analysis of Phenolic Composition

Ten milligrams of the CB extract was dissolved in 5 mL 70% ethanol and filtered through a membrane filter (0.45 μm, PTFE, Whatman Inc., Piscataway, NJ, USA). The extract was then used to analyze the phenolic composition using high-performance liquid chromatography (System: 1200 series, Agilent Technologies, Santa Clara, CA, USA; column: Fusion hydro-RP, 250 × 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA) with a UV-Visible detector. The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The following conditions were used: 0 min (2% A/B), 0–5 min (2–2%

A/B), 5–12 min (2–5% A/B), 12–17 min (5–8% A/B), 17–65 min (8–30% A/B), 65–68 min (30–30% A/B), 68–78 min (30–50% A/B), 78–100 min (50–100% A/B), and 100–110 min (100–100% A/B). The injection volume, flow rate, and detection wavelength were set at 10 μL, 1.0 mL/min, and 340 nm, respectively.

2.6. Statistical Analysis

Data obtained for multiple trials are presented as mean ± standard deviation. Significance was determined by one-way ANOVA with post hoc Tukey's multiple comparison test.

3. Results

3.1. Effects of the CB Extract on NO Production and Cell Proliferation in BV2 Cells

The effects of the CB extract on NO production and cell viability were evaluated in LPS-treated BV2 cells. Treatment of the cells with the CB extract (5–40 μg/mL, final concentration) significantly and dose-dependently inhibited NO production by 8.6–31.0%, compared to that observed in the untreated group (0.0%) (Figure 1A, $p < 0.05$). Furthermore, CB extract also inhibited IL-6 release in a dose-dependent manner by 18.8–50.4% compared to the untreated group in LPS-treated BV2 cells (Figure 1B, $p < 0.05$). The CB extract dose-dependently increased BV2 cell proliferation by 63.8–115.2%, compared to that observed in the control (100.0%) (Figure 1C, $p < 0.05$). These results suggested that the CB extract has anti-inflammatory efficacy and may improve memory.

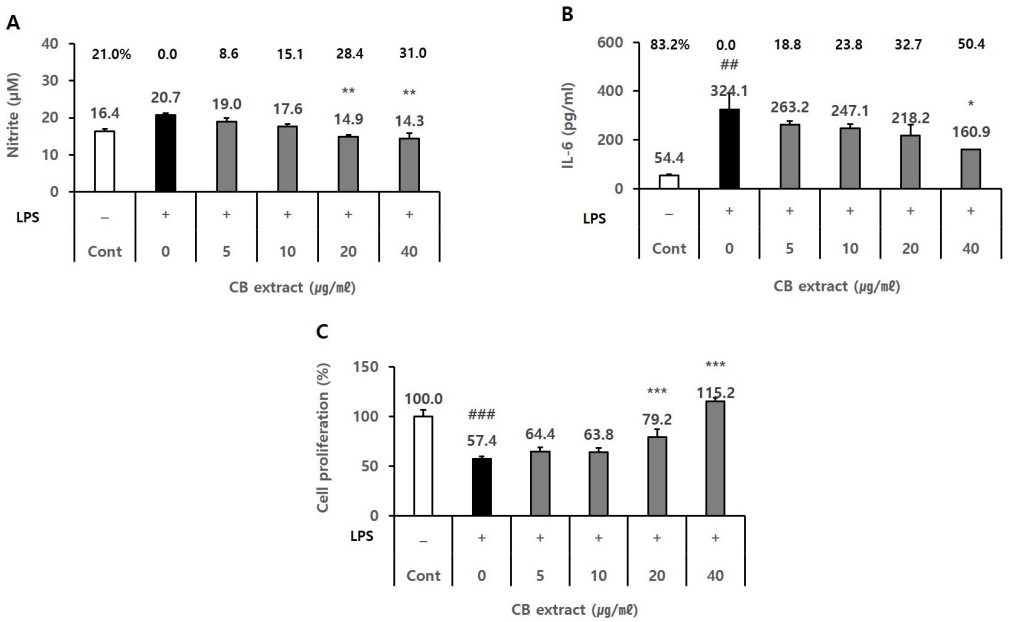

Figure 1. Effect of CB extract on (**A**) NO level, (**B**) IL-6 level, and (**C**) cell proliferation in LPS-treated BV2 cells. BV-2 cells (2×10^5 cells/mL) in 48-well plate were treated with sample extracts (at a final concentration of 5, 10, 20, 40 μg/mL) for 2 h and with serum free media as control. The wells were reacted with 0.5 μg/mL LPS for 24 h. The optical density of the reaction supernatant for the nitrite assay was measured at 520 nm. Supernatant (100 μL) from each well was also used to measure the effect on IL-6 release using an ELISA kit at the wavelength of 450 nm. An MTT assay on the remnant cells that were treated with CB extract (5–40 μg/mL) was conducted at 540 nm. (−), LPS-untreated experiment; (+), LPS-treated experiment. LPS, lipopolysaccharide. Cont, control. Significance was determined by one-way ANOVA with post hoc Tukey's multiple comparison test. Data are presented as means ± standard errors of the mean (SEM). ## $p < 0.01$, ### $p < 0.001$ significance compared with the control (white bar). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significance compared with LPS-treated cells (black bar).

3.2. Inhibitory Effect of the CB Extract on p-Tau Production in SH-SY5Y Cells

The effects of the CB extract on p-Tau production and cell viability were evaluated in A$\beta_{1–42}$-treated SH-SY5Y cells. Treatment with the CB extract at final concentrations of 1.25, 2.5, and 5 μg/mL suppressed the production of p-Tau by 4.9%, 12.9%, and 35.0%, respectively, compared to that in the control (Figure 2A, $p < 0.05$). CB extract treatments (2.5 and 5 μg/mL) of SH-SY5Y cells not treated with A$\beta_{1–42}$ resulted in cell proliferation rates of 108.5% and 106.3%, respectively, compared to that in the control (100%) (Figure 2B, $p < 0.05$). These results suggested that the CB extract could also have the potential to positively affect memory improvement in an in vivo AD model.

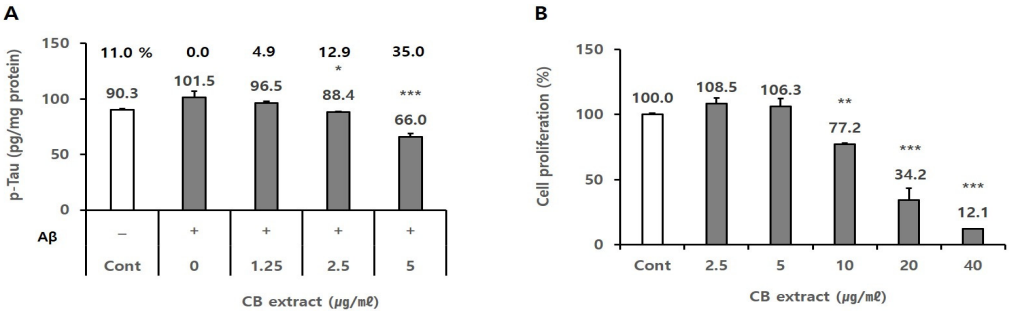

Figure 2. Effect of CB extract on (**A**) p-tau production and (**B**) cell proliferation in Aβ-treated SH-SY5Y cells. SH-SY5Y cells (1×10^5 cells/mL) in 6-well plate were treated with sample extracts (at the final concentrations of 1.25, 2.5, or 5 μg/mL) for 2 h and with serum free (SF) media as control. The wells were reacted with 0.1 μM A$\beta_{1\text{-}42}$ for 21 h. p-Tau sampling for the ELISA assay was conducted using the manufacturer's instructions. An MTT assay on the remnant cells that were treated with CB extract (2.5–40 μg/mL) and not treated with A$\beta_{1\text{-}42,}$ was conducted at 540 nm. (−), Aβ-untreated experiment; (+), Aβ-treated experiment; Cont, control. Significance was determined by one-way ANOVA with post hoc Tukey's multiple comparison test. Data are presented as means ± standard errors of the mean (SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significance compared with Aβ-treated cells (black bar).

3.3. Effect of the CB Extract on the Biomarker Levels in Rats Treated Chronically with Sco

In the next step of the study, we analyzed the hippocampal ChAT and Ach levels, to evaluate the effect of the CB extract on the cholinergic system. The hippocampal ChAT level in the NC group (1.00 as relative fold-change) was lower than that in the normal group (1.97), while the CBH and CBL groups displayed higher ChAT levels (1.47 and 1.62, respectively) than the Donepezil group (1.78) (Figure 3A, $p < 0.05$). The serum ACh level of the NC group (100.0%) was lower than that of the normal group (106.90%), while the ACh level of the CBH group (114.4%) was higher than that of the NC group (Figure 3B, $p < 0.05$). These results suggested that the CB extract plays a positive role in cholinergic signal transmission, which is related to the memory-enhancing process.

The effects of the CB extract on the expression of p-ERK1/2 and BDNF were analyzed in rats treated chronically with Sco, using Western blot. In the hippocampus, the p-ERK/ERK ratio of the NC group (1.04 ± 0.04; 0.0% as inhibition), which was treated only with Sco and without CB extract, was higher than that of the normal group (0.45 ± 0.05; 56.8%). The p-ERK/ERK ratios of the CB-extract-treated groups, CBL and CBH, were 0.55 ± 0.11 (47.8%) and 0.50 ± 0.06 (52.0%), respectively, which were lower than that of the Donepezil group (0.77 ± 0.17; 26.0%) (Figure 4A, $p < 0.05$). With respect to the expression of BDNF, the NC group showed a lower value (0.72 ± 0.07; 100%) than the normal group (0.91 ± 0.05; 126.7%, relative to the NC group). However, the BDNF expression levels in the CB-extract-administered CBL and CBH groups (1.20 ± 0.12; 166.4% and 1.34 ± 0.23; 186.1%, respectively) and Donepezil group (1.11 ± 0.09, 154.6%) were significantly higher than those in the NC group (Figure 4B, $p < 0.05$).

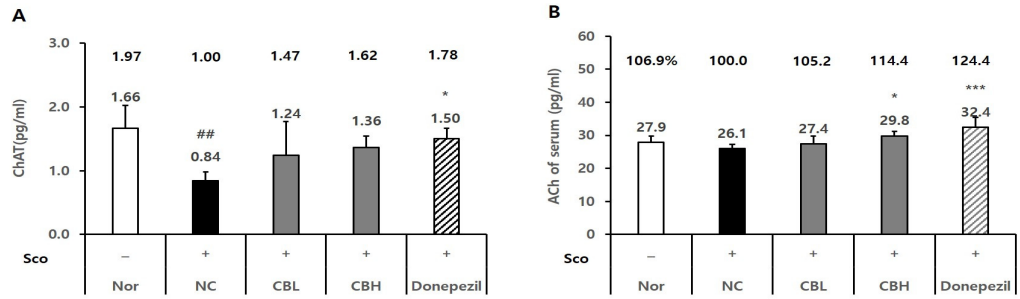

Figure 3. Effects of CB extract on the levels of (**A**) hippocampal ChAT and (**B**) serum ACh level of chronically scopolamine-treated rats. Rats were randomly divided into five groups. Nor; normal, commercial diet + saline; NC, negative control (Sco), commercial diet + Sco in saline; CBL, CB extract 0.07% diet + Sco in saline; CBH, CB extract 0.14% diet + Sco in saline; Do, commercial diet + 1 mg/kg Donepezil in saline + Sco in saline (n = 3 for ChAT and ACh). Sco at 1 mg/kg was administered to rats one time per day for 21 days (i.p). Significance was determined by one-way ANOVA with post hoc Tukey's multiple comparison test; Data are means ± standard errors of the mean (SEM). ## $p < 0.01$ significance compared with the control (white bar). * $p < 0.05$, *** $p < 0.001$ significance compared with Sco-treated groups (black bar).

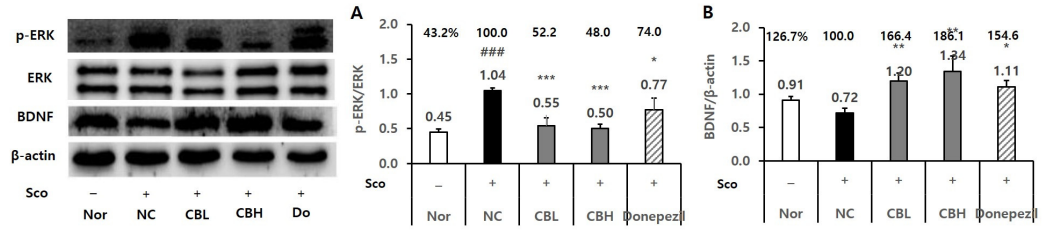

Figure 4. Effects of CB extract on the expression of (**A**) hippocampal p-ERK/ERK, and (**B**) BDNF of chronically Sco-treated rats. Rats were randomly divided into five groups. Nor, normal, commercial diet + saline; NC, negative control (Sco), commercial diet + Sco in saline; CBL, CB extract 0.07% diet + Sco in saline; CBH, CB extract 0.14% diet + Sco in saline; Donepezil; commercial diet + 1 mg/kg Donepezil in saline + Sco in saline (n = 3 for p-ERK/ERk and BDNF). Sco at 1 mg/kg was administered to rats one time per day for 21 days (i.p). Significance was determined by one-way ANOVA with post hoc Tukey's multiple comparison test. Data are presented as means ± standard errors of the mean (SEM). ### $p < 0.001$ significance compared with the control (white bar). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significance compared with Aβ-treated groups (black bar).

3.4. Effects of the CB Extract on the Behavior of Animals Treated Chronically and Acutely with Sco

In the Y-maze test of rats treated chronically with Sco, administration of a commercial diet with 0.07% (CBL) and 0.14% (CBH) CB extract caused slightly increased spontaneous alterations (30.4% and 29.6%, respectively), compared to those observed in the NC rats (26.3%). Furthermore, the CB-extract-administered groups showed the same levels of alterations as those observed in the normal and Do groups (29.9% and 29.1%, respectively) (Figure 5A, $p < 0.05$). In the PAT carried out on rats treated chronically with Sco, the escape latency of the NC group (29.6 s; 1.00 as the relative fold-change) was lower than those of the normal and Donepezil groups (218.9 s; 7.4 and 179.1 s; 6.0, respectively) and the CBL and CBH groups (110.6 s; 3.7 and 197.8 s; 6.7, respectively) (Figure 5B, $p < 0.05$). These results indicated that the CB extract increases the memory-enhancing tendencies in chronic amnesia rat models.

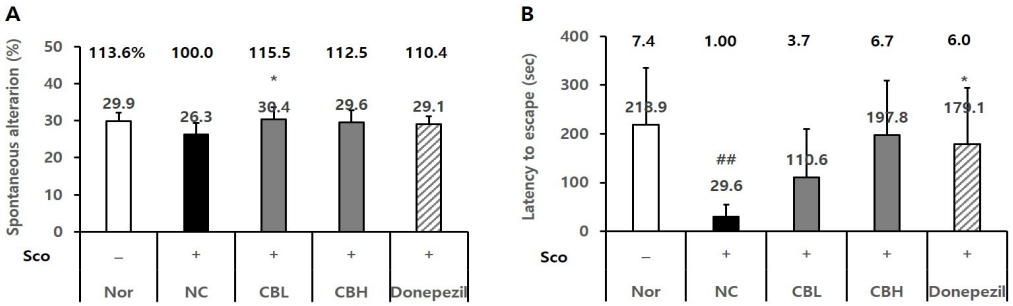

Figure 5. Learning and memory improving effect of CB extract in chronically scopolamine-treated rats in (**A**) Y-maze test and (**B**) passive avoidance test (PAT). Rats were randomly divided into five groups. Nor, normal, commercial diet + saline; NC, negative control (Sco), commercial diet + Sco in saline; CBL; CB extract 0.07% diet + Sco in saline; CBH, CB extract 0.14% diet + Sco in saline; Donepezil; commercial diet + 1 mg/kg Donepezil in saline + Sco in saline (n = 8 for Y-maze test and PAT). Sco at 1 mg/kg was administered to rats one time per day for 21 days (i.p). Significance was determined by one-way ANOVA with post hoc Tukey's multiple comparison test; Data are presented as means ± standard errors of the mean (SEM). ## $p < 0.01$, significance compared with the control (white bar). * $p < 0.05$ significance compared with Sco-treated groups (black bar).

In the mice treated acutely with Sco, the learning test was conducted between the 17th and 20th days of the MWM test. The times taken by the NC group, which were administered 1 mg/kg (i.p.) Sco for the test days, to reach the escape platform were significantly different from those taken by the CBL (orally administered 112.7 mg/kg CB extract + 1 mg/kg Sco), CBH (orally administered 328.0 mg/kg CB extract + 1 mg/kg Sco), normal, and Donepezil [1 mg/kg (i.p.) Donepezil + 1 mg/kg (i.p.) Sco] groups, with these groups showing a decreasing tendency (Figure 6A, $p < 0.05$). On the 4th day, the escape times of the normal, NC, CBL, CBH, and Donepezil groups were 29.8 ± 6.9, 49.5 ± 5.8, 35.9 ± 13.5, 29.8 ± 12.7, and 32.1 ± 9.3 s, respectively (Figure 6A, $p < 0.05$). Therefore, the escape times of the CB-extract-administered groups were reduced to the same levels as those observed in the normal and Donepezil groups. This result implies that the CB extract increases learning capacity in acute amnesia mouse models. In addition, a memory potency test was carried out on the 5th day, in which the platform was removed, and the free-swimming (FS) times of the mice were measured in the target quadrant for 60 s, using EthoVision®. The FS time of the NC group (12.8 ± 0.7 s) was lower than that of the normal group (18.3 ± 0.9 s), while those of the CBL and CBH groups were higher at 20.0 ± 1.2 and 19.5 ± 0.8 s, respectively, similar to that observed in the Donepezil group (18.7 ± 1.1 s) (Figure 6B, $p < 0.05$). These results implied that the CB extract improves both long-term and spatial memories.

3.5. Phenolic Composition

The phenolic composition of the CB extract is given in Figure 7 and Table 1. The major phenolic components identified in the CB extract were caffeoylquinic acids (isochlorogenic acid A and chlorogenic acid) and flavonoids (linarin and luteolin), which had contents of 34.79 ± 1.043, 7.37 ± 0.286, 24.03 ± 1.853, and 3.85 ± 0.137 mg/g extract, respectively. Linarin, a flavone glycoside, is abundant in *Circium*, *Micromeria*, and *Buddleja* species and has exhibited remedial effects on nervous system disorders [Mottaghipisheh, 2021-[37]. Because the compositions of flavonoids and caffeoylquinic acids in the same Compositae family were markedly different, it is expected that the phenolics of CB extract could be significantly related to its physiological activity (Lai—2007 [38]).

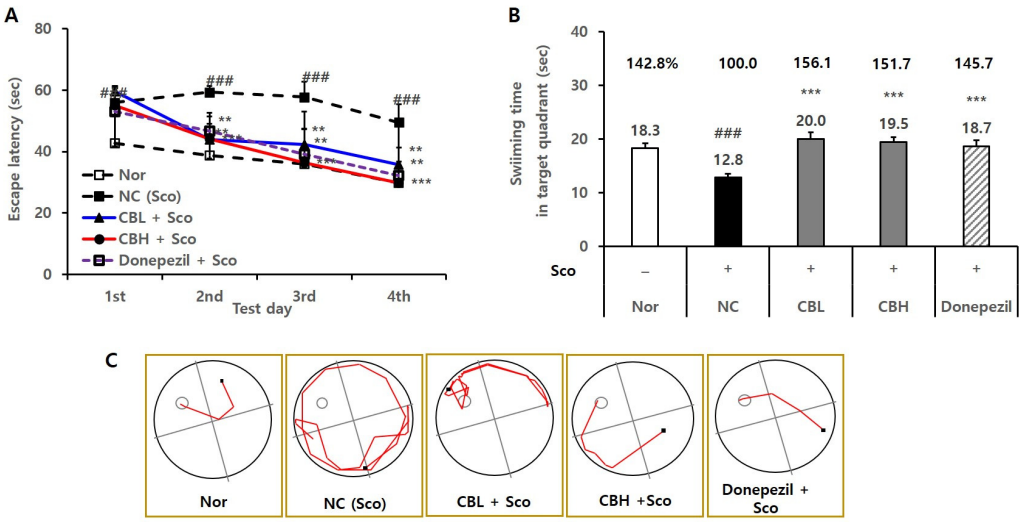

Figure 6. Effects of CB extract on (**A**) the escape latency on days 1–4, (**B**) swimming time on day 5, and (**C**) representative search strategy on day 4 of acutely Sco-treated mice in the Morris water maze test. Mice were randomly divided into five groups. Nor, normal, commercial diet + saline; NC, negative control (Sco), commercial diet + Sco in saline; CBL, 112.7 mg/kg CB extract + Sco in saline; CBH, 328.0 mg/kg CB extract + Sco in saline; Donepezil, commercial diet + 1 mg/kg Donepezil in saline (p.o.) + Sco in saline (n = 10). Sco at 1 mg/kg was administered to the mice one time per day for 5 days (i.p.). Significance was determined by one-way ANOVA with post hoc Tukey's multiple comparison test. Data are presented as means ± standard errors of the mean (SEM). ### $p < 0.001$ significance compared with the control (white bar). *** $p < 0.001$, ** $p < 0.01$, significance compared with Sco-treated groups (black bar).

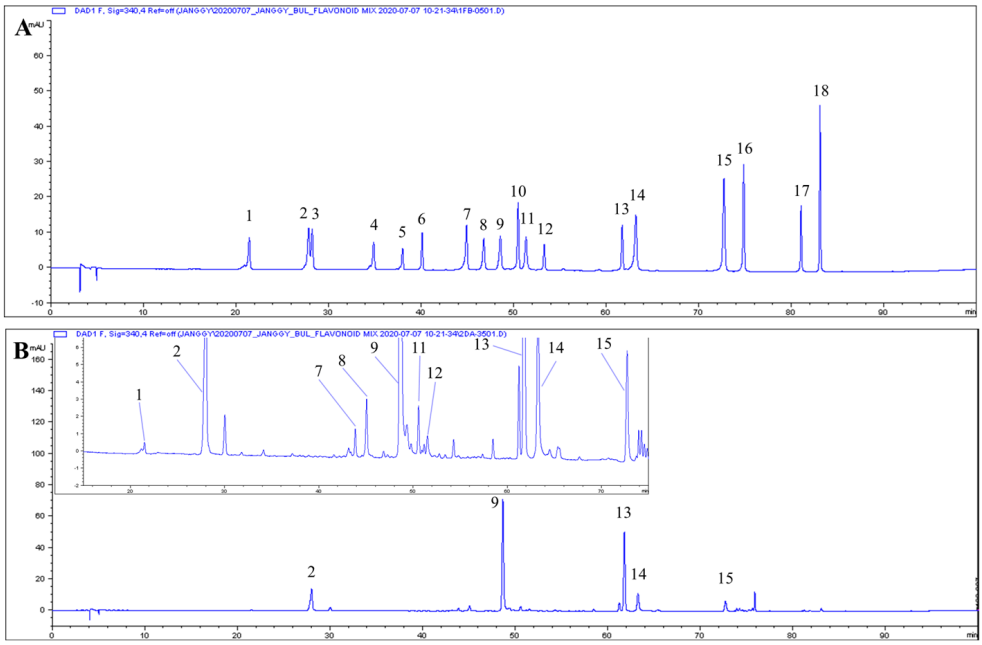

Figure 7. Typical chromatogram of extract of *Chrysanthemum boreale* (Makino) Makino (CB) flower. Samples: (**A**), standard mixture; (**B**), extract from CB. Standards: 1, neochlorogenic acid; 2, chlorogenic acid; 3, 4-*O*-caffeoylquinic acid; 4, 1,3-di-*O*-caffeoylquinic acid; 5, vicenin-1, 6, isochftoside; 7, luteolin 7-*O*-glucoside; 8, isochlorogenic acid B; 9, isochlorogenic acid A; 10, apigenin 7-*O*-glucoside; 11, isochlorogenic acid C; 12, diosmetin 7-*O*-glucoside; 13, linarin; 14, luteolin; 15, apigenin; 16, diosmetin; 17, eupatorine, 18, acacetin.

Table 1. Phenolic composition of flower extract of *Chrysanthemum boreale* (Makino) Makino.

RT (min)	Phenolic	Content (mg/g Extract, d.b.)
21.40	Neochlorogenic acid	0.14 ± 0.002
27.88	Chlorogenic acid	7.37 ± 0.286
44.92	Luteolin 7-*O*-glucoside	1.39 ± 0.082
46.78	Isochlorogenic acid B	0.21 ± 0.018
48.58	Isochlorogenic acid A	34.79 ± 1.043
50.46	Apigenin 7-*O*-glucoside	0.88 ± 0.060
51.38	Isochlorogenic acid C	0.72 ± 0.013
53.28	Diosmetin 7-*O*-glucoside	0.14 ± 0.007
61.67	Linarin	24.03 ± 1.853
63.16	Luteolin	3.85 ± 0.137
72.65	Apigenin	1.53 ± 0.173
74.79	Diosmetin	0.15 ± 0.015
83.02	Acacetin	0.15 ± 0.020

4. Discussion

AD is a neurodegenerative disorder characterized by cognitive decline and memory loss as the disease progresses [1,3,4]. Factors such as neuroinflammation and Aβ/tau proteins are involved in the pathogenesis of the disease [5,6]. Inflammatory reactions are related to the accumulation of Aβ proteins and the activation of microglia. Aβ activates microglia, which in turn releases neurotoxic factors such as NO and tumor necrosis factor-α [7,11]. The administration of inflammatory cytokines released from microglia causes deficits in spatial memory, and the increased expression of cytokines such as tumor necrosis factor-α leads to decreased performance in memory tasks such as passive avoidance [39]. Soluble Aβ and amyloid plaques, which contribute to inflammation, could also induce oxidative stress, hyperphosphorylation of tau protein, and excitotoxicity, leading to cell death and neurotransmitter deficits, such as that of ACh [3]. Under pathological conditions, aberrant post-translational modifications, including phosphorylation, produce pathological tau, which leads to conformational changes, aggregation, formation of neurofibrillary tangles, and synaptic dysfunction in AD [38]. In early disease processes, hyperphosphorylated tau localized in dendritic spines can affect the trafficking of postsynaptic receptors and accompany the formation of neurofibrillary tangles, which are closely associated with cognitive decline [3].

This study investigated the potential of CB extract as a cognition-/memory-improving functional material. Treatment of LPS-treated BV2 cells with CB extract (at a final concentration of 6.25–50 μg/mL) decreased the levels of NO by 6.5–27.1%, as compared to that observed upon no treatment. In addition, treatment with CB extract (at final concentrations of 1.25, 2.5, and 5 μg/mL) inhibited the production of p-Tau in Aβ-treated SH-SY5Y cells by 4.9%, 12.9%, and 35.0%, respectively, compared to that observed upon no treatment. Thus, the in vitro experiments indicated that the CB extract has an inhibitory effect on inflammation and tau phosphorylation in cells, which in turn could have some potential to suppress neurodegenerative disorders.

Dysfunction, loss of synapses, and cholinergic neurons are involved in AD progression and loss of memory and attention in the condition [3,40,41]. Deficiency in the brain neurotransmitter Ach and loss of cortical and hippocampal ChAT activity has been correlated with the severity and duration of the disease. Some studies have shown that the hippocampal ChAT activity in Aβ-treated rats is increased by luteolin [42,43]. Therefore, it is considered that biologically active components could significantly influence the cholinergic system.

In the present study, the memory-enhancing potential of the CB extract was evaluated in animals chronically and acutely treated with Sco as AD models. Upon analysis of the effect of CB extract on the cholinergic system, the NC group showed a decrease in the hippocampal ChAT level (1.00), compared to the normal group (2.52); however, the CB extract-treated groups had higher hippocampal ChAT values (2.19 and 2.16). The serum

ACh level in the NC group was lower than that in the normal group, whereas the value of CBH increased higher than in the NC group. These results implied that CBH treatment may play a positive role in cholinergic signal transmission.

Among the synaptic activity-related proteins in the brain, ERK1/2 is known to play a critical role in hippocampal synaptic plasticity, learning, and memory. As such, abnormal ERK1/2 activation in the hippocampus may impair hippocampal function and contribute to memory deficits in AD patients [44]. BDNF, which is located in the cortex and hippocampus as a growth factor in the nervous system, is transported from the entorhinal cortex to the hippocampus and is associated with memory [45]. After the loss of basal forebrain cholinergic neurons, hippocampal BDNF levels subsequently decrease. Decreased levels of BDNF in the hippocampus and partial cortex of the AD group, compared to that in the control group, have been reported [39,46].

In the present study, when the expression of proteins related to synaptic function was measured in a rat model chronically administered Sco, the p-ERK/ERK ratio in the hippocampus of the NC group was higher than that in the hippocampus of the normal group. The level in the CB-extract-administered group, especially the CBH group, was close to that in the normal group, which was lower than that in the Donepezil group. With respect to BDNF expression, the CBL, CBH, and Donepezil groups showed an increasing tendency, compared to the normal and NC groups. The CBH rats displayed the highest values among the experimental groups. These results indicated that CB extract may enhance memory through actions related to synaptic protein expression.

Three tests, namely, the Y-maze, PAT, and MWM tests, were used to measure the efficacy of the CB extract on behavior related to memory and learning. While the Y-maze and PAT tests were conducted in rats chronically treated with Sco, the MWM test was performed in mice acutely treated with Sco. The Y-maze test, which is used to observe immediate short-term spatial memory and alteration tasks, indicated that both the CB-extract-administered groups ameliorated the spontaneous alteration of NC rats to the same levels as those observed in the normal and Donepezil groups. In the PAT, which is a behavioral task widely employed to assess learning and memory [47], the reduced escape latency observed in the NC rats, compared to that in the normal rats, was alleviated in the CB-extract-administered groups, especially CBH. Furthermore, the MWM test was conducted via two routes: the learning test conducted between the 17th and 20th days and the FS time test conducted on the 5th day. In the learning test, the escape time of all the groups, except the NC group, decreased as the days progressed. In the FS time test, the CB-extract-administered groups displayed significantly increased FS times (156.1% and 151.7%), compared to the NC group (100%). These results indicated that the CB extract could exert activity related to spatial memory in an acute amnesia AD model [34].

In addition, measurements using high-performance liquid chromatography were quantitatively conducted to evaluate the components of the CB extract that affect its activity. From the evaluation, it was confirmed that the CB extract contained good amounts of isochlorogenic acid A (3,5-dicaffeoylquinic acid, 3,5-DCQA), linarin, chlorogenic acid, and luteolin (34.79, 24.03, 7.37, and 3.85 mg/g extract, respectively). It is reported that 3,5-dicaffeoylquinic acid isolated from *Ligularia fischei* leaves showed anti-inflammation activity in Raw264.7 cells and CQA treatment alleviated cognitive impairment in APP/PS1 mice by affecting the activation of some signaling pathways [48,49]. It has been suggested that caffeoylquinic acids, including isochlorogenic acid A and chlorogenic acid, could improve cognitive functions to some degree, and an intake of chlorogenic acids for six months may enhance memory function in patients suffering from memory loss [50,51]. It is also reported that CQAs inhibited Aβ transformation into β-sheets [52]. Linarin, a flavonoid, is known to down-regulate pro-inflammatory cytokine production and to exhibit the remedial effects on nervous system disorders and to inhibit AChE activity [37,53–55]. Therefore, it is considered that isochlorogenic acid A, linarin, and chlorogenic acid, the major components of the CB extract, could play important roles related to the activity of the CB extract in memory improvement.

5. Conclusions

The present study demonstrated that the CB flower extract, which has inhibitory effects on inflammation and p-Tau production in cells, can alleviate memory deficits by modulating neurotransmitter and protein expression related to synaptic function in Sco-treated animal models, functions seemingly mediated by CQAs including isochlorogenic acids and a flavonoid such as linarin. In conclusion, the CB flower is a potential candidate for use as a functional material for improving cognition or memory.

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