

Black Currant Extract Protects against Oxidative Stress in SH-SY5Y Cells and High-Fat Diet-Induced Cognitive Impairment in C57BL/6 Mice

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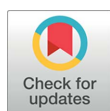
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Black currant (*Ribes nigrum* L.) is rich in anthocyanins and other phenolic compounds with diverse health benefits. This study investigated the neuroprotective effects of black currant extract (BCE) using integrated *in vitro* and *in vivo* approaches. UHPLC-MS/MS analysis identified four major anthocyanins in BCE: delphinidin 3-O-glucoside, delphinidin 3-O-rutinoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside. *In vitro*, BCE protected SH-SY5Y neuroblastoma cells against H₂O₂-induced oxidative stress in a dose-dependent manner, restoring cell viability to 77% of control levels at 50 mg/l. BCE also exhibited dose-dependent inhibition of acetylcholinesterase and butyrylcholinesterase, demonstrating cholinergic-enhancing properties. *In vivo*, C57BL/6 mice were fed a high-fat diet (HFD) for 4 weeks to induce obesity, followed by 4 weeks of oral BCE administration (50 or 200 mg/kg/day) with continued HFD feeding. Cognitive function was assessed using the Y-maze, passive avoidance, and Morris water maze tests. BCE treatment (50 mg/kg/day) significantly increased the time spent in the target quadrant compared with the HFD-only group ($p < 0.05$), suggesting that BCE mitigates HFD-induced cognitive impairment by enhancing spatial memory consolidation. BCE also showed trends toward improved performance in Y-maze and passive avoidance tests, though these did not reach statistical significance. These findings demonstrate that BCE exerts multi-target neuroprotective effects through antioxidant activity, cholinesterase inhibition, and amelioration of obesity-associated cognitive deficits, supporting its potential as a functional food ingredient for cognitive health in metabolic syndrome.

Keywords: Anthocyanin, Cholinesterase, Morris water maze, Passive avoidance, Y-maze



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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by neuronal loss and cognitive decline, including memory impairment and executive dysfunction [1, 2]. The pathophysiology of AD involves multiple interconnected mechanisms, including oxidative stress, protein aggregation, and neurotransmitter deficits. Excessive reactive oxygen species (ROS) generation plays a central role in AD pathogenesis, exacerbated by amyloid- β plaque deposition, tau protein hyperphosphorylation, and acetylcholine depletion [3, 4]. Elevated ROS induces neuronal damage through multiple pathways, including lipid peroxidation, DNA oxidation, mitochondrial dysfunction, and apoptosis [3, 5, 6]. The brain is particularly vulnerable to oxidative stress due to its high metabolic rate, abundant polyunsaturated fatty acids susceptible to lipid peroxidation, dense mitochondrial content, limited neuronal regenerative capacity, and relatively low antioxidant enzyme levels [3, 4, 7, 8].

The global prevalence of obesity has increased dramatically, emerging as a critical public health challenge with far-reaching consequences beyond metabolic dysfunction. Emerging evidence

identifies obesity as a critical risk factor for AD, demonstrating its role in accelerating both the onset and clinical progression of neurodegeneration [9, 10]. Chronic inflammation resulting from obesity drives neuroinflammation through peripheral cytokine signaling that activates brain microglial cells, which in turn generate NADPH oxidase-mediated ROS [6, 10, 11]. Concurrently, obesity-associated metabolic disturbances, including mitochondrial dysfunction, impaired glucose metabolism, and insulin resistance, further amplify oxidative stress in the brain [10, 12]. This convergence of peripheral metabolic dysfunction and central nervous system oxidative damage establishes obesity as a modifiable risk factor for cognitive decline, highlighting the therapeutic potential of interventions targeting obesity-related neurodegeneration.

Dietary phenolic compounds, including phenolic acids and flavonoids, show significant promise as neuroprotective agents against AD. Preclinical studies demonstrate that phenolic compounds protect neurons from oxidative stress through multiple mechanisms: ROS scavenging, antioxidant enzyme activation, neuroinflammation suppression, and cholinesterase inhibition [12-14]. Compared with conventional single-target drugs, phenolic compounds offer advantages such as multiple pathway modulation, favorable safety profiles, and dietary accessibility [15-18]. This substantiates plant-derived phenolic compounds as attractive candidates for preventing and treating neurodegenerative diseases, warranting systematic identification and characterization of botanical sources rich in these bioactive compounds [17, 18].

Black currant (*Ribes nigrum* L.) is rich in bioactive compounds, particularly anthocyanins (delphinidin and cyanidin glycosides), phenolic acids, and vitamins, conferring diverse health benefits [19-22]. Its anthocyanins exhibit robust antioxidant activity, cholinesterase inhibition, and neuroprotective effects, including amelioration of scopolamine-induced cognitive deficits [23-25]. Although black currant has been investigated in the context of cancer [26], diabetes [27], and obesity [22], its potential to ameliorate memory and learning deficits under high-fat diet (HFD)-induced metabolic stress, a clinically relevant model of metabolic syndrome-associated neurodegeneration, remains unexplored.

This study investigated the neuroprotective effects of black currant extract (BCE) using complementary *in vitro* and *in vivo* approaches. We identified the major anthocyanins in BCE, evaluated its cholinesterase inhibitory activity and protection against H₂O₂-induced oxidative stress in SH-SY5Y neuroblastoma cells, then assessed its efficacy in ameliorating HFD-induced cognitive impairment in mice. Cognitive functions were assessed using three behavioral tests: the Y-maze (spatial working memory), passive avoidance (fear-associated memory), and Morris water maze (spatial learning and long-term memory). This integrated approach was designed to establish whether the neuroprotective properties of BCE translate to measurable cognitive benefits in the context of obesity-associated neurodegeneration.

Materials and Methods

Chemicals and Reagents

Hydrogen peroxide (H₂O₂), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acetylcholinesterase (AChE), butyrylcholinesterase (BChE), acetylthiocholine iodide (ATCI), butyrylthiocholine chloride (BTCC), 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate (tacrine), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Dulbecco's phosphate-buffered saline (DPBS), and phosphate-buffered saline (PBS) were purchased from Sigma Aldrich Co., LLC (USA). Minimum Essential Medium Eagle (MEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Welgene Inc. (Republic of Korea). All other chemicals were of analytical or high-performance liquid chromatography (HPLC) grade. BCE was obtained from Artemis International (USA). According to the manufacturer, the extract contains approximately 25% anthocyanins.

Identification of Anthocyanins in Black Currant Extract (BCE)

Anthocyanins were identified using HPLC with diode array detection (HPLC-DAD; Agilent 1100 series, Agilent Technologies, Inc., USA). Separation was performed on a reversed-phase C18 column (ZORBAX Eclipse XDB-C18, 150 mm × 4.6 mm, 5 μm; Agilent Technologies, Inc.) using 8% (v/v) formic acid in water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 1 ml/min with an injection volume of 20 μl. The gradient elution program followed a previously established method [28]. Anthocyanins were detected at 520 nm.

For structural confirmation, anthocyanins were analyzed by UHPLC coupled with Orbitrap XL mass spectrometry (Accela UHPLC system, Thermo Fisher Scientific, USA). Separation was achieved using a reversed-phase C18 column (ACQUITY BEH C18, 2.1 mm × 150 mm, 1.7 μm; Waters Corp., Ireland) using 4% (v/v) formic acid in water (mobile phase C) and 4% (v/v) formic acid in methanol (mobile phase D) at a flow rate of 0.5 ml/min and an injection volume of 2 μl. The gradient program was: 95% C (0-2 min), 95-80% C (2-6 min), 80% C (6-10 min), 80-77% C (10-12 min), 77-70% C (12-15 min), 70-55% C (15-18 min), 55-47% C (18-20 min), 47-20% C (20-25 min), 20-0% C (25-25.1 min), and 0% C (25.1-27 min). Mass spectrometry (MS) was performed in positive ion mode using heated electrospray ionization (HESI) with a spray voltage of 5.0 kV, capillary voltage of 35 V, and source temperature of 275°C. Data were acquired using an Orbitrap analyzer with Fourier transform MS at a resolution of 60,000 at *m/z* 400. Anthocyanins were identified by comparing retention times, UV spectra, high-resolution *m/z* values, and MS/MS fragment ions with authentic standards and published data.

Determination of Cholinesterase Inhibition of BCE

Cholinesterase inhibition activity was evaluated using AChE and BChE enzymes with their respective substrates, ATCI and BTCC. DTNB served as the chromogenic reagent. For the AChE inhibition assay, BCE (20 μl at various concentrations) was mixed with DPBS (150 μl), ATCI substrate (20 μl, 15 mM), and DTNB (30 μl, 10 mM). After incubation at 37°C for 10 min, AChE (20 μl, 0.2 U/ml) was added to initiate the reaction. The BChE inhibition assay followed the same procedure, except BChE (0.065 U/ml) and BTCC (10 mM) were used instead of AChE and ATCI. After 30-min reaction at 37°C, absorbance was measured at 415 nm using a microplate reader (Infinite M200; Tecan Austria GmbH, Austria). DPBS replaced the sample as a control, and tacrine was used as the positive control. Inhibitory activities were calculated as follows: Inhibitory activity (%) = [1 - (absorbance_{sample} / absorbance_{control})] × 100

Neuroprotective Effect of BCE in SH-SY5Y Cells

SH-SY5Y human neuroblastoma cells (ATCC, USA) were cultured in MEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂ (BB 15 CO₂ incubator; Thermo Electron LED GmbH, Germany).

Neuroprotective effects against oxidative stress were assessed using the MTT assay. SH-SY5Y cells were seeded at 1 × 10⁴ cells/well in 96-well plates and incubated for 24 h. After medium removal, cells were treated with various concentrations of BCE in serum-free MEM for 24 h, followed by exposure to 20 μM H₂O₂ in serum-free MEM for an additional 24 h to induce oxidative stress. MTT reagent was then added and incubated for

3 h. The resulting formazan crystals were dissolved in DMSO (50 μ l), and absorbance was measured at 570 nm (test wavelength) and 630 nm (reference wavelength) using a microplate reader (Infinite M200; Tecan Austria GmbH). Cell viability was calculated as a percentage relative to untreated control cells.

Animals and Experimental Design

Male C57BL/6 mice ($n = 32$, 4 weeks old) were obtained from the Animal Facility of Aging Science at the Korea Basic Science Institute (Republic of Korea) and housed in groups of 2-3 per cage under standard conditions (12-h light/dark cycle, 23°C, and 50% humidity). Mice were randomly assigned to four experimental groups ($n = 8$ /group): (1) control (chow diet), (2) HFD (HFD only), (3) BCE50 (HFD + 50 mg BCE/kg body weight [BW]/day), and (4) BCE200 (HFD + 200 mg BCE/kg BW/day).

After a one-week acclimation period, all mice except the control group were fed an HFD (D12492; Research Diets, USA; composition detailed in Table S1) for four weeks to induce obesity. Beginning in week 5, BCE was administered orally by gavage at the specified doses in tap water daily for four consecutive weeks (Fig. 1). Control and HFD groups received an equivalent volume of tap water. BCE solutions were prepared fresh daily. BW was recorded weekly throughout the study. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Gyeongsang National University (approval number: GNU-190530-M0028; approval date: May 30, 2019) and conducted in accordance with the guidelines of the Policy of the Ethical Committee of the Ministry of Health and Welfare, Republic of Korea.

Y-Maze Test

The Y-maze test was conducted to evaluate short-term spatial working memory and exploratory behavior. The apparatus consisted of three identical arms (33 cm length $\times$ 15 cm height $\times$ 10 cm width) arranged at 120° angles and randomly labeled A, B, and C. Each mouse was placed at the end of one arm and allowed to explore freely for 8 min while being recorded by an overhead camera. Mouse movements were tracked using the SMART video tracking system (SMART v3.0; Panlab, Spain).

Arm entry was recorded when all four paws of the mouse entered an arm. Spontaneous alternation was defined as consecutive entries into all three different arms in overlapping triplet sets (e.g., ABC, BCA, CAB in the sequence ABCABAB). The alternation percentage was calculated as: Alternation (%) = [Number of spontaneous alternations / (Total arm entries - 2)] $\times$ 100

Passive Avoidance Test

The step-through passive avoidance test was performed to assess fear-associated learning and long-term avoidance memory. The apparatus consisted of two compartments separated by an automated guillotine door: a brightly lit chamber and a dark chamber equipped with a stainless steel grid floor capable of delivering electric shocks.

The test consisted of two phases conducted over two consecutive days. On day 1 (training trial), each mouse was placed in the lit chamber and allowed to acclimate for 2 min. The door to the dark chamber was then opened. When the mouse entered the dark chamber, the door closed automatically, and an electric foot shock (0.5 mA, 3 s) was delivered through the grid floor. Mice were immediately returned to their home cages after shock delivery.

On day 2 (retention trial, 24 h post-training), each mouse was again placed in the lit chamber, and the door to the dark chamber was opened. The step-through latency (the time taken to re-enter the dark chamber) was recorded with a maximum cutoff time of 300 s. Longer latencies indicate better retention of the aversive memory.

Morris Water Maze

The Morris water maze was used to assess spatial learning and long-term memory. The apparatus consisted of a circular stainless-steel pool (90 cm diameter) filled with water ($22 \pm 2^\circ\text{C}$) made opaque with skim milk. The pool was divided into four equal quadrants (N, S, E, W) with distinct visual cues positioned on the surrounding walls to aid spatial navigation. A circular escape platform (6 cm diameter) was submerged 1 cm below the water surface in the center of the W (target) quadrant and remained in this fixed location throughout training.

During the training phase, mice underwent four consecutive days of training, with four trials per day. In each trial, mice were placed facing the pool wall in one of the four quadrants (start positions varied across trials) and allowed to swim for a maximum of 60 sec to locate the hidden platform. Upon finding the platform, mice were allowed to remain on it for 20 sec before being returned to their home cages. Mice that failed to locate the platform within 60 sec were gently guided to it and allowed to remain for 20 sec. Escape latency (time to reach the platform) was recorded for each trial.

Twenty-four hours after the final training session, the platform was removed, and a single 90-sec probe trial was conducted. Mice were placed in the quadrant opposite to the target quadrant and allowed to swim freely. Swimming paths were recorded using an overhead camera and analyzed with the SMART video tracking system (SMART v3.0; Panlab). Time spent in the target quadrant was quantified as an index of memory retention.

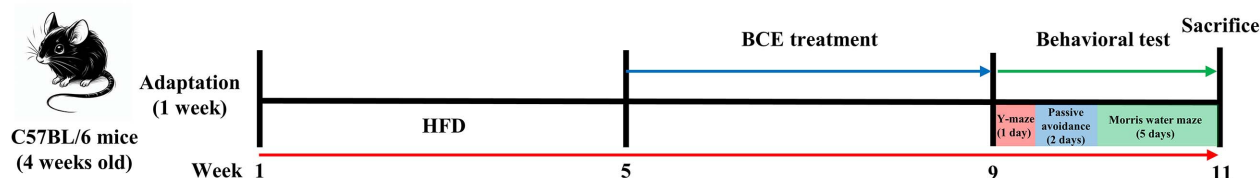


Fig. 1. Experimental timeline for the study. Male C57BL/6 mice were fed HFD for 4 weeks to induce obesity, followed by 4 weeks of BCE treatment (50 or 200 mg/kg/day, *p.o.*) with continued HFD feeding. Behavioral tests were conducted during weeks 9 and 10. BCE, black currant extract; HFD, high-fat diet; *p.o.*, *per os* (oral administration).

Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics (version 23.0; IBM Corp., USA). Group differences were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was set at $p < 0.05$.

Results and Discussion

Identification of Anthocyanins in BCE using HPLC-DAD and LC-MS/MS

HPLC-DAD analysis revealed four major anthocyanins in BCE (Fig. 2): delphinidin 3-*O*-glucoside (peak 1), delphinidin 3-*O*-rutinoside (peak 2), cyanidin 3-*O*-glucoside (peak 3), and cyanidin 3-*O*-rutinoside (peak 4). Structural identities were confirmed by LC-MS/MS analysis (Table 1). The 3-*O*-glucosides of delphinidin and cyanidin (peaks 1 and 3) exhibited characteristic MS/MS fragmentation patterns with loss of the hexosyl moiety (-162 Da), yielding aglycone fragments at m/z 303 (delphinidin) and m/z 287 (cyanidin), respectively. The 3-*O*-rutinosides of delphinidin and cyanidin (peaks 2 and 4) displayed fragmentation corresponding to loss of a rhamnosyl unit (-146 Da) and the entire rutinosyl moiety (-308 Da). These MS/MS fragmentation patterns are consistent with a previous report [29], confirming the structural identities of the four major anthocyanins in BCE.

The relative proportions of the four major anthocyanins in the BCE were calculated based on their respective peak areas (Table S2). Delphinidin 3-*O*-rutinoside was identified as the most prominent component, accounting for 36.2%, followed by cyanidin 3-*O*-rutinoside at 33.6%, delphinidin 3-*O*-glucoside at 20.7%, and cyanidin 3-*O*-glucoside at 9.5%. This distribution pattern aligns with the anthocyanin profile of standardized black currant extract reported in a previous study [22], where these four major anthocyanins were quantified at 120.7, 81.3, 71.1, and 25.0 mg/g dry weight, respectively, resulting in a total anthocyanin content of approximately 298.1 mg/g dry weight. Although absolute quantification was not performed in the present study, the similarity in the relative peak area percentages indicates that our BCE sample retains a representative anthocyanin profile typical of this standardized material.

Cholinesterase Inhibition by BCE

BCE exhibited dose-dependent AChE inhibitory activity, ranging from 9.6% to 54.2% at concentrations of 16 to 250 mg/l (Fig. 3A). BChE inhibition increased dose-dependently up to 500 mg/l (46.6%), but showed no further significant increase at 1,000 mg/l (48.3%), indicating saturation of the inhibitory effect at higher concentrations (Fig. 3B). These results demonstrate that BCE possesses substantial cholinesterase inhibitory activity.

Cholinesterase inhibition is closely linked to cognitive function, as elevated cholinesterase activity accelerates the degradation of acetylcholine, which contributes to cognitive dysfunction [4]. Phenolic compounds, including anthocyanins, are known to inhibit AChE through competitive, noncompetitive, and mixed-type mechanisms [16, 25]. Notably, cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside, major anthocyanins identified in BCE (Fig. 2, Table 1), have been shown to inhibit both AChE and BChE [25]. The cholinesterase inhibitory effects observed in this study are consistent with previous reports on black currant extracts [19, 30], further supporting the potential of BCE as a source of bioactive compounds for cognitive health.

Neuroprotective Effects of BCE against H₂O₂-Induced Oxidative Stress in SH-SY5Y Cells

The protective potential of BCE was evaluated by measuring cell viability in SH-SY5Y human neuroblastoma cells exposed to oxidative stress. Treatment with H₂O₂ (20 μ M) significantly reduced cell viability to 34.1% compared with untreated controls (Fig. 3C). However, treatment with

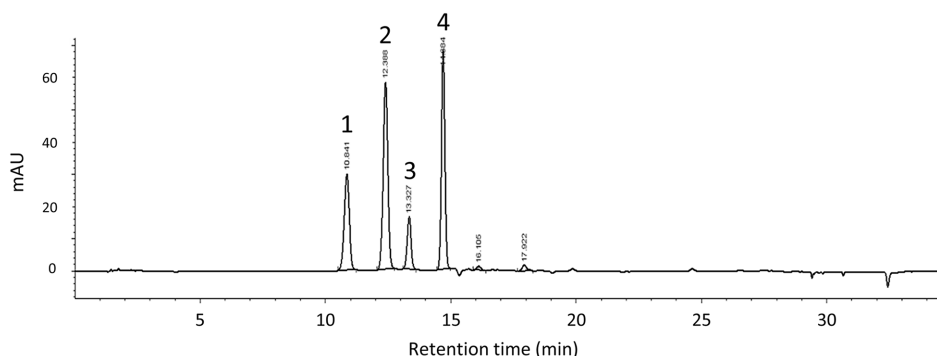


Fig. 2. HPLC chromatogram of anthocyanins in black currant extract at 520 nm. Peaks: 1, delphinidin 3-*O*-glucoside; 2, delphinidin 3-*O*-rutinoside; 3, cyanidin 3-*O*-glucoside; 4, cyanidin 3-*O*-rutinoside.

Table 1. Identification of anthocyanins in black currant extract determined by UHPLC-Orbitrap-mass spectrometry.

Peak	Compound	Molecular formula	Theoretical m/z	Measured m/z	Mass error (δ ppm)	MS/MS fragment
1	Delphinidin 3- <i>O</i> -glucoside	[C ₂₁ H ₂₁ O ₁₂] ⁺	465.1028	465.1032	0.860	303
2	Delphinidin 3- <i>O</i> -rutinoside	[C ₂₇ H ₃₁ O ₁₆] ⁺	611.1607	611.1610	0.491	465, 303
3	Cyanidin 3- <i>O</i> -glucoside	[C ₂₁ H ₂₁ O ₁₁] ⁺	449.1078	449.1083	1.113	287
4	Cyanidin 3- <i>O</i> -rutinoside	[C ₂₇ H ₃₁ O ₁₅] ⁺	595.1658	595.1661	0.504	449, 287

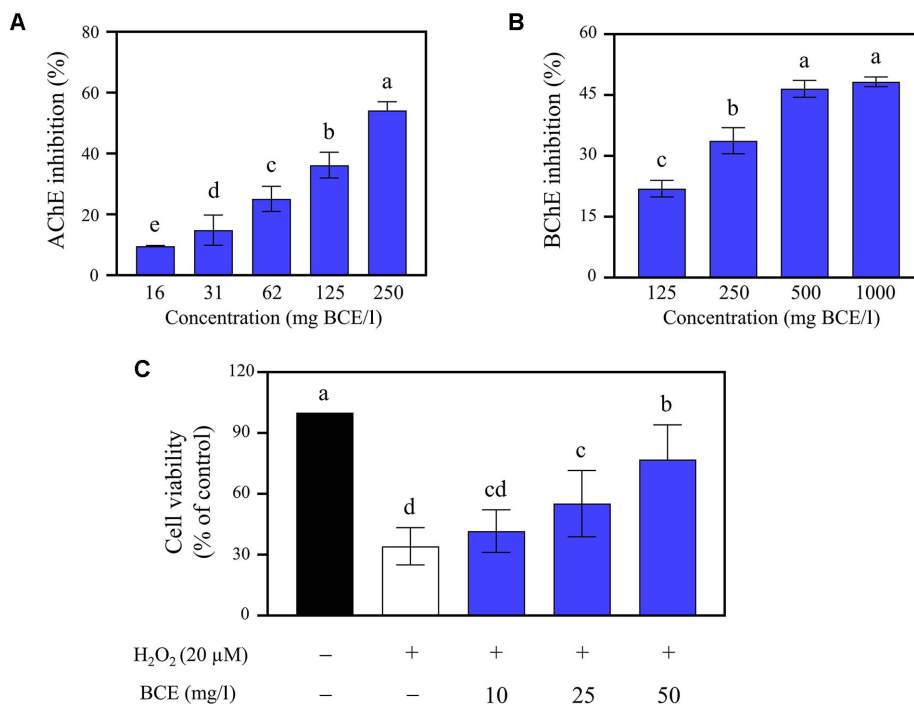


Fig. 3. Effects of BCE on cholinesterase activity and cell viability. (A) AChE inhibition, (B) BChE inhibition, and (C) protective effect against H₂O₂-induced oxidative stress in SH-SY5Y cells. The control group was normalized to 100%. Data are presented as mean ± SD. Bars that do not share any letters indicate significant differences among groups, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$). AChE, acetylcholinesterase; BCE, black currant extract; BChE, butyrylcholinesterase.

BCE at 10, 25, and 50 mg/l restored cell viability to 41.6%, 55.2%, and 77.0% of control levels, respectively (Fig. 3C), demonstrating significant protection against oxidative stress.

These findings are consistent with previous studies demonstrating neuroprotective effects of black currant extracts in SH-SY5Y cells against oxidative stress [31, 32]. The cytoprotective activity observed may be attributed to the anthocyanin content of BCE, particularly delphinidin and cyanidin glycosides (Fig. 2, Table 1), which possess potent antioxidant and free radical scavenging properties. Based on these *in vitro* results and considering variations in phytochemical composition and bioavailability between *in vitro* and *in vivo* systems, doses of 50 and 200 mg/kg BW/day were selected as the low and high doses, respectively, for subsequent animal studies to evaluate the neuroprotective efficacy of BCE.

Changes in Body Weight in HFD-Fed C57BL/6 Mice

BW changes throughout the experimental period are presented in Table 2. At baseline (week 1), no significant differences in BW were observed among the four groups. After 4 weeks of HFD feeding (week 5, before BCE administration), the three HFD-fed groups (HFD, BCE50, and BCE200) exhibited significantly higher BWs (37.8, 36.2, and 36.2 g, respectively) compared with the control group (27.0 g; Table 2), confirming successful induction of diet-induced obesity.

At the end of the experiment, BWs in the HFD, BCE50, and BCE200 groups were approximately 1.5-, 1.3-, and 1.4-times higher than the control group, respectively (Table 2). Notably, BCE administration attenuated HFD-induced weight gain: the BCE50 and BCE200 groups exhibited BWs that were 84.6% and 95.4% of the HFD group, respectively, with the BCE50 group showing a statistically significant reduction compared with the HFD group. These results indicate that BCE, particularly at the lower dose, partially mitigates HFD-induced weight gain.

High-calorie diets promote excessive weight gain and fat accumulation, leading to insulin resistance and elevated inflammatory cytokine

Table 2. Effects of black currant extract (BCE) on body weight in high-fat diet (HFD)-fed C57BL/6 mice.

Group	Body weight (g)		
	Baseline (week 1)	After HFD feeding (week 5)	After BCE treatment (week 9)
Control	22.3 ± 0.8 aC	27.0 ± 1.1 bB	30.0 ± 1.5 cA
HFD	24.5 ± 0.8 aC	37.8 ± 1.9 aB	45.4 ± 2.6 aA
BCE50	24.6 ± 1.5 aB	36.2 ± 0.8 aA	38.4 ± 1.5 bA
BCE200	24.4 ± 1.5 aC	36.2 ± 2.5 aB	43.3 ± 3.6 abA

Data are expressed as mean ± SD ($n = 8$ per group). Means that do not share lowercase and uppercase letters within the same column and row, respectively, are significantly different according to Tukey's multiple comparison test ($p < 0.05$). BCE50 and BCE200: HFD-fed mice receiving 50 and 200 mg BCE/kg/day, respectively.

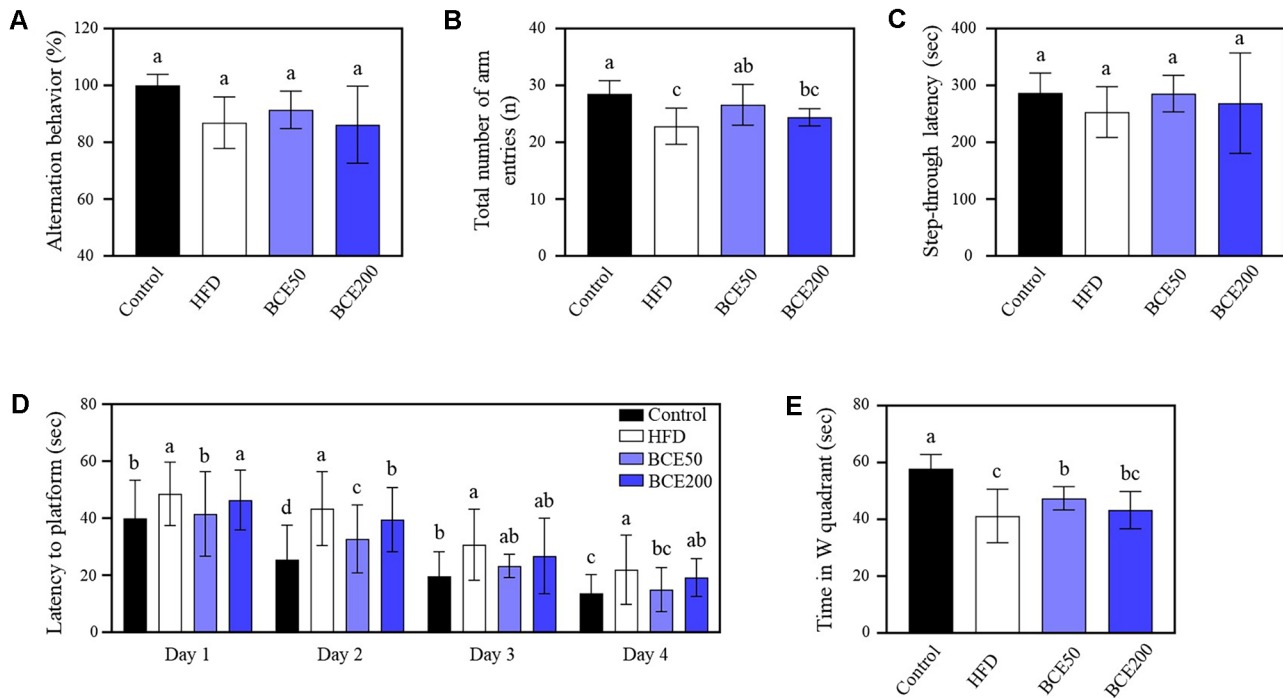


Fig. 4. Effects of BCE on cognitive function in high-fat diet-fed mice. (A) Spontaneous alternation and (B) total arm entries in the Y-maze test; (C) step-through latency in the passive avoidance test; (D) escape latency during the training session and (E) time in the target (W) quadrant during the probe test in the Morris water maze. Data are presented as mean $\pm$ SD ($n = 8$ per group). Bars (means) that do not share any letters indicate significant differences among groups, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$). BCE, black currant extract; HFD, high-fat diet only; BCE50, HFD + 50 mg BCE/kg/day; BCE200, HFD + 200 mg BCE/kg/day

secretion [9, 10, 33]. These metabolic disturbances are closely linked to cognitive impairment [9]. In the present study, HFD feeding resulted in significant weight gain; however, BCE administration, especially at 50 mg/kg BW/day, significantly attenuated this effect (Table 2). The BW-lowering effect of BCE may contribute to alleviating obesity-associated metabolic dysfunction, potentially preserving cognitive function through reduction of systemic inflammation and metabolic stress.

Effects of BCE on Short-Term Spatial Memory: Y-Maze Test

Based on the neuroprotective effects observed *in vitro*, we hypothesized that BCE could ameliorate cognitive impairment *in vivo*. To test this hypothesis, cognitive dysfunction was evaluated in HFD-fed C57BL/6 mice using three behavioral paradigms: the Y-maze test, passive avoidance test, and Morris water maze (Fig. 4).

Short-term spatial working memory was assessed using the Y-maze spontaneous alternation test. Although no statistically significant differences in alternation behavior were observed among groups (Fig. 4A), the HFD group exhibited numerically lower alternation (86.9%) compared with the control group. Treatment with BCE at 200 mg/kg BW/day resulted in similar alternation (86.2%) to the HFD group, whereas BCE at 50 mg/kg BW/day resulted in higher alternation (91.4%), approaching control levels. These trends suggest a potential dose-related effect of BCE on spatial working memory, though statistical significance was not achieved.

The total number of arm entries, an index of locomotor activity and exploratory behavior, was reduced in HFD-fed groups compared with controls (Fig. 4B). The HFD, BCE50, and BCE200 groups exhibited 80.1%, 93.3%, and 85.6% of control arm entries, respectively. Notably, the BCE50 group showed significantly higher arm entries compared with the HFD group ($p < 0.05$), reaching levels comparable to controls, indicating that low-dose BCE preserves exploratory activity in HFD-fed mice.

The Y-maze test exploits the innate tendency of rodents to explore novel arms rather than recently visited ones, providing a measure of short-term spatial working memory. HFD-induced obesity impairs cognitive function through multiple mechanisms, including cholinergic dysfunction and oxidative stress-mediated disruption of antioxidant enzyme systems [14]. These impairments manifest as reduced spontaneous alternation, reflecting a failure to remember recently visited locations.

In the present study, the HFD group exhibited trends toward impaired short-term spatial working memory, evidenced by numerically lower alternation behavior and significantly reduced total arm entries compared with the control group (Fig. 4A and 4B). Although alternation differences did not reach statistical significance, BCE treatment, particularly at 50 mg/kg BW/day, showed numerical improvement in alternation behavior (91.4% vs. 86.9% in HFD), and significantly increased arm entries, suggesting partial restoration of exploratory activity and spatial memory function.

Effects of BCE on Long-Term Aversive Memory: Passive Avoidance Test

Long-term fear-associated memory was assessed using the step-through passive avoidance test (Fig. 4C). During the acquisition phase (day 1), all mice readily entered the dark chamber upon door opening, where they received an electric foot shock. In the retention test 24 h later (day 2), step-through latency served as an index of aversive memory retention.

The control group exhibited the longest latency (286.7 sec), indicating strong memory retention of the aversive experience (Fig. 4C). The HFD group showed reduced latency (253.1 sec, 88.3% of control), suggesting impaired long-term memory, though this difference did not reach statistical significance. BCE treatment resulted in numerical improvements: the BCE50 group (285.6 sec) showed latency nearly identical to controls, while BCE200 (268.7 sec) exhibited intermediate values. However, no statistically significant differences were detected among groups ($p > 0.05$).

The passive avoidance test exploits rodents' innate preference for dark environments while assessing their ability to suppress this behavior based on aversive memory. HFD-induced obesity has been consistently associated with impaired performance in this task, reflecting deficits in hippocampal- and amygdala-dependent emotional memory [34]. Phytochemical interventions, particularly those with antioxidant and anti-inflammatory properties, have shown promise in ameliorating HFD-induced cognitive deficits in this paradigm [12-14]. In the present study, the HFD group exhibited a trend toward reduced step-through latency, and BCE treatment, particularly at 50 mg/kg BW/day, showed numerical improvement approaching control levels (Fig. 4C). Although these effects did not achieve statistical significance, the consistent directional trend across behavior tests (Y-maze and passive avoidance) suggests potential cognitive-enhancing properties of BCE that warrant further investigation with larger sample sizes or extended treatment duration.

Effects of BCE on Long-Term Spatial Learning and Memory: Morris Water Maze

The Morris water maze evaluates hippocampus-dependent long-term spatial learning and reference memory based on the ability of mice to locate a hidden platform using extramaze visual cues. During the 4-day acquisition phase, escape latency progressively decreased in all groups, indicating spatial learning (Fig. 4D). Throughout training, the HFD group showed impaired spatial learning, with escape latencies that were 21.5%, 70.4%, 56.5%, and 60.3% longer than controls on days 1-4, respectively (Fig. 4D). BCE treatment improved performance, with the lower dose showing greater efficacy. The BCE50 group showed reductions of 14.5%, 24.5%, 24.3%, and 31.9% relative to the HFD group on days 1-4, respectively, while the BCE200 group exhibited comparatively smaller reductions of 4.5%, 8.9%, 12.7%, and 12.5% on the corresponding days (Fig. 4D). By day 4, the BCE50 group performed comparably with controls, suggesting effective restoration of spatial learning capacity.

In the probe test conducted 24 h after the final training session, spatial memory retention was assessed by measuring time spent in the target quadrant where the platform had been located during training. The HFD group spent significantly less time in the target quadrant (28.8% reduction) compared with controls ($p < 0.05$; Fig. 4E), indicating impaired spatial memory consolidation. Notably, BCE50 treatment significantly increased time in the target quadrant compared with the HFD group ($p < 0.05$; Fig. 4E), demonstrating preserved spatial memory retention. The BCE200 group showed intermediate effects that did not reach statistical significance.

Phenolic compounds exert pleiotropic effects by modulating multiple pathological pathways simultaneously, offering a promising multi-target therapeutic strategy for AD that may be more effective than single-mechanism drugs [16]. AD is characterized by complex, interconnected pathologies including: (1) extracellular deposition of amyloid- β plaque and intracellular formation of neurofibrillary tangles composed of hyperphosphorylated tau protein; (2) hippocampal synaptic disruption and impaired long-term potentiation; (3) neuroinflammation driven by activated microglia and astrocytes; (4) oxidative stress and ROS-mediated neuronal damage; (5) dysregulation of antioxidant defense systems; and (6) cholinergic deficits resulting from reduced acetylcholine availability [1, 4, 7, 16].

While single-target drugs can potentially modulate individual pathways, they often fail to address the multifactorial nature of AD, limiting their therapeutic efficacy. In contrast, phenolic compounds act as multi-target ligands, simultaneously engaging multiple disease-relevant mechanisms. Accumulating evidence demonstrates that phenolic compounds can inhibit amyloid- β aggregation and tau hyperphosphorylation, suppress neuroinflammatory responses, enhance cholinergic neurotransmission through acetylcholinesterase inhibition, scavenge free radicals, upregulate endogenous antioxidant enzymes, and promote synaptic plasticity [14, 15, 17-19, 25, 35, 36]. This multi-pronged approach positions phenolic compounds, particularly anthocyanins, as valuable candidates for preventing or slowing AD progression.

In the present study, HFD-induced obesity resulted in relatively mild cognitive impairment compared with chemically induced neurotoxicity or transgenic AD models. Despite this, BCE consistently improved short-term spatial working memory (Y-maze), long-term aversive memory (passive avoidance), and spatial learning and long-term memory (Morris water maze) compared with the HFD group, with statistically significant effects observed in select parameters, particularly at 50 mg/kg BW/day. Notably, this lower dose consistently outperformed BCE at 200 mg/kg BW/day (Fig. 4), a pattern consistent with previously reported non-dose-dependent cognitive benefits of anthocyanin extracts in APP/PS1 mice [32]. Importantly, parent anthocyanins generally exhibit limited systemic availability after oral intake and are extensively transformed into phenolic metabolites; human pharmacokinetic studies using isotopically labeled cyanidin-3-glucoside have identified protocatechuic acid (and its downstream conjugates) along with higher-abundance phenolic acids such as hippuric acid and ferulic acid in circulation, often at concentrations exceeding those of parent anthocyanin-related conjugates [37, 38]. These metabolites also have independent evidence for cognitive protection *in vivo*: protocatechuic acid ameliorated cognitive deficits and reduced amyloid deposition and neuroinflammation in A β PP/PS1 mice [39], vanillic acid improved scopolamine-induced learning and memory impairment in rats in association with reduced oxidative stress and restored synaptic plasticity and cholinergic signaling [40], and ferulic acid attenuated AD-like pathology and cognitive decline in APP/PS1 mice [41]. Taken together, the superior efficacy of BCE at 50 mg/kg BW/day may reflect an optimal dosing window in which the balance between metabolic conversion and systemic exposure to neuroactive phenolic metabolites is most favorable, whereas higher dosing may not translate into proportionally greater exposure to these metabolites, thereby attenuating net benefit [37, 38].

In conclusion, this study demonstrates that BCE exerts neuroprotective effects through multiple mechanisms, supporting its potential as a phytochemical intervention for obesity-associated cognitive decline. *In vitro*, BCE protected SH-SY5Y cells against oxidative stress and inhibited AChE and BChE activity. *In vivo*, BCE ameliorated HFD-induced cognitive impairment in C57BL/6 mice, with significant improvement in spatial memory retention in the Morris water maze probe test and favorable trends in Y-maze and passive avoidance performance. These cognitive benefits likely reflect BCE's multi-target actions, including cholinesterase inhibition and antioxidant activity mediated by its four major anthocyanins (delphinidin and cyanidin glycosides). However, these findings are limited by the use of a single animal model, a narrow dose range, and the absence of direct measurements of *in vivo* anthocyanin or phenolic metabolite exposure and brain biomarkers. Future research should explore a broader range of dosing strategies and longer intervention periods, integrating pharmacokinetic analyses, biomarker and metabolite assessments, and detailed phytochemical profiling to confirm the robustness of these effects and elucidate the underlying mechanisms. Overall, the findings suggest that BCE may be a promising functional food ingredient for preventing and mitigating obesity-associated cognitive dysfunction.

Author Contributions

Sunghin Im: Conceptualization, Writing – original draft, Formal analysis, Investigation, Methodology. **Kwan Joong Kim:** Data curation, Writing – original draft. **Gyo-Ha Hwang:** Data curation, Formal analysis. **Jong Suk Lee:** Investigation, Data curation. **Tae Gyu Nam:** Writing – Review & editing, Conceptualization. **Ho Jin Heo:** Writing – Review & editing, Conceptualization, Methodology, Resources. **Dae-Ok Kim:** Writing – Review & editing, Conceptualization, Supervision, Project administration.

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