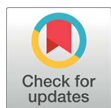


Aster yomena Alleviates Chronic Unpredictable Mild Stress (CUMS)-Induced Depressive Cognitive Dysfunction by Regulating the HPA Axis and TLR4/NF- κ B Pathway

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The purpose of this study was to assess the effects of a 60% ethanolic extract of *Aster yomena* (EAY) on chronic unpredictable mild stress (CUMS)-induced depressive cognitive dysfunction. The results showed that EAY mitigated CUMS-induced depressive-like behaviors, as confirmed by the sucrose preference test (SPT), open field test (OFT), tail suspension test (TST), and forced swimming test (FST). In addition, EAY showed protective effects on cognitive function in the Y-maze and the Morris water maze (MWM) tests. In this regard, EAY alleviated hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis through regulation of corticotropin-releasing factor (CRF), adrenocorticotrophic hormone (ACTH), and cytochrome P450 family 11 subfamily B member 1 (CYP11B1), thereby improving the levels of serum cortisol. It suppressed neuroinflammation, oxidative stress, and mitochondrial dysfunction by inhibiting the Toll-like receptor 4 (TLR4)/nuclear factor κ -light-chain-enhancer of the activated B cells (NF- κ B) pathway. As a result, cognitive dysfunction was ameliorated through modulation of the cholinergic system, including acetylcholinesterase (AChE), acetylcholine (ACh), and choline acetyltransferase (ChAT), and synaptic plasticity-related factors such as postsynaptic density protein 95 (PSD-95) and growth-associated protein 43 (GAP-43). Based on these results, EAY could potentially be used as a natural therapeutic for prevention of major depressive cognitive impairment.

Keywords: *Aster yomena*, Chronic unpredictable mild stress, Cognitive dysfunction

Introduction

Major depressive disorder (MDD), a serious mental illness affecting over 350 million individuals globally, is recognized as a major global public health concern [1, 2]. This condition presents with affective symptoms, including depressed mood, anhedonia, and guilt [2]. In addition, it is associated with numerous physical conditions, including cancer, cardiovascular disease, and cognitive dysfunction [3]. In particular, patients with MDD experience difficulty fulfilling social and occupational roles due to cognitive dysfunction, which has been identified as a major factor contributing to reduced overall quality of life [4, 5]. Indeed, structural and functional abnormalities have been consistently observed in brain regions associated with cognitive functions, such as attention, working memory, and executive function in MDD patients [5]. As a result, cognitive dysfunction has been recognized as a core symptom of MDD, prompting research aimed at elucidating pathological mechanisms [3, 5]. The cognitive dysfunction observed in MDD is attributed to various pathophysiological factors, among which hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis is considered as a central factor [3]. This condition results from chronic exposure to stressors, which disrupts the negative feedback mechanism and leads to sustained secretion of cortisol [6]. In addition, it triggers Toll-like receptor 4 (TLR4)/nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) pathway via microglial activation, which promotes the release of pro-inflammatory cytokines, including interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) [7]. The resulting neuroinflammation

compromises antioxidant defense systems, including catalase and superoxide dismutase (SOD), thereby promoting oxidative stress and mitochondrial dysfunction, ultimately reducing neuronal survival [1]. The advancement of these pathological processes culminates in cholinergic dysfunction, structural synaptic alterations, and neuro-plasticity deficits, thereby contributing to cognitive dysfunction [6, 8]. There is currently no medication for treatment of depressive cognitive dysfunction, and synthetic drugs, including serotonin-norepinephrine reuptake inhibitors (SNRIs) and selective serotonin reuptake inhibitors (SSRIs), used in treatment of MDD, primarily target a single mechanism, such as the monoamine hypothesis [2, 4]. As a result, the rate of treatment success remains low at approximately 30%, and current therapies have been reported to cause adverse effects, including sexual dysfunction, gastrointestinal disturbances, and neuropsychiatric symptoms [1, 9]. Therefore, development of a treatment strategy for depressive cognitive dysfunction that targets multiple pathological mechanisms while minimizing side effects is necessary.

Aster yomena (*A. yomena*), a perennial herb belonging to the Asteraceae family, has wide distribution across Korea, China, Japan, and Siberia [10]. *A. yomena* has been reported to contain various bioactive compounds, including polyphenolic substances such as caffeic acid, chlorogenic acid, and rutin as well as terpenoids and saponins [11, 12]. These compounds modulate diverse molecular pathways, contributing to the suppression of pathological progression in various chronic disorders [13]. A previous study reported that *A. yomena* extract exerted protective effects against cerulein-induced pancreatitis by suppressing neutrophil infiltration and the production of pro-inflammatory cytokines [10]. In another study, *A. yomena* extract alleviated ovalbumin-induced allergic responses by altering the balance of type 1 and type 2 T helper cytokines [14]. In addition, in a recent study an ethyl acetate fraction of *A. yomena* extract ameliorated high-fat diet (HFD)-induced cognitive impairment by modulating the insulin receptor substrate (IRS)/protein kinase B (Akt) pathway [15]. However, research on the protective effects of *A. yomena* against chronic unpredictable mild stress (CUMS)-induced depressive cognitive impairment remains limited. Therefore, this study attempted to determine whether *A. yomena* can attenuate depression-related cognitive impairment in the CUMS model.

Materials and Methods

Procurement and Extraction of *A. yomena*

A. yomena was obtained from Gurye Local Foods (Republic of Korea) in May 2023 for use in this study. EAY was prepared according to the previously described protocol [16]. Briefly, powdered *A. yomena* was mixed with 60% ethanol at a 1:50 (w/v) ratio and reflux extracted at 40°C for 2 h. The extract was filtered, concentrated, and lyophilized. It was subsequently maintained at –20°C until required for experimentation.

Phytochemicals Analysis

EAY was solubilized in 50% methyl alcohol and filtered using a syringe filter. The sample was subjected to LC-QTOF/MS analysis (Vion IMS QTOF, Waters, USA). The components were resolved via the Acquity UPLC BEH C₁₈ column (2.1 × 100 mm, 1.7 μm, Waters) with the flow rate and column temperature adjusted to 0.35 ml/min and 40°C, respectively. The eluent was constituted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B), and the gradient profile was as follows: 0–1 min, 99% A; 1–8 min, 99–0% A; 8–9 min, 0% A; 9–9.5 min, 0–99% A; and 9.5–12 min, 99% A. MS analysis was performed in negative ESI mode over an *m/z* range of 50–1500. Collision energies were applied at 6 and 10–30 eV. Data acquisition and processing were carried out using UNIFI software (Waters).

Animal Experimentation

All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of Gyeongsang National University (approval No. GNU-230314-M0047; approved on 14 March 2023). C57BL/6 mice (4 weeks, male) were purchased from Koatech (Republic of Korea) and adapted for 1 week under standard laboratory conditions (temperature: 22 ± 1°C, humidity: 55 ± 5%, and 12 h light/dark cycle). Thereafter, the mice were randomly allocated into four groups (*n* = 12 per group): NC group (maintained under normal conditions + water), CUMS group (maintained under CUMS conditions + water), EAY50 group (maintained under CUMS conditions + EAY50 mg/kg of body weight), and EAY100 group (maintained under CUMS conditions + EAY100 mg/kg of body weight). They were orally administered either drinking water (NC and CUMS groups) or EAY dissolved in drinking water (EAY50 and EAY100 groups) and concurrently exposed once daily for 4 weeks to a randomly selected stressor.

CUMS Model Building

The CUMS procedure was performed based on a modification of a previous study [17]. Except for the control group, all experimental groups were exposed to seven different stressors once daily for a period of 4 weeks. The stressors included: (1) cage swap for 24 h with cages from different groups, (2) 45° cage tilting for 24 h, (3) empty cage exposure without bedding for 24 h, (4) food or water deprivation for 24 h, (5) mild restraint in an acrylic box (10.5 × 10.5 × 5.5 cm) with ventilation holes for 2 h, (6) light exposure during the 12 h dark cycle, and (7) exposure to wet bedding for 24 h. These stressors were applied in a non-sequential and unpredictable manner to avoid habituation and ensure the mice could not anticipate the onset of the stressor. These stressors were applied in a non-sequential and unpredictable manner to avoid habituation and ensure that the mice could not anticipate the onset of the stressor, with the order randomized using the RANDARRAY function in Excel (Microsoft 365, Microsoft Corp., USA).

Depressive and Cognitive Behavioral Assessments

Sucrose Preference Test (SPT). The SPT consisted of 4 days. On the first day, the mice were individually housed and supplied with 1% sucrose solution in two bottles for 24 h. On the second day, the mice were provided with two bottles in which one contained a 1% sucrose solution and the other contained drinking water, with the bottle positions switched after 12 h. On the third day, the mice had no access to food and water for 24 h. On the final day, animals were given one bottle containing 1% sucrose solution and another containing a non-sucrose solution, and intake from each was recorded.

Open Field Test (OFT). The mice were introduced into an acrylic box (50 × 50 × 50 cm) partitioned into a central zone (25 × 25 cm) and a peripheral zone for 5 min. A video tracking system (Smart 3.0, Panlab, Spain) was used to monitor the locomotor activity of the mice.

Tail Suspension Test (TST). The mice were suspended from a stick by the tail using adhesive tape for 5 min. A video tracking system (Smart 3.0, Panlab) was employed to record immobility time during the final 4 min.

Forced Swimming Test (FST). The mice were positioned in a transparent cylindrical container (10 × 50 cm) containing 15 cm of water for 5 min. A video tracking system (Smart 3.0, Panlab) was used to record the immobility time.

Y-maze test. The mice were placed at the end of one arm of the Y-maze (33 × 10 × 15 cm) and permitted to navigate freely for 8 min. A video tracking system (Smart 3.0, Panlab) was used to monitor arm entries and alternation behaviors.

Morris Water Maze (MWM) Test. The MWM (90 × 30 cm) test consisted of 6 days. The maze was partitioned into four quadrants (N, E, S, and W) and visual cues were provided for each quadrant. An escape platform was located centrally within the W quadrant, and the maze was prepared with water containing non-toxic white ink was dissolved. On the initial training day, the mice were allowed to swim for 1 min with the escape platform exposed 1 cm above the water surface. From the second to the fifth day, the mice were trained to navigate to the submerged escape platform. On the final day, the mice were allowed to swim for 1 min with the escape platform removed. A video tracking system (Smart 3.0, Panlab) was employed to monitor the locomotor activity.

Cortisol Analysis

The serum was collected after centrifuging blood samples at 13,000 ×g for 15 min. It was vortexed in 50% methyl alcohol and centrifuged at 18,472 ×g for 10 min to isolate the supernatant. The sample was concentrated under vacuum using a centrifugal evaporator (NB-503CIR, N-Biotek, Republic of Korea). Subsequently, it was dissolved in 50% methyl alcohol and re-centrifuged at 18,472 ×g for 10 min to collect the supernatant. It was quantified using an LC-MS/MS system (Xevo TQ-S, Waters). The samples were resolved via the Acquity UPLC BEH C₁₈ column (Waters) with the flow rate and column temperature adjusted to 0.35 ml/min and 40°C, respectively. The eluent was constituted of solvent A and solvent B, and the gradient profile was as follows: 0–1 min, 99% A; 1–5 min, 99–0% A; 5–6 min, 0% A; 6–6.2 min, 0–99% A; and 6.2–8 min, 99% A. Calibration curves were established using cortisol standards under MRM mode. The optimized transition for cortisol was from *m/z* 363.11 to 363.00, with a collision energy of 6 V, and the retention time was approximately 3.57 min.

Measurement of Antioxidant System

Malondialdehyde (MDA) content. The homogenized brain tissues in PBS underwent centrifugation at 2,356 ×g for 10 min. Then, a mixture composed of supernatants, 0.67% thiobarbituric acid, and 1% phosphoric acid was subjected to heating in a 95°C water bath. The MDA content in the samples was measured at 532 nm with a UV-1800 spectrophotometer (Shimadzu, Japan).

SOD levels. The homogenized brain tissues in PBS underwent centrifugation at 400 ×g for 10 min. The pellets were re-centrifuged at 10,000 ×g for 10 min after being mixed with a buffer containing WST solution, 200 mM phenylmethylsulfonyl fluoride, and 20% Triton X-100 to obtain the supernatants. The SOD levels were assessed using the SOD kit (Dojindo Molecular Tech., USA) on an Epoch 2 microplate spectrophotometer (BioTek Instruments, USA), following the instructions of the manufacturer.

Reduced GSH levels. The homogenized brain tissues in 10 mM phosphate buffer supplemented with 1 mM edetic acid (EDTA) underwent centrifugation at 10,000 ×g for 15 min. The collected supernatants were subjected to re-centrifugation at 2,000 ×g for 2 min after mixing with 5% metaphosphoric acid. The supernatants were combined with 0.26 M Tris-hydrochloride, 0.65 M sodium hydroxide, and 1 mg/ml phthalaldehyde. The reduced GSH levels in the samples were determined using an Infinite F200 fluorescence microplate reader (Tecan, Switzerland) with excitation and emission wavelengths set at 360 nm and 430 nm, respectively.

Measurement of Mitochondrial Activity

Isolation of mitochondria. Mitochondrial isolation buffer was prepared by dissolving 0.1% bovine serum albumin, 20 mM HEPES sodium salt, 215 mM mannitol, and 75 mM sucrose. The homogenized brain tissues in buffer supplemented with 1 mM egtazic acid (EGTA) were centrifuged at 1,300 ×g for 5 min. Subsequently, the supernatants underwent re-centrifugation at 13,000 ×g for 10 min. The pellets were incubated with the buffer supplemented with 0.1% digitonin, followed by the addition of the buffer supplemented with 1 mM EGTA. It was centrifuged at 13,000 ×g for 15 min, and the pellets were re-centrifuged at 10,000 ×g for 10 min after the addition of the buffer. It was subjected to centrifugation at 13,000 ×g for 15 min, and the pellets were subjected to re-centrifugation at 10,000 ×g for 10 min after buffer addition. The pellets were resuspended in the buffer and used for the ROS and MMP assay. After assessing ROS and MMP, the extracts underwent centrifugation at 13,000 ×g for 10 min. The pellets were re-centrifuged at 10,000 ×g for 15 min after being mixed with 1% trichloroacetic acid buffer. The supernatants were used for the ATP assay.

Reactive Oxygen Species (ROS) production. The assay buffer was prepared by dissolving 0.5 mM EGTA, 1 mM magnesium chloride, 2 mM monopotassium phosphate, 2.5 mM malic acid, 5 mM pyruvic acid, 20 mM HEPES, and 125 mM potassium chloride. The extracts were mixed with the assay buffer supplemented with 2',7'-dichlorofluorescein diacetate. The ROS levels in the samples were measured on an Infinite F200 fluorescence microplate reader (Tecan) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The ROS levels in the samples were determined using an Infinite F200 fluorescence microplate reader (Tecan) with excitation and emission wavelengths set at 485 nm and 535 nm, respectively.

Mitochondrial Membrane Potential (MMP). The assay buffer was prepared by supplementing the mitochondrial isolation buffer with 1 mM EGTA, 5 mM malic acid, and 5 mM pyruvic acid. The extracts were mixed with the assay buffer supplemented with JC-1. The MMP in the samples was determined using an Infinite F200 fluorescence microplate reader (Tecan) with excitation and emission wavelengths set at 535 nm and 590 nm, respectively.

ATP content. The ATP content was assessed via the ATP kit (Promega Co., USA) with a microplate reader (Glomax®, Promega Co.), following the instructions of the manufacturer.

Measurement of Cholinergic System

Acetylcholine (ACh) Content. The brain tissues were processed in PBS by homogenization and centrifuged at 13,572 ×g for 30 min. The supernatants were combined with 2 M hydroxylammonium chloride, 3.5 M sodium hydroxide, 0.5 M hydrochloric acid, and 0.37 M ferric chloride hexahydrate. ACh content in the samples was assessed from absorbance recorded at 405 nm on an Epoch 2 microplate spectrophotometer (BioTek Instruments).

Acetylcholinesterase (AChE) activity. The brain tissues were processed in PBS by homogenization and centrifuged at 13,572 ×g for 30 min. The supernatants were combined with 50 mM sodium phosphate buffer, 1 mM Ellman's reagent, and 500 μM acetylthiocholine iodide. AChE activity in the samples was assessed from absorbance recorded at 405 nm on an Epoch 2 microplate spectrophotometer (BioTek Instruments).

Western Blotting

The brain tissues were processed by homogenization in ProtinEx Animal cell/tissue (GeneAll Biotechnology, Republic of Korea) supplemented with 1% (v/v) protease inhibitor cocktail (Quartett, Germany). The homogenates were subjected to centrifugation at 16,000 ×g for 10 min. The supernates were combined with 4× sample buffer (Bio-Rad, USA) and resolved using SDS-PAGE. The proteins were blotted onto a polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked in 5% skim milk and then subjected to primary antibody incubation. Then, the washing was carried out using Tris-buffered saline supplemented with 0.1% Tween 20, after which the membrane was incubated with the secondary antibody. It was washed and visualized using ECL kit (TransLab, Republic of Korea) and the iBright CL1500 imaging system (Thermo Fisher Scientific, USA). Quantification of protein was conducted via ImageJ software (version 1.54d, NIH, USA). Descriptions of the antibodies utilized in this study are outlined in Table S1.

Statistical Analysis

Data were first analyzed using the Shapiro–Wilk test to assess the normality of distribution and the Levene's test to evaluate the homogeneity of variance. Depending on the results, either an ordinary one-way analysis of variance (ANOVA) followed by Duncan's multiple range test or the nonparametric Kruskal–Wallis test followed by Dunn's post hoc test was performed. Statistical difference ($p < 0.05$) of each group was shown by different lowercase letters. Principal component analysis (PCA) was performed using MetaboAnalyst 6.0.

Results

Phytochemicals Analysis of EAY

Phytochemicals identified in a EAY using UPLC-QTOF/MS include the following: chlorogenic acid, caffeic acid, rutin, kaempferol-3-O-rutinoside, and dicaffeoylquinic acid (Fig. 1 and Table 1).

Effects of EAY on Depression-Related Behaviors

In the SPT, the CUMS group (52.35%) showed attenuated sucrose preference relative to the NC group (67.47%) (Fig. 2A). However, the EAY groups (EAY50, 67.57%; EAY100, 71.16%, respectively) exhibited an increase in sucrose preference relative to the CUMS group.

In the OFT, the CUMS group (1.61%) exhibited reduced time spent in the center zone relative to the NC group (4.97%) (Fig. 2B and 2C). However, the EAY groups (EAY50, 3.80%; EAY100, 5.29%, respectively) exhibited an increase in time spent in the center zone relative to the CUMS group.

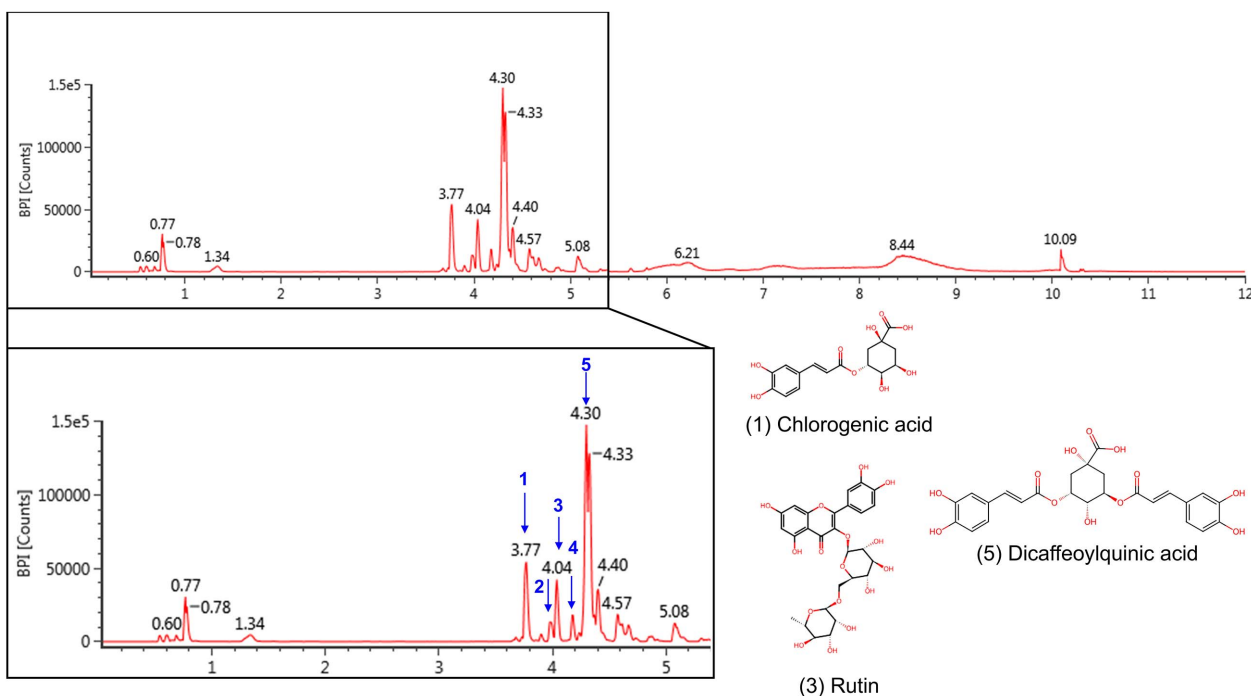


Fig. 1. UPLC-QTOF/MS chromatogram of EAY. 1. Chlorogenic acid; 2. Caffeic acid; 3. Rutin; 4. Kaempferol-3-O-rutinoside; 5. Dicaffeoylquinic acid

Table 1. Phytochemical compounds identified in EAY via UPLC-QTOF/MS.

No.	Retention time	Proposed compound	<i>m/z</i>	MS fragments	Area (%)
1	3.77	Chlorogenic acid	353	135, 173, 179, 191	8.95
2	3.91	Caffeic acid	179	109	0.62
3	4.04	Rutin	609	255, 271, 300, 301	5.80
4	4.18	Kaempferol-3-O-rutinoside	593	255, 285	2.51
5	4.30	Dicaffeoylquinic acid	515	135, 173, 179, 191, 353	20.68

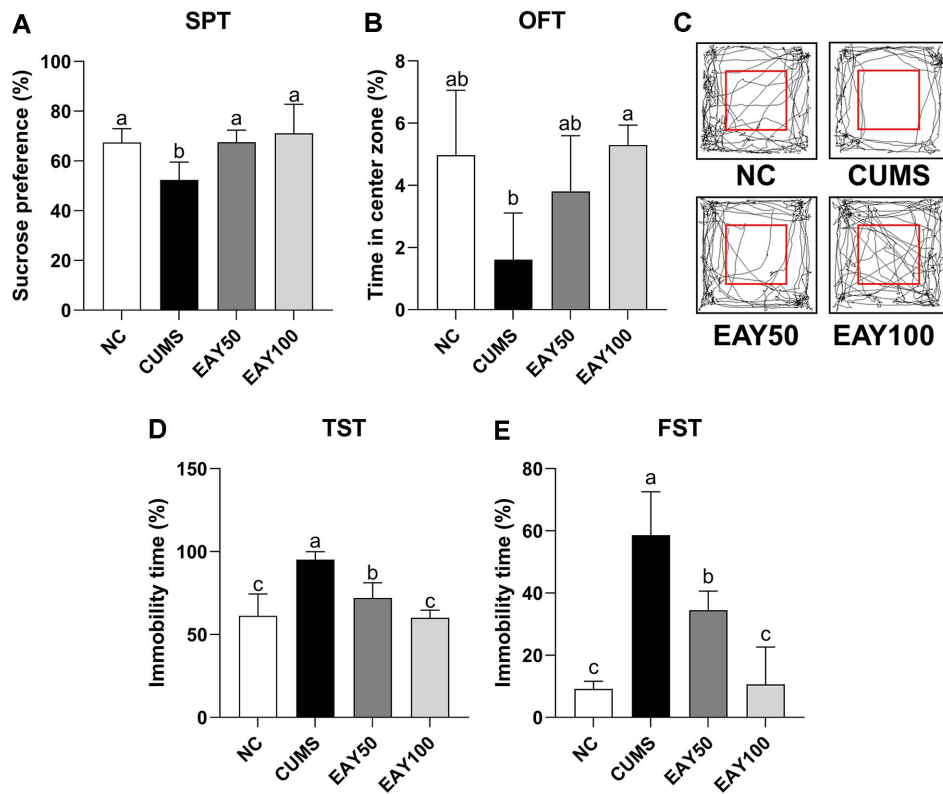


Fig. 2. Effects of EAY on depressive-like behaviors in CUMS-induced mice. (A) Sucrose preference in SPT, (B) time in center zone in OFT, (C) tracing image in OFT, (D) immobility time in TST, and (E) FST. Results are presented as the mean $\pm$ SD ($n = 6$). Different letters indicate statistically significant differences between groups ($p < 0.05$) based on one-way ANOVA followed by Duncan's multiple range test or Kruskal–Wallis test followed by Dunn's post hoc test.

In the TST, the CUMS group (95.14%) showed increased immobility time relative to the NC group (61.33%) (Fig. 2D). However, the EAY groups (EAY50, 72.00%; EAY100, 60.11%, respectively) exhibited decreased immobility time relative to the CUMS group.

In the FST, the CUMS group (58.66%) showed increased immobility time relative to the NC group (9.14%) (Fig. 2E). However, the EAY groups (EAY50, 34.49%; EAY100, 10.64%, respectively) demonstrated reduced immobility time relative to the CUMS group.

Effects of EAY on HPA Axis Hyperactivation

The CUMS had increased expression of corticotropin-releasing factor (CRF) (1.78), adrenocorticotrophic hormone (ACTH) (1.82), and cytochrome P450 family 11 subfamily B member 1 (CYP11B1) (1.55) relative to the NC group (1.00) (Fig. 3A and 3B). In contrast, the EAY100

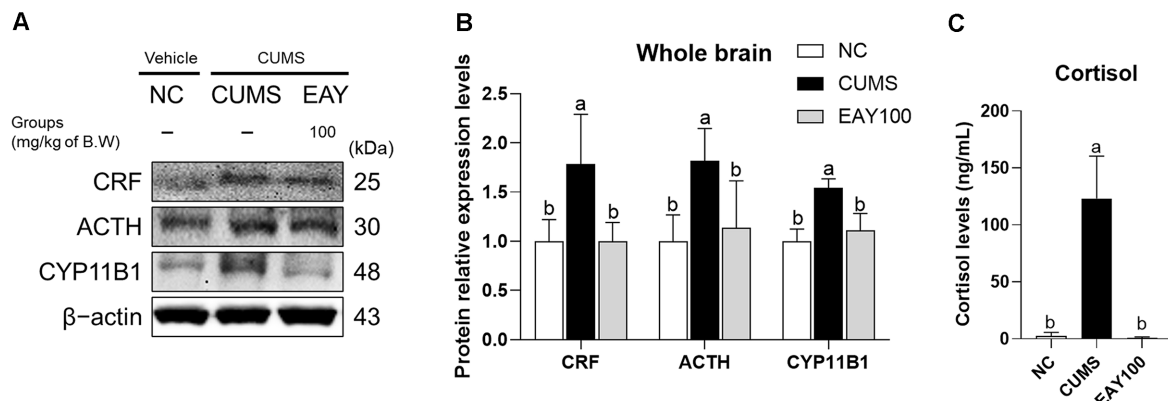


Fig. 3. Effects of EAY on HPA axis dysregulation in CUMS-induced mice. (A) Western blot images, (B) protein relative expression levels of CRF, ACTH, and CYP11B1 in the whole brain, and (C) cortisol levels in serum. Results are presented as the mean $\pm$ SD ($n = 3$). Different letters indicate statistically significant differences between groups ($p < 0.05$) based on one-way ANOVA followed by Duncan's multiple range test or Kruskal–Wallis test followed by Dunn's post hoc test.

group had decreased expression of CRF (1.00), ACTH (1.13), and CYP11B1 (1.11) relative to the CUMS group.

The CUMS group (123.20 ng/ml) had increased serum cortisol levels relative to the NC group (2.52 ng/ml) (Fig. 3C). In contrast, the EAY100 group (0.91 ng/ml) had decreased serum cortisol levels relative to the CUMS group.

Effects of EAY on Cognitive Impairments

In the Y-maze test, total arm entries showed no appreciable differences between groups (Fig. 4A). The CUMS group (27.28%) displayed reduced alternation behavior relative to the NC group (42.66%) (Fig. 4B and 4C). However, the EAY groups (EAY50, 36.43%; EAY100, 42.36%, respectively) showed enhanced alternation behavior relative to the CUMS group.

In the Morris water maze (MWM) test, on the fourth day of the training phase, the CUMS group (45.63 sec) showed an increase in escape latency relative to the NC group (20.64 sec) (Fig. 4D-4F). However, the EAY groups (EAY50, 40.77 sec; EAY100, 24.73 sec, respectively) exhibited a decrease in escape latency relative to the CUMS group. In the test phase, the CUMS group (10.18 sec) showed a reduction in the time spent in the target zone relative to the NC group (48.58 sec). In contrast, the EAY groups (EAY50, 18.36 s; EAY100, 37.72 s, respectively) exhibited an increase in the time spent in the target zone relative to the CUMS group.

Effects of EAY on Neuroinflammation

In the whole brain, the CUMS group showed elevated expression of TLR4 (1.63), p-NF- κ B (1.80), TNF- α (1.76), and IL-1 β (1.52) relative to the NC group (1.00) (Fig. 5A and 5B). In contrast, the EAY100 group showed decreased expression of TLR4 (0.95), p-NF- κ B (1.26), TNF- α (1.03), and IL-1 β (0.75) relative to the CUMS group.

In the cortex, the CUMS group showed elevated expression of TLR4 (1.37), p-NF- κ B (1.43), TNF- α (1.83), and IL-1 β (1.47) relative to the NC group (1.00) (Fig. 5C and 5D). In contrast, the EAY100 group showed decreased expression of TLR4 (1.13), p-NF- κ B (1.01), TNF- α (1.18), and IL-1 β (1.03) relative to the CUMS group.

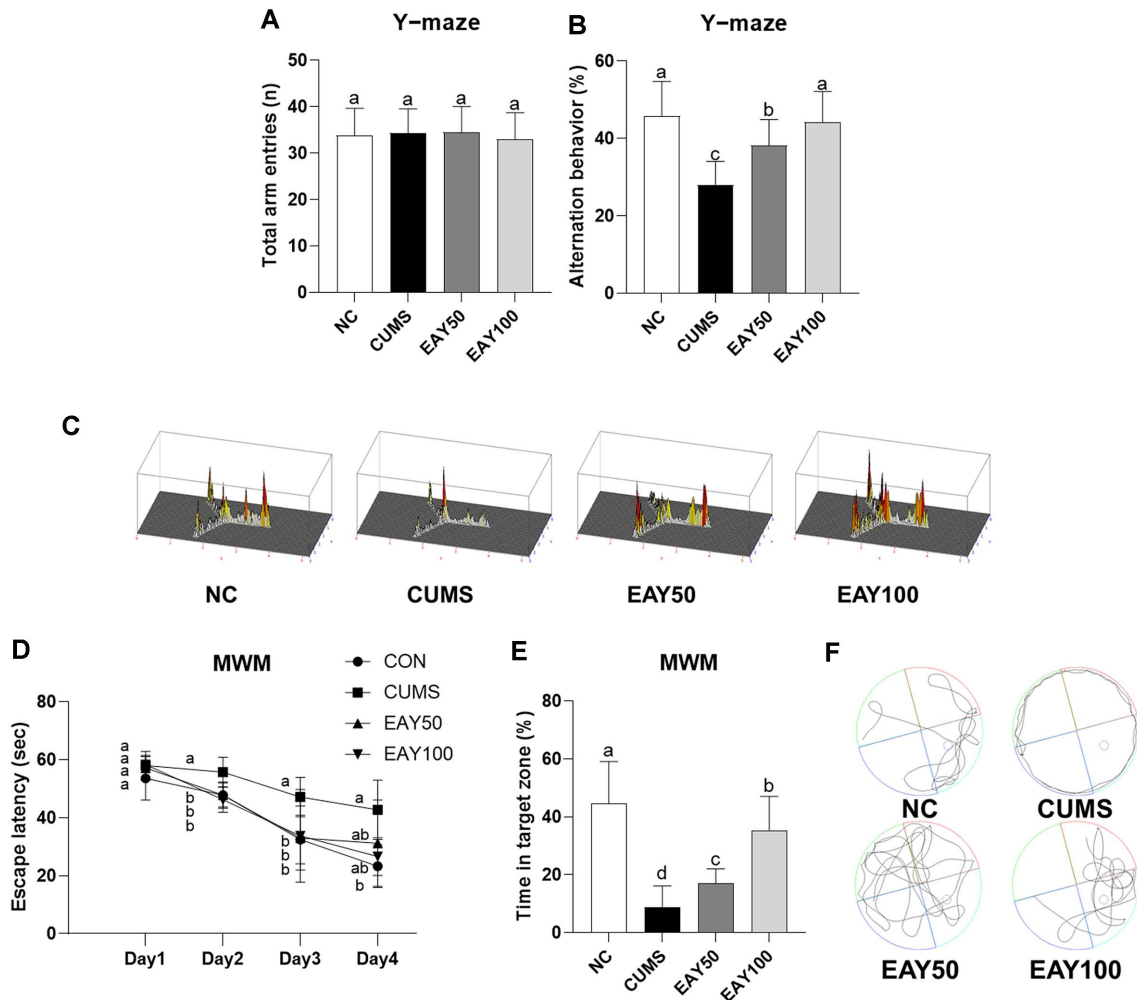


Fig. 4. Effects of EAY on cognitive function in CUMS-induced mice. (A) Total arm entries, (B) alternation behavior, (C) tracing images in Y-maze, (D) escape latency, (E) time in target zone, and (F) tracing image in MWM. Results are presented as the mean $\pm$ SD ($n = 6$). Different letters indicate statistically significant differences between groups ($p < 0.05$) based on one-way ANOVA followed by Duncan's multiple range test or Kruskal–Wallis test followed by Dunn's post hoc test.

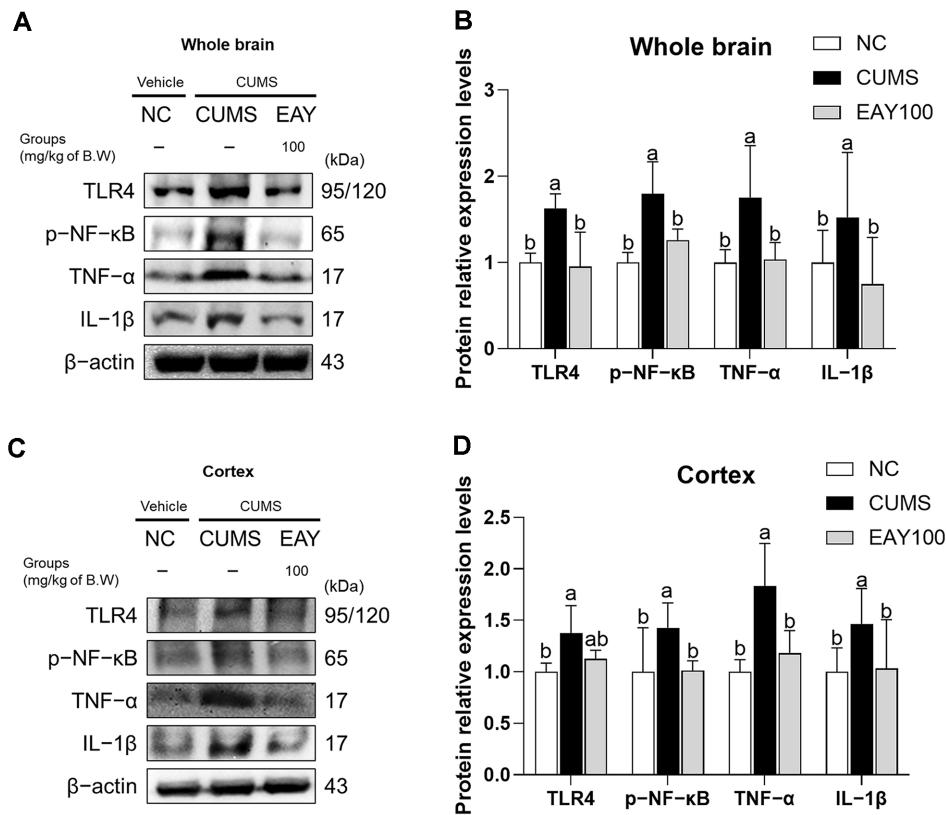


Fig. 5. Effect of EAY on neuroinflammation in CUMS-induced mice. (A, C) Western blot images and (B, D) protein relative expression levels of TLR4, p-NF-κB, TNF-α, and IL-1β in the whole brain and cortex, respectively. Results are presented as the mean ± SD ($n = 3$). Different letters indicate statistically significant differences between groups ($p < 0.05$) based on one-way ANOVA followed by Duncan's multiple range test or Kruskal–Wallis test followed by Dunn's post hoc test.

Effects of EAY on the Antioxidant System

The CUMS group (4.45 nmol/mg of protein) showed elevated MDA content relative to the NC group (4.00 nmol/mg of protein) (Fig. 6A). However, the EAY groups (EAY50, 3.64 nmol/mg of protein; EAY100, 3.54 nmol/mg of protein, respectively) showed reduced MDA content relative to the CUMS group.

The CUMS group (0.20 unit/mg of protein) showed reduced SOD levels relative to the NC group (0.27 unit/mg of protein) (Fig. 6B). However, the EAY groups (EAY50, 0.32 unit/mg of protein; EAY100, 0.33 unit/mg of protein, respectively) showed elevated SOD levels relative to the CUMS group.

The CUMS group (87.19%) had decreased reduced GSH levels relative to the NC group (100.00%) (Fig. 6C). However, the EAY groups (EAY50, 88.58%; EAY100, 96.76%, respectively) exhibited increased reduced GSH levels relative to the CUMS group.

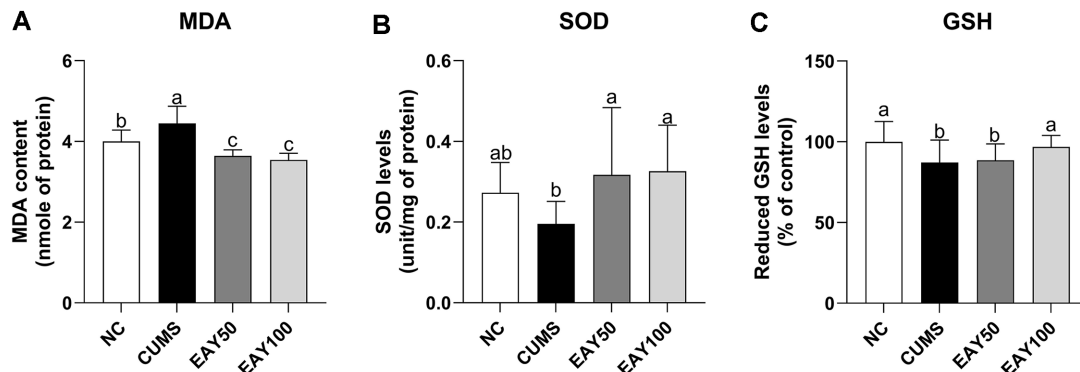


Fig. 6. Effect of EAY on oxidative stress in CUMS-induced mice. (A) MDA content, (B) SOD levels, and (C) reduced GSH levels in whole brain tissues. Results are presented as the mean ± SD ($n = 5$). Different letters indicate statistically significant differences between groups ($p < 0.05$) based on one-way ANOVA followed by Duncan's multiple range test or Kruskal–Wallis test followed by Dunn's post hoc test.

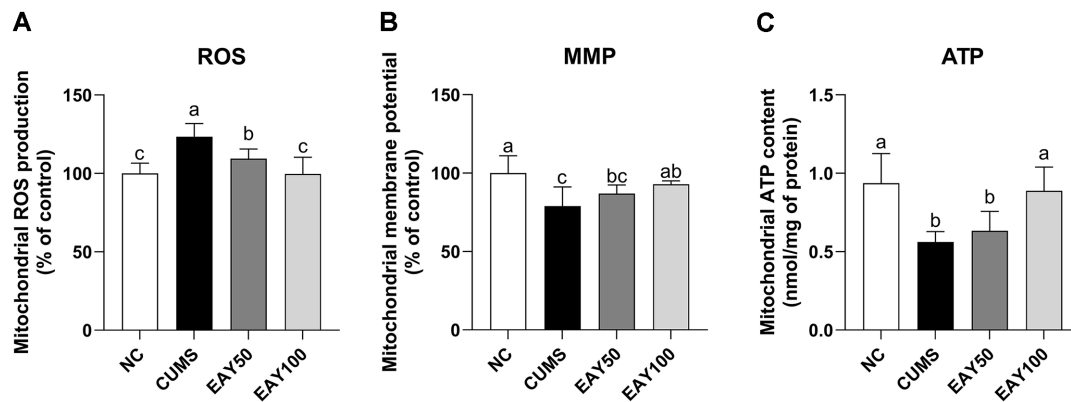


Fig. 7. Effect of EAY on mitochondrial function in CUMS-induced mice. (A) Mitochondrial ROS production, (B) MMP, and (C) mitochondrial ATP content in whole brain tissues. Results are presented as the mean $\pm$ SD ($n = 3$). Different letters indicate statistically significant differences between groups ($p < 0.05$) based on one-way ANOVA followed by Duncan's multiple range test or Kruskal–Wallis test followed by Dunn's post hoc test.

Effects of EAY on Mitochondrial Function

The CUMS group showed elevated ROS levels (123.50%) relative to the NC group (100.00%) (Fig. 7A). However, the EAY groups (EAY50, 109.38%; EAY100, 99.66%, respectively) showed reduced ROS levels relative to the CUMS group.

The CUMS group had reduced MMP (78.99%) relative to the NC group (100.00%) (Fig. 7B). However, the EAY groups (EAY50, 86.96%;

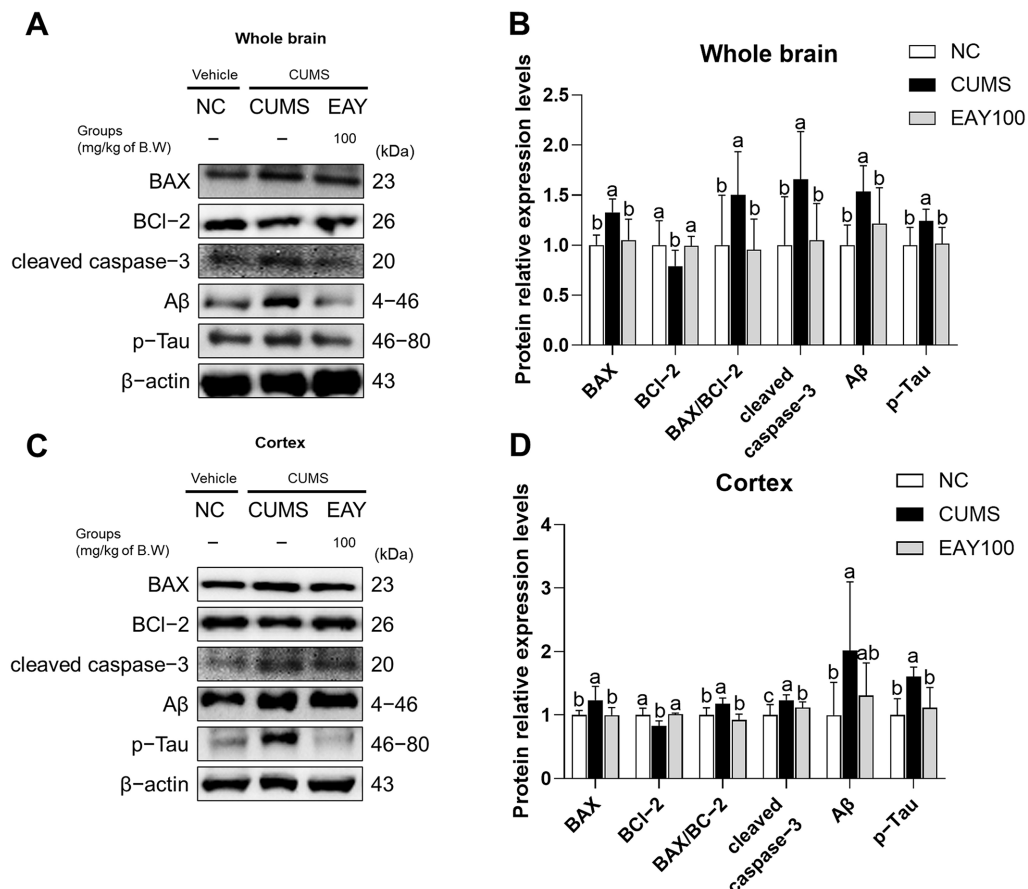


Fig. 8. Effect of EAY on neurotoxicity in CUMS-induced mice. (A, C) Western blot images and (B, D) protein relative expression levels of BAX, BCL-2, cleaved caspase-3, Aβ, and p-Tau in the whole brain and cortex, respectively. Results are presented as the mean $\pm$ SD ($n = 3$). Different letters indicate statistically significant differences between groups ($p < 0.05$) based on one-way ANOVA followed by Duncan's multiple range test or Kruskal–Wallis test followed by Dunn's post hoc test.

EAY100, 92.92%, respectively) showed increased MMP relative to the CUMS group.

The CUMS group had reduced ATP content (0.56 nmol/mg of protein) relative to the NC group (0.94 nmol/mg of protein) (Fig. 7C). However, the EAY groups (EAY50, 0.63 nmol/mg of protein; EAY100, 0.89 nmol/mg of protein, respectively) showed increased ATP content relative to the CUMS group.

Effects of EAY on Neuronal Apoptosis and A β /p-Tau Accumulation

In the whole brain, the CUMS group exhibited decreased expression of B-cell leukemia/lymphoma 2 (BCL-2) (0.79) and increased expression of BCL-2 associated X (BAX) (1.33), BAX/BCL-2 ratio (1.50), caspase-3 (1.60), amyloid β (A β) (1.54), and p-Tau (1.24) relative to the NC group (1.00) (Fig. 8A and 8B). In contrast, the EAY100 group showed restored expression of BCL-2 (1.00), BAX (1.05), BAX/BCL-2 (0.93), caspase-3 (1.09), A β (1.22), and p-Tau (1.01) relative to the CUMS group.

In the cortex, the CUMS group exhibited decreased expression of BCL-2 (0.83) and increased expression of BAX (1.23), BAX/BCL-2 ratio (1.18), caspase-3 (1.24), A β (2.02), and p-Tau (1.61) relative to the NC group (1.00) (Fig. 8C and 8D). In contrast, the EAY100 group showed restored expression of BCL-2 (1.01), BAX (1.00), BAX/BCL-2 (0.92), caspase-3 (1.12), A β (1.31), and p-Tau (1.11) relative to the CUMS group.

Effects of EAY on the Cholinergic System and Synaptic Plasticity

The CUMS group showed decreased ACh content (0.45 nmole/mg of protein) relative to the NC group (1.06 nmole/mg of protein) (Fig. 9A). However, the EAY groups (EAY50, 0.62 nmole/mg of protein; EAY100, 1.18 nmole/mg of protein, respectively) showed increased ACh content relative to the CUMS group.

The CUMS group showed elevated AChE activity (130.82%) relative to the NC group (100.00%) (Fig. 9B). However, the EAY groups (EAY50,

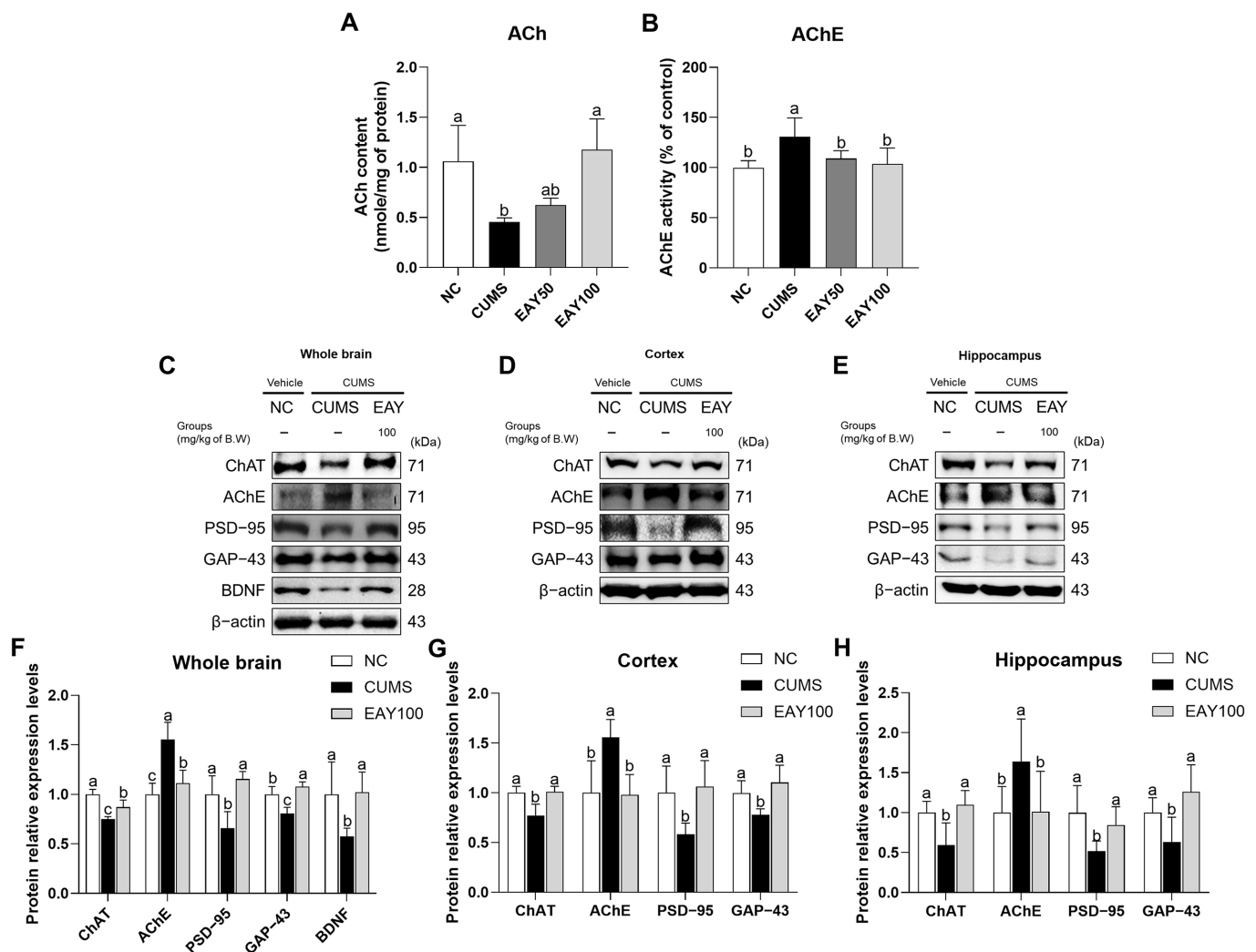


Fig. 9. Effect of EAY on the cholinergic system and synaptic function in CUMS-induced mice. (A) ACh content and (B) AChE activity in brain tissue, (C–E) western blot images, and (F–H) protein relative expression levels ChAT, AChE, PSD-95, GAP-43, and BDNF in the whole brain, cortex, and hippocampus, respectively. Results are presented as the mean $\pm$ SD (a–b; $n = 5$ and c–h; $n = 3$). Different letters indicate statistically significant differences between groups ($p < 0.05$) based on one-way ANOVA followed by Duncan's multiple range test or Kruskal–Wallis test followed by Dunn's post hoc test.

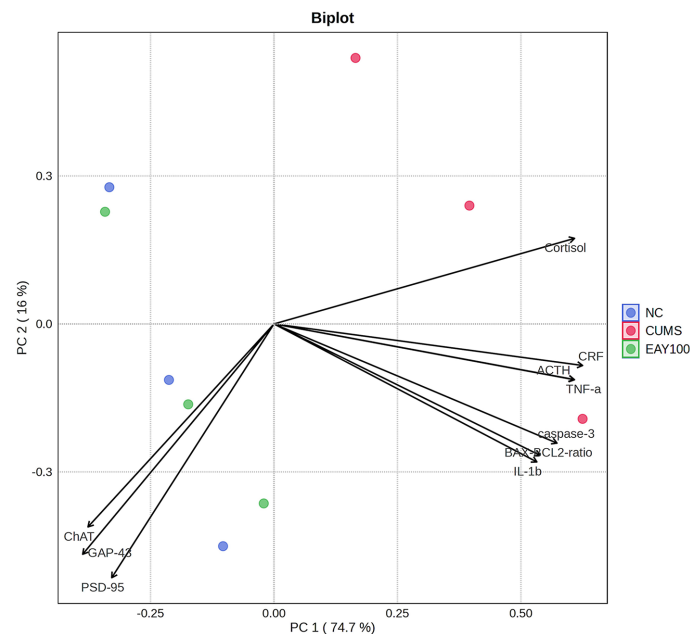


Fig. 10. PCA biplot of the NC, CUMS, and EAY100 groups.

109.07%; EAY100, 103.59%, respectively) showed reduced AChE activity relative to the CUMS group.

In the whole brain, the CUMS group showed elevated expression of AChE (1.56) and decreased expression of choline acetyltransferase (ChAT) (0.87), postsynaptic density protein 95 (PSD-95) (0.66), growth-associated protein 43 (GAP-43) (0.81), and brain-derived neurotrophic factor (BDNF) (0.57) relative to the NC group (1.00) (Fig. 9C and 9F). In contrast, the EAY100 group showed restored expression of AChE (1.11), ChAT (1.04), PSD-95 (1.15), GAP-43 (1.08), and BDNF (1.02) relative to the CUMS group.

In the cortex, the CUMS group showed elevated expression of AChE (1.56) and decreased expression of ChAT (0.77), PSD-95 (0.58), and GAP-43 (0.78) relative to the NC group (1.00) (Fig. 9D and 9G). In contrast, the EAY100 group showed restored expression of AChE (0.98), ChAT (1.01), PSD-95 (1.06), and GAP-43 (1.10) relative to the CUMS group.

In the hippocampus, the CUMS group showed elevated expression of AChE (1.64) and decreased expression of ChAT (0.59), PSD-95 (0.52), and GAP-43 (0.64) relative to the NC group (1.00) (Fig. 9E and 9H). In contrast, the EAY100 group showed restored expression of AChE (1.01), ChAT (1.10), PSD-95 (0.85), and GAP-43 (1.26) relative to the CUMS group.

Multivariate Analysis of Depressive and Cognitive Dysfunction Biomarkers

Principal component analysis (PCA) revealed that the principal components accounted for 90.7% of the total variance, with PC1 and PC2 explaining 74.7% and 16.0% of the variance, respectively (Fig. 10). The CUMS group was distributed along the positive axis of PC1, while the NC and EAY100 groups were positioned on the negative axis of PC1, demonstrating clear separation between the groups along this component. Moreover, the CUMS group was characterized by greater influence from CRF, ACTH, cortisol, p-NF-κB, TNF-α, IL-1β, BAX/BCL-2, caspase-3 and AChE ratio in contrast to the NC and EAY100 groups, which were associated with higher levels of GAP-43, PSD-95, and ChAT.

Discussion

MDD is a psychiatric disorder characterized by structural and functional alterations in the brain, frequently accompanied by cognitive dysfunction [2, 4]. Several pathophysiological mechanisms have been implicated in the pathogenesis of depressive cognitive dysfunction, particularly hyperactivation of the HPA axis, neuroinflammation, oxidative stress, and changes in synaptic plasticity [6]. Therefore, the goal of this study was to assess the protective effects of EAY against cognitive impairment associated with depression in a CUMS model.

A. yomena has traditionally been used for treatment of sore throat and fever and its antioxidant, anti-inflammatory, and neuroprotective properties have been confirmed in recent studies, thereby positioning it as a promising therapeutic alternative [10]. The therapeutic effects of natural products have been attributed to their constituent phytochemicals [1]. Therefore, analysis of EAY was performed using LC-MS for evaluation of its potential therapeutic effects, resulting in the identification of chlorogenic acid, caffeic acid, rutin, kaempferol-3-O-rutinoside, and dicaffeoylquinic acid (Fig. 1 and Table 1). Dicaffeoylquinic acid has demonstrated inhibitory effects on the activity of monoamine oxidase A (MAO-A) and B (MAO-B), thereby reducing corticosterone-induced production of ROS and ameliorating memory impairment in murine models of depression [18]. Rutin attenuated hyperactivation of the HPA axis through modulation of ACTH and expression of corticosterone, while concurrently mitigating depressive symptoms and cognitive deficits via downregulation of NF-κB expression [19]. In addition, chlorogenic acid and caffeic acid, polyphenolic compounds sharing a cinnamic acid structural motif, have been reported to exert neuroprotective properties through inhibition of the activity of AChE and butyrylcholinesterase (BChE) [20]. These findings support our hypothesis that *A. yomena* has preventive effects against cognitive dysfunction associated with depression. Therefore, the goal of this study was to examine the protective effects of EAY against CUMS-induced depressive cognitive disorder in mice.

Stress is a physiological and psychological response to external stimuli or changes [21]. Specifically, chronic stress exceeding the psychological

resilience of the individual results in elevated secretion of cortisol, commonly known as the stress hormone, thereby contributing to the development of depression characterized by symptoms such as sleep disturbances, anxiety, and cognitive dysfunction [22]. The CUMS model, which simulates clinical syndromes of depression including anhedonia, despair, and social withdrawal by exposing animals to various stressors, is widely employed to investigate the pathophysiology of depression and screen for antidepressant agents [17]. Previous studies have reported that chlorogenic acid, a major bioactive compound of EAY, produces antidepressant-like effects by decreasing immobility time in the TST and FST [23]. Rutin, another bioactive compound identified in EAY, was reported to alleviate depressive-like behaviors by reducing time spent in the periphery in the OFT and increasing sucrose preference in the SPT [19]. In this study, the group treated with EAY containing rutin and chlorogenic acid exhibited improvements in depressive-like behaviors in the SPT, OFT, TST, and FST relative to the CUMS group (Fig. 2). These results indicate the potential of EAY to attenuate chronic stress-induced depressive-like behaviors.

Hyperactivation of the HPA axis induced by CUMS is widely accepted in modern psychiatry as a crucial mechanism underlying the pathogenesis of depression [17]. It begins with the secretion of CRF from the hypothalamus, which stimulates the release of ACTH from the pituitary gland, resulting in elevated cortisol secretion from the adrenal glands [2]. This process is normally regulated by negative feedback mechanisms, however, chronic exposure to stress impairs the function of the HPA axis, leading to hypercortisolemia [19]. Elevated cortisol levels overactivate dopamine D2 autoreceptors, suppressing the intrinsic excitability and excitatory synaptic transmission of ventral tegmental area (VTA) dopaminergic neurons, which attenuates mesolimbic dopamine signaling and consequently diminishes reward-seeking while increasing anxiety-like behavior [24]. In addition, structural changes in the prefrontal cortex are induced, including decreased dendritic spine density and reduced synaptic plasticity, while simultaneously causing dendritic growth in the amygdala, leading to weakened logical emotional regulation and strengthened primitive emotional responses [25]. Chlorogenic acid, rutin, and dicaffeoylquinic acids, which are found in *Synurus deltoides*, modulated HPA axis-related expression levels of CRF, ACTH, and CYP11B1 in a CUMS mouse, thereby reducing corticosterone levels and ameliorating neurofunctional impairments [26]. In addition, the results showed that rutin alleviated depression-like behaviors in a CUMS-induced rat model through modulation of the HPA axis hormone ACTH, thereby normalizing cortisol levels associated with the development of depression [27]. Similarly, the EAY group containing caffeic acid and rutin was found to attenuate hyperactivation of the HPA axis relative to the CUMS group (Fig. 3). Therefore, these results demonstrate that EAY may have potential promise as a candidate for modulating stress levels by regulating the HPA axis.

Most MDD patients experience cognitive decline, including impairments in memory, calculation, sense of direction, and spatial perception, which is associated with hyperactivation of the HPA axis [4]. Excessive secretion of cortisol promotes neuroinflammation, diminishes neuroplasticity in the prefrontal cortex and hippocampus, and consequently induces cognitive dysfunction. This process may ultimately function as a pathological mechanism facilitating neurodegenerative disorders such as Alzheimer's and Parkinson's [3]. However, a previous study reported that the ethyl acetate fraction of *A. yomena* improved learning and memory abilities in the T-maze and MWM tests by downregulating inflammation-related proteins such as NF- κ B and IL-1 β in HFD-induced mice [15]. Similarly, *Ficus erecta* Thunb. extract, which contains chlorogenic acid, rutin, and kaempferol-3-O-rutinoside identified in EAY, was found to enhance BDNF expression and alleviate neuroinflammation, thereby reducing escape latency in the MWM test in A β -induced cognitive impairment mice [27]. In this study, consistent with previous findings, the EAY group showed improved learning and memory abilities relative to the CUMS group (Fig. 4). These results demonstrate the beneficial effect of EAY on improving CUMS-induced depressive cognitive dysfunction. Therefore, we investigated biological markers related to neuroinflammation, oxidative stress, mitochondrial dysfunction, neuronal apoptosis, and synaptic plasticity to elucidate the neuroprotective effects of EAY.

Neuroinflammation in MDD patients is associated with microglial hyperactivation, in which the TLR4/NF- κ B pathway contributes to the amplification of inflammatory responses [7]. Microglia, the resident macrophage-like cells of neural tissue, perform neuroprotective functions and eliminate damaged neurons under normal physiological conditions [6, 19]. However, cortisol during hyperactivation of the HPA axis stimulates microglia, leading to increased expression of TLR4, a pattern recognition receptor (PRR) [29]. This leads to activation of the NF- κ B pathway, resulting in enhanced secretion of TNF- α , IL-6, and IL-1 β , which induces chronic neuroinflammation and inhibits neuronal survival and growth, thereby precipitating cognitive dysfunction [6, 7]. A previous study has shown that caffeic acid isolated from *A. yomena* exhibits inhibitory activity against IL-6, a key factor of the inflammatory response, in TNF- α -stimulated MG-63 fibroblast cells *in vitro* [12]. The methanol extract of *A. yomena* containing chlorogenic acid was shown to attenuate lipopolysaccharide (LPS)-induced microglial activation by moderating the NF- κ B and mitogen-activated protein kinase (MAPK) pathways, thereby decreasing the levels of inflammatory cytokines such as IL-1 β and TNF- α [30]. Similar to previous findings, this study demonstrated that the EAY group alleviated CUMS-induced neuroinflammation by downregulating the TLR4/NF- κ B pathway (Fig. 5). These results suggest a potential role of EAY in the prevention of depressive cognitive dysfunction through alleviation of CUMS-induced neuroinflammation.

Chronic neuroinflammation promotes excessive production of ROS and impairs the antioxidant system, thereby inducing oxidative stress and increasing the risk of neurodegenerative disorders. Overactivated microglia release inflammatory cytokines such as TNF- α and IL-1 β , which activate NADPH oxidase (NOX) on the neuronal membrane, promoting the conversion of molecular oxygen into superoxide anions (O $_2^{\cdot-}$) [7, 19]. It causes direct cellular damage by reacting with biomolecules while simultaneously being converted to H $_2$ O $_2$ and hydroxyl radicals (OH $\cdot$), exacerbating oxidative stress and promoting neuronal damage [1]. In particular, ROS induce lipid peroxidation by reacting with polyunsaturated fatty acids that constitute neuronal membranes, thereby compromising their structural integrity and leading to cellular dysfunction and neuronal degeneration [1, 19]. Brain tissue possesses endogenous antioxidant defense systems, including GSH, SOD, catalase, and glutathione peroxidase, although their expression levels are significantly lower than in other tissues [2]. Rutin, a compound present in EAY, was found to improve depressive neurofunctional impairment by regulating the levels of SOD, catalase, glutathione, and MDA in the brain tissue of social defeat stress (SDS)-induced mice [31]. In addition, previous research reported that the ethyl acetate fraction of *A. yomena* attenuated oxidative stress-induced memory deficits by reducing elevated MDA levels in the brain tissue of HFD-induced mice [15]. Similarly, in this study the EAY group alleviated the imbalanced levels of MDA, SOD, and GSH in the brain tissue of CUMS-induced mice (Fig. 6). This result suggests that EAY can enhance the antioxidant defense system for alleviation of oxidative stress, thereby potentially contributing to the prevention of cognitive dysfunction.

Brain functions, including cognitive function, require substantial energy, with mitochondria serving as the primary source of energy production [1, 3]. The mitochondrial electron transport chain (ETC) converts approximately 2% of oxygen consumed into ROS under normal conditions [32]. However, mitochondrial DNA (mtDNA), which encodes multiple protein subunits of the ETC complexes but has limited repair capacity, accumulates oxidative base lesions, strand breaks, and deletions under oxidative stress, thereby impairing electron transport and increasing electron leak, which consequently accelerates generation of ROS [33]. Excessive ROS peroxidize cardiolipin, a major phospholipid of

the inner mitochondrial membrane, thereby weakening the structural stability of respiratory supercomplexes and the binding of cytochrome c, which in turn dissipates the MMP [32]. In addition, oxidative stress drives the sustained opening of the mitochondrial permeability transition pore (mPTP), disrupting membrane selectivity and increasing non-selective permeability, thereby impairing ATP synthesis and accelerating accumulation of ROS, establishing a positive feedback loop of mitochondrial dysfunction [34]. Consequently, mitochondrial dysfunction leads to neuronal metabolic disturbances, potentially resulting in cognitive impairment [35]. Chlorogenic acid and caffeic acid identified in *A. yomena* have therapeutic potential for neurodegenerative diseases associated with neurotoxicity, as they restored glutamate-induced accumulation of ROS and depolarization of MMP in 8-DIV cortical neurons derived from the cortical lobes of E18 Sprague-Dawley rat embryos and cultured for 8–10 days before use [36]. Similarly, treatment with the ethanol extract of *A. yomena* reduced ROS levels in H₂O₂-treated SK-N-MC and HT-22 neuronal cells [16]. In this study, EAY alleviated CUMS-induced mitochondrial dysfunction, including excessive production of ROS, collapse of MMP, and a decrease in ATP levels (Fig. 7). These findings suggest that EAY can improve mitochondrial dysfunction, leading to the prevention of cognitive impairment.

Mitochondrial impairment activates the apoptosis pathway, promoting neuronal cell death, which is a key factor accelerating the progression of neurological disorders [34, 35]. An interaction occurs between the anti-apoptotic protein BCL-2 and the pro-apoptotic protein BAX under basal conditions, inhibiting the oligomerization and activation of BAX, thereby preventing the initiation of apoptosis [37]. However, mitochondrial dysfunction, which is associated with the overproduction of ROS, promotes oxidative modification and conformational activation of BAX, disrupting the interaction between BAX and BCL-2 and driving translocation of BAX to the mitochondrial outer membrane, where BAX oligomerizes to form pores [38]. This results in release of cytochrome C, subsequently leading to the activation of caspase-3 which cleaves structural and DNA repair proteins such as poly ADP-ribose polymerase (PARP), thereby inducing apoptotic cell death [38, 39]. In addition, the activity of β -secretase and γ -secretase is increased by caspase-3, promoting cleavage of amyloid precursor protein (APP) and accumulation of A β [40, 41]. In addition, Tau protein is cleaved by caspase-3, inducing p-Tau aggregation and formation of neurofibrillary tangles (NFT), leading to instability of microtubules [42]. In this process the accumulated A β and p-Tau further inhibit the mitochondrial ETC complex, particularly complexes I and IV, leading to energy deficiency and oxidative stress, thereby forming a pathological loop that ultimately accelerates neuronal cell death [43]. The ethyl acetate fraction of *A. yomena* exerted neuroprotective effects by inhibiting apoptosis in SH-SY5Y neuronal cells through the downregulation of H₂O₂-induced expression of apoptosis-related proteins such as the caspase-9, BAX/BCL-2 ratio, and PARP [44]. Dicafeoylquinic acid, a compound found in *A. yomena*, was shown to modulate the expression of apoptosis-related proteins, including caspase-3 and cytochrome c, in the brain tissue of HFD-fed C57BL/6 mice, thereby ameliorating the expression levels of p-Tau and A β , which are associated with cognitive impairment [45]. Consistent with previous findings, the EAY group attenuated the elevated expression of apoptosis-related proteins in the brain tissue of CUMS-induced mice (Fig. 8). These findings suggest that EAY may prevent neurodegenerative diseases by inhibiting apoptosis mediated by the BCL-2 protein family.

The neurobiological changes observed in MDD cause damage to the cholinergic nervous system and synaptic plasticity, which are closely associated with cognitive function [6, 8, 35]. The cholinergic system, which utilizes ACh as a neurotransmitter, is essential for cognitive functions such as memory and learning [45]. ACh is produced by ChAT and influences neuronal activity in the hippocampus and prefrontal cortex [46, 47]. Thus, a decrease in ChAT activity and an increase in AChE activity weaken cholinergic neurotransmission, which directly impairs cognitive function [46]. In addition, this condition leads to decreased synaptic function, which negatively affects cognitive flexibility and memory formation [46]. GAP-43, located in the presynaptic terminal, serves a pivotal role in neuronal growth, axonal plasticity, and synaptogenesis, which are directly linked to enhanced learning and memory capabilities [48]. PSD-95, a critical postsynaptic scaffolding protein, stabilizes synaptic connections and maintains neural circuitry [49]. The decreased expression of these proteins ultimately compromises synaptic connectivity, impairing memory formation [50]. In addition, downregulation of PSD-95 leads to inhibition of the tropomyosin receptor kinase B (TrkB)/cyclic AMP response element binding protein (CREB) pathway, thereby decreasing the expression of BDNF, which is essential for synaptic plasticity, neuronal development, and survival [19, 51]. BDNF, the principal ligand of TrkB, is reduced, further impairing TrkB signaling, thereby diminishing expression of extracellular signal-regulated kinase (ERK)-mediated GAP-43 and exacerbating deficits in synaptic plasticity [52]. This condition contributes to a range of central nervous system dysfunctions, including MDD and cognitive impairment [2]. Caffeic acid and chlorogenic acid, present in *A. yomena*, have been documented to prevent neurodegeneration by inhibiting the activity of AChE and BChE, enzymes that hydrolyze acetylcholine in the brain, thereby increasing its synaptic availability and enhancing inter-neuronal signaling [20]. Rutin was reported to alleviate cognitive impairment in Tau-P301S mice through modulation of oxidative stress and neuroinflammation, thereby restoring the expression of synaptic proteins such as synaptophysin and PSD-95 [53]. In addition, dicafeoylquinic acid regulated expression of BDNF in Pb-induced neurotoxicity mice, thereby ameliorating depressive cognitive dysfunction via the CREB/phosphoinositide 3-kinase (PI3K)/Akt and TLR4/myeloid differentiation primary response gene 88 (MyD88) pathways [54]. In this study, EAY containing these bioactive compounds was found to improve CUMS-induced impairments in the cholinergic system and synaptic plasticity (Fig. 9). In addition, PCA demonstrates that EAY treatment shifts the multivariate profile toward that of the NC group, indicating mitigation of these maladaptive interactions (Fig. 10). In conclusion, these findings indicate that EAY confers protection against cognitive deficits by modulating HPA-axis-driven neuroinflammation, neurotoxicity, cholinergic dysfunction, and impaired synaptic plasticity. However, this study has a technical limitation because it did not provide direct quantitative or visual evidence for ROS and apoptosis using flow cytometry or confocal microscopy, which are implicated in the pathophysiology of depression-related cognitive impairment. In addition, the mitochondrial analysis did not include a comparison of cytochrome c levels between the cytosolic and mitochondrial fractions, which limits the interpretation of the results to a specific fraction. Therefore, future studies should complement the current findings on ROS and apoptosis and assess cytochrome c distribution between fractions to refine the mechanistic basis for the neuroprotective effects of EAY.

Author Contributions

J.M.K. and **H.J.H.**: Conceptualization, **I.Y.K.** and **J.M.K.**: Methodology, **H.S.L.**: Software, **H.J.C.** and **H.J.H.**: Validation, **H.S.L.** and **J.H.K.**: Formal analysis, **J.H.K.** and **I.Y.K.**: Investigation, **J.M.K.**: Data curation, **I.Y.K.**: Writing—original draft preparation, **H.L.L.** and **H.J.H.**: Writing—review and editing, **I.Y.K.**: Visualization, **H.J.H.**: Supervision and Project administration.

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