Ficus Erecta Thunb. Leaves Protect Against Cognitive Deficit and Neuronal Damage in a Mouse Model of Amyloid- β -induced Alzheimer's Disease

Eunjin Sohn

Korea Institute of Oriental Medicine <https://orcid.org/0000-0002-9410-4788>

Yu Jin Kim

Korea Institute of Oriental Medicine

Joo-Hwan Kim

Gachon University

Soo-Jin Jeong (✉ sjjeong@kiom.re.kr)

Clinical Medicine Division, Korea Institute of Oriental Medicine, Daejeon 34054, South Korea;
<https://orcid.org/0000-0001-6529-1767>

Research

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Abstract

Background: Alzheimer's disease (AD) pathogenesis is linked to amyloid plaque accumulation, neuronal loss, and brain inflammation. *Ficus erecta* Thunb. is a food and medicinal plant used to treat inflammatory diseases.

Methods: we investigated the neuroprotective effects of *F. erecta* Thunb. against cognitive deficit and neuronal damage in a mouse model of amyloid- β (A β)-induced AD.

Results: First, we confirmed the inhibitory effects of ethanol extracts of *F. erecta* (EEFE) leaves on A β aggregation *in vivo* and *in vitro*. Next, behavioral tests (passive avoidance task and Morris water maze) revealed EEFE markedly improved cognitive impairment in A β -injected mice. Furthermore, EEFE reduced neuronal loss and the expression of neuronal nuclei (NeuN), a neuronal marker, in brain tissues of A β -injected mice. EEFE significantly reversed A β -induced suppression of cAMP response element-binding protein (CREB) phosphorylation and brain-derived neurotrophic factor (BDNF) expression, indicating neuroprotection was mediated by CREB/BDNF signaling. Moreover, EEFE significantly suppressed the inflammatory cytokines interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), and expression of ionized calcium-binding adaptor molecule 1 (Iba-1), a marker of microglial activation, in brain tissues of A β -injected mice, suggesting anti-neuroinflammatory effects.

Conclusions: Taken together, EEFE protected against cognitive deficit and neuronal damage in AD-like mice via activation of CREB/BDNF signaling and upregulation of inflammatory cytokines.

Background

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder that leads to memory loss and learning deficit. The main hallmark of AD pathology is accumulation of amyloid- β (A β) peptide and the formation of large aggregates (plaques) in the brain [1, 2]. Therefore, prevention of A β aggregation and accumulation is a potential strategy for the treatment of AD. Although anti-A β treatment has failed in clinical trials, A β is still considered a key target molecule for developing novel AD therapies [3, 4]. However, U.S. Food Drug Administration (FDA)-approved drugs for AD, namely tacrine, galantamine, rivastigmine, donezil, and memantine, target acetylcholinesterase or the *N*-methyl-d-aspartate (NMDA) receptor, not A β ; those drugs do not provide a cure and have low efficacy and severe side effects. In the face of these limitations, complementary and alternative therapies could offer a solution to block the progression of AD.

Many studies have reported the potential of natural products or medicinal plants to prevent or treat AD or AD-related diseases [5–7] by exerting anti-amyloidogenesis, anti-inflammation, and anti-oxidation effects [5], inhibiting A β aggregation, and improving A β -induced neurotoxicity *in vivo* and *in vitro* [8–12]. More recent studies have reported that extracts of natural plants enhance cognitive memory and reduce neuroinflammation in a A β -induced AD rat model [13], and attenuate neuroinflammation in microglial cells [14]. If proven to be effective and safe, these natural plants could be used in patients with AD [8].

Ficus erecta Thunb. (hereinafter referred to simply as *F. erecta*) is a native plant distributed in seashore areas of Korea, Japan, and China. *F. erecta*, which is resistant to fungus, has been used as a food and medicine for nephritis and arthritis, such as inflammatory diseases. [15, 16]. Recent studies have reported that *F. erecta* leaves inhibit osteoporotic inflammatory factors [17] and that *F. erecta* fruits exert anti-oxidant and thrombolytic activities [18]. Despite the traditional uses of *F. erecta*, scientific evidence of its pharmacological activities has been limited to the two above-mentioned articles. In the current study, we assessed the neuroprotective effects of *F. erecta* against cognitive deficit and neuronal damage in A β -induced AD mouse model. Here, we report that EEFE has the potential to inhibit A β aggregation and protect against neuronal damage and inflammation via activation of CREB/BDNF signaling and upregulation of inflammatory cytokine levels in A β -injected (AD-like) mice.

Materials And Methods

Plant extracts

The dried leaves and branches of *F. erecta* were kindly provided by the Korean Seed Association (Seongnam, Korea) and identified by Professor Joo-Hwan Kim (Gachon University, Seongnam, Korea). Voucher specimens (SCD-A-114-1 and SCD-A-114-2) were deposited at the Clinical Medicine Division, Korea Institute of Oriental Medicine (Daejeon, Korea). The dried leaves (3.4 kg) and branches (4 kg) of *F. erecta* were extracted twice with aqueous ethanol using an electric extractor (COSMOS-660, Kyungseo Machine Co., Incheon, Korea) for 3 h at $80 \pm 2^\circ\text{C}$. The filtered extracted solution was concentrated using a rotary evaporator under vacuum and freeze-dried to obtain powdered leaf and branch extracts (636.13 and 293.31 g, respectively). The yield of EEFE was 18.71% (leaf extracts) and 7.33% (branch extracts).

In vitro A β_{1-42} aggregation assay

The A β_{1-42} aggregation assay was performed as described in our previous report [19] using the Thioflavin T β -Amyloid Aggregation kit (Cat. AS-72214, Anaspec, Inc., Ferment, CA, USA). The mixture of A β_{1-42} peptide solution and EEFE was combined with thioflavin T. Then, fluorescence signals were read at an excitation/emission wavelength = 440/484 nm at 37°C using a Multi-Mode microplate reader (SpectraMax i3, Molecular Devices, Sunnyvale, CA, USA). Morin (Cat. M4008-2G, Sigma-Aldrich, St. Louis, MO, USA) was used as an A β aggregation inhibitor, positive control. Experiments were performed in triplicate and repeated three times. The inhibition (%) of A β_{1-42} aggregation was calculated by following equation:

$$\text{Inhibition of A}\beta \text{ aggregation (\%)} = \left(1 - \frac{\text{Fluorescence of A}\beta - \text{treated sample}}{\text{Fluorescence of untreated sample}}\right) \times 100$$

Animals and intracerebroventricular injection of A β aggregates

Male C57BL6 mice weighing 24 ± 2 g were obtained from Orient Bio (Seoul, South Korea), and acclimated for 1 week prior to the study. Animals were given standard rodent diet (2918C; ENVIGO, Blackthorn, UK) and water *ad libitum*. They were maintained in individual acrylic cages at a temperature of $23 \pm 3^\circ\text{C}$, relative humidity of $55 \pm 15\%$, ventilation frequency of 10–20 times/h, illumination intensity of 150–300 lux, and lights on from 8 AM until 8 PM (12:12 h light:dark cycle). The experiments were approved by the Institutional Animal Care and Use Committee of Nonclinical Research Institute, Chemon Inc. (IACUC Approval No. 18-M111) and was performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

The $\text{A}\beta_{1-42}$ peptide was obtained from Tocris Bioscience (Cat. 1428, Pittsburgh, PA, USA), dissolved to a concentration of $1 \mu\text{g}/\mu\text{L}$ in sterilized 0.1 M phosphate-buffered saline (PBS; pH 7.4), and incubated at -20°C for 4 weeks to encourage the formation of $\text{A}\beta$ aggregates. Mice were anesthetized with mixed zoletil (tiletamine–zolazepam; Verbac, Carros, France) and rompun (Bayer, Leverkusen, Germany) (4:1 v/v) at dose of 1 mL/kg before $\text{A}\beta$ injection. The incubated $\text{A}\beta$ aggregates were injected in the intracerebroventricular (ICV) area using a stereotaxic apparatus (Harvard Apparatus, Panlab, San Diego, CA, USA) fixed at the following coordinates: anterior/posterior (AP) -1.0 mm, dorsal/ventral - 2.5 mm, and mediolateral/lateral + 1.0 mm. Aggregated $\text{A}\beta_{1-42}$ ($5 \mu\text{L}$) was infused at a rate of $2 \mu\text{L}/\text{min}$ speeds [20]. Saline was used as a control. At 24 h after the injection, experimental mice were assorted into five groups ($n = 8$ per group): normal control mice (NOR), $\text{A}\beta_{1-42}$ -injected mice ($\text{A}\beta$), $\text{A}\beta$ + EEFE-injected mice at 50 or 150 mg/kg/day (EEFE-50 or EEFE-150), and $\text{A}\beta$ + morin-injected mice at 10 mg/kg/day (M-10). Animals received a nontoxic concentration of EEFE for 20 days. The experimental schedule, including $\text{A}\beta$ injection, EEFE administration, and behavioral testing, is shown in Fig. 1.

Preparation of brain tissue sections for immunohistochemistry and Nissl staining

At the end of experimental period, mouse from each group were sacrificed under deep anesthesia. Three mouse brain from each group were perfused transcardially with saline and then fixed in 4% paraformaldehyde. Each of hippocampal and cortical tissues isolated from five mouse of each group were immediately stored at -80°C until further analysis. Brain paraffin blocks were sliced into $4\text{-}\mu\text{m}$ -thick sections. The slides were deparaffinized and hydrated with xylene and sequentially of ethanol solutions, respectively. Slides were treated with 0.3% hydrogen peroxide in methanol for 25 min and briefly rinsed in PBS for quenching endogenous peroxidase activity. For antigen retrieval, slides were boiled in citrate buffer (pH 6.0) in a microwave for 15 min. Slides were incubated with horse serum for blocking for 1 h at 37°C and then probed with primary anti- $\text{A}\beta$ (AB_10988723, 1:250 dilution; Santa Cruz Biotechnology Inc., Dallas, TX, USA), anti-neuronal nuclei (NeuN) (AB_10711153, 1:250 dilution; Abcam, Cambridge, UK), and anti-ionized calcium-binding adaptor molecule 1 (Iba-1) (AB_839504, 1:100 dilution; Wako Pure Chemicals, Osaka, Japan) antibodies for overnight at 4°C . To detect $\text{A}\beta$, NeuN, and Iba-1, the slides were incubated with labeled streptavidin–biotin for 30 min and visualized using diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA, USA). Slides were immersed in 1% cresyl violet acetate solution for Nissl staining, washed with water, and dehydrated with 90% and 100% ethanol

for 5 min before mounting in xylene. Mounted slides were then captured at 400 × magnification using a microscope (Olympus DP71, Tokyo, Japan). Image analysis was performed by blinded investigators using Image J software program (Java-based image processing program, NIH, Bethesda, MD).

Western blot analysis

Homogenized brain lysates (30 µg) were resolved on polyacrylamide gels and then transferred on 0.2-µm PVDF membranes using the Trans-Blot transfer system (Bio-Rad, Hercules, CA, USA). Blocked membranes with 5% nonfat milk washed with Tris-buffered saline with 0.1% tween 20 (TBST) and then incubated with primary anti-BDNF (AB_10862052), anti-phospho-CREB (AB_731734), anti-total CREB (AB_2827810, Abcam), and anti-Aβ (1:3000 dilution; Santa Cruz Biotechnology Inc.) antibodies for overnight at 4°C. Washed membranes with TBST were incubated with secondary antibodies anti-rabbit-horseradish peroxidase (HRP). Immuno-reactive membrane was developed using the chemiluminescent substrate reagents (Cat. 34577, Amersham Bioscience, Piscataway, NJ, USA). Membranes were also incubated with anti-β-actin (AB_476743, Sigma-Aldrich, Saint Louis, MO, USA) for loading control. Protein bands were detected by using imaging analyzer (Las-4000 MINI; Fuji Photo, Tokyo, Japan) and quantified with Image J software (NIH).

Behavioral analysis

As we have described previously [21], memory and learning function of experimental mice was conducted using passive avoidance task (PAT) and Morris water maze (MWM) tests. The PAT was performed using an electronic shock generator with lightened and darkened compartments (Jeungdo Bio & Plant Co. Ltd., Seoul, Korea) on days 8–10 after Aβ injection. Transfer latency time was recorded as the amount of time (within 5 min) the mice remained in the lightened compartment. The MWM was conducted on days 15–21 after Aβ injection. Mice were put in a water pool containing four designated release points and allowed to find the escape platform for 60 s. After finding the platform, the animals were allowed to rest for 30 s on the platform. If mice failed to find the platform within 60 s, they were guided to rest on the platform for 30 s. After all animals finished the first trial, the next trial was started. The release points were randomly chosen every time without overlap. Time taken to find the platform (escape latency) was monitored (training: 2 days, behavioral tests: 4 days, and probe trial: 1 day). In the last day (day 7), the platform was removed 1 h after EEFE administration and the probe trial was performed for 60 sec to measure the number of times the mice crossed the platform. Experimental mice were given EEFE or morin prior to behavioral test.

Quantitative analysis of inflammatory cytokines in brain tissues

Mouse pro-inflammatory cytokines, TNF-α and IL-1β were measured in brain lysates using commercial competitive ELISA (Cat. MBS2500421, MyBioSource, San Diego, CA, USA) and (Cat. AB100705, Abcam), respectively. Supernatants of brain lysates were collected by according to manufacture's protocol. The concentration of TNF-α and IL-1β was calculated from the standard curves. The optical density (OD) was measured using a microplate reader (BioTek Instrument, Winooski, VT, USA) at 450 nm.

Chemicals, Reagents, And Sample Preparation For Quantitative Analysis

Three standard compounds (rutin, chlorogenic acid, and kaempferol-3-*O*-rutinoside) and Biopurify Phytochemicals were purchased from ChemFaces Biochemical (Wuhan, China) and (Chengdu, China) (purity > 98% by high-performance liquid chromatography [HPLC]), respectively. Acetonitrile and water of HPLC grade (J. T. Baker Chemical, Phillipsburg, NJ, USA), and trifluoroacetic acid (TFA, Sigma-Aldrich) were used for quantitative analysis, respectively. For quantitative analysis, powdered EEFE leaves were dissolved in 80% aqueous methanol to a final concentration of 10 mg/mL and filtered through a syringe filter (0.45- μ m pore size). To make a standard mixture, the stock solutions of the three standard compounds (1.0 mg/mL) were mixed with methanol to final concentration of 0.1 mg/mL and diluted to obtain various concentrations of standard solution before chromatographic analysis.

Chromatographic Conditions

For HPLC analysis, we used the HPLC system equipped with a photodiode array (PDA) detector (Waters Alliance e2695, PDA #2998, Waters Corp., Milford, MA, USA). The data were acquired and processed using Empower software (version 3; Waters Corp.). For chromatographic separation of the three compounds, we used a Sunfire C₁₈ analytical column (250 $\times$ 4.6 mm, 5 μ m, Waters Corp.), which we maintained at 35 °C. The gradient conditions were 10–23% B for 30 min, 23–100% B for 10 min, and 100% B for 10 min. The mobile phases consisted of two solvents: 0.1% (v/v) TFA in water (A) and acetonitrile (B). For scanning chromatograms, PDA detection was performed at 210–400 nm. Column were used at flow rate of 1.0 mL/min and injected volume of 10 μ L, respectively.

Statistical analysis

All data were expressed as the mean $\pm$ SEM. A value of $p < 0.05$ was considered to indicate statistical significance. Prism software (Graph Pad, version 8.4.1, San Diego, CA, USA) was used for all analyses. Statistically significant differences were evaluated with one-way analysis of variance (ANOVA) for comparison three or more groups, and with an unpaired or paired Student's *t*-test for comparison between two groups. All experiments were performed individually at least three times.

Results

Inhibitory effects of EEFE on A β aggregation

A β accumulation is a pivotal pathogenic factor in AD progression [22]. We assessed the effects of EEFE leaves or branches on A β aggregation *in vitro*. In contrast to the branch extracts, the leaf extracts inhibited A β aggregation more dramatically. The half maximal inhibitory concentration (IC₅₀) values of leaf and branch extracts were 43.43 and > 100 μ g/mL, respectively (Fig. 2A and B).

Inhibitory effects of EEFE on A β aggregation in A β -injected mice

The effects of EEFE leaf extracts on A β aggregation were further investigated using A β -injected mice. A β aggregates were delivered through ICV injection into the mouse brain, and EEFE was administered orally for 20 days at 50 or 150 mg/kg/day. Compared with the NOR group, the A β group had remarkably more A β -positive cells in both hippocampal and cortical regions (Fig. 3A–C). In contrast, the EEFE-150 group, but not the EEFE-50 group, had markedly fewer A β -positive cells than the A β group. Consistently, the EEFE-150 group had significantly decreased expression of A β protein in brain tissues compared to the A β group (Fig. 3D and E). Morin, positive control, was used for an inhibitor of A β aggregation.

Effects of EEFE on cognitive deficit in A β -injected mice

To investigate whether EEFE protects against A β -induced cognitive deficit in mice, we carried out the PAT and MWM behavioral tests. In the PAT, the transfer latency time was significantly shorter in the A β group than in the NOR group. In contrast, the transfer latency time was significantly higher in the EEFE-150 group than in the A β group (Fig. 4A). In the MWM, the mean escape latency time to find the hidden platform was significantly higher in the A β group than in the NOR group over the five training days. In contrast, the EEFE-150 group exhibited a marked decline of the latency time compared to the A β group (Fig. 4B). In the probe trial, the time spent in the target quadrant and platform crossings number were significantly lower in the A β group than in the NOR group. In contrast, A β -induced negative effects were remarkably reversed in the EEFE-150 group (Fig. 4C and D). These results demonstrate that EEFE improved spatial memory impairment in mice with A β -induced cognitive deficit.

Effects of EEFE on neuronal loss in A β -injected mice

To determine whether EEFE prevents neuronal loss in mice with A β -induced cognitive deficit, we performed Nissl staining and immunohistochemistry for NeuN, a neuronal marker. The number of surviving neurons in the CA1 area of the hippocampus and cortex was markedly reduced in the A β group compared to the NOR group, a feature that was accompanied by neuronal shrinkage or loss (Fig. 5A). The number of Nissl-stained cell bodies in the CA1 area of the hippocampus (Fig. 5B) and cortex (Fig. 5C) was significantly higher in the EEFE-150 and M-10 groups than in the A β group. Moreover, the number of NeuN-positive cells in the hippocampus and cortex was significantly lower in the A β group than in the NOR group (Fig. 5D–F). In contrast, neuronal cell loss in the A β group was markedly reversed in the EEFE and M groups.

Effects of EEFE on the CREB/BDNF pathway in A β -injected mice

The CREB/BDNF pathway is critical in the promotion of neuronal cell survival related with memory and learning ability in AD [23]. We determined the expression of BDNF and phosphorylation of CREB by western blot analysis. In the A β group, the levels of phospho-CREB and BDNF in brain tissue lysates were

markedly reduced compared to that in the NOR group. However, A β -induced suppression of CREB phosphorylation and BDNF expression was significantly reverted in the EEFE-150 group (Fig. 6A–C). Positive control morin also had inhibitory effects on the CREB/BDNF signaling pathway.

Effects of EEFE on inflammatory protein expression in A β -injected mice

Neuroinflammation plays an essential role in the pathogenesis of AD [24]. We investigated whether EEFE protected against neuroinflammation in A β -injected mice. Immunohistochemistry and ELISA were carried out to assess the levels of Iba-1, known as maker of microglia cell activation, and pro-inflammatory cytokines IL-1 β and TNF- α in the brain, respectively. Iba-1 positive cells were markedly enriched in the hippocampus and cortex region of the A β group compared with the NOR group (Fig. 7A–C). Consistently, the levels of IL-1 β and TNF- α in the brain lysates were significantly elevated in the A β group compared with the NOR group. In contrast, the EEFE-150 group significantly reversed the levels of Iba-1, IL-1 β , and TNF- α compared to the A β group (Fig. 7). Morin showed anti-neuroinflammatory effects.

Determination of the three standard compounds in EEFE

The simultaneous determination of the three standard compounds in EEFE was performed by an optimized HPLC method. The chromatograms showed good separation using mobile phases consisting of 0.1% (v/v) TFA in water and acetonitrile. The ultraviolet (UV) wavelengths to detect compounds were 320 nm for chlorogenic acid and 260 nm for rutin and kaempferol-3-*O*-rutinoside. The retention times of chlorogenic acid, rutin, and kaempferol-3-*O*-rutinoside were 10.71, 23.48, and 27.97 min, respectively. The three compounds were resolved within 30 min. The HPLC chromatograms of EEFE and the standard mixture are presented in Fig. 8. The calibration curves for the three compounds were calculated by the linear relationships between the concentration (x, μ g/mL) ranging from 3.125–100 μ g/mL and peak area (y) for each standard compound and presented as regression equations ($y = ax + b$) in Table 1. All correlation coefficient values showed good linearity ($r^2 \geq 0.9999$). The limit of detection (LOD) for the three compounds ranged from 0.185–0.526 μ g/mL and the limit of quantitation (LOQ) ranged from 0.562–1.595 μ g/mL. The amounts of chlorogenic acid, rutin, and kaempferol-3-*O*-rutinoside in EEFE were 1.80, 5.49, and 1.38 mg/g, respectively (Table 1).

Discussion

In current study, we explored for the first time the pharmacological effects of *F. erecta* leaves in AD or AD-related diseases. We found that EEFE prevented A β aggregation, improved memory and cognitive dysfunction, and had neuroprotective and neuroinflammatory effects in a mouse model of A β -induced AD.

A β , a key molecule in the AD pathogenesis, leads to neurotoxicity and neuronal loss [25], and increases production of Iba-1, TNF- α , and IL-1 β level, known as inflammatory factors, in the brain of patients with AD [26]. The main symptom of AD is memory and cognitive deficit [1, 20]. A β -induced AD is a well-

characterized AD-like model that is characterized by learning and memory impairment as well as neuroinflammation and neuronal damage [27, 28], supporting its validity as a model for AD [29]. Accumulating behavioral data demonstrate that direct injection of neurotoxic A β aggregates into the mouse brain causes memory and cognitive deficit [3, 30]. On the basis of these results, we conducted PAT and MWM behavioral tests in mice with A β -induced AD treated with or without EEFE for 20 days. Consistent with previous reports [3], our results demonstrate that A β injection significantly enhanced memory and cognitive deficits in the MWM and PAT whereas EEFE treatment markedly reversed A β -mediated cognitive deficit, suggesting that EEFE could protect against memory and cognitive deficits in AD-like mice.

Memory and cognitive deficits are associated with the interplay between hippocampal and cortical regions caused by A β accumulation [31, 32]. Death of neuronal cells by A β accumulation in the prefrontal brain area has been regarded as an early event in patients with AD [33, 34]. That is, hippocampal and cortical damage might contribute to memory and cognitive deficits in AD. In the present study, we found that EEFE inhibited A β aggregation in the brain of A β -injected mice. A β aggregates accumulation in the mouse brain was visualized using immunohistochemistry and western blotting. EEFE treatment significantly reduced the number of A β -positive cells in hippocampal and cortical regions and decreased the level of A β protein in brain lysates of A β -injected mice. The inhibitory effects of EEFE on A β aggregation were further confirmed in an *in vitro* A β aggregation assay using thioflavin T. Moreover, Nissl staining and NeuN immunohistochemistry revealed neuronal damage and loss in hippocampal and cortex regions exposed to A β ICV injection. However, EEFE treatment significantly reversed neuronal/NeuN-positive cell loss in brain tissues. These results indicate the protective effects of EEFE against A β -induced neurotoxicity in an AD mouse model.

The CREB/BDNF signaling pathway is a major neuroprotective mechanism in AD. Changes in CREB level are associated with the pathophysiology of various neurodegenerative diseases [23, 35]. CREB phosphorylation is responsible for transcriptional activation, leading to the production of BDNF, which in turn plays key roles in cognitive functions and synaptic plasticity involved in short- and long-term memory formation. Thus, reducing BDNF/CREB levels is thought to be an important component of AD pathology mediated by neurotoxic A β production [36–38]. In this study, we found that EEFE treatment reverted the reduction in phospho-CREB and BDNF protein levels in brain lysates of AD-like mice. These results suggest that EEFE treatment enhanced memory and cognitive function through activation of CREB/BDNF signaling. Actually, many studies have reported that CREB/BDNF activation in the brain of experimental mice facilitates successful memory retrieval [35, 39, 40], implying that enhancers of CREB/BDNF pathway may have the potential for treatment of AD. In this respect, EEFE may be considered one of these candidate drugs.

Neuronal inflammation plays an essential role in facilitating A β deposition, neuronal loss, and cognitive deficit [41–43]. Many studies suggested that inhibitors of pro-inflammatory cytokines may be a valuable strategy for subverting AD progression in preclinical and clinical trials [44]. In addition, recent studies have reported that inflammatory cytokines TNF- α , IL-1 β , and IL-6 are produced by activated microglia,

which are linked to A β -mediated AD pathology. Pro-inflammatory cytokines are pivotal contributors to early-stage AD pathogenesis; they can lead to neuronal injury and cell death [43, 45]. In this regard, upregulation of neuroinflammation is considered a pathological factor of AD that may be targeted by drugs to overcome cognitive deficits. Our results showed that the levels of Iba-1, an activation marker of microglia, and pro-inflammatory cytokines TNF- α and IL-1 β , were increased in A β -injected brain tissues. EEFE treatment markedly reduced the number of Iba-1-positive cells in hippocampal and cortical regions, and the level of inflammatory cytokine in brain lysates of AD-like mice. These results demonstrate that the beneficial effects of EEFE against neuroinflammation might reduce neuronal injury in AD-like mice.

EEFE was effective at 150 mg/kg, but not at 50 mg/kg. However, EEFE was not associated with toxic side effects during the experimental period—not even at 150 mg/kg. The activity of EEFE-150 was comparable to that of morin, a positive control of A β inhibitors. Crude plant extracts or medicinal plants with various flavonoids and alkaloids compounds have been shown to have neuroprotective effects against AD or AD-like conditions [8], and *F. erecta* is one such medicinal plant [15–18]. Our study firstly reports the advantageous effects of EEFE on memory improvement and neuroprotection in AD-like mice. By using HPLC analysis, we identified rutin, chlorogenic acid, and keampferol-3-*O*-rutinoside (nicotiflorin) as standard compounds of EEFE. The most abundant compound in EEFE was rutin (5.49 ± 0.02 mg/g), which of the three standard compounds in EEFE by identified using the quantitative analysis of HPLC–PDA method. We and others have reported that rutin has strong neuroprotective effects due to anti-oxidant, anti-amyloidogenic activities, and anti-inflammatory in neurodegenerative diseases, including AD [3, 46]. Keampferol-3-*O*-rutinoside also attenuates memory dysfunction via neuroprotective effects against oxidative stress and inflammation [47, 48]. Chlorogenic acid has the potential to protect against neurological degeneration involved with oxidative stress and neurotoxicity in the brain [49, 50]. Based on these findings, effects of EEFE against neurodegeneration might be thought of as a result of the synergistic interaction of the constituent components (chlorogenic acid, rutin, and keampferol-*O*-3-rutinoside). Further study will be needed to find more compounds in unidentified peaks of HPLC chromatograms and determine the potential bioactive compound(s) of EEFE.

Conclusions

In summary, EEFE protected against cognitive deficit and neuronal damage in mice with A β -induced neurotoxicity and inflammation. The pharmacological effects of EEFE occurred via activation of the CREB/BDNF signaling pathway and upregulation of inflammatory cytokines. Overall, our findings support EEFE as a promising drug candidate for the treatment or prevention of AD or AD-related neurodegenerative conditions.

Abbreviations

AD Alzheimer's disease

NeuN Neuronal nuclei

PBS Phosphate-buffered saline

TBST Tris-buffered saline with Tween 20 reagent

pCREB Phosphorylated cAMP response elements binding protein

BDNF Brain-derived neurotrophic factor

HPLC High-performance liquid chromatography

TNF- α Tumor necrosis factor alpha

Iba-1 Ionized calcium-binding adaptor molecule 1

IL-1 β Interleukin 1 beta

Declarations

Ethic declarations

Consents for publication: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Availability of data and materials: The data supporting the findings in this research are provided within the manuscript.

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Author Contributions: ES and S-JJ conceptualized the study. ES performed experiments, analyzed, interpreted the data, and wrote original the manuscript. YJK and J-HK performed validation of biochemical analysis and wrote the manuscript. ES, YJK, J-HK, and S-JJ reviewed the result. S-JJ interpreted the data, edited the manuscript, and supervised the study. All authors reviewed and approved the final manuscript.

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Tables

Table 1
Regression equation, linearity, LOD, LOQ, and content of three compounds.

Compound	Linear range ($\mu\text{g/mL}$)	Regression equation ($y = ax + b$) ^{a)}		r^2	LOD ^{b)} ($\mu\text{g/mL}$)	LOQ ^{c)} ($\mu\text{g/mL}$)	Content (mg/g)
		Slope (a)	Intercept (b)				
Chlorogenic acid	3.125–100	33760	-85.066	0.9999	0.185	0.562	1.80 ± 0.01
rutin	3.125–100	21010	8588.4	0.9999	0.443	1.343	5.49 ± 0.02
Kaempferol-3-O-rutinoside	3.125–100	21232	8269.3	0.9999	0.526	1.595	1.38 ± 0.01
^{a)} $y = ax + b$, y means peak area and x means concentration ($\mu\text{g/mL}$)							
^{b)} LOD : $3.3 \times$ (standard deviation (SD) of the response / slope of the calibration curve)							
^{c)} LOQ : $10 \times$ (SD of the response / slope of the calibration curve)							

Figures

Figure 1

Timeline for EEFE administration and behavioral tests in the animal experiments. Amyloid- β aggregates were injected at intracerebroventricular (ICV) area with stereotaxic apparatus into male C57BL6 mice.

EEFE (50 or 100 mg/kg) or morin (10 mg/kg) were orally treated daily for 20 days. The passive avoidance task was performed on the 8th - 10th day. Morris water maze test (MWM) was conducted on the 15th - 21st day. EEFE: ethanol extracts of *Ficus erecta*.

Figure 2

Effects of EEFE leaves and branches on amyloid-β (Aβ) aggregation. (A and B) Aβ aggregation assay was performed by reaction with 6.25, 12.5, 25, 50, or 100 µg/mL concentrations of EEFE leaves (A) and branches (B) with Aβ peptides, followed by the addition of Thioflavin T. Thioflavin T Fluorescence was assessed at an excitation 440 nm and an emission 485 nm, and inhibition rate (%) of Aβ aggregation was calculated. Percentage in the Aβ1-42 peptide solution without sample was set as 100%. Morin (M) was used as a positive control. EEFE: ethanol extracts of *Ficus erecta*.

Figure 3

Effects of EEFE on amyloid- β (A β) aggregation in A β -injected mice. (A) Multiple 4- μ m paraffin sections of hippocampus and cortex regions were prepared from the sacrificed mouse brains. A β expression was determined by immunohistochemistry with A β antibody in hippocampal and cortex region of brain sections. Images were captured using a microscope at 400 x magnification (Olympus DP71) (B and C). Quantitative analysis of A β positive immunostained cells was performed in hippocampal (B) and cortex (C) regions of brain sections. The staining intensities are reported in arbitrary unit (AU). (D) Western blot analysis was conducted to detect levels of A β in brain lysates. (E) Quantitative analysis was conducted to assess the band intensities of A β in the western blotting. Data are presented as the mean $\pm$ SD (n=5). **p<0.01 vs NOR group and #p<0.05 vs A β group. NOR: normal control mice, A β : A β 1-42-injected mice, EEFE: ethanol extracts of *Ficus erecta*, and M: morin.

Figure 4

Effects of EEFE on memory and cognitive deficits in A β -injected mice. (A) The passive avoidance task (PAT) was performed on the 8th-10th day after A β injection. Transfer latency time was recorded the time mice remain in lighted compartment within 5 min. A β injection, Morris water maze test (MWM) was conducted on the 15th- 21st day (B-D). Graphs display the time of spent to find the escape platform during for 5 training days (B). The time spent in the target quadrant was measured in the probe trials (C). The number of platform area crossing was evaluated in the probe trials (D). The data are presented as the mean $\pm$ SD (n=8). **p<0.01 vs NOR group and #p<0.05 vs A β group. NOR: normal control mice, A β : A β 1-42-injected mice, EEFE: ethanol extracts of *Ficus erecta*, and M: morin.

Figure 5

Effects of EEFE on neuronal damage in A β -injected mice. Multiple 4- μ m paraffin sections of hippocampus and cortex regions were prepared from the sacrificed mouse brains. (A) Nissl staining was conducted using cresyl violet solution. (B and C) Graphs display the quantitative analysis of Nissl stained cell bodies in hippocampus (B) and cortex (C). (D) The expression of NeuN, as a neuronal marker, was determined by immunohistochemistry. (E and F) Graphs display the quantitative analysis of NeuN

positive cells in hippocampus (E) and cortex (F). Representative photomicrographs are shown at magnification of x 400. The staining intensities are reported in arbitrary unit (AU). The data are presented as the mean $\pm$ SD (n=3). **p<0.01 vs NOR group and #p<0.05 vs A β group. NOR: normal control mice, A β : A β 1-42-injected mice, EEFE: ethanol extracts of *Ficus erecta*, and M: morin.

Figure 6

Effects of EEFE on the CREB/BDNF signal dysfunction in A β -injected mice. (A) Homogenized brain lysates were applied to western blot analysis. CREB phosphorylation and BDNF expression were detected with anti-phospho-CREB and BDNF antibodies. Images were visualized using a ChemiDox imaging analyzer. (B and C) Quantitative analysis was performed to assess the band intensities of phospho-CREB (B) and BDNF (C) in the western blotting. The protein levels were normalized to β -actin for BDNF and total CREB for phospho-CREB. The results are presented as the mean $\pm$ SD (n=5). *p<0.05 vs NOR group and #p<0.05 vs A β group. NOR: normal control mice, A β : A β 1-42-injected mice, EEFE: ethanol extracts of *Ficus erecta*, and M: morin.

Figure 7

Effects of EEFE on the neuroinflammation in A β -injected mice. (A) Multiple 4- μ m paraffin sections of hippocampus and cortex regions were prepared from the sacrificed mouse brains. Expression of Iba-1, known as marker of microglial activation, was determined by immunohistochemistry with Iba-1 antibody in hippocampal and cortex region of brain sections. (A) Images were captured using a microscope at 400 x magnification (Olympus DP71). (B and C) Quantitative analysis of Iba-1 positive immunostained cells was performed in hippocampal (B) and cortex (C) regions of brain sections. The staining intensities are reported in arbitrary unit (AU). The data are presented as the mean $\pm$ SD (n=3). (D and E) Levels of inflammatory cytokines IL-1 β and TNF- α in brain lysates were determined using ELISA kits according to the manufacturers' instructions. The data of three independent experiments are expressed as mean $\pm$ SEM. (n=5). *p<0.05 or **p<0.01 vs NOR group and #p<0.05 vs A β group. NOR: normal control mice, A β : A β 1-42-injected mice, EEFE: ethanol extracts of *Ficus erecta*, and M: morin.

Figure 8

HPLC chromatograms of EEFE (A) and standard mixture (B) at 260 and 320 nm. chlorogenic acid (1), rutin (2), and keampferol-3-O-rutinoside (3)