



Modulation of the A β -Peptide-Aggregation Pathway by Active Compounds From *Platycladus orientalis* Seed Extract in Alzheimer's Disease Models

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Alzheimer's disease (AD) is a neurodegenerative disease characterized by neuronal loss, cognitive impairment, and aphasia. Aggregation of β -amyloid (A β) peptide in the brain is considered a key mechanism in the development of AD. In the past 20 years, many compounds have been developed to inhibit A β aggregation and accelerate its degradation. *Platycladus orientalis* seed is a traditional Chinese medicine used to enhance intelligence and slow aging. We previously found that *Platycladus orientalis* seed extract (EPOS) inhibited A β -peptide aggregation in the hippocampus and reduced cognitive deficits in 5 \times FAD mice. However, the mechanisms of these effects have not been characterized. To characterize the protective mechanisms of EPOS, we used a transgenic *Caenorhabditis elegans* CL4176 model to perform Bioactivity-guided identification of active compounds. Four active compounds, comprising communic acid, isocupressic acid, imbricatolic acid, and pinusolide, were identified using ¹³C- and ¹H-NMR spectroscopy. Furthermore, we showed that isocupressic acid inhibited A β generation by modulating BACE1 activity *via* the GSK3 β /NF- κ B pathway in HEK293-APPsw cells. These findings showed that EPOS reduced cognitive deficits in an AD model *via* modulation of the A β peptide aggregation pathway.

Keywords: A β -peptide aggregation, *Platycladus orientalis* seed, cognitive function, isocupressic acid, β -secretase 1

INTRODUCTION

Alzheimer's disease (AD) is a chronic neurodegenerative disease characterized by abnormal amyloid- β (A β) peptide accumulation, tau hyperphosphorylation, and neuronal loss. These neuropathologies affect brain regions involved in memory and cognition (Montine et al., 2012). Several hypotheses have been proposed for the mechanisms of AD pathology, but the A β cascade hypothesis has received the most attention (Frautschy and Cole, 2010).

Abnormal metabolism of A β plays a key role in the pathogenesis of AD (LaFerla et al., 2007). An imbalance between A β production and clearance results in A β aggregation and deposition, which triggers a pathophysiological cascade of events that includes synaptic dysfunction, tau hyperphosphorylation, and neuronal loss, which results in cognitive impairment (Janus et al., 2000; Kamenetz et al., 2003; Huang and Jiang, 2009). Amyloid- β is formed by sequential cleavage events of β -amyloid precursor protein (APP) by β -secretase 1 (BACE1) and γ -secretase (Vassar, 2004; Agostinho et al., 2015). Chen et al found that levels of nuclear factor kappa-B (NF- κ B) p65 and BACE1 were higher in brains of patients with AD, and also showed that BACE1 gene expression and APP metabolism were facilitated by NF- κ B p65 (Chen et al., 2012). Chen et al also showed that overexpression of NF- κ B p65 increased human BACE1 promoter activity (Chen et al., 2012). Another study found that the GSK3 β /NF- κ B signaling pathway regulated BACE1 gene transcription (Ly et al., 2013). This finding led to a contribution that AD may develop through GSK3 β /NF- κ B-mediated BACE1 expression.

Medicinal herbs and natural products are significant sources of compounds that protect against AD (Kim et al., 2010). For example, curcumin, huperzine A, and EGb 761 (Ginkgo Biloba extract) have been hypothesized to protect against AD through anti-oxidative, anti-amyloidogenic, and anti-inflammatory effects (Lim et al., 2001; Zhang and Tang, 2006; Bate et al., 2008). The seed of *Platyclusus orientalis* is a traditional Chinese medicine that slows aging and enhances intelligence (Inoue et al., 1985). Modern studies have also shown that it can induce sedative and hypnotic effects, and ameliorate cognitive deficits caused by amygdala lesions in mice (Sun et al., 2010). The seed of *Platyclusus orientalis* has also been shown to slow physiological aging and to prevent age-related memory deficits in humans. In a previous study, *Platyclusus orientalis* seed extract (EPOS) significantly prolonged the lifespan of *Caenorhabditis elegans* and protected the transgenic nematode CL4176 strain against A β toxicity (Liu et al., 2013).

In this study, EPOS was verified that it could significantly improve spatial working memory and reduce A β 42 plaque deposition and alter the dendritic spine density of neurons in the hippocampus. Then, we isolated and identified the major fractions of EPOS that protected against A β peptide aggregation using bioassay-guided fractionation. This screening resulted in the identification of four compounds that were further evaluated for anti-A β aggregation effects in HEK293-APPsw cells. Among these compounds, isocupressic acid showed the best anti-A β generation activity by modulating BACE1 activity *via* the GSK3 β /NF- κ B pathway in HEK293-APPsw cells.

MATERIALS AND METHODS

Plant Material and Extraction of *Platyclusus orientalis* Seed

Platyclusus orientalis seeds were purchased from the Guangzhou Daxiang, Limited (Guangdong, China) and were authenticated

by Guangdong Institute for Drug Control (GDGID No. 2018A03174). *Platyclusus orientalis* seed (0.5 kg) was powdered for 5 min and extracted three times with 2.5 volumes of petroleum ether (PE) at room temperature. The combined PE extract was concentrated to dryness in a vacuum (PE extract weight: 0.14 kg), and the resulting residue was dried on an iron plate for 1 day. The residue was refluxed with 95% ethanol (v/v) for 2 h. The combined ethanol extracts were then concentrated using a rotary evaporator at 50°C to produce EPOS.

Animals

Transgenic mice (5 \times FAD) that overexpress human APP and PS1 with 5 familial AD (FAD) mutations [APP K670N/M671L (Swedish) + V717I (London) + I716V (Florida) and presenilin [(PS1) M146L + L286V] (Oakley et al., 2006) were purchased from Model Animal Research Center of Nanjing University [Certification No. SCXK-(Su) 2010-0001] and housed in a specific pathogen-free environment at the Ocean and Traditional Chinese Medicine Laboratory of Sun Yat-sen University [License No. SCXK-(Yue) 2014-0020]. All animal experimental procedures in this study were approved by “The Animal Care and Use Committee of the School of Life Sciences of Sun Yat-sen University” (Permission No.150207). The resulting progeny [hybrid 5 \times FAD transgenic and non-transgenic wild type (WT)] were used in this study. Genotyping was performed by polymerase chain reaction (PCR) of mouse tail DNA (Oakley et al., 2006). In this study, mice were used and handled according to the guidelines of China for animal care. Animals were kept in a temperature-controlled room at 20°C \pm 2°C and a 12/12 h light/dark cycle (light 0 at 6 AM). Food and water were available *ad libitum*.

Experimental Animal Model

Twenty-four 5 \times FAD Tg mice were separated into the following groups: Tg, EPOS (100 mg/kg/day), and EGb761 (100 mg/kg/day). Eight WT mice were used as controls. The mice were administered EPOS by gavage once daily for 30 consecutive days. The WT group and the Tg group were treated with the same volume of normal saline.

Behavioral Test

The Y maze is a three-part radial box, which consists of three arms of the same length. The mice were placed at the center of the three arms and allowed to explore freely for 5 min. When the limbs of the mice were completely inserted into one arm, this was considered one entry. The total number of times and the order of arm entry were recorded. Spontaneous alternations were defined as consecutive triplets of different arm choices.

The Morris water maze consists of a circular pool made of stainless steel with a diameter of 100 cm and a height of 50 cm, with a movable platform. The platform and the pool wall were white. During the experiment, the pool and the surrounding environment remained unchanged. The pool was monitored using a computer to track and record the swimming trajectory. The Morris water maze was divided into four quadrants, and the platform was placed in the middle of the first quadrant.

Swimming was recorded and data were analyzed using JLabBehv-MWMM. The navigation test was conducted for 5 days, and the mice were trained once per day. The mice were placed in water for 60 s in order of consecutive quadrants. The mice were placed in the water facing the pool wall. The instrument automatically recorded the mice took looking for and climbing the platform, and the time from which the mice entered the water to the time at which the found and climbed the platform (escape the incubation period). Mice that did not find the platform within 60 s were placed on the platform for 30 s, and the time to finding the platform was recorded as 60 s. After the training period was completed, the mice were returned to their cages. On the sixth day, the spatial exploration experiment was performed. The platform in the first quadrant was removed, and the mice were placed in the water in the third quadrant and allowed to swim for 60-s. The time spent in the first quadrant was recorded for 60 s.

Immunofluorescence Staining of Frozen Sections

Three mice per group were used for immunofluorescence staining. Mice were transcranial perfused with 4% paraformaldehyde (PFA) in PBS, and the brains were carefully dissected. The brains were fixed in 4% PFA at 4°C overnight and dehydrated using 10%, 20%, and 30% sucrose solutions for 1 day each. The brains were sliced into 20- μ m sections and immunostained with rabbit anti-A β (1–42) IgG antibody (1:500) at 4°C overnight, then incubated with Goat Anti-Rabbit IgG (Alexa Fluor® 594). Images were captured using a Nikon confocal microscope.

Golgi Staining

Mouse brains were dissected and prepared using the FD Rapid Golgi Stain Kit (FD Neuro Technologies, Shanghai, China) according to the manufacturer's instructions. Slices were visualized using a Nikon confocal microscope (Nikon, A1R HD25). Images of Golgi-stained hippocampal CA1 pyramidal neurons were captured. We used three animals per group to

do Golgi-staining. Thirty neurons from three mice (10 slices of every sample) were analyzed for dendritic spine density by NIS-Elements Viewer (Nikon). The analyzed method was followed as previous research (Du, 2019).

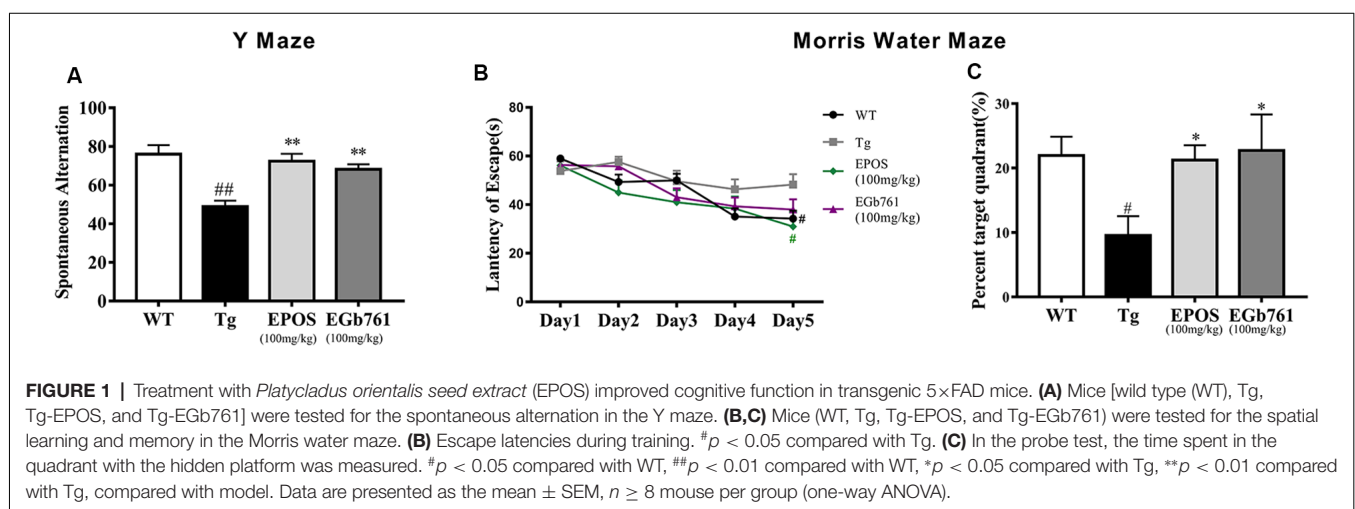
Isolation and Characterization of EPOS

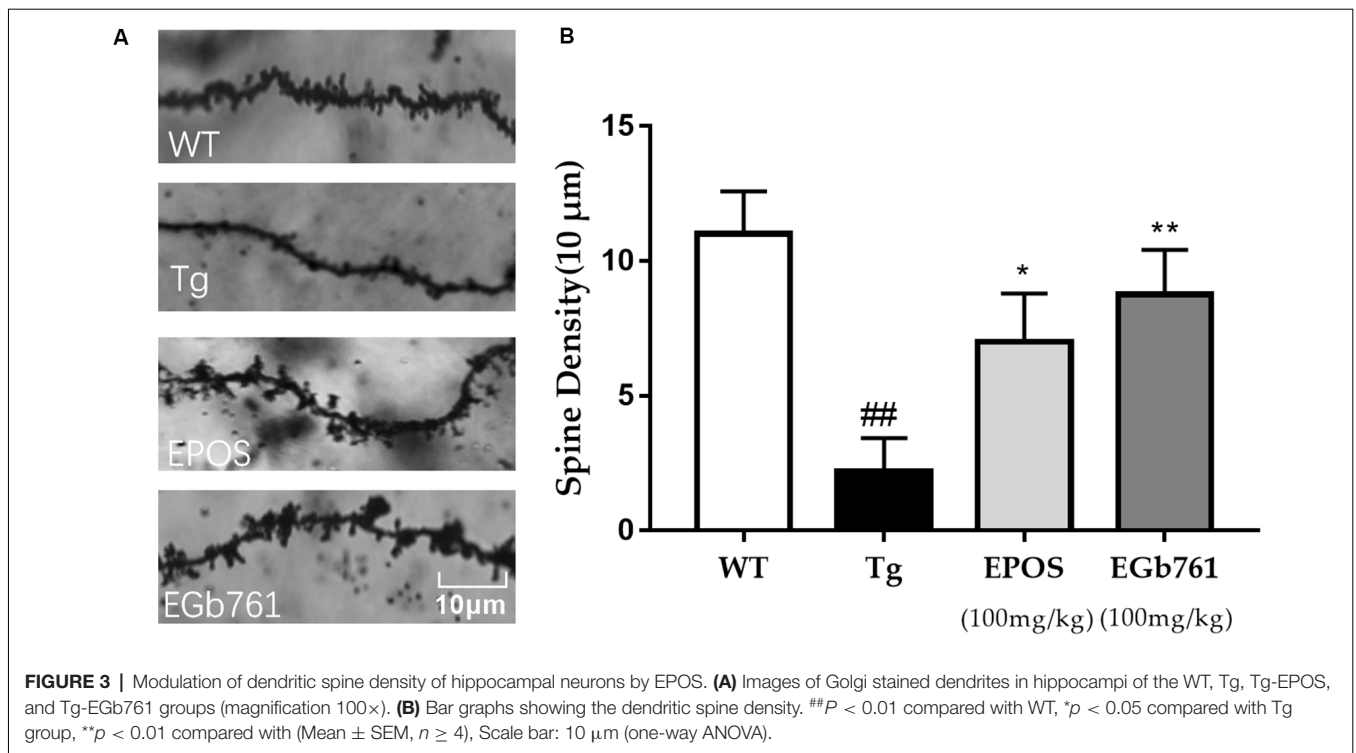
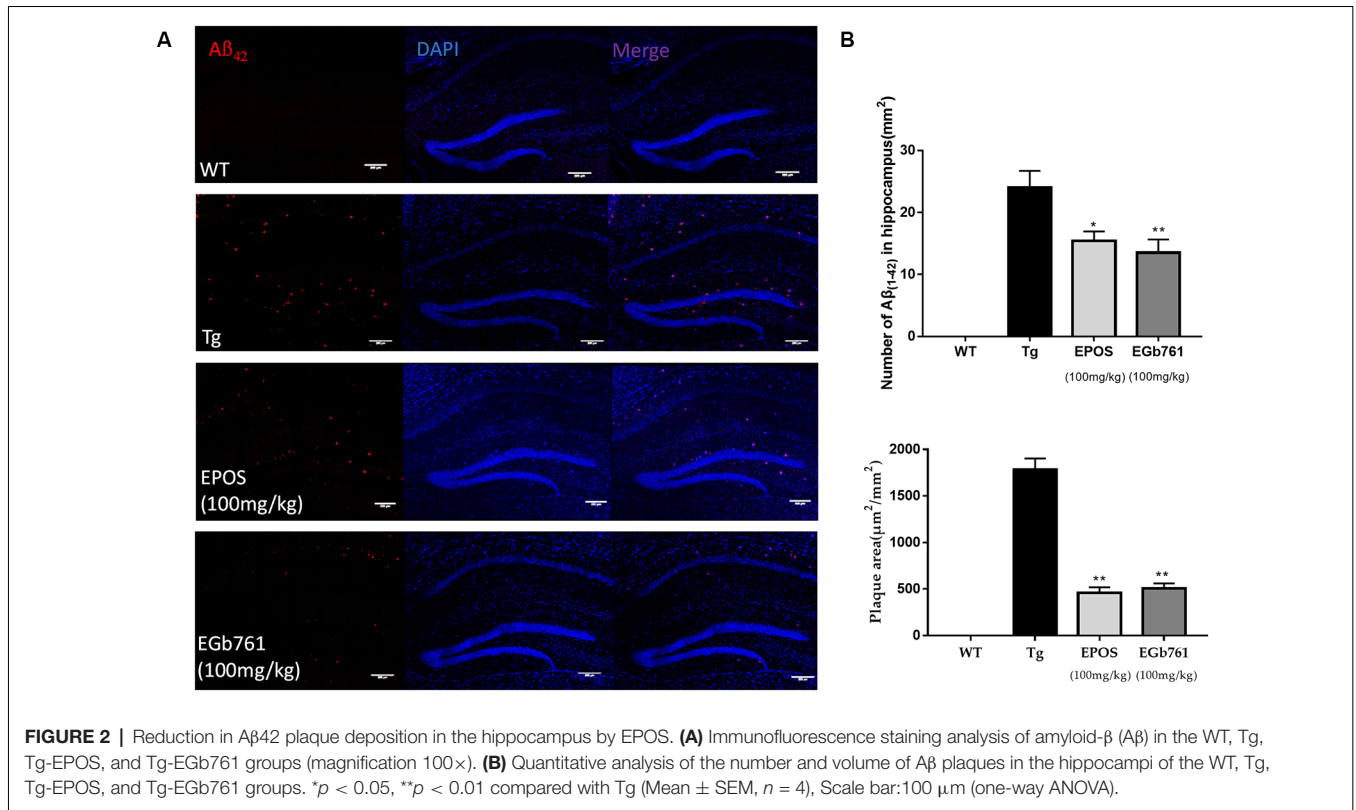
Sixty-five grams of EPOS was isolated using silica gel low pressure column chromatography (Teledyne ISCO RF, USA; column: Buch, ϕ 10 \times 25 cm, USA) with chloroform:methanol (100:0, 98:2, 95:5, 90:10, 80:20; 70:30, 60:40, and 0:100). Ten fractions were obtained (Fr1, 32.49g, 50%; Fr2, 3.48g, 5.4%; Fr3, 3.12g, 4.8%; Fr4, 1.96g, 3.0%; Fr5, 4.79g, 7.4%; Fr6, 7.24g, 11.1%; Fr7, 4.42g, 6.8%; Fr8, 2.95g, 4.5%; Fr9, 3.21g, 4.9%; Fr10, 1.34g, 2.1%).

Fraction 3 (1.5 g) was fractionated using an octadecyl silane column (50 μ m, Ultimate XB-C18, USA) and eluted with methanol:H₂O (30:70, 50:50, 30:70, 0:100). Preparative HPLC (Shimadzu, SHIMADZU-20A, Japan; column: 10 \times 250 mm, Xterra C18, 5 μ m, Waters, USA) was used to isolate four compounds. The structures of these compounds were determined by ¹³C-NMR (100 MHz) spectra and ¹H-NMR (400 MHz) using a Bruker Avance 400 spectrometer. Tetramethylsilane (TMS) was used as an internal standard and CDCl₃ as the solvent.

Caenorhabditis elegans Strains and Maintenance

The *C. elegans* CL4176 strain [genotype: smg1(cc546ts); dvIs27] and *Escherichia coli* OP50 strain were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA; Sutphin and Kaerberlein, 2009). The CL4176 nematode model expresses human A β 1–42 in body wall muscle cells by activating the myo-3 body wall-specific myosin promoter. When A β is sufficiently expressed, the nematodes produce sputum until death (Ewald and Li, 2010). We maintained *C. elegans* on nematode growth medium (NGM) agar plates with *Escherichia coli* OP50 at 16°C.





Paralysis Assay

The above strain was egged synchronized onto the culture mediums with or without different ingredients. Transgenic

expression was induced by adjusting the temperature (16–26°C). Induction occurred within 36 h after spawning and was sustained until the last worm became paralyzed. For the paralysis

assay, Nematodes were marked as dead when they got the worst of responding to repeated touching with a platinum wire. Each experiment was double-blinded and performed in triplicate.

Cell Culture, ELISA Assay, and Western Blot

WT human embryonic kidney 293 cells (HEK293/WT) and HEK293 cells stably transfected with human APP Swedish mutant (HEK293sw) were gifts from Professor Zhong Li (Sun-Yat-sen University, Guangzhou, China). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 5% fetal bovine serum in a 5% CO₂ atmosphere at 37°C.

Following 24 h drug treatment, A β (1–42) in the medium was measured using an ELISA kit (CUSABIO, Wuhan, China) according to the manufacturer's protocol. Protein content was determined using a BCA Protein Assay Kit (Beyotime Biotechnology). Levels of APP, BACE1, A β (1–42), ADAM10, PSEN1, NF- κ B p68, GSK-3 β (Tyr 216), and GSK-3 β were analyzed using western blot (primary antibodies: anti-APP, 1:1,000, CST; anti-BACE1, 1:1,000, CST; anti-A β (1–42), 1:2,000; CST; anti-ADAM10, 1:1,000, CST; anti-PSEN1, 1:1,000, CST; anti-NF- κ B p68, 1:1,000, CST; anti-GSK-3 β , 1:1,000, CST; anti-GSK-3 β (Tyr 216, 1:1,000, Thermo Fisher). Densitometry was performed using a Tanon5200.

Statistical Analysis

All data are presented as the mean \pm standard error of the mean (SEM), and experiments were performed in triplicate. Statistical analyses were determined using one-way ANOVA, followed by Fisher's protected least significant difference for *post hoc* comparisons. *P*-values < 0.05 were considered significant. GraphPad Prism 7 was used for all statistical analyses.

RESULTS

Platycladus orientalis Seed Extract Ameliorated Cognitive Deficits in 5 \times FAD Tg Mice

To investigate whether EPOS ameliorated cognitive deficits in Tg mice, we used the Y maze and Morris water maze. The Tg group showed a decreased percentage of triple alternations compared to the WT group in the Y maze test ($p < 0.01$). The EPOS group had a significantly increased percentage of triple alternations compared with the Tg group ($p < 0.01$), and did not differ from the EGb761 group (Figure 1A), which indicated that EPOS treatment improved spontaneous spatial working memory in 6-month-old 5 \times FAD Tg mice.

To evaluate spatial learning and memory, we performed the Morris water maze test. During 5 days of training, the mice shortened their time to find the submerged platform, but the escape latency in the Tg group was significantly longer than that in the WT and EPOS groups (Figure 1B, $p < 0.05$). We removed the hidden platform and performed the probe test on the 6th day. Compared with the WT group, the Tg group spent less time in the target quadrant.

Treatment with EPOS significantly increased the length of time Tg mice spent in the target quadrant ($p < 0.05$), and time spent in the target quadrant did not differ between the EPOS and EGb761 groups (Figure 1C). These results showed that EPOS ameliorated cognitive deficits in 6-month-old 5 \times FAD mice.

Treatment With EPOS Reduced A β Plaque Deposition in the Hippocampus

To study the effect of EPOS on A β accumulation, immunofluorescence staining of A β was performed in the hippocampi of 6-month-old mice. The results showed no obvious A β plaques in the WT group. In contrast, many plaques were observed in the CA1 of hippocampi in the Tg group (Figure 2A). Compared with the Tg group, the number and area of A β plaques were decreased in the EPOS group ($*p < 0.05$, $**p < 0.01$; Figure 2B). These results showed that EPOS reduced the size and number of A β plaques in the CA1 region of the hippocampus.

Treatment With EPOS Altered Dendritic Spine Density of Neurons in the Hippocampus

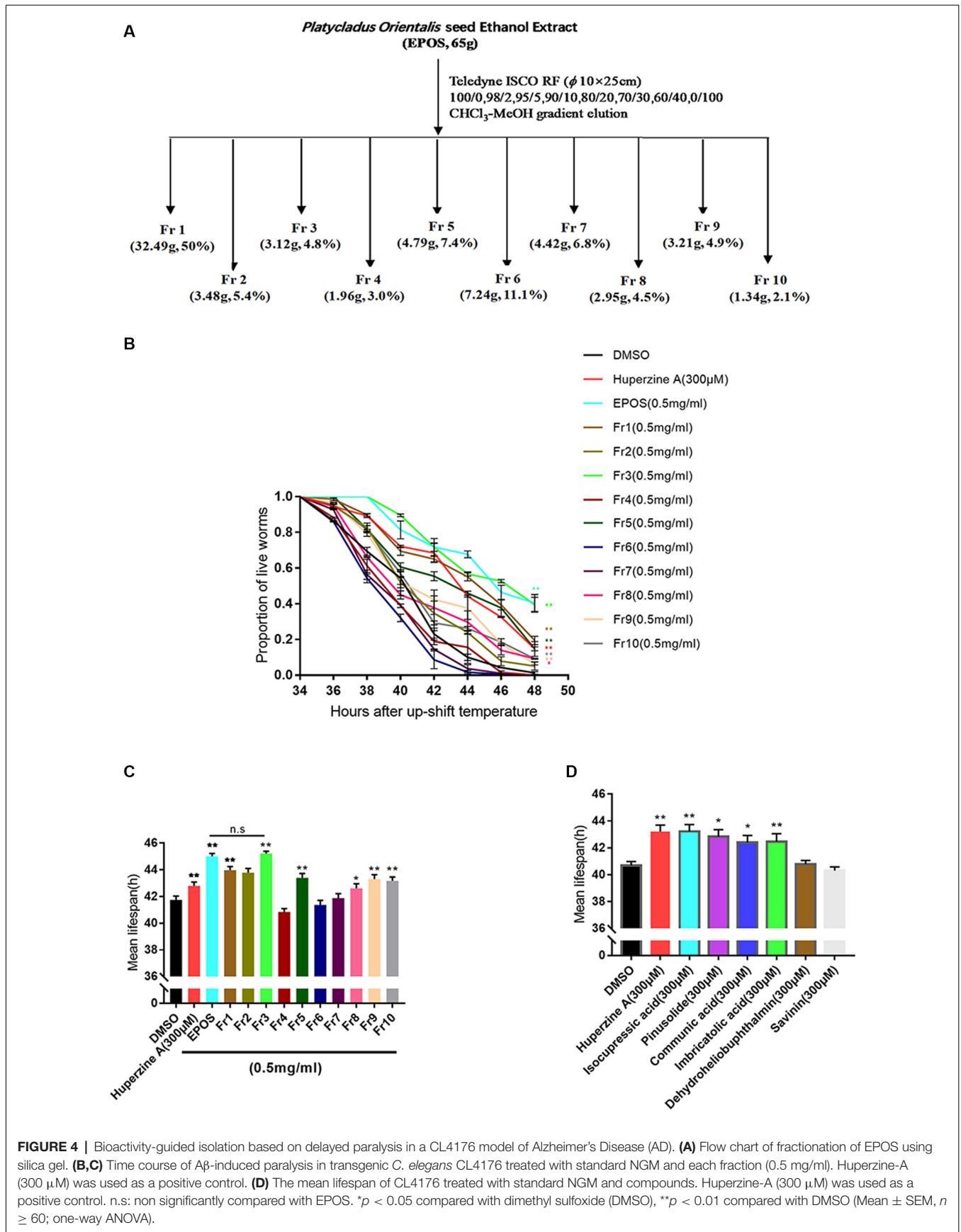
Dendritic spines are small protrusions on dendrites that play a critical role in synaptic plasticity and cognitive function. Dendritic spine density was calculated from a single nerve in a randomly selected 10- μ m region of the hippocampal CA1 region. The morphology of dendritic spines was abnormal in the Tg group (Figure 3A). The densities of dendritic spines were lower in the Tg group compared with those in the WT group. Treatment with EPOS resulted in increased numbers of dendrite spines compared to the 5 \times FAD Tg group. There were no differences between the EPOS group and the EGb761 group ($p < 0.05$; Figure 3B).

Fraction 3 of EPOS Most Effectively Delayed Paralysis in the CL4176 *C. elegans* Model of AD

Silica gel column chromatography was performed to prepare EPOS fractions (Figure 4A). The activities of the fractions were evaluated using the transgenic *C. elegans* CL4176 strain. As shown in Figures 4B,C, Fr1, Fr2, Fr3, Fr5, Fr8, Fr9, and Fr10 significantly protected worms from A β -induced rapid paralysis compared with the untreated control group ($p < 0.05$). The activity of Fr3 did not differ from that of EPOS.

Compounds in Active Fraction 3 Were Determined Using 1H-NMR (400 MHz), 13C-NMR (100 MHz), and High-Resolution (HR) Electrospray Ionization (ESI)-Mass Spectrometry (MS)

We used ODS column chromatography and preparative HPLC to separate active compounds. We used 1H-NMR (400 MHz), 13C-NMR (100 MHz), and HR-ESI-MS for structural characterization. Communic acid (Kuo and Chen,



1999), isocupressic acid (Fang et al., 1996), imbricatolic acid (Wang et al., 2008), and pinusolide (Kuo and Chen, 1999) were identified (Figure 5, Supplementary Tables S1–S4).

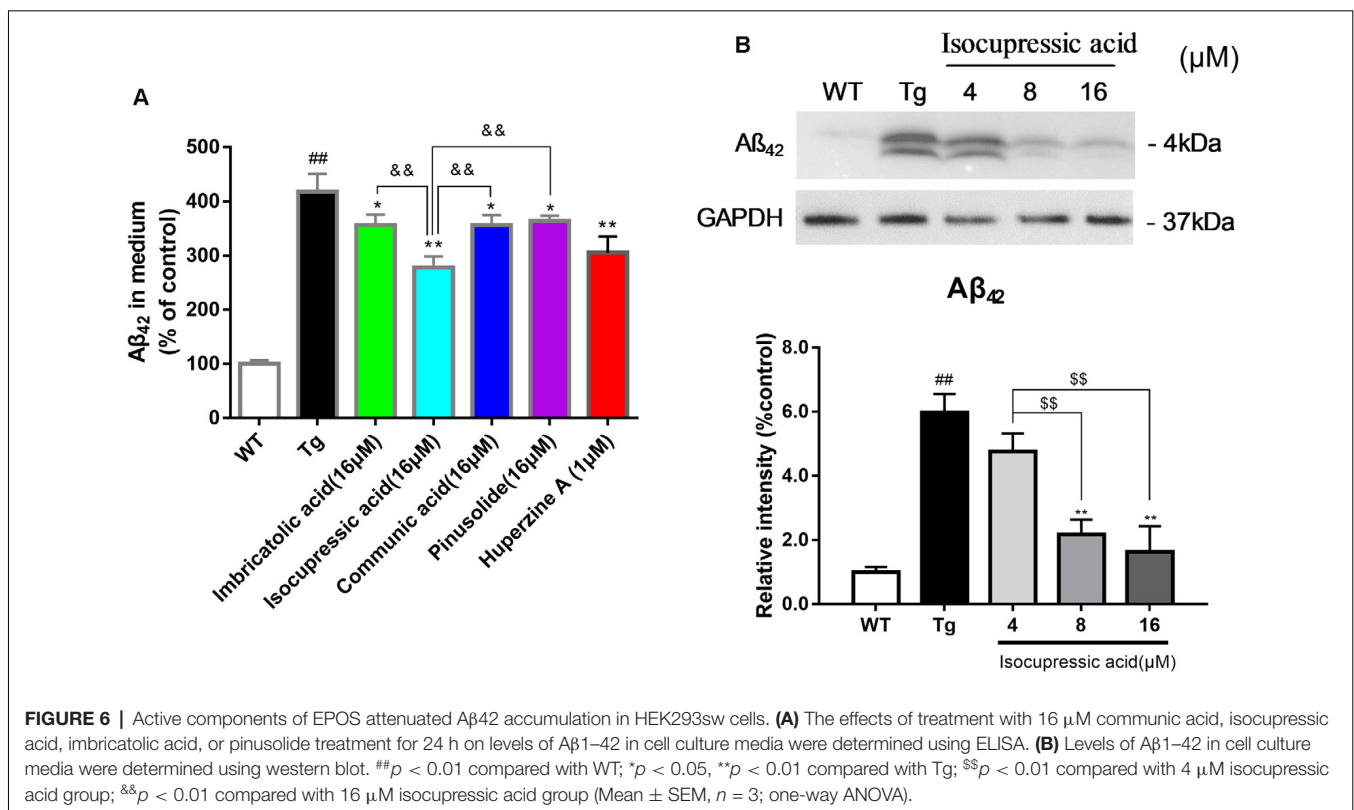
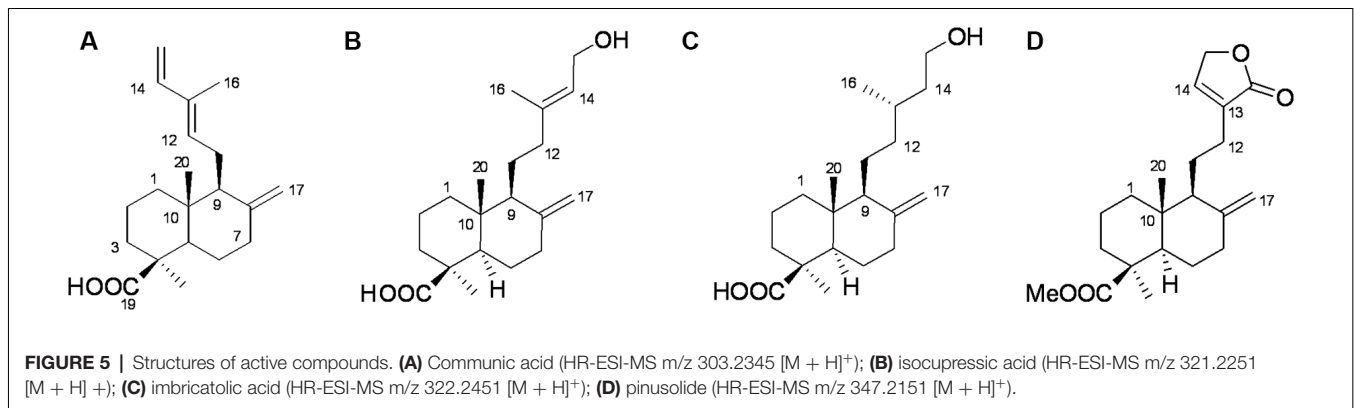
Active Compounds Isolated From EPOS Attenuated A β (1–42) Accumulation in HEK293sw Cells

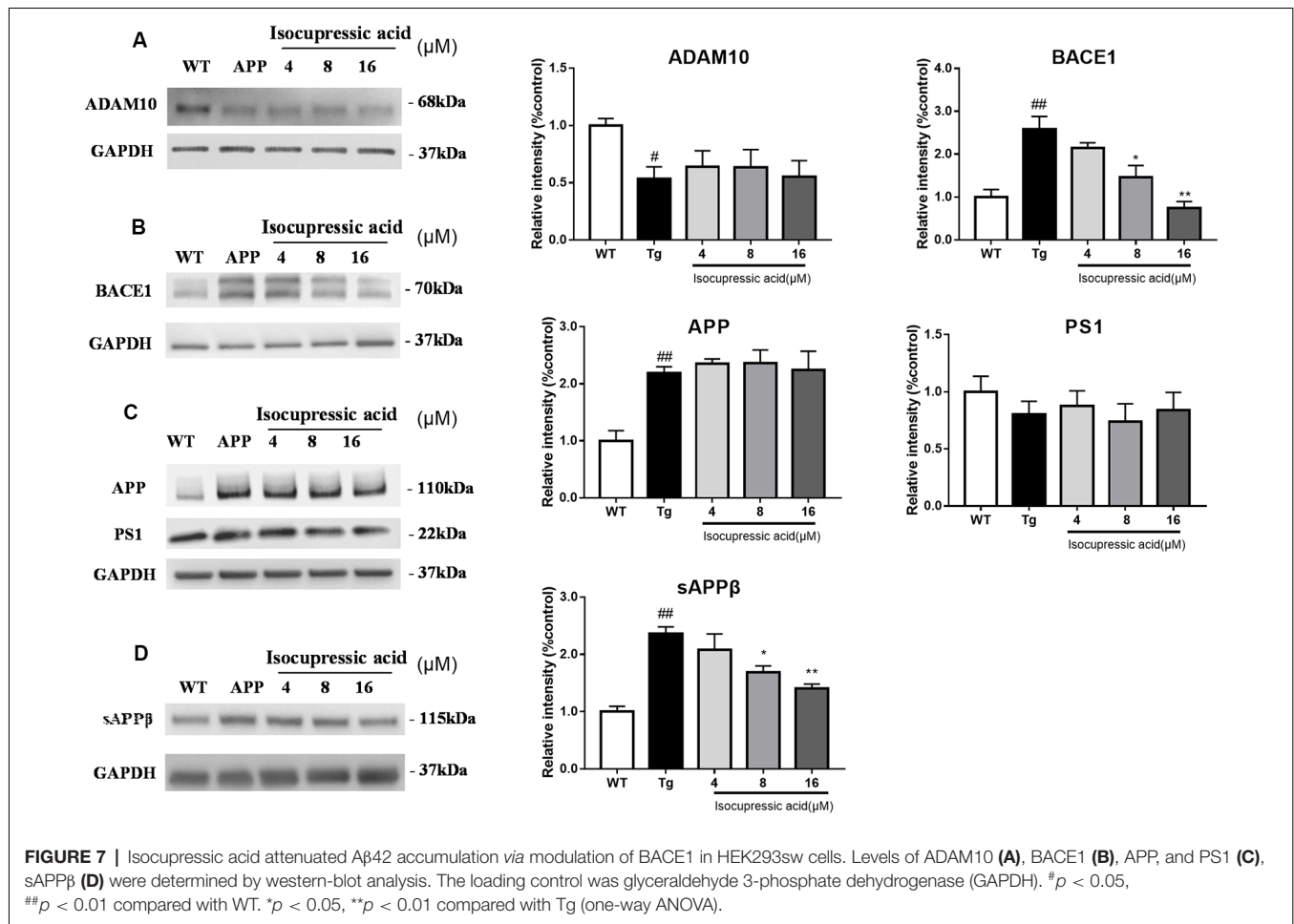
WT Human Embryonic Kidney 293 cells (HEK293/WT) and HEK293 cells stably transfected with human APP Swedish mutant (HEK293sw) were used to evaluate the effect of isolated active compounds. Treatment with 16 μ M communic acid, 16 μ M isocupressic acid, 16 μ M imbricatolic acid, or 16 μ M pinusolide for 24 h resulted in significantly reduced

A β (1–42) protein levels in HEK293sw cells. Isocupressic acid decreased the levels of A β 42 to the greatest extent (Figure 6A). Furthermore, Isocupressic acid decreased A β (1–42) protein level in a dose-dependent manner ($P < 0.05$; Figure 6B).

Isocupressic Acid Attenuated A β 42 Formation via BACE1 In HEK293sw Cells

To investigate the effect of isocupressic acid on APP processing, we measured the levels of APP, ADAM10, BACE1, and PS1 using western blot. As shown in Figure 7, the level of BACE1 and sAPP β protein was higher in HEK293sw cells than in HEK293/WT cells ($P < 0.01$). Furthermore,





HEK293sw cells treated with 8 and 16 μ M isocupressic acid reduced BACE1 and sAPP β protein levels ($P < 0.05$). In contrast, treatment with isocupressic acid did not alter the expression of ADAM10, PS1, or APP. These findings indicated that isocupressic acid downregulated A β by activating BACE1.

Isocupressic Acid Reduced BACE1 Activity Through GSK3 β /NF κ B Pathway in HEK293sw Cells

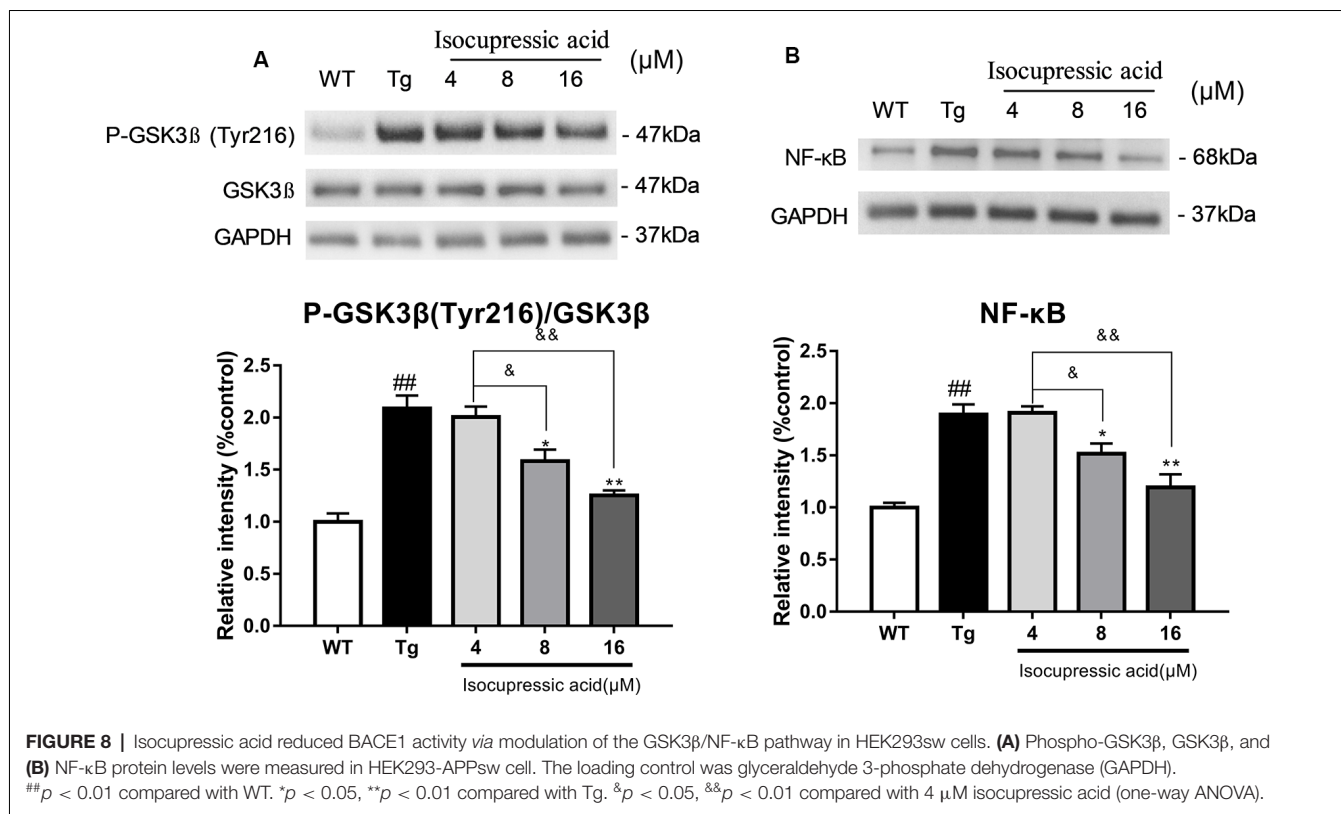
Western blot was used to evaluate the mechanisms by which isocupressic acid downregulated A β and BACE1 in HEK293sw cells. The results showed that 8 μ M and 16 μ M isocupressic acid significantly inhibited GSK-3 β activity and NF- κ B expression in HEK293sw cells ($P < 0.01$; **Figure 8**). These results indicated that isocupressic acid downregulated A β and BACE1 in the HEK293sw cells through inhibition of the GSK3 β /NF- κ B pathway.

DISCUSSION

Alzheimer's disease (AD) is the most common age-related progressive neurodegenerative disorder and is characterized

by cognitive deficits (Montine et al., 2012). Overproduction of A β peptide or reduced clearance is believed to cause AD. The development of strategies to inhibit A β aggregation and increase A β degradation has received significant attention. Traditional Chinese medicines (TCM), which contain various medicinal components, produce comprehensive effects *via* multiple pathways (Liu et al., 2015). Studies have shown that TCM can be used to prevent and treat neurodegenerative diseases (Wei, 2016). We isolated EPOS and treated 5 \times FAD mice with this extract to evaluate the anti-AD efficacy of EPOS in comparison to EGb761, which is a well-defined extract of Ginkgo biloba used to treat dementia (von Gunten et al., 2016).

We showed that EPOS significantly improved spatial working memory in 5 \times FAD mice, as evidenced by a higher spontaneous alternation ratio in the Y maze, and higher quadrant dwell time and shorter escape latency in the Morris water maze (**Figure 1**). Golgi staining showed that dendritic spine morphology was improved in 5 \times FAD Tg mice treated with EPOS. The dendritic axes of neurons were thinner and the number of dendritic spines was significantly decreased in 5 \times FAD Tg mice (Šišková et al., 2014). Treatment with EPOS treatment increased the number of neuronal dendritic spines and improved the morphology



of neuronal dendrites, which indicated that EPOS exerted neuroprotection (Figure 3). Studies have shown that A β is a neurotoxic protein, and excessive accumulation of A β in the brain can lead to neuronal damage, neuronal loss, and destruction of neural networks (Buggia-Prévoit et al., 2013). Treatment with EPOS reduced A β deposition. Our results showed that EPOS improved cognition and reduced A β deposition, which indicated that EPOS may be an effective treatment for AD (Figure 2).

We used modern pharmacodynamic evaluation tools, such as the CL4176 *C. elegans* model and the *in vitro* HEK293sw model, to isolate and identify active compounds. HEK293sw cells were stably transfected with the human APP Swedish mutant (Buggia-Prevot et al., 2008). The transgenic nematode CL4176 strain is a temperature-sensitive mutant strain that expresses human A β 42 at certain temperatures. Communic acid, isocupressic acid, imbricatolic acid, and pinusolide, which are components isolated from Fr3, have anti-A β 42 toxicity effect in CL4176 strain (Figure 4D). Besides, these four active compounds inhibit A β 42 formation in HEK293sw cell (Figure 6A). Furthermore, western blot analysis results showed that isocupressic acid reduced A β 42 formation via decreased expression of amyloidogenic APP pathway (Peineau et al., 2010; Chami and Checler, 2012; Figures 6B, 7).

We then characterized the molecular mechanisms underlying decreased BACE1 expression by isocupressic acid. We investigated whether isocupressic acid influenced BACE1

via the GSK-3 β /NF- κ B pathway. Previous studies showed that NF- κ B transcriptionally regulated BACE1 expression (Chen et al., 2012). Also, the activation of GSK3 β has been shown to upregulate BACE1 (Ly et al., 2013). Phosphorylation of GSK-3 β residues can result in changes in substrate binding affinity. Phosphorylation of Ser9 of GSK-3 β results in decrease active site availability, phosphorylation at Tyr216 results in enhanced enzymatic activity. Our results showed that isocupressic acid inhibited GSK3 β by decreasing the ratio of pTyr216-GSK3 β to total GSK3 β , and also reduced NF- κ B activity. These results suggested that isocupressic acid suppressed BACE1 expression and A β generation via the GSK3 β /NF- κ B pathway (Figure 8).

In summary, for the first time, we found that isocupressic acid is a BACE1 inhibitor, which could inhibit A β generation via the GSK3 β /NF- κ B pathway signaling. These findings suggested that *Platycladus orientalis* seed might be a potential source of new anti-AD drugs. However, these conclusions were based on the responses of the HEK293 APPsw cell line and so might not reflect processes in the neuron or neuroblastoma cell lines. In future research, we will study more kinds of neuron cells to further investigate the active compounds in EPSO to improve AD mice cognitive function.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Materials.

ETHICS STATEMENT

All animal experimental procedures in this study were reviewed and approved by the Animal Care and Use Committee of the School of Life Sciences of Sun Yat-sen University (Permission No. 150207).

AUTHOR CONTRIBUTIONS

LY conceived and designed the experiments. XH, YJ, JW, FL and RP performed the experiments. XH and YJ analyzed the data. LY and XH prepared the manuscript. PL, YW and WS critically revised this manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnagi.2020.00207/full#supplementary-material>.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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