

Multiple Bioactivities of Traditional Medicinal Herbs for Treatment of Neurodegenerative Diseases

Lead Guest Editor: Gunhyuk Park Guest Editors: Yong-ung Kim and Irawan W. Kusuma



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Editorial

Multiple Bioactivities of Traditional Medicinal Herbs for Treatment of Neurodegenerative Diseases

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Neurodegenerative diseases are debilitating and incurable conditions of the central nervous system. Characterized by neuronal dysfunction, they are often associated with atrophy of the affected nervous system structures. Age-related dementia constitutes a major subset of neurodegenerative diseases. Alzheimer's disease (AD) is the most prevalent clinical form of dementia in aging populations: 43% of individuals ≥85 years of age are thought to have AD in the United States. Parkinson's disease (PD), another prevalent neurodegenerative disease among the elderly, affects 1%-3% of the population over 60 years of age. By 2050, the United Nations predicts that, worldwide, 400 million individuals ≥80 years of age will have a neurodegenerative disease. Owing to the financial, societal, and personal impact of these diseases, the etiologies, prevention, and treatment have become the major focus of basic and clinical research. Associations between neurodegenerative diseases and genetic factors are reported; however, variations exist within populations for one disease state. AD is also linked to mutations in a specific gene; however, the downstream effects of its protein product on symptoms, including dementia, are not fully understood. Multiple signaling pathways govern the molecular basis of the effects of genetic variation, lifestyle, and environmental factors, including trauma and infection, in neurodegenerative diseases. The neuropathological hallmarks of dementia include β -amyloid plaques and neurofibrillary tangles in

AD and Lewy body inclusions in PD. However, although misfolded protein aggregation clearly contributes to neurodegenerative disease, it is only a signature of neuronal damage and additional causative factors remain to be identified. Inflammation and nitric oxide signaling are active areas of investigation. The effects of these, and other key factors, on the transcriptional regulation and initiation of apoptosis and neurotoxicity continue to be explored. The pathogenesis of neurodegenerative diseases remains unclear; the present "one-drug, one-target" paradigm for neurodegenerative diseases appears clinically unsuccessful. The pathological effects of neurodegenerative diseases result from altered activity in multiple pathways. Thus, new pharmacological therapeutic strategies, using natural compounds or medicinal herbal extracts comprising multiple compounds, are specifically designed to act on multiple neural and biochemical targets.

In this special issue, we invited investigators to contribute original research articles and reviews that will help understand the basic mechanisms and development of multiple bioactivities and potential herbal medicinal strategies in the treatment of neurodegenerative diseases. Seven studies regarding the multiple bioactivities of traditional medicinal herbs in treating neurodegenerative diseases are included.

C.-Y. Kim et al. reported the therapeutic effects and mechanisms of 6-gingerol, a pungent ingredient of ginger, on scopolamine-induced learning and memory impairments

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in C57BL/6 mice. They suggested that 6-gingerol may result from the elevated protein expression of brain-derived neurotrophic factor mediated via the activation of the protein kinase B (Akt)/Akt- and cyclic adenosine monophosphateresponse element binding protein signaling pathway; thus, this may be a candidate for the treatment of AD.

Y. Yang et al. demonstrated the anti-tau effects of Fuzheng Quxie Decoction (FQD), a Chinese herbal complex clinical formula, on learning and memory impairments in senescence-accelerated mice prone-8. Their results suggested that the effects of FQD, which may be a candidate for the treatment of AD, are mediated through the inhibition of hippocampal tau hyperphosphorylation via N-methyl-D-aspartate receptor/protein phosphatase 2A-associated proteins.

M. Liu et al. demonstrated the anti-amyloid precursor protein (APP) cleavage and amyloid β effects of Huannao Yicong Decoction (HYD) extract, a Chinese herbal formula, on the spatial learning and memory ability of transgenic APP/PS1 mice. Their study suggested that HYD extract clearly inhibited γ -secretase activity resulting from the inhibition of cyclic adenosine monophosphate-response elements activity and subsequent downregulation of presenilin enhancer 2 expressions.

Y. J. Kim and and H.-S. Lim et al. reported the effect and possible mechanism of Jodeungsan (JDS), a traditional herbal formula, on lipopolysaccharide or H_2O_2 -induced neurotoxicity in hippocampal neurons and microglia and suggested that JDS extracts might suppress tumor necrosis factor- α and interleukin-6 production, supporting the antineuroinflammatory activity.

G.-Y. Lee et al. demonstrated the antioxidative effects of α -pinene on scopolamine-induced learning and memory impairments in C57BL/6 mice and suggested that the effects may be due to elevated expression of choline acetyltransferase and antioxidant enzymes, mediated via activation of the NF-E2-related factor 2 (Nrf2) pathway. Thus, α -pinene may be a candidate for the treatment of AD.

N.-Y. Chen et al. demonstrated the antiaging effects of *Fructus Cannabis* (EFC) from hempseed on D-galactose-induced learning and memory impairments. Their results suggested that the effect of EFC was mediated via inhibition of glia activation, tau phosphorylation, and the expression of presenilin 1 via reactive oxygen species generation, which would be useful to relieve age-related memory impairments

L. Wu et al. reported the antioxidative effects of salidroside, a major component extracted from *Rhodiola rosea* L., on MPP⁺-induced neurotoxicity. Their study suggested that the effects of salidroside were mediated via upregulation of the PD protein 7/Nrf2-antioxidant pathway and associated antioxidant enzymes; therefore, it may be a candidate for the treatment of PD and associated neurodegenerative diseases.

L. E. M. Brandão et al. reported the antioxidative effects of *Passiflora cincinnata* Masters (PAS), a native Brazilian species of passionflower, on reserpine-induced neurotoxicity; they suggested that PAS delays the development of motor impairment and prevents dopaminergic neuronal loss and thus may be a potential candidate for PD treatment.

J. Chen et al. reviewed the potential of the fruits of *Ziziphus jujuba*, known as jujube or Chinese date, and assessed their phytochemical, ethnobotanical, phytotherapeutical, and pharmacological properties, combining a traditional perspective with recent neurological and scientific observations. Their efforts will assist future researchers in studying the dynamic ethnobotanical and phytotherapeutical roles of *Ziziphus jujuba* and develop novel ideas regarding the therapeutic roles of health supplements in the prevention and/or treatment of neurological diseases.

We hope that the readers will be interested in neurodegenerative diseases and that this special issue will attract the interest of the scientific community, thereby supporting further investigations that lead to the discovery of novel strategies and therapeutic medicinal herbs for use in the field of neurodegenerative diseases.

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> Gunhyuk Park Irawan W. Kusuma Yong-ung Kim

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Research Article

Neuroprotective Effect and Molecular Mechanism of [6]-Gingerol against Scopolamine-Induced Amnesia in C57BL/6 Mice

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We have investigated the neuroprotective and memory enhancing effect of [6]-gingerol (GIN), a pungent ingredient of ginger, using an animal model of amnesia. To determine the neuroprotective effect of GIN on cognitive dysfunction, scopolamine (SCO, 1 mg/kg, i.p.) was injected into C57BL/6 mice, and a series of behavioral tests were conducted. SCO-induced behavior changes and memory impairments, such as decreased alteration (%) in Y-maze test, increased mean escape latency in water maze test, diminished step-through latency in passive avoidance test, and shortened freezing time in fear condition test, were significantly prevented and restored by the oral administration of GIN (10 or 25 mg/kg/day). To further verify the neuroprotective mechanism of GIN, we have focused on the brain-derived neurotrophic factor (BDNF). The administration of GIN elevated the protein expression of BDNF, which was mediated via the activation of protein kinase B/Akt- and cAMP-response element binding protein (CREB) signaling pathway. These results suggest that GIN may have preventive and/or therapeutic potentials in the management of memory deficit and cognitive impairment in mice with amnesia.

1. Introduction

Neurodegeneration in individuals with Alzheimer's disease (AD), Parkinson's disease (PD), and other neurodegenerative disorders is multifactorial, of which a complex set of toxic reactions, including inflammation, glutaminergic neurotoxicity, increased iron levels, accumulation of reactive oxygen and/or nitrogen species (ROS/RNS), depletion of endogenous antioxidant, reduced expression of neurotropic factors, and increased expression of apoptosis-related proteins, lead to neuronal cell death and damage [1]. Learning and memory are generated by an experience-dependent and long-lasting modification of the central nervous system (CNS), which involve the activation of diverse neurotransmitters, such as dopamine and serotonin, and the receptor-linked enzymes that are responsible for the activation of intercellular second messengers [2]. Particularly, cholinergic neurons in the brain of patients with AD are vulnerable, and cholinergic function plays an important role in the learning and memory process.

Cholinergic neurons in the CNS are degenerated in patients with AD, which is correlated with senile dementia severity and degree of degeneration [3].

In this study, because the regulation of the cholinergic system is a key process in memory function, scopolamine (SCO), an experimental tool that induces cognitive dysfunction, was utilized, which is a tropane alkaloid exhibiting muscarinic antagonistic effects. The administration of SCO induces central cholinergic blockade and produces a reversible and well-described impairment in maintaining attention, processing information, and acquiring new knowledge in rodents and humans [4]. In *in vivo* animal models, SCO induced deficits in learning and memory as assessed by contextual and cued fear conditioning, inhibitory avoidance, and spatial learning tasks [4, 5]. Recently, studies showed that memory impairment in an SCO-treated animal model is associated with the altered status of brain oxidative stress and subsequent degenerative changes within the forebrain cholinergic system [6].

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Biologically active phytochemicals in plants or plant products and herbal ingredients that can be used to develop practical natural medicines have been a topic of interest [7]. Traditional oriental herbs contain a wide variety of biologically active compounds, which is being used for the treatment of various types of chronic and acute diseases for thousands of years. Increasing evidence has suggested that traditional herbs can prevent and/or treat pathological outcomes from neurodegenerative disorders, and herbal medicine has been used by the general population because of these effects [8].

Ginger (Zingiber officinale Roscoe, Zingiberaceae) is utilized as a traditional oriental medicine that alleviates conditions such as headache, nausea, and colds [9]. It also has gastroprotective effects against motion sickness and slowwave dysrhythmias [10]. Gingerols are pungent compounds in the rhizome of ginger. Under conditions with elevated temperature, gingerols can be converted into less pungent chemicals, such as shogaols and zingerone. Shogaols are not found in fresh ginger and are normally generated by the dehydration of gingerol during heating or long-term storage [11]. Particularly, gingerols have been found to possess diverse pharmacological and physiological activities, including antipyretic, analgesic, cardiotonic, antioxidant, and anti-inflammatory effects [11–14]. The combination of ginger extract and Ginkgo biloba (G. biloba) [15] or ginger and Cyperus rotundus [16] improves cognitive dysfunction and protects against oxidative stress in age-related animal models with dementia. However, the memory enhancing effect of ginger extract alone or its active ingredients has not been investigated and verified. Therefore, this study aimed to examine the effect of ginger extracts and [6]-gingerol (GIN) in C57BL/6 mice with SCO-induced learning and memory impairments and to elucidate the underlying molecular mechanisms.

2. Materials and Methods

- 2.1. Materials. GIN was purchased from Wako (Osaka, Japan), and SCO was obtained from Sigma-Aldrich (MO, USA). Anti-phospho-cAMP-response element binding protein (p-CREB) and anti-phosphoprotein kinase B/Akt (p-Akt) antibodies were obtained from Cell Signaling Technology (TX, USA). Anti-Akt and anti-brain-derived neurotrophic factor (BDNF) antibodies were provided by Santa Cruz Biotechnology (CA, USA), and anti-actin antibody and other chemical reagents were bought from Sigma-Aldrich. Fresh and dried ginger was purchased from Sejong pharmaceutical manufacturer (Daegu, Korea), and 600 g of fragmented ginger was extracted in 2.6 L of water at 70°C for 1.5 h. The supernatant was filtered, concentrated (Rotavapor R-220, Buchi, Switzerland), and freeze-dried to powder form (FreeZone 6, Labconco, MO, USA).
- 2.2. Animals and Drug Administration. Twelve-week-old male C57BL/6 mice were used in this study (20 ± 5 g; Charles River Orient Inc., Seongnam, Gyeonggi-do, Korea). They were kept in a room with the following conditions: temperature at $23 \pm 1^{\circ}$ C and a 12-h light and 12-h dark cycle (lights turned on at 06:00 am). Fresh and dried ginger extracts (FGE

and DGE) and GIN were suspended in normal saline and 1% ethanol, respectively. The mice were pretreated orally with FGE (100 or 200 mg/kg), DGE (100 or 200 mg/kg), or GIN (10 or 25 mg/kg) for 3 days before starting memory tests and continuously administered the drug during behavior tests. All experimental procedure was conducted in accordance with the National Institutes of Health and Daegu Haany University guidelines for Laboratory Animals, which was approved by the Committee for the Care and Use of Laboratory Animals in the Daegu Haany University.

2.3. Y-Maze Task. Y-maze task is frequently used in monitoring spatial learning. Animals were allowed to learn alternation between arms based on their memory of the previously visited arms. The experimental apparatus consisted of a white-painted Y-maze that is made from acryl. Each arm of the Y-maze was 30 cm long, 14 cm high, and 8 cm wide, and it is positioned at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. Spontaneous alteration behavior was defined as the consecutive entry into all three arms in overlapping triplet sets. During each trial, spontaneous alternations were recorded using the EthoVision System (Noldus, Wageningen, Netherlands). The percentage (%) of spontaneous alternation behavior was determined by dividing the total number of alternations by the total number of arm entries, subtracting 2, and then multiplying by 100 according to the following equation: % alternation = [(number of alternations)/(total number of arm entries -2)] $\times 100$. One hour before the test, mice were orally administrated with vehicle, FGE (100 or 200 mg/kg), DGE (100 or 200 mg/kg), or GIN (25 mg/kg), and 30 min later, the mice were injected with vehicle or SCO (1 mg/kg, i.p.). The Y-maze arms were thoroughly cleaned in between tests to remove residual odors.

2.4. Morris Water Maze Test. The Morris water maze test is a behavioral procedure designed to test spatial memory. In this experiment, a stainless circular pool (120 cm in diameter and 45 cm in height) with a featureless inner surface that is filled with white colored water (21 \pm 1°C) was used. A white platform (30 cm in height and 10 cm in diameter) was placed in one of the pool quadrants 2 cm below the surface. Four external cues to the maze were visible from the pool and used by the mice for spatial memory. The position of the cues remained unchanged throughout the experiments. During the four consecutive training days, the mice were subjected to three trials per day. One hour before the test, the mice were treated with vehicle, FGE (200 mg/kg), DGE (100 mg/kg), or GIN (25 mg/kg), and 30 min before the test, the mice were injected with vehicle or SCO (1 mg/kg). For each training trial, the rodents were placed in the water facing the pool wall at one of the pool quadrants in a different order each day. The time interval between each trial was 10 min. When the mice located the platform, they remained on it for another 10 sec. If the mice did not find the platform within 120 sec, they were guided to and placed on it for 10 sec. During each trial, the time taken for the mice to find the platform was recorded using the EthoVision System (Noldus, Wageningen, Netherlands). On the 4th day after the final training trial, the mice were subjected to the probe test in which the platform was removed from the pool. They were allowed to swim to search for the platform for 90 sec, and the time spent to find the platform quadrant was monitored.

2.5. Passive Avoidance Task. The passive avoidance task is a fear-motivated avoidance task in which the mice learn to refrain from stepping through a door to a dark compartment that is safer but one in which they were previously punished. This test was carried out in identical illuminated and nonilluminated boxes (16 cm long, 10 cm high, and 11 cm wide) that are connected by guillotine door (Gemini Avoidance System, SD, USA). The floor of both compartments was composed of 2-mm stainless rods that are 1 cm apart. An hour prior to the behavioral test, the mice were treated with vehicle or GIN (10 or 25 mg/kg), and 30 min after, they were injected with vehicle or SCO (1 mg/kg). For the acquisition trials, the mice were initially placed in the illuminated compartment, and the door between the two compartments was opened 20 sec later. When the mice entered the dark compartment, the door automatically closed, and an electrical foot shock (0.5 mA, 5 sec) was delivered through the stainless-steel rods. Twelve hours after the acquisition trial, the mice were replaced in the illuminated compartment for the retention test. The time taken for a mouse to enter the nonilluminated compartment after opening the door was defined as a step-through latency for both acquisition and retention trials. The maximum stepthrough latency to enter the dark compartment was restricted to 300 sec.

2.6. Contextual Fear Conditioning Task. Contextual fear conditioning is the method by which organisms learn to fear new stimuli. It is a form of learning in which fear is associated with a particular neutral context or stimulus. One hour before this test, the mice were orally administrated with vehicle or GIN (25 mg/kg), and after 30 min, they were intraperitoneally injected with vehicle or SCO (1 mg/kg). During training, the mice were placed in the conditioning cage (26 cm long, 28 cm wide, and 15 cm high) for 60 sec for adaptation. Sound tone (68–80 dB) was delivered for 15 sec as a conditioned stimulus, and after the last 1 sec of the tone stimulus, an electrical foot shock (0.5 mA) was delivered for 1 sec. This procedure was repeated two times at 150-sec intervals. One day after the training test, the mice were placed in the conditioning cage, and the freezing response was continuously measured for 5 min, which is defined as the absence of mouse paw moving and breathing.

2.7. Immunohistochemistry. After completing the behavioral tests, the mice were anesthetized with Zoletil with lompun and perfused with 4% paraformaldehyde (PFA) in 0.1-M phosphate buffer saline (PBS). The brains were removed and postfixed with 4% PFA at 4 $^{\circ}$ C overnight and then incubated in a series of 10%, 20%, and 30% sucrose solution for cryoprotection. In addition, they were embedded in OCT compound and quickly frozen using liquid nitrogen. The frozen brains were cut into 30 μ m-thick sections using a

cryostat (Leica, Wetzlar, Germany), placed onto the slides, and then incubated with 1% Triton X-100 in PBS for 10 min at room temperature (RT). After washing, sections were incubated in 0.6% hydrogen peroxide (H₂O₂) in DDW for 30 min in the dark to remove endogenous peroxidase activity, washed twice with PBS, and further treated with 5% bovine serum albumin (BSA) in PBS for 60 min at RT, followed by incubation with anti-p-CREB antibody in PBS containing 3% BSA overnight at 4°C. The sections were rinsed with PBS, treated with biotinylated anti-rabbit secondary antibody for 60 min at RT, and then treated with avidin-biotin complex reagent (Santa Cruz Biotechnology) for 30 min. The sections were washed again with PBS and developed using 0.025% 3,3diaminobenzidine (DAB) with 0.003% H₂O₂ (Vector Laboratories Inc., CA, USA) in PBS and images were visualized under a light microscope (Leica, Germany).

2.8. Western Blot Analysis. After the behavioral tests, the mice were anesthetized with Zoletil and lompun, and the cortical tissues were dissected for western blot analysis. The isolated cortex tissues were homogenized in RIPA buffer that contains 50 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄, and Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics, IN, USA). The protein concentrations of the samples were determined via the BCA protein assay (Pierce, IL, USA). The protein samples were separated on 12.5% SDS polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane (Pall Co., MI, USA) for further processing. After blocking with 0.05% Tween 20 in PBS (PBST) that contains 5% nonfat dried milk for 1h at RT, the membranes were incubated with primary antibodies in PBS that contains 3% skim milk at 4°C overnight. After three times of washing with PBST, the blots were further reacted with horseradish peroxidase- (HRP-) conjugated anti-rabbit or anti-mouse secondary antibody (1:5000, Enzo Life Sciences Inc., NY, USA) in PBS containing 3% skim milk for 1h. After washing with PBST for three more times, the specific bands were detected with enhanced chemiluminescence (ECL) western blotting detection reagent (Amersham Biosciences, NJ, USA) and visualized by using an ImageQuant LAS 4000 Multi Gauge software (Fujifilm, Tokyo, Japan).

2.9. Statistical Analysis. The data were expressed as means \pm SEM, and a statistical analysis for multiple comparisons was performed using one-way ANOVA, followed by the Tukey test using the SPSS software (SPSS 12.0 KO for windows). A p value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of Ginger Extracts on SCO-Induced Memory Loss in C57BL/6 Mice. Immediate working memory performance was assessed by recording spontaneous alteration behavior in a single session of a Y-maze task. In this test, SCO (1 mg/kg, i.p.) significantly decreased the percentage of spontaneous alteration (Figure 1(a)). The treatment of C57BL/6 mice with FGE (200 mg/kg) effectively improved memory impairment

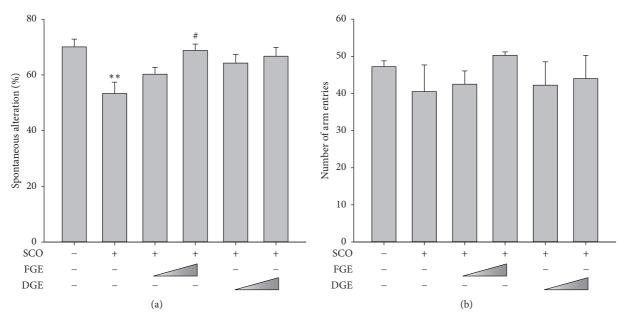


FIGURE 1: Memory enhancing effects of ginger extracts based on the Y-maze task. One hour before the test, the mice were treated with vehicle or ginger extracts (FGE, 100 or 200 mg/kg; DGE, 100 or 200 mg/kg; p.o.), and 30 min later, the mice were injected with vehicle or scopolamine (SCO, 1 mg/kg, i.p.). (a) Effects of ginger extracts on the SCO-induced spontaneous alternation. (b) Total number of arm entries drug during the 8-min trials of the Y-maze task. Data were presented as mean \pm SEM (n=4). Significant difference between the groups: ** p<0.01, vehicle-treated control versus SCO group; *p<0.05, SCO group versus ginger extract-treated group in combination with SCO.

that is induced by SCO (Figure 1(a)). However, the total number of arm entries was similar in each experimental group (Figure 1(b)).

In another experiment, the Morris water maze was conducted to compare spatial memory. The mean escape latency in finding the hidden platform during consecutive training days was calculated and compared. The mice in the vehicle-treated sham control group rapidly learned the hidden submerged platform, which they reached within 40 sec by the 3rd day of the training period (Figure 2(a)). In contrast, the performance of the SCO-treated group was significantly impaired. Repeated daily administration of FGE (200 mg/kg) reduced the mean escape latencies, particularly on the 3rd day (Figure 2(a)). The representative swimming-tracking paths of each group on the 3rd day are shown in Figure 2(b).

3.2. Restoration of SCO-Induced Learning and Memory Impairment due to the Use of GIN in the Passive Avoidance and Fear Conditioning Tests. In the passive avoidance task, particularly during the acquisition trial, no significant difference was observed in the latency time among the experimental groups. In addition, no apparent difference in the vocalization of mice in each treatment group was observed when they received a foot shock. However, during retention trial, the step-through latency was significantly decreased in the SCO-treated group compared with the vehicle-treated control group. The SCO-reduced step-through latency was recovered by repeated oral administration of GIN with a dose of 25 mg/kg (Figure 3(a)).

In the contextual fear conditioning task, during the training trial, no significant difference was observed in

each treatment group when they received a foot shock. However, after 24 h, a significant decrease in the freezing time was observed in the SCO-treated group compared with the vehicle-treated control group (Figure 3(b)). SCO effectively disrupted the conditioned fear to the context in C57BL/6 mice. Moreover, the freezing time was increased almost to control level by the repeated administration of GIN (25 mg/kg, p.o.) (Figure 3(b)).

3.3. Improvement in SCO-Induced Learning and Memory Deficit with the Use of GIN in the Y-Maze Task. Immediate working memory performance was assessed by recording spontaneous alteration behavior in a single session of the Y-maze task. In the Y-maze task, the spontaneous alternation in SCO-treated mice was significantly lower than that of the vehicle-treated control mice (Figure 4(a)). The reduced alternation via i.p. injection of SCO was significantly reversed by the oral administration of GIN (Figure 4(a)). SCO slightly increased the total number of arm entries, which was not much affected by GIN treatment (Figure 4(b)).

3.4. Attenuation of SCO-Induced Cognitive Dysfunction with the Use of GIN in the Morris Water Maze Test. In the Morris water maze test, the SCO-treated group exhibited longer escape latencies, the time taken to find the platform, compared with the vehicle-treated control group throughout the training days. However, the oral administration of GIN (25 mg/kg) significantly reduced the mean escape latency, thereby ameliorating the learning and memory loss caused by SCO (Figure 5(a)). To further verify the spatial memory, the time spent in the platform quadrant during the probe

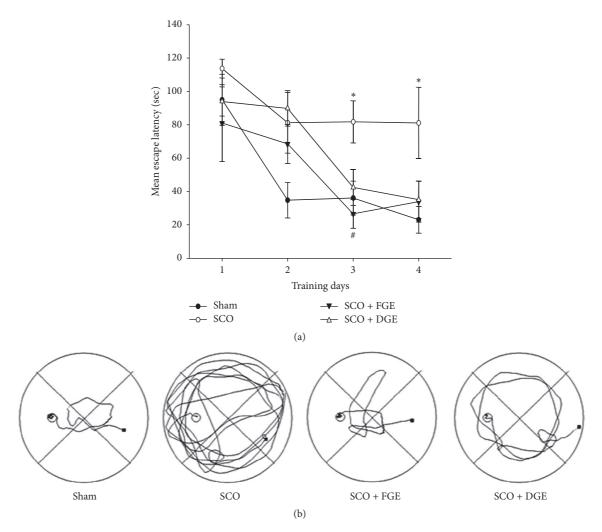


FIGURE 2: Cognitive enhancing effects of ginger extracts based on the Morris water maze test. One hour before the test, the mice were orally administered with vehicle or ginger extracts (FGE, 200 mg/kg; DGE, 100 mg/kg; p.o.), and after 30 min, the mice were injected with vehicle or SCO (1 mg/kg, i.p.). (a) Effects of ginger extracts on the SCO-induced spatial memory impairment that was represented by the mean escape latency. Data were presented as mean \pm SEM (n=4). Significant difference between the groups: *p<0.05, vehicle-treated control versus SCO group; *p<0.05, SCO group versus ginger extract-treated group in combination with SCO. (b) The representative water maze paths that were selected from each group on the 3rd day of training trials.

trial was measured after the last training trial on the 4th day. Swimming times of the SCO-treated group within the platform quadrant were significantly lower than those of the vehicle-treated group (Figure 5(b)). The time spent reflects the retention of learning and memory. The sham and GIN-treated groups spent more time searching for the removed platform in the target quadrant than the SCO-treated amnesic group (Figure 5(b)).

3.5. Restoration of BDNF Expression with the Use of GIN Possibly through the Activation of Akt-CREB Pathway. To verify the neuroprotective mechanisms of GIN against SCO-induced deficits in learning and memory, the expression of BDNF and its upstream regulators were examined. The SCO-treated amnesic group exhibited decreased levels of BDNF protein in the cortex, which was increased by the oral administration of GIN (25 mg/kg) (Figure 6(a)). Moreover, the

increased expression of BDNF seemed to be mediated by the activation of Akt-CREB signaling pathway. The amount of the phosphorylated form of CREB was remarkably reduced in the cortical tissues of the SCO-treated mice, and GIN treatment also increased the p-CREB levels in the SCO-treated group comparable to the sham control group (Figure 6(b)). Under the same experimental condition, the SCO-reduced relative ratio of p-Akt/Akt, a possible upstream kinase of CREB, was upregulated by GIN treatment (Figure 6(c)).

4. Discussion

As the average human lifespan has increased dramatically over the last decades, the human society has been more concerned with age-associated memory loss and neurodegenerative diseases, such as AD. Neurodegenerative disorders are a group of disease conditions that resulted from chronic

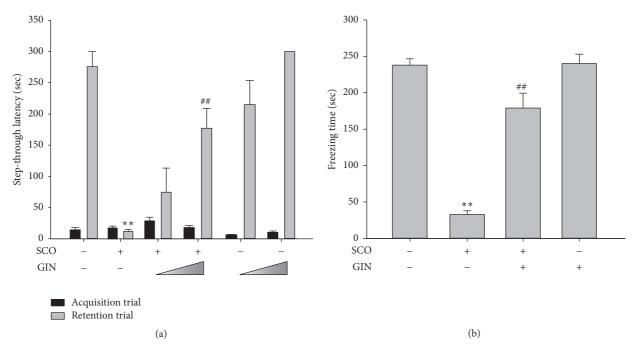


FIGURE 3: Protective effect of GIN on the learning and memory deficits caused by SCO based on the passive avoidance and contextual fear conditioning tests. One hour before this test, the mice were treated with vehicle or GIN (10 or 25 mg/kg, p.o.), and 30 min later, they were treated with vehicle or SCO (1 mg/kg, i.p.). (a) Effect of GIN on the SCO-diminished step-through latency was monitored during the retention trial. (b) Effect of GIN on the SCO-decreased immovable time without any movement for the freezing response was examined. Data were presented as mean \pm SEM (n = 7). Significant difference between the groups: ** p < 0.01, vehicle-treated control versus SCO group; ## p < 0.01, SCO alone group versus GIN-treated group in combination with SCO.

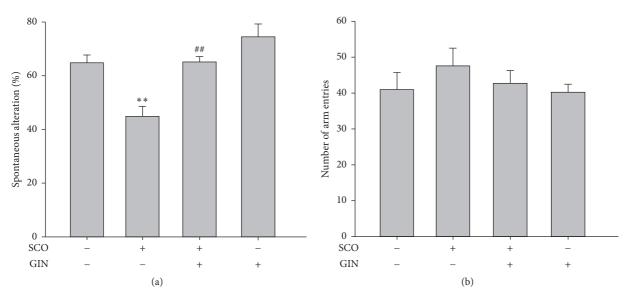


FIGURE 4: Protective effect of GIN on the learning and memory impairment induced by SCO in the Y-maze task. One hour before this test, mice were orally administered with vehicle or GIN (25 mg/kg, p.o.), and after 30 min, they were injected with vehicle or SCO (1 mg/kg, i.p.). (a) Effect of GIN on the SCO-decreased spontaneous alternation. (b) Total number of arm entries during the 8-min trials of the Y-maze task. Data were presented as mean \pm SEM (n=7). Significant difference between the groups: ** p<0.01, vehicle-treated control versus SCO group; ## p<0.01, SCO group versus GIN-treated group in combination with SCO.

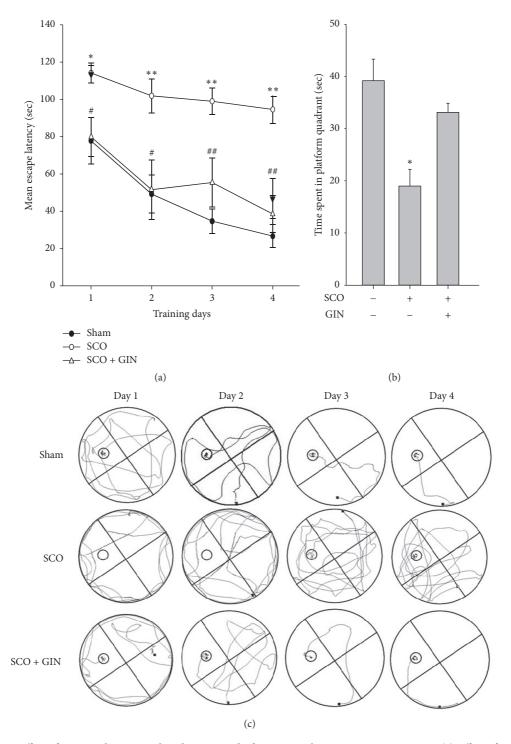


FIGURE 5: Protective effect of GIN on the SCO-induced cognitive dysfunction in the Morris water maze test. (a) Effect of GIN on the SCO-induced spatial memory impairment that was represented by the mean escape latency. (b) On the 4th day after the final training trials, the mice were subjected to probe trials in which the platform was removed from the pool, and they were allowed to swim for 90 sec to search for the platform. Data were presented as mean \pm SEM (n=7). Significant difference between the groups: *p < 0.05 and **p < 0.01, vehicle-treated control versus SCO (1 mg/kg, i.p.) group; *p < 0.05 and **p < 0.01, SCO alone group versus GIN-treated (25 mg/kg, p.o.) group in combination with SCO.

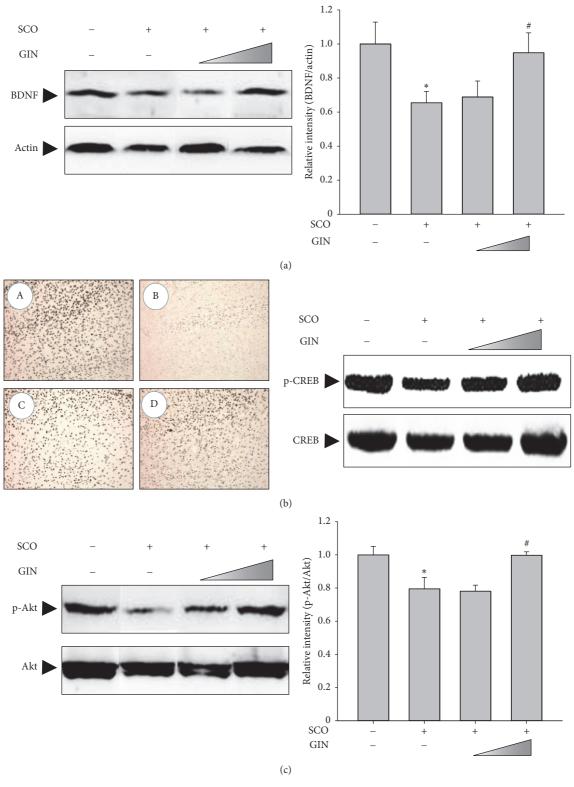


FIGURE 6: Effect of GIN on the SCO-reduced protein expression of BDNF in the cortex. Mice were pretreated with GIN for 3 days before the start of the test and then continuously administered during the behavior tests. The protein expression ratios of BDNF/actin (a) and p-Akt/Akt (c) were monitored via western blot analysis. Data were presented as mean \pm SEM (n=3). Significant difference between the groups: p < 0.05, vehicle-treated control versus SCO group; p < 0.05, SCO alone group versus GIN-treated group in combination with SCO. (b) Effect of GIN on the activation of CREB via phosphorylation in the cortex was examined via immunohistochemistry by staining with anti-p-CREB antibody (right panel). (A) sham control, (B) SCO (1 mg/kg), (C) SCO (1 mg/kg) + GIN (10 mg/kg), (D) SCO (1 mg/kg) + GIN (25 mg/kg). The protein levels of p-CREB and CREB were also verified by western blot analysis (left panel).

breakdown and progressive functional and structural loss of the neurons due to neural damage and cell death. Naturally occurring phytochemicals can strongly develop biologically active compounds with antiamnesic and anti-AD activities. However, only some natural sources, such as *G. biloba* and *Huperzia serrata*, have been extensively studied because of their natural therapeutic properties to treat patients with AD. Therefore, in this study, we have investigated the neuroprotective and memory enhancing effects of ginger extracts and one of its major constituents (GIN) against SCO-induced cognitive dysfunction in C57BL/6 mice.

Increasing evidence from experimental as well as clinical studies clearly indicates the major roles of acetylcholine (ACh), choline acetyltransferase (ChAT), and acetylcholine esterase (AChE) in the regulation of cognitive functions [3]. Based on a cholinergic hypothesis, many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity with the use of acetylcholinesterase (AChE) inhibitors, acetylcholine precursors, or cholinergic agonist. The therapeutic strategies for the prevention and/or treatment of cognitive disorder aimed to improve ACh activity. Therefore, the cholinergic receptor agonists (muscarinic and nicotinic) and enhancer of endogenous levels of ACh have been used in patients with Alzheimer-type senile dementia [17–19]. Based on this result, the commonly prescribed AChE inhibitors, such as donepezil, rivastigmine, and galantamine, have been developed. Although AChE inhibitors can diminish the severity of AD, their effects on memory function is still not verified and elucidated.

First, to induce learning and memory impairment, we have intraperitoneally injected SCO in C57BL/6 mice and performed Y-maze, Morris water maze, passive avoidance, and contextual fear conditioning tests. Numerous studies reported the compromised cognitive performance after systemic treatment with SCO, for example, deficits in contextual and cued fear conditioning, impairments in inhibitory avoidance, and alterations in short-term and long-term spatial learning [20, 21].

In the Y-maze task, SCO-treated mice showed decreased spontaneous alteration compared with the vehicle-treated mice, which was increased by FGE and GIN pretreatments. In the Morris water maze task, the SCO-treated mice took a longer time to find the platform than the sham control group, and the FGE- or GIN- and SCO-treated group in combination easily found the location of the hidden platform. In the passive avoidance and contextual fear conditioning tests, SCO treatment reduced the step-through latency as well as freezing time, which were effectively restored by the daily oral administration of GIN.

To support our findings, the administration of Zingicomb, a mixture of Zingiber officinale and G. biloba extracts, had been shown to improve inhibitory avoidance learning and spatial learning in old Wistar rats [15]. Naturally occurring phytochemicals, which can restore the cognitive dysfunction caused by SCO, were investigated and considered as promising candidates in other studies. G. biloba extract, a well-known neuroprotective natural medicine, has been reported to improve memory impairments in the SCO-treated rodent models [22, 23] and humans with AD [24, 25].

G. biloba has cholinergic actions, including the modulation of presynaptic choline uptake and acetylcholine release, upregulation of postsynaptic muscarinic receptors, and indirect modulation of the serotonergic system [25]. Huperzine A, which is another neuroprotective phytochemical candidate with the most selective AChE inhibitory activity, significantly restored memory deficits caused by SCO or the normal aging process as determined by the water maze test [26, 27]. In another study, the alcoholic extract of ginger improved memory impairment and infarct volume caused by focal cerebral ischemia by suppressing oxidative stress and fortifying antioxidant enzymes in Wistar rats [28].

As a molecular mechanism underlying the memory enhancing effect of GIN against SCO-induced amnesia, the expression of BDNF was examined. It has been suggested that neurodegeneration is associated with a lack of neurotrophic support, such as BDNF in cholinergic neurons. BDNF is a family of neurotrophins that interact with high-affinity receptor TrkB and play an important role in neuronal survival and maintenance of different types of neurons, synaptic plasticity, and cognitive processes [29]. The oral administration of GIN significantly upregulated the SCO-reduced protein expression of BDNF in the cortex of C56BL/6 mice, which seemed to be mediated by the activation of CREB via phosphorylation. CREB is a transcription factor with diverse roles in neural functions, such as the regulation of neural plasticity, learning and memory, and neurogenic process and is activated by phosphorylation of upstream kinases, such as ERK1/2 and Akt. In this study, the phosphorylation levels of CREB and its upstream regulator Akt were significantly increased in the cortical regions of GIN-treated mice compared with the SCO-treated amnesic group.

Fermented *Curcuma longa* [30], *Asparagus officinalis* [31], *Aronia melanocarpa* [32], and pine needle [33] extracts improved memory dysfunction in SCO-induced amnesia models by increasing the expression of CREB (or p-CREB) and BDNF. Oleanolic acid is a naturally occurring triterpenoid that ameliorated SCO-induced memory impairment by modulating the BDNF-ERK1/2-CREB pathway [34]. Zguggulsterone, a compound extracted from the resin of the plant *Commiphora wightii*, improved SCO-induced memory loss through the enhancement of the CREB-BDNF signal by the activation of ERK1/2 as well as Akt via phosphorylation [35].

In summary, GIN improved the SCO-induced learning and memory impairment and cognitive dysfunction in C57BL/6 mice based on the results of diverse behavior tests, such as Y-maze, Morris water maze, passive avoidance, and contextual fear conditioning tests. As a neuroprotective molecular mechanism, the oral administration of GIN upregulated the protein levels of neurotrophic factor BDNF, which seemed to be mediated by the activation of Akt-CREB pathway. These results suggested that GIN may have therapeutic potentials in preventing and/or treating memory loss in individuals with amnesia and neurodegenerative diseases, such as AD. However, further studies should be conducted to elucidate the molecular targets of GIN in diverse brain regions and their cross-talk in cellular signaling cascades.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Fuzheng Quxie Decoction Ameliorates Learning and Memory Impairment in SAMP8 Mice by Decreasing Tau Hyperphosphorylation

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Hyperphosphorylation of the microtubule-associated protein, tau, is critical to the progression of Alzheimer's disease (AD). Fuzheng Quxie Decoction (FQD), a Chinese herbal complex, is an effective clinical formula used to treat AD. In the current study, we employed high-performance liquid chromatography and liquid chromatography tandem mass spectrometry to identify the components of FQD. Three major components (ginsenoside Rg1, ginsenoside Re, and coptisine) were detected in the brain of FQD-fed mice, indicating their ability to cross the blood-brain barrier. We further evaluated the efficacy of FQD on Senescence-Accelerated Mice Prone-8 (SAMP8) mice. FQD significantly ameliorated learning and memory deficits in SAMP8 mice on the Morris Water Maze, decreasing escape latency (p < 0.01) and increasing swim time within the original platform-containing quadrant (p < 0.05). Further, FQD increased the number of neurons and intraneuronal Nissl bodies in the hippocampal CA1 region. FQD also decreased the expression of phosphorylated tau protein and increased the expression of protein phosphatase 2A (PP2A) and the N-methyl-D-aspartate receptor subunit, NR2A (p < 0.01). Our results indicate that FQD improves the learning and memory ability of SAMP8 mice. Moreover, our findings suggest that the protective effect of FQD is likely mediated through an inhibition of hippocampal tau hyperphosphorylation via NMDAR/PP2A-associated proteins.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease, characterized by memory loss, cognitive dysfunction, and decreased quality of life. Intraneuronal neurofibrillary tangles caused by hyperphosphorylation of the microtubule-associated tau protein and extraneuronal plaques composed of amyloid- β (A β) protein are the main pathological hallmarks of AD. Due to the failure of immunotherapy against A β to effectively treat individuals with AD, recent research has focused on developing interventions to target aberrant phosphorylation of tau protein [1].

Tau is the main microtubule-associated protein in neurons. Normal phosphorylation of tau is required for microtubule assembly and binding activity; therefore, this protein

is crucial in maintaining physiological function [2]. Hyperphosphorylation of tau protein leads to partial or complete loss of its biological activity [3]. AD pathophysiology is characterized by aggregates of abnormally phosphorylated tau in the entorhinal cortex, which spread to the hippocampus and entire cerebral cortex. The progression of this irregular protein diffusion is correlated with a decrease in cognitive function in AD [4]; thus, tau hyperphosphorylation is the central link of AD toxic effects. Both the National Institute on Aging and Alzheimer's Association (NIA-AA; 2011) and the International Working Group (IWG-2; 2014) have proposed using tau protein and p-tau protein levels in cerebrospinal fluid as a diagnostic biomarker of AD [5]. In terms of molecular mechanisms leading to the hyperphosphorylation

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Pinyin name	Source plants	Latin name	Amount/weight ratio
Renshen	Panax ginseng C. A. Mey.	Ginseng Radix et Rhizoma	9
Huanglian	Coptis chinensis Franch.	Rhizoma Coptidis	6
Chuanxiong	Ligusticum chuanxiongHort.	Rhizoma Ligustici Chuanxiong	5

TABLE 1: Composition of the Chinese herbal formula FQD.

of tau protein, both the upregulation of protein kinases and downregulation of protein phosphatases have been implicated [6]. In the human brain, PP2A is the major tau phosphatase and has been reported to account for 71% of the total tau phosphatase activity [7]. PP2A inactivation prevents tau dephosphorylation and promotes activation of protein kinase A [8].

Fuzheng Quxie Decoction (FQD) is a clinical traditional herbal formula composed of Ginseng Radix et Rhizoma (Renshen), Rhizoma Coptidis (Huanglian), and Rhizoma Ligustici Chuanxiong (Chuanxiong). Numerous studies have shown that biocomponents of FQD, including the Rg1 and Rb1 ginsenosides of Renshen, berberine of Huanglian, and ligustilide of Chuanxiong, can ameliorate learning and memory impairments in models of AD [9–15]. In this study, we aimed to investigate the effect of early FQD administration on learning and memory in Senescence-Accelerated Mice Prone-8 (SAMP8) and its regulation on tau hyperphosphorylation in the hippocampus.

2. Materials and Methods

2.1. Animals. The present study employed SAMP8 mice, which exhibit age-related cognitive dysfunction accompanied by $A\beta$ and tau protein abnormalities, as a mouse model of AD. Senescence-resistant-1 (SAMR1) mice were used as controls. Mice in both groups were 3-month-old males, weighing 26–30 g, reared under specific pathogen-free conditions and obtained from the Department of Experimental Animal Science, Peking University Health Science Center, permit number, SCXK (Jing) 2011-0012. Throughout the experiments, animals were housed in a controlled environment, with a 12-h light/dark cycle; temperature, 23 ± 2°C; relative humidity, 50–70%. Animals received food and water ad libitum, and mice were allowed to adapt to their environment for 1 week before experiment onset.

2.2. Drug Preparation. FQD, composed of Renshen, Huanglian, and Chuanxiong, with a weight ratio of 9:6:5 (Table 1), was prepared into an extract by the Department of Pharmaceutical Preparation of Xiyuan Hospital, China Academy of Chinese Medical Sciences. One gram of FQD extract was equivalent to 3.71 g of crude drug. High-performance liquid chromatography (HPLC) was used to determine the main components of FQD, employing a combination of qualitative and quantitative methods for quality control. During the study, the extract was mixed with varying ratios of distilled water, resulting in concentrations of 0.7 g/ml and 3.5 g/ml (extract).

Memantine hydrochloride tablets (Ebixa 10 mg, batch number: H20120268) produced by H. Lundbeck A/S were

suspended in distilled water, resulting in a concentration of 2.6 mg/ml.

2.3. Reagents. Rabbit monoclonal anti-Tau s396 phosphorylated antibody, mouse monoclonal anti-tau antibody (TAU-5), rabbit monoclonal anti-PP2Aα Y307 phosphorylated antibody, rabbit monoclonal anti-N-methyl-D-aspartate receptor (NMDAR) 2A antibody, and mouse monoclonal anti-NMDAR2B antibody were acquired from Abcam (Cambridge, GRB). Rabbit anti-PP2Ac subunit antibody was purchased from Neuron Signaling Technology (Massachusetts, USA). Beta-actin mouse monoclonal antibody was provided by ImmunoWay (California, USA). Goat anti-rabbit IgG (H+L)-HRP and goat anti-mouse IgG (H+L)-HRP were provided by Beijing TDY Biotech (Beijing, CHN). BCA protein quantification kit, Coomassie Brilliant Blue staining solution, and ponceau red staining solution were provided by the Beijing Sinoble Biotechnology Center. Protease and phosphatase inhibitors were provided by Roche (Basel, CHE).

2.4. Animal Groups and Drug Administration. Forty male SAMP8 mice were randomly divided into four groups by a computer-generated random number table. Groups included model (equal volume distilled water), memantine (2.6 mg/kg/d), FQD low-dose (FQD-L, 0.7 g/kg/d, extract), and FQD high-dose (FQD-H, 3.5 g/kg/d, extract) groups. The control group comprised 10 male SAMR1 mice with the same genetic background as experimental mice, which were fed with distilled water. Each group received intragastric 0.1 ml/10 g/d distilled water or liquid-suspended memantine or FQD once a day for 12 weeks. All efforts were made to minimize the number of animals used and their suffering during this study. Animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals and the Beijing Laboratory Animal Welfare Ethics Review Guidelines issued by the Ministry of Science and Technology of China. This study was approved by the Ethics Committee of Xiyuan Hospital, China Academy of Chinese Medical Sciences (Permit Number: CACMS/20141220X21).

2.5. Morris Water Maze. After 3 months of intragastric water or drug administration, spatial learning and memory were assessed using the Morris Water Maze (MWM) video analysis system (Beijing ZS Dichuang Technology Development Co., Ltd., Beijing, China). The apparatus consisted of a white pool (120 cm in diameter and 50 cm in height), platform (10 cm in diameter and 50 cm in height), video camera, and computer. The pool was divided into four quadrants, with the visible cues outside the pool. The pool was filled with opaque water, maintained at 19°C [16]. The platform was fixed in one quadrant of the pool, 1 cm below the surface of the water, and

the test environment was kept quiet with a stable light source. One day before the experiment, mice were habituated to the pool environment for 120 s without the platform. The MWM experiment consisted of two parts: navigation trials and a spatial probe trial. The trials were conducted between 9:00 and 10:30 every morning to exclude any impact of circadian rhythm.

In the navigation trials, mice were placed into the water facing the wall of the pool at the 1/2 radian position in one of the four quadrants. Mice were allowed 60 s to find and mount the platform, where they remained for at least 5 s. If mice failed to find the platform within 60 s, they were guided to the platform, where they remained for 10 s before being removed, dried, and returned to their home cages. During the 5-day training period, mice underwent four trials per day, with an intertrial interval of 15 min. Time to find the platform (escape latency, EL) and search strategies were recorded using video tracking software. The maximum value for escape latency was regarded as 60 s. Search strategies were classified as spatial strategies, nonspatial strategies, or repetitive looping paths [17].

The spatial probe trial was performed on the 6th day. During this trial, the platform was removed from the pool, and mice were placed into the water facing the pool wall. Mice were allowed to swim freely for 60 s. The number of times mice crossed the original platform location, as well as total swimming time and distance in the original platform quadrant, was recorded.

2.6. Sample Preparation. Mice were fasted overnight after the MWM test. Five mice were randomly selected from each group and anesthetized with chloral hydrate (0.9 mL/100 g body weight). Blood samples from the retroorbital sinus were collected in heparin-treated EP tubes and centrifuged (3000 r/min, 10 min). Plasma was then collected from the samples and stored at -80°C. Next, mice were transcardially perfused with ice-cold saline, and brains were quickly removed and sagittally cut into left and right hemispheres on blocks of ice. The left half of the brain was fixed in 10% neutral formaldehyde and paraffin-embedded for pathological and immunohistochemical staining. The right half of the brain was dissected into the hippocampus and cortex. Hippocampal tissue was weighed and stored in liquid nitrogen for protein immunoblotting, while the right cerebral cortex was homogenized in 0.15:1 (wt/vol) ice-cold saline solution. The homogenate was centrifuged (2000 r/min, 15 min) and the supernatant was used to measure the content of brain tissue.

2.7. Liquid Chromatography Tandem Mass Spectrometry. Solid phase extraction (SPE) was used to prepare the plasma sample, while the acetonitrile precipitation method was used to prepare supernatant obtained from the above described method brain tissue. We then employed liquid chromatography tandem mass spectrometry (LC-MS/MS) to identity the individual components separated out by LC and to establish a standard calibration curve. If samples exhibited a good fit with the standard curve, they were determined to be accurately quantified.

2.8. Hematoxylin Eosin Staining. Fixed, paraffin-embedded brain tissue was sectioned and underwent hematoxylin eosin (HE) staining according to the following procedure. Sections were deparaffinized, washed in distilled water, and incubated in hematoxylin solution for 5 min; excess hematoxylin solution was washed off with running tap water. To remove background staining, sections underwent a differentiation step in hydrochloric acid alcohol, after which they were fully washed in running tap water. Sections were then counterstained in eosin solution for 2-3 min, washed in running tap water, dehydrated through graded alcohol, and mounted with neutral resin. Pathological changes in neurons were observed at 400x under a light microscope.

2.9. Nissl Staining. Paraffin-embedded, fixed brain tissue was deparaffinized, washed 1-2 min in distilled water, dipped in 1% thionin lyosol at 37°C for 30 min, and washed again for 1-2 min in distilled water. In order to moderately differentiate the nucleus, sections were incubated in 0.5% hydrochloric acid alcohol, washed back to blue, and differentiated using 95% alcohol until the Nissl substance was visualized. Sections were then dehydrated (twice for 5 min each) in 100% anhydrous alcohol, permeabilized with xylene twice (5 min each), and mounted with neutral gum. Changes observed in neurons and Nissl bodies of the hippocampal CA1 region were detected at 400x under a light microscope.

2.10. Immunohistochemistry. Sections were deparaffinized and incubated in a 3% H₂O₂-deionized water solution for 5-10 min to block endogenous peroxidase activity. Sections were then washed three times (2 min each) in phosphatebuffered saline (PBS), and incubated in primary antibodies overnight at 4°C. The next day, sections were washed in PBS three times (3 min each), incubated 20 min in secondary antibodies, and again washed in PBS three times (3 min each). Sections were then stained using 3,3'-diaminobenzidine, dehydrated in graded alcohol, cleared in xylene, and mounted with neutral gum. The CA1 region of the hippocampus in three adjacent fields on each section was viewed under DpxView Pro, a light microscope (DeltaPix, DEN). Tau-5 and p-tau-positive neurons were counted with Image-Pro Plus 6.0 image processing software using mean optical density (MOD). All evaluations were done by one researcher that was blind to the experimental design.

2.11. Western Blot Analysis. Hippocampal tissue samples (5/group) were lysed with RIPA buffer to extract protein and centrifuged at 13,000g for 20 min at 4°C (3-18K, Sigma). The supernatant was collected, and total protein was quantified using a BCA kit (Beijing Sinoble Biotechnology Center, Beijing, China). The protein concentration was adjusted to 4 mg/ml with RIPA and 5x reduced sample buffer and boiled for 5 min. According to the molecular weight of the target protein, we prepared either 8% or 12% acrylamide resolving gels, with the concentration of the stacking gel remaining at 5%. Samples containing 20 μ g of protein were loaded on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis (90-V for the stacking gel ~20 min; 160 V for the separating gel, timed in accordance with the

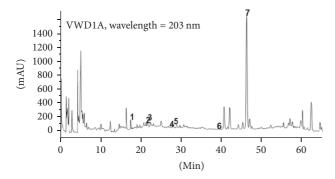


FIGURE 1: Fingerprint map of Fuzheng Quxie Decoction (FQD) extract detected with HPLC. 1: ferulic acid; 2: ginsenoside Rg1; 3: ginsenoside Re; 4: coptisine; 5: ginsenoside Rb1; 6: berberine; 7: ligustilide.

protein marker) and transferred to $0.45\,\mu\mathrm{m}$ nitrocellulose filter membranes (300 mA, 1-2 h). Membranes were blocked with 3% bovine serum albumin-tris-buffered saline for 30 min and incubated in primary antibody solution overnight at 4°C. After incubation with horseradish peroxidase-conjugated antibody anti-rabbit or anti-mouse IgG antibodies for 40 min, protein complexes were detected using enhanced chemiluminescence Western blotting detection reagents (Millipore). Band intensity was determined using Gel-Pro Analyzer 4.0 software.

2.12. Statistical Analysis. Values of all experiments are represented as mean \pm standard error of the mean (SEM). Statistical analyses were performed using SPSS 19.0 software. A oneway analysis of variance (ANOVA) with a least significant difference test or a one-way ANOVA with post hoc Dunnett's T3 (multiple comparisons) was used to compare betweengroup values. p < 0.05 was considered statistically significant.

3. Results

- 3.1. Bioactive Components of FQD. Using HPLC, ginsenoside Rg1 (9.86%), ginsenoside Re (2.86%), ginsenoside Rb1 (0.47%), coptisine (7.12%), berberine (2.19%), ligustilide (21.40%), and ferulic acid (0.21%) were identified as the seven main bioactive components of FQD. Their corresponding fingerprint was established using joined qualitative and quantitative methods for quality control (Figure 1).
- 3.2. Determination of FQD Components That Cross the Blood-Brain Barrier. The calibration curves of ginsenoside Rgl, ginsenoside Re, ginsenoside Rbl, coptisine, berberine, and ferulic acid in plasma and brain tissue homogenates were established using the LC-MS/MS detection method. A curve for ligustilide could not be constructed, as this component is not compatible with LC-MS/MS. Results showed a good linear relationship of the six standard curves; thus, all components could be quantified. All six components were detected in the plasma, while only ginsenosides Rgl and Re and coptisine were detected in the brain (Table 2).
- 3.3. FQD Ameliorates Spatial Learning and Memory Deficits in SAMP8 Mice. On the first day of MWM testing, there was no

TABLE 2: FQD components detected via LC-MS/MS (ng/mL).

Component	Plasma level	Brain level
Ginseng		
Ginsenoside Rg1	2.63	1.58
Ginsenoside Re	2.63	4.47
Ginsenoside Rb1	10.7~282	ND
Huanglian		
Coptisine	0.793~94.2	0.767~3.3
Berberine	9.54~13.9	<lloq< td=""></lloq<>
Chuanxiong		
Ferulic acid	0.36~22.5	ND

 $\it Note.$ ND, not detected; <LLOQ, lower limit of quantitation. Ginsenoside Rg1 and ginsenoside Re were detected in one plasma sample and one brain tissue homogenate sample.

statistically significant difference between groups in terms of escape latency (p > 0.05). For each group, the escape latency on the fifth day of testing was shorter than that observed on the first day (p < 0.01 or p < 0.05), indicating that all mice were able to learn. On the last 2 days of training, the model group exhibited longer latencies to reach the platform than the control group (p < 0.01). Moreover, when compared to the model group, memantine, FQD-L, and FQD-H groups exhibited shorter latencies to reach the platform on the fifth (p < 0.05), fourth (p < 0.01), and second (p < 0.05) days of training (Figure 2(a)).

Differences in search strategy may explain the observed improvements in performance during the 5 days of navigation trials. Mice of the control group utilized spatial strategies (direct: swimming directly to the platform), while those of the model group utilized nonspatial strategies (scanning: searching the interior portion of the tank without spatial bias). Performance was worse among mice of the memantine than among those of the control group. In contrast to the model group, the memantine, FQD-L, and FQD-H groups tended to adopt spatial strategies (indirect: swimming to the platform with at most one loop) (Figure 2(b)). No significant differences in performance were observed between mice of the FQD groups and those of the memantine group.

The spatial probe trial was performed on the 6th day. Values representing the number of crossings, swim time, and the percentage of the target quadrant in the total distance were significantly lower in the model group than in the control group (p < 0.05). Both the number of crossings exhibited by the memantine group and the swim time and the percent distance in target quadrant of the FQD-H group were significantly higher (p < 0.05) than the corresponding values in the model group. No between-group difference in total swim distance was observed throughout the test (p > 0.05) (Figure 2(c)).

3.4. Morphological Changes in CA1. HE staining revealed that neurons in the hippocampal CA1 region of the control group were arranged in an organized, compact pattern with clear boundaries. In the model group, however, neurons were scattered with no clear boundaries. Moreover, the number of

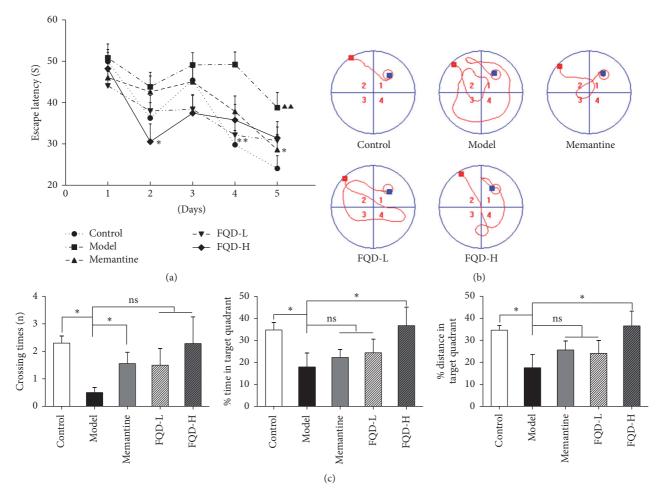


FIGURE 2: FQD ameliorated learning and memory deficits of SAMP8 mice. (a) All the group displayed a spatial learning effect (p < 0.01) or p < 0.05). On the last 2 days of training, the model group exhibited longer latencies to reach the platform than the control group (p < 0.01). Moreover, compared to the model group, memantine, FQD-L, and FQD-H groups exhibited shorter latencies to reach the platform on the fifth (p < 0.05), fourth (p < 0.01), and second (p < 0.05) days of training. (b) The strategies for searching platform on the fifth day of navigation trial. (c) Values representing the number of crossings, swim time, and percent distance in the target quadrant were all significantly lower in the model group compared to the control group (p < 0.05). Compared to the model group, the number of crossings exhibited by the memantine group and the swim time and percent distance in target quadrant of the FQD-H group were significantly higher (p < 0.05). No between-group difference was observed in terms of total swim distance throughout the test (p > 0.05). *p < 0.05, **p < 0.05 between model and drug-treated groups. Black triangle, p < 0.01 between model and control group.

free neurons was increased and neuron layers were decreased. The nuclei of these neurons appeared as deep pyknotic masses or, in some cases, were not even present. After 3 months of drug administration, all aspects of pathological neuron morphology were improved (Figure 3).

Nissl staining revealed that neurons in the CA1 region of the hippocampus of control mice were large in number, arranged in neat rows, and rich in Nissl bodies and exhibited pale blue nuclei and dark blue backgrounds. In contrast, neurons in the model group were lower in number, disordered, and swollen, lacked clear Nissl body boundaries, and exhibited light blue cytoplasm and dark blue pyknotic nuclei. Drug administration ameliorated the histopathology of the SAMP8 mice in the hippocampal CA1 region: neurons significantly increased in number, appeared neatly arranged,

and exhibited a greater number of Nissl bodies following treatment (Figure 4).

3.5. FQD-Mediated Inhibition of Tau Hyperphosphorylation. Western blot and immunohistochemistry experiments were used to assess the expression of t-tau and p-tau at the Ser396 epitope (S396). Immunohistochemistry revealed a significant increase in the MOD of CA1 region p-Tau (S396) in the model group, relative to values observed in the control group (p < 0.05). However, no increases in the MOD of t-tau were observed (p > 0.05). After treatment with memantine or FQD, the MOD of CA1 region p-tau (S396) was significantly decreased (p < 0.01 and p < 0.05, resp.) relative to levels observed in the untreated model group. No significant decreases in the MOD of t-tau were observed (p > 0.05) (Figures 5(a)-5(c)).

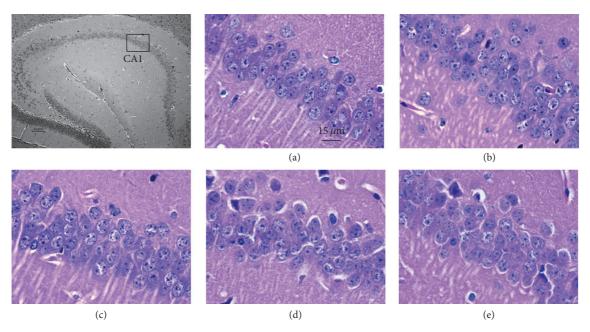


FIGURE 3: *HE staining of hippocampal CA1* (×400). (a) Control group. (b) Model group. (c) Memantine group. (d) FQD-L group. (E) FQD-H group.

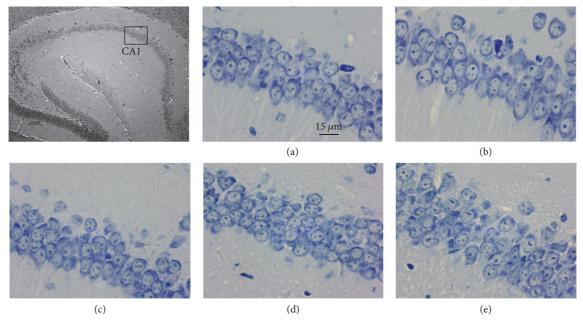


FIGURE 4: Nissl staining of hippocampal CA1 ($\times 400$). (a) Control group. (b) Model group. (c) Memantine group. (d) FQD-L group. (e) FQD-H group.

Western blot analyses confirmed our immunohistochemistry results, revealing a significant increase in the level of p-tau (S396) in model mice relative to levels observed in control mice (p < 0.05). The expression of hippocampal p-tau (S396) was significantly decreased following treatment with memantine and high doses of FQD (p < 0.05) (Figure 5(d)).

3.6. Expression of NMDAR/PP2A-Associated Proteins. Western blot was used to assess the expression of p-PP2A, PP2Ac,

NR2A, and NR2B. Compared to the control group, the expression of p-PP2A, PP2Ac, and NR2A was significantly decreased in hippocampal tissues of model group mice (p < 0.01, p < 0.05, and p < 0.01, resp.). However, NR2B expression was not significantly different in model mice (p > 0.05). The expression of hippocampal p-PP2A and NR2A was significantly elevated in drug-treated mice (p < 0.01); NR2A was significantly elevated in memantine and high doses of FQD treated mice (p < 0.01 and p < 0.05, resp.), while

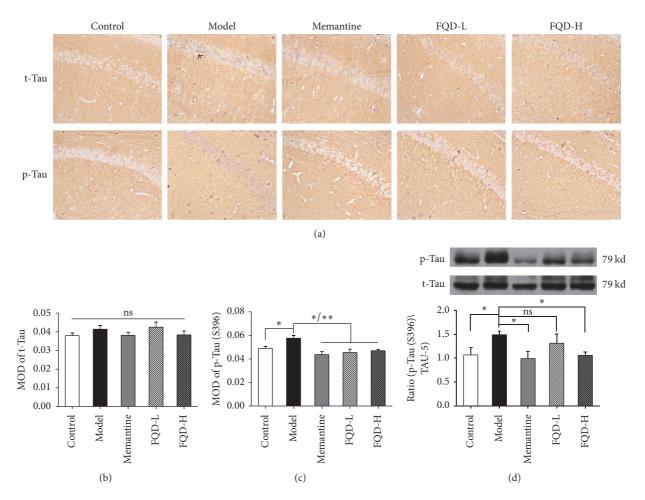


FIGURE 5: FQD inhibited tau protein hyperphosphorylation in SAMP8 mice. (a) Immunohistochemistry of t-Tau and p-Tau (S396) (×100). (b-c) Compared to the control group, the model group showed a significant increase in the MOD of CA1 region p-Tau (S396) (p < 0.05). However, the MOD of t-Tau was not increased (p > 0.05). After treatment with memantine and FQD, the MOD of CA1 region p-Tau (S396) was significantly decreased (p < 0.01 and p < 0.05, resp.) compared to the untreated model group. The MOD of t-Tau was not statistically decreased (p > 0.05). (d) Western blot analyses, showing a significant increase in the level of p-Tau (S396) in model mice compared to control group (p < 0.05). The expression of hippocampal p-Tau (S396) was significantly decreased after administration with memantine and high doses FQD (p < 0.05). *p < 0.05, **p < 0.05.

PP2Ac and NR2B were not significantly different (p > 0.05) (Figure 6).

4. Discussion

The findings of the present study demonstrated that FQD can ameliorate learning and memory impairments in SAMP8 mice, increase the number of intraneuronal Nissl bodies and the total number of neurons in the hippocampal CA1 region, and inhibit tau hyperphosphorylation in the hippocampus. Further, our findings indicated that the main bioactive components of FQD include ginsenosides Rg1, Re, and Rb1, as well as coptisine, berberine, ligustilide, and ferulic. We also observed that ginsenoside Rg1, ginsenoside Re, and coptisine can cross the blood-brain barrier. These findings are in agreement with those of several studies, which have revealed that the major components of FQD (i.e., ginsenosides Rg1,

Rb1, and Re, as well as ferulic acid, coptisine, berberine, and ligustilide) exert antidementia effects in models of AD via one or several of the following mechanisms: reducing A β content [15, 18–20], inhibiting tau hyperphosphorylation [9, 11–15], reducing the occurrence of inflammatory reactions [21], regulating neurotransmitter expression and signaling [22, 23], inhibiting hippocampal glial fibrillary acidic protein expression [19], and promoting resistance to oxidative stress [23].

As SAMP8 mice exhibit rapid senescence, they are commonly used as an animal model of AD. The rapid senescence displayed by SAMP8 can be seen at 4 months, with the total lifespan of these mice limited to approximately 12-13 months [24]. SAMP8 mice are characterized by an age-related spontaneous deterioration in learning and memory, similar to the symptoms associated with AD [25, 26]. Research has revealed that these age-related behavioral alterations occur

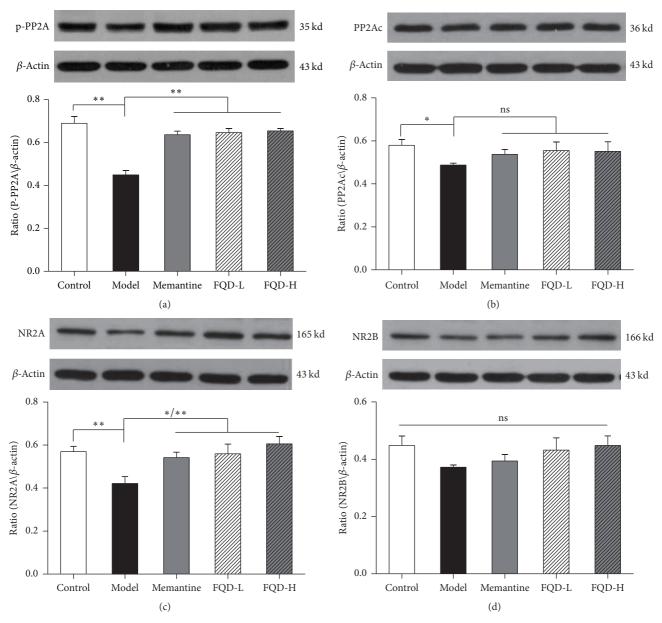


FIGURE 6: Western blot assessed the expression of p-PP2A (a), PP2Ac (b), NR2A (c), and NR2B (d). Compared to the control group, the expression of p-PP2A, PP2Ac, and NR2A was significantly decreased in hippocampal tissues of model group mice (p < 0.01 and 0.05 and p < 0.01, resp.). However, NR2B expression was not significantly different in model mice (p > 0.05). The expression of hippocampal p-PP2A and NR2A was significantly elevated in drug-treated mice (p < 0.01); NR2A was significantly elevated in memantine and high doses of FQD treated mice (p < 0.01 and 0.05, resp.), while PP2Ac and NR2B were not significantly different (p > 0.05). *p < 0.05, **p < 0.05.

as early as 4 months of age [27]. Spatial learning and memory capacity begin to decline in these mice as early as 2–4 months, as shown by impaired performance on the MWM [28, 29].

The MWM is extensively used to evaluate spatial learning and memory in rodents. In the MWM of this study, 6-monthold SAMP8 mice exhibited significantly prolonged escape latencies, as well as a reduction in the number of crossings and total time spent in the quadrant of the maze that contained the platform location during training sessions. These findings indicate that learning and memory capacity were significantly decreased in these mice, which is consistent with findings

reported by Huang et al. [25, 28, 29]. After 3 months of early FQD intervention, escape latency was significantly decreased and swim time in the original platform-containing quadrant was significantly increased, which indicates that FQD improved learning and spatial memory capacity in SAMP8 mice.

Tau hyperphosphorylation is critical in AD pathology [30]. In SAMP8 mice, tau phosphorylation at Ser202 and Ser396 has been observed in the hippocampus at 3 months of age [31]. In contrast, phosphorylation at Ser422 has been reported in the cortex of these animals at 5 months of age

[32], with abnormal phosphorylation of tau appearing at 11 months [33]. Canudas et al. [34] reported that, compared to 5-month-old SAMRI mice, tau hyperphosphorylation in the cortex, striatum, and hippocampus can be found in age-matched SAMP8 mice. In the present study, levels of hippocampal p-tau (S396) were significantly increased in 6-month-old SAMP8 mice relative to those observed in SAMRI mice, consistent with the aforementioned findings. Our results further indicated that these increases in p-Tau (S396) were inhibited following FQD or memantine intervention. Thus, higher doses of FQD may promote greater protein dephosphorylation than lower doses.

The entorhinal cortex and CA1 region of the hippocampus correspond to the first two brain regions in which AD pathological changes occur. Indeed, both regions exhibit significant reductions in neurons at the early stages of the disease, resulting in impaired spatial memory [35]. Nissl bodies are characteristic structures in a neuron's cytoplasm, serving as structural proteins that synthesize the organelles and enzymes required for neurotransmitter production. In this study, 6-month-old SAMP8 mice displayed a significant reduction in the number of neurons and intraneuronal Nissl bodies located in the CA1 region of the hippocampus; such morphological changes indicate neuronal degeneration and impaired function and are in agreement with the results of Sureda et al. [32]. After 3 months of FQD and memantine intervention, the structure and quantity of neurons and intraneuronal Nissl bodies in the CA1 have improved. Thus, both FQD and memantine prevented the age-related neuronal degeneration that these mice have typically exhibited.

Activation of NMDARs is essential for synaptic plasticity and necessary for long-term potentiation (LTP) [36]. Several studies have demonstrated that localization of the major NMDAR subunits, NR2A and NR2B, correlates with opposite functions; synaptic NR2A provides neuroprotection and mediates LTP, while extrasynaptic NR2B is linked to damageinduced toxicity and long-term depression (LTD) [37-41]. PP2A is composed of structural subunit A, regulatory subunit B, and catalytic subunit C (PP2Ac). PP2Ac plays an important role in the regulation of PP2A activity [42]. Downregulating the expression or activity of PP2A phosphatase is one of the major causes of tau hyperphosphorylation [43]. Zhang et al. showed that phosphorylation of the PP2A catalytic subunit at Y307 efficiently inactivates PP2A in vitro [44]. PP2A and NMDAR can form a complex such that once NMDARs are overactivated, PP2A dissociates from the complex and gets inactivated, leading to tau hyperphosphorylation [45, 46]. Mondragón-Rodríguez et al. [47] considered that inhibition of PP2A activity leads to NMDAR overactivation and then leads to tau hyperphosphorylation. NMDAR overactivation leads tau protein toxicity, which ultimately causes apoptosis [48]. As a noncompetitive antagonist of NMDARs, memantine can act as a neuroprotective agent to inhibit NR2B overactivation and block the inhibition of PP2A activity [47].

In the present study, hippocampal levels of PP2Ac, P-PP2A, and NR2A were significantly decreased in SAMP8 mice, relative to those observed in SAMR1 mice. Following FQD or memantine intervention, both p-PP2A (Y307) dephosphorylation and decreases in NR2A were significantly

inhibited. The p-PP2A expression observed in the present study was similar to those described by Amadoro et al. [49]. Specifically, Liu et al. reported that p-PP2A (Y307) was decreased in an acute hypoxia-induced AD neuron model and that an increase in PP2A activity was correlated with the amount of dephosphorylated PP2A (Y307). Another in vitro study by Liu et al. [50] revealed that blocking intraneuronal tyrosine protein kinase Src for 12 hours significantly reduces p-PP2A (Y307) and decreases total PP2A activity. It has been shown that the expression of NR2A and NR2B in 6-monthold SAMP8 mice is significantly lower than age-matched SAMR1 [51]. This study showed that abnormal function and expression of synaptic NMDARs led to the inhibition of PP2A expression, enhancing tau phosphorylation and inhibiting dephosphorylation. These mechanisms in turn caused synaptic abnormalities and tau hyperphosphorylation in mouse hippocampal neurons, thereby affecting learning and memory capacity. We further found that FQD administration can regulate the expression of NMDAR/PP2Aassociated proteins to delay neurodegenerative disease.

In conclusion, our findings demonstrated that FQD may exert protective effects against learning and memory impairments in SAMP8 mice via inhibition of tau hyperphosphorylation in the hippocampus. Although the mechanisms underlying the effects of FQD in AD models remain to be fully elucidated, our findings provide a solid foundation for future investigation of the role of tau protein phosphorylation in AD progression. As such, the traditional Chinese medicine FQD is a promising candidate for the development of novel AD treatments.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Effects and Mechanism of Huannao Yicong Decoction Extract on the Ethology of Transgenic APP/PS1 Mice

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To investigate the mechanism of Huannao Yicong Decoction (HYD) extract on improving of learning memory of transgenic amyloid precursor protein (APP)/presenilin 1 (PS1) mice, we randomly divided 60 transgenic APP/PS1 mice of 3 months old into 4 groups: the model group, the Donepezil group, the HYD-L group, and the HYD-H group, with 15 C57BL/6J mice of the same genetic background as the control group. These mice were gavaged for 6 months in a row. The results showed that the latency was significantly shortened and the number of passing through the original platform was increased. HYD extract can increase the amount of neurons and improve the morphological structure of Nissl body obviously. The γ -secretase activity and the expression of phosphorylated APP, A β 1-40, and A β 1-42 in hippocampal CA1 were significantly decreased. The expressions of protein and mRNA of PEN-2 and CREB in hippocampal were significantly downregulated. These results demonstrated that HYD extract can improve the memory ability of transgenic APP/PS1 mice, which was related to the protection of neurons and structure of Nissl body, reducing cleavage of APP and production of A β and inhibiting the activity of γ -secretase by decreasing CREB activity because of downregulated expression of PEN-2.

1. Introduction

The aging of population has become an irreversible trend in 21st Century. With memory and cognitive impairment as the main manifestation of Alzheimer's disease (AD) and its incidence rising, it is a serious threat to the health and life quality of elderly people. Composing of Radix polygonum multiflorum preparata, Panax ginseng, Acorus gramineus, Coptis chinensis, and Ligusticum wallichii, Huannao Yicong Decoction (HYD) is a clinical protocol, which is used to treat the elderly patients with cognitive impairment and Alzheimer's disease in Xiyuan Hospital of China Academy of Chinese Medical Sciences and has obtained the national invention patent [1] and has been applied in clinic for more than 10 years. The previous clinical studies had confirmed its effect on improving memory and cognition [2]. Basic research showed that HYD can improve the behavior of AD model rats, pathological morphology, and cholinergic system, inhibit the apoptosis of nerve cells, regulate the protein expression related to APP and A β , and inhibit inflammatory factors and have the effect of antioxidation [3]. This study is mainly to investigate the mechanism of HYD extract on improving the learning memory of transgenic amyloid precursor protein (APP)/presenilin 1 (PS1) mice as animal model simulating AD through the detection of ethology, pathological morphology, and related indicators of γ -secretase.

2. Materials and Methods

2.1. Animals. 60 transgenic APP/PS1 mice and 15 C57BL/6J mice of a congenic genetic background, 3 months old, clean grade (SPF grade), male and female combination, weight (25–35) g, were obtained from Chinese Academy of Medical Sciences (license number: SCXK (Beijing) 2009-0007). The experiment was carried out after 7 days of adaptive feeding in the room where the temperature was controlled at 23°C~25°C and the humidity was 50%~70%.

2.2. Drugs and Reagents. Donepezil hydrochloride (Aricept, 5 mg/tablet, batch number: 100806A) was produced by

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Eisai Pharmaceutical Co. Ltd. (China). HYD extract (3.2 g crude drug/g) was prepared in the drug preparation room, Beijing University of Chinese Medicine. Chinese herbal pieces, including Radix polygonum multiflorum preparata, Panax ginseng, Acorus gramineus, Coptis chinensis, and Ligusticum wallichii, were obtained from Hebei Shennong Pharmaceutical Co. Ltd. (Beijing). Rabbit anti-phosphorylation APP/ABPP (Thr668) (batch number: 990182w), Rabbit anti- $A\beta$ 1-40 (CT) (batch number: 20120810), Rabbit anti- $A\beta$ 1-42 (batch number: YE0907), Immunohistochemical staining kit (batch number: 999098), and DAB staining kit (batch number: 980579) were purchased from Beijing Bioss Technology Co. Ltd. Rabbit anti-anterior pharynx defective protein-1 (APH-1) (model: AB9214, Millipore), Rabbit anti-hypoxia inducing factor-la (HIF-la) (model: ab51608, Abcam), Rabbit anti-presenilin enhancer-2 (PEN-2) (model: SC-32946, Santa), Rabbit anti-cyclic adenosine monophosphate response element binding protein (CREB) (model: 06-863, Millipore), Mouse anti- β -actin antibody (model: TDY041, TiandeYue), Goat anti-rabbit IgG (H + L) (model: s004, FanxingBoao), and Luciferase assay kits (product code: E1910) were purchased from Promega company. Ultrapure RNA extraction kit (product code: CW0581), HiFi-MMLV cDNA first chain synthesis kit (product code: CW0744), Real Super Mixture (with Rox) (product code: CW0767), and DNase 1 (product code: CW2090) were purchased from Beijing Kangwei century biological technology Co. Ltd.

2.3. HYD Extract Preparation. The first step is to extract the volatile oil from Acorus gramineus, after which the drug liquid and residue together with Radix polygonum multiflorum preparata were extracted with water and precipitated with alcohol, and then the alcohol deposit liquid was obtained. Panax ginseng, Coptis chinensis, and Ligusticum wallichii were extracted together with alcohol to get the alcohol extract. The alcohol deposit liquid and alcohol extract were rotary evaporated together and then were mixed with the volatile oil, form which the final HYD extract was prepared. The HYD extract was detected by high performance liquid chromatography (HPLC) according the best chromatographic conditions which were the wavelength of 203 nm and 280 nm, mobile phase of acetonitrile - 0.05% phosphoric acid water, Apollo C18 (150 mm \times 4.6 mm), column temperature 30 $^{\circ}$ C, and the flow rate of 1.0 ml/min. The main effective components of HYD extract include ferulic acid, stilbene glycoside, berberine hydrochloride, ginseng saponin Rg1, Rb1, and Re, and emodin whose concentrations were 2.4183, 0.8765, 1.0424, 0.3993, 0.5816, 0.6932, and 0.8045 mg/g, respectively, reaching the requirements of Chinese Pharmacopoeia (2010 version). The quality was controlled with the combination of qualitative and quantitative methods, and the corresponding fingerprint was established (Figure 1).

2.4. Animal Grouping and Treatments. 60 transgenic APP/PS1 mice were randomly (applying SPSS software) divided into 4 groups with 15 in each: model group (given the equal volume of distilled water), Donepezil group (given the Donepezil 0.65 mg/kg/d which is clinical equivalent dose), the HYD-L group (1.7 g/kg/d which is clinical equivalent

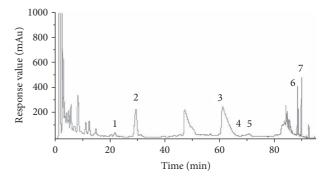


FIGURE 1: Fingerprint map of HYD extract detected with HPLC. (1) Ferulic acid; (2) styrene glycosides; (3) berberine hydrochloride; (4) ginseng saponin Rg1; (5) ginseng saponin Rb1; (6) ginseng saponin Re; (7) emodin.

dose), and the HYD-H group (3.4 g/kg/d which is twice the clinical equivalent dose), with 15 of C57BL/6J mice as control group (given the equal volume of distilled water). The mice of the five groups were gavaged for 6 months in a row, with the liquids of the equal volume and corresponding concentration diluted by distilled water. There were 6 mice dead for the gavage reason during the experiment, 2 in the Donepezil group, 1 in HYD-L group, and 3 in HYD-H group. The experiment was in accordance with the animal ethics requirements.

2.5. Morris Water Maze Test. The Morris water maze test was carried out after 6-month gavage, to assess the learning memory ability of the transgenic APP/PS1 mice. The Morris water maze apparatus (120 cm in diameter and 50 cm in depth) was produced by Institute of Pharmacology, Chinese Academy of Chinese Medicine. The water in the pool was 30 cm deep and approximately 1 cm above the platform surface, which was located in the fourth quadrant, with the temperature of $25\pm1^{\circ}$ C. Some milk powder was added in the water to make it opaque white, making the mice (black) unable to distinguish the platform in the tank. The whole experiment was divided into two parts: place navigation test and spatial probe test. For the place navigation test, at the marked entry point from the second quadrant 1/2 radian of the pool wall, the mice were put into the water against the pool wall one by one. Record the time (name as escape latency) the mice spend finding the platform (until the limbs on the platform) and the swimming distance by an autotracking system. Animals were allowed to swim for 3 min to search for the platform at each trial and to take a rest on platform for 10 s if they found it. For those animals who failed the mission, the escape latency was recorded as 180 s. All the mice were trained once a day and for 4 consecutive days. On the fifth day, the escape latency as an evaluation index of the learning memory ability of mice was recorded in the morning. In the afternoon the spatial probe test was performed to assess the spatial memory ability with removal of the platform. Choose an entry point, put the mice into the water with mice facing the pool wall, and record the times of crossing the platform-site in 3 min.

All mice were sacrificed by decapitation after the Morris water maze test. The brain was removed immediately after the

transcardial perfusion with saline. The brains were sagittally cut into brain specimens of left or right sides allocated randomly on the ice. Six specimens in each group were fixed in 4% neutral paraformaldehyde solution and were sliced after paraffin embedded, which were used to observe after Nissl Staining and immunohistochemistry. The hippocampi of other specimens were isolated and stored in refrigerator at -80° C, in order to detect the related molecules by dual-luciferase reporter, Western blotting, and real-time fluorescent quantitative-polymerase chain reaction (RT-PCR).

2.6. Nissl Staining. The paraffin-embedded brain slices were deparaffinized and washed routinely, washed 1-2 min with distilled water, dipped for 30 min in 1% thionin water solution at 37°C incubator, then washed 1-2 min with distilled water, differentiated to see clear Nissl body with 95% alcohol, dehydrated for 8–10 min with anhydrous alcohol, permeabilized for 8–10 min with xylene, and sealed with neutral gum. Neurons and Nissl bodies in hippocampus CA1 area were observed by BH-2 biological microscope (produced by Olympus of Japan).

2.7. Immunohistochemistry. All paraffin-embedded brain slices were deparaffinized routinely and washed with distilled water, dipped in PBS for 5 min, incubated for 20 min in 3% H₂O₂ deionized water to eliminate the endogenous peroxidase activity, and incubated for 20 min at room temperature after working solution of normal goat serum was dropped for sealing; then the liquid was poured out with no washing. Then the slices were incubated overnight at 4°C with primary antibodies, which were diluted by appropriate proportion. On the second day, the slices were incubated at room temperature for 20 min with the secondary antibodies, before and after which the slices were washed 3 times with PBS (each for 3 min). After incubation at room temperature for 20 min with the working solution of horseradish peroxidase labeled streptavidin, the slices were washed 3 times with PBS, each for 3 min, stained with DAB, and then washed fully with tap water, redyed, dehydrated, transparentized, and sealed finally. The hippocampus CA1 area of mice was observed with BH-2 biological microscope. The positive result was brown yellow cytoplasm or prominence. Two slices from each mouse were observed and taken pictures of, applying the Image Pro Plus 6.0 image analysis software (produced by American Media Cybernetics corporation) to calculate the average optical density (OD) value of positive expression in each high power (×600) view.

2.8. Dual-Luciferase Reporter. Five hippocampus tissue specimens in each group were chosen and grinded with 200 ul lysates of luciferase assay kits, centrifuged for 5 min with the speed of 15000g. Then 20 ul of the supernatant was sucked out, which was blended with 100 ul Luciferase Assay Buffer II, The luciferase value was read using multifunctional microplate reader (produced by Molecular Devices corporation). Then 100 ul of stop&Glo Buffer was added and mixed well; then the Renilla value was recorded. Luciferase/Renilla value indicated γ-secretase activity with Renilla value as internal reference.

2.9. Western Blotting. Six samples in each group were used to extract the tissue proteins and protein quantifying by BCA kit. According to the loading quantity of sample of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the protein concentrations were adjusted with RIPA and 5x buffer solution of restored sample and the protein solution were boiled for 5 min. According to the molecular weights of target proteins, separation gels of 8% and 12% were prepared, respectively; the concentration of spacer gel was made at 5%. For electrophoresis, spacer gel constant voltage was set at 90 v for about 20 min. Separation gel constant voltage was set at 160 v, and the stop time of the electrophoresis was determined by predyed marker protein. Then the proteins were transferred onto the membranes, sealed, and incubated with primary antibodies and the membranes were washed. The proteins were incubated with the internal reference, which was β eta-actin mouse monoclonal antibody diluted with 3% BSA-TBST (rate 1:5000), for 2h at room temperature. After incubating with the secondary antibody, the membranes were washed again. After the ECL reaction, the film was exposed, developed, and fixed.

2.10. RT-PCR. Six samples in each group were selected and the total RNA were extracted with ultrapure RNA extraction kit. Electrophoresis was performed on 5 ul of the RNA liquid with 1% agarose gel with DYY-GC electrophoresis apparatus (produced by Beijing Liuyi Instrument Factory). Primers were synthesized by Beijing Huateng Shengwei Biological Technology Co. Ltd., with β -actin as internal reference. The upstream of APH-1a mRNA primer was 5'-ATGCACCTTCGTCGCGTTCG-3' and the downstream was 5'-AGAAGGACAGAGACAGCACCAA-3' (amplified length was 222 bp). The upstream of HIF-1 α mRNA primer was 5'-GATGAATCAAAAACAGAGACGAAGG-3' and the downstream was 5'-CTGATGCCTTAGCAGTGG-TCGT-3' (amplified length was 109 bp). The upstream of PEN-2 mRNA primer was 5'-ACACAGAGCAGAGCC-AAATCAAAGG-3' and the downstream was 5'-CAGGGG-AATGGTGAAGGAGGAGGTAG-3' (amplified length was 155 bp). The upstream of CREB mRNA primer was 5'-ACC-ATTGCCCCTGGAGTTGTTAT-3' and the downstream was 5'-CTCTTGCTGCCTCCCTGTTCTTC-3' (amplified length was 115 bp). The upstream of internal reference β actin mRNA primer was 5⁷-GCCTTCCTTGGGTAT-3' and the downstream was 5'-GGCATAGAGGTCTTTACGG-3' (amplified length was 97 bp). Reverse transcription was conducted using the first strand synthesis kit of HiFi-MMLV cDNA and the amplification was conducted with RealSuper Mixture (with Rox) with the procedure of 95°C for 10 min, 40 cycles with melting for 15 s at 95°C and annealing and extension for 1 min at 60°C. The melting curve was analyzed at 60-95°C, after which the standard curve of the target gene and internal reference gene was drawn. At last the data was relatively quantified and analyzed using the method of $2^{-\Delta\Delta CT}$

2.11. Statistical Analyses. The data were expressed as the mean \pm SEM. All statistical analyses were done by using the statistical package of SPSS 17.0. One-way ANOVA and

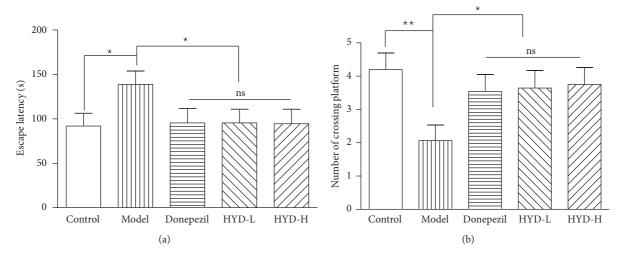


FIGURE 2: HYD extract improved the ability of learning and memory of transgenic APP/PS1 mice. (a) Escape latency, (b) number of crossing platform. $^*P < 0.05, ^{**}P < 0.01$.

q-test were used to compare among groups after all the data were tested with the normality test and homogeneity test of variance. P values of less than 0.05 were considered significant and those less than 0.01 were considered obviously significant.

3. Results

3.1. Effects of HYD Extract on the Learning Memory Ability of Transgenic APP/PSI Mice. As shown in Figure 2, compared with the control group, the escape latency of model group mice was significantly prolonged (P < 0.05); the number of crossing platform was decreased significantly (P < 0.01); after intervention, compared with the model group, the latencies of Donepezil group, HYD-L group, and HYD-H group were shortened (P < 0.05); the number of crossing platform was increased (P < 0.05) (Figure 2).

3.2. Effects of HYD Extract on Neurons and Nissl Body of Hippocampal CA1 Area of Transgenic APP/PS1 Mice. As shown in Figure 3, with Nissl Staining, the neurons of control group mice in hippocampus CA1 area were in a larger quantity, arranged in neat rows, rich in Nissl bodies (dark blue), with pale blue nucleus and light blue background; the neurons of model group mice in the hippocampal CA1 area decreased in quantity, arranged disorderedly, with obscure or disappeared boundary of Nissl bodies, karyopyknosis, or karyolysis which were shaped in elliptic or triangle and dyed dark blue; the neurons of Donepezil group, HYD-L group, and HYD-H group mice in the hippocampal CA1 area increased significantly in quantity, arranged neatly, with more Nissl bodies, little karyopyknosis, and karyolysis (Figure 3).

3.3. Effects of HYD Extract on the Expression of APP and $A\beta$ in Hippocampal CA1 Area of Transgenic APP/PS1 Mice. Compared with the control group, the expressions of phosphorylated APP and $A\beta$ of model control group mice in hippocampal CA1 area were significantly upregulated

(P < 0.01); after drug administration, compared with the model group, the expression of phosphorylated APP and A β 1-42 of Donepezil group, HYD-L group, and HYD-H group and the expression of phosphorylated A β 1-40 of HYD-L group, and HYD-H group were significantly downregulated (P < 0.01); and the expressions of phosphorylated APP and A β 1-40 of the HYD-H group were significantly lower than the Donepezil group and the HYD-L group (P < 0.01) (Figures 4(a), 4(b), and 4(c)).

3.4. Effects of HYD Extract on the γ -Secretase Activity of Transgenic APP/PS1 Mice. Compared with the control group, the γ -secretase activity of model group significantly increased (P < 0.01); after drug administration, compared with the model group, the γ -secretase activity of HYD-L group and HYD-H group decreased (P < 0.05); compared with the Donepezil group, the γ -secretase activity of HYD-H group decreased (P < 0.05) (Figure 5).

3.5. Effects of HYD Extract on the Protein Expression of APH-1a, HIF-1 α , PEN-2, and CREB of Transgenic APP/PS1 Mice. Compared with the control group, the protein expressions of APH-1a, HIF-1 α , PEN-2, and CREB were increased significantly (P < 0.05, P < 0.01); after drug administration, compared with the model group, the protein expressions of PEN-2 and CREB of HYD-L group and HYD-H group significantly decreased (P < 0.05, P < 0.01); compared with the Donepezil group, the protein expressions of PEN-2 of HYD-L group and HYD-H group decreased (P < 0.05) (Figures 6(a), 6(b), 6(c), and 6(d)).

3.6. Effects of HYD Extract on the mRNA Expression of APH-1a, HIF-1 α , PEN-2, and CREB of Transgenic APP/PS1 Mice. Compared with the control group, the mRNA expression of APH-1a, HIF-1 α , PEN-2, and CREB of model group increased significantly (P < 0.05, P < 0.01); after drug administration, compared with the model group, the mRNA expression of

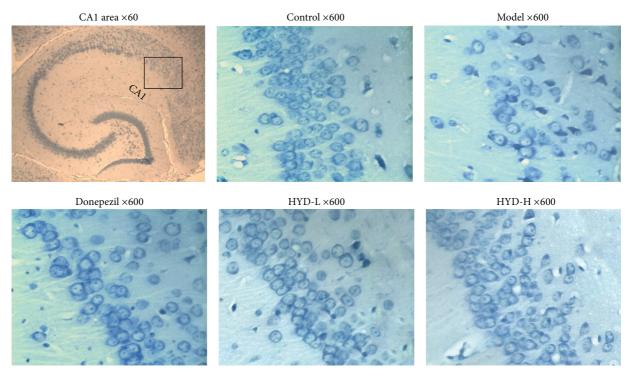


FIGURE 3: HYD extract protected the neuron and Nissl body of transgenic APP/PS1 mice (Nissl Staining, ×60 and ×600).

PEN-2 of HYD-L group and the mRNA expression of PEN-2 and CREB of HYD-H group significantly decreased (P < 0.05, P < 0.01); compared with the Donepezil group, the mRNA expression of PEN-2 of HYD-L group and HYD-H group significantly decreased (P < 0.05, P < 0.01) (Figures 7(a), 7(b), 7(c), and 7(d)).

4. Discussion

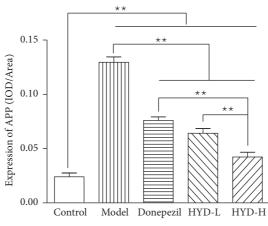
Alzheimer's disease is a neurodegenerative disease characterized by progressive memory decline and cognitive impairment. The main pathological features of Alzheimer's disease include senile plaques, nerve fiber entanglement, and neuronal loss. The production and deposition of A β (especially for A β 1-42) play an important role in the pathological process. The A β deposition in extracellular space directly results in the senile plaques and then further induces tau protein hyperphosphorylation in the cells, forming the neurofibrillary tangles, both of which can cause the corresponding neurotoxic effects, eventually making the structure and function of neurons injured and lost [4, 5]. A β is produced through hydrolyzing of amyloid precursor protein (APP) by β and γ secretase. The γ -secretase is the final key of the formation of $A\beta$, whose activity is closely related to the occurrence of Alzheimer's disease. So the intervention in above pathological process is very important for the prevention and treatment of Alzheimer disease [6].

APP mutation can cause the structural change of APP binding sites with β -secretase, which increased β -secretase activity, so as to increase the amount of A β , and intensifies the formation of senile plaques. PS1 mutation can change γ -secretase activity, resulting in the change of the metabolic

process of APP, selectively causing A β 1-42 production and increase. So the transgenic APP/PS1 mice can well simulate human Alzheimer's disease of graduated progress [7]. According to the reports, the 2.5-month-old mice began to show A β deposition, around 3-month-old mice began to show cognitive and behavioral changes of early stage, 7-month-old mice showed cognitive impairment, and 9-month-old mice showed dysfunction of spatial memory [8–10]. The pathological mechanism of the model is associated with activation of γ -secretase. It is an ideal model for the study of drugs in the regulation of γ -secretase pathway and is also a successful transgenic AD model domestically recognized [11].

 γ -secretase is kind of membrane integrated protease complexes with large molecular weight, which is composed of four subunits: presenilin (PS, catalytic activity center of γ -secretase), nicastrin (NCT, the assembly bracket and binding site of substrate of γ -secretase), anterior pharynx defective-1 (APH-1, stent partner of NCT), and presenilin enhancer-2 (Pen-2, the assembly molecular wedge of γ -secretase complex). The expression changes of the four essential proteins play an important role in γ -secretase activity, the production of $A\beta$, and the onset of AD [12–16]. The research group studied all the four proteins. This thesis mainly summarizes the research results of APH-1 and PEN-2 and their related factors and the research results of NCT and PS were published in Chinese Journal of Pharmacology and Toxicology [17].

APH-1 is one of the essential constituent proteins of γ -secretase in mammalian cells, with two highly homologous proteins: APH-la and APH-lb. APH-la is the main isomer of γ -secretase in the process of embryonic development, and lacking APH-la can reduce the production of A β . HIF-1 is a transcription regulatory factor of APH-la, which is composed



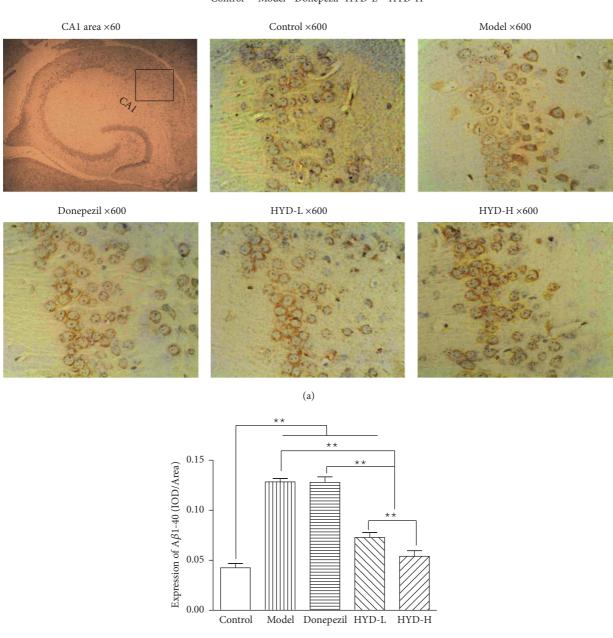


FIGURE 4: Continued.

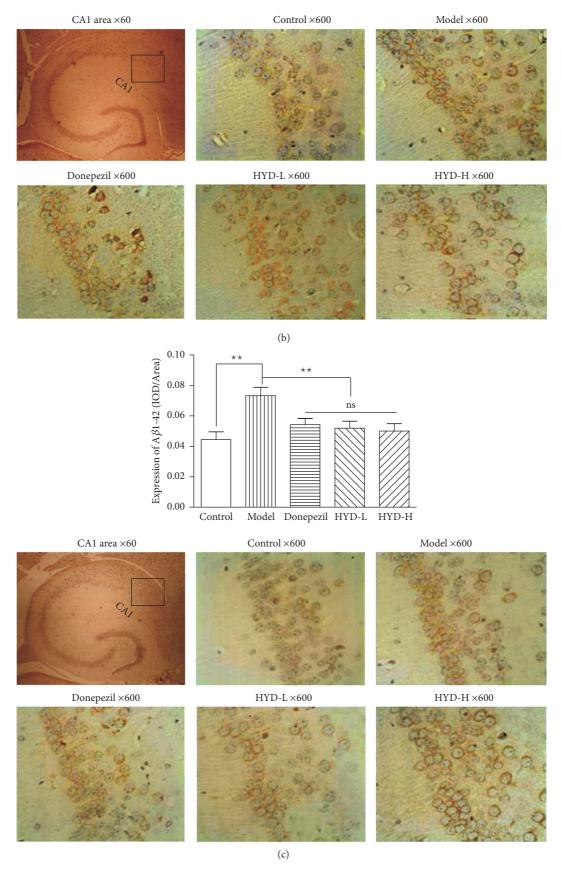


FIGURE 4: HYD extract regulated protein expression of APP, A β 1-40, and A β 1-42 in the hippocampus CA1 area of transgenic APP/PS1 mice (immunohistochemistry, ×60 and ×600). (a), (b), and (c) are the protein expressions of APP, A β 1-40, and A β 1-42. **P < 0.01.

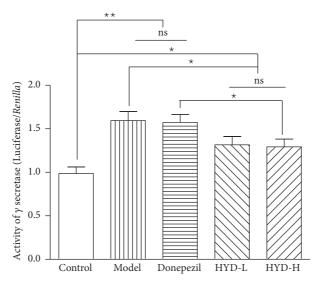


FIGURE 5: HYD extract regulated activity of γ -secretase of transgenic APP/PS1 mice. *P < 0.05, **P < 0.01.

of subunits HIF-1 α and HIF-1 β . The physiological activity of HIF-1 mainly depends on the activity and expression of HIF-1 α [18, 19]. Studies had found that APH-1a promoter contains the binding sites of HIF-1, and when HIF-1 is activated, the levels of mRNA and protein of APH-1a, γ -secretase activity and A β production are significantly increased [20].

PEN-2 is widely expressed in specific nerve cells of most brain areas of transgenic APP/PS1 mice, including the olfactory bulb, basal ganglia, striatum, cortex, hippocampus, amygdala, thalamus, hypothalamus, cerebellum, brainstem, and spinal cord. It is not only the main component of γ -secretase, but also the modulator of γ -secretase activity, playing a key role in the production of A β 1-42 [21–23]. CREB, a transcription regulatory factor of PEN-2, which can activate some specific genes to produce proteins, strengthens the connection between cells and is closely related to the formation and maintaining of long-term memory [24]. The study found that the promoter of PEN-2 gene contains CREB binding site and can bind to each other specifically, consequently decreasing γ -secretase activity and A β formation through inhibiting CREB activity and reducing the expression of PEN-2 [25].

The results of this study show that the latency of 9month-old transgenic APP/PS1 mice in the Morris water maze test was significantly prolonged, and the platform-site crossing times were significantly decreased, which showed that the spatial learning and memory ability were significantly decreased. On the pathological morphology, neurons and Nissl body in hippocampal CA1 area were significantly decreased in quantity and significantly impaired in structure. Meanwhile, γ -secretase activity and the expression of APP, $A\beta$ 1-40, and $A\beta$ 1-42 in hippocampus CA1 area were significantly increased. Furthermore, the APH-la, HIF-1α, PEN-2, CREB protein, and their mRNA expression of 9-monthold transgenic APP/PS1 mice were significantly increased. It suggested that HIF-1 α binding sites of APH-1a promoter and CREB binding sites of PEN-2 promoter in the hippocampal tissue of transgenic APP/PS1 mice were activated, which resulted in a significant increase in APH-1a and PEN-2

expression and γ -secretase activity and caused APP dissociation and production of a large number of A β . Neurons damage and loss were caused by further accumulation of A β , which affected the spatial learning and memory. It was one of the main reasons for the mice to become AD model.

In Traditional Chinese medicine theory, AD is thought as amnesia or dementia which was caused by deficiency of the kidney essence in origin and blood stasis and turbidity in superficiality. So our team proposed that treatment regimens of AD were reinforcing deficiency and supplementing body essence mainly and promoting blood circulation to remove blood stasis and relieving toxicity and transforming turbid secondly. HYD was formulated through above regimen that was composed of Radix polygonum multiflorum preparata, *Panax ginseng, Acorus gramineus, Coptis chinensis*, and *Ligusticum wallichii*. Early toxicity experiments had confirmed that HYD extract was safe and had no obvious side effects.

After drug administration of six months, HYD extract can significantly shorten the latency and increase the crossing times of transgenic APP/PS1 mice in Morris water maze test; can obviously protect neurons and Nissl body, improve their quantity and structure, and promote the protein synthesis in hippocampus CA1 area; can significantly increase γ -secretase activity and the expression of APP, A β 1-40, and A β 1-42; can obviously decrease PEN-2, CREB protein, and their mRNA expression and its effect is better than or equivalent to Donepezil. The experiment showed that HYD extract can down-regulate PEN-2 expression and γ -secretase activity through inhibiting the CREB binding site activation of PEN-2 gene promoter, intervene in APP metabolic pathways, reduce the production of A β 1-40 and A β 1-42, restrain the formation of senile plaques, reduce the loss of neurons, improve memory, and then inhibit AD occurring and developing. So it had a positive meaning for early applying HYD extract to prevent and treat AD. However, HYD extract could not obviously change APH-la, HIF-lα protein, and their mRNA expression, which indicated that APH-la and HIF-l α are not HYD targets in treating AD.

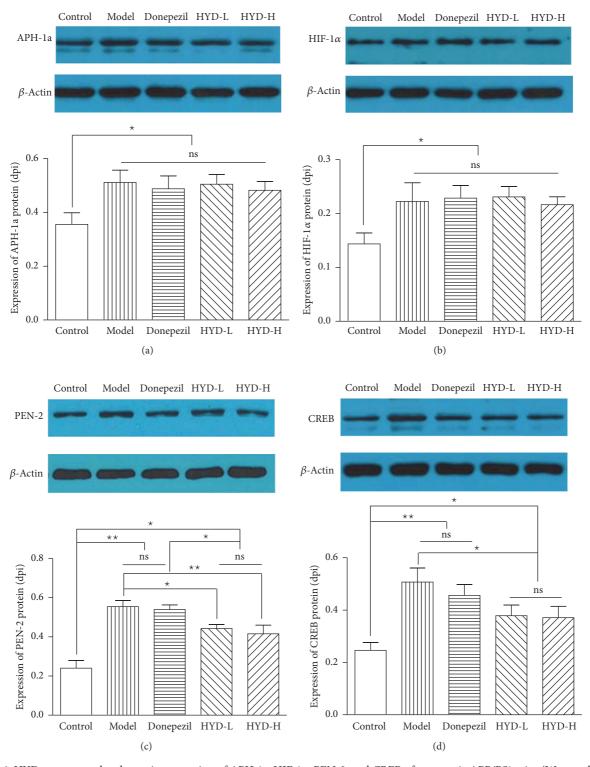


FIGURE 6: HYD extract regulated protein expression of APH-1a, HIF-1 α , PEN-2, and CREB of transgenic APP/PS1 mice (Western blotting, APH-1a 1:1000, HIF-1 α 1:1000, PEN-2 1:200, CREB 1:2000, β -actin 1:5000). (a), (b), (c), and (d) are the protein expressions of APH-1a, HIF-1 α , PEN-2, and CREB. *P < 0.05, ***P < 0.01.

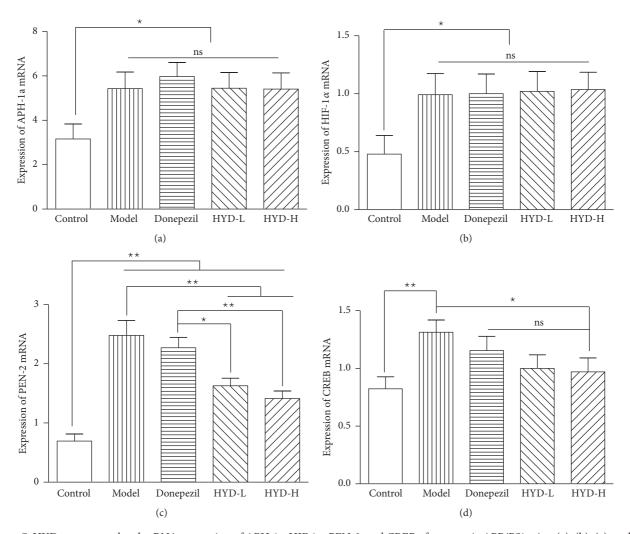


FIGURE 7: HYD extract regulated mRNA expression of APH-1a, HIF-1 α , PEN-2, and CREB of transgenic APP/PS1 mice. (a), (b), (c), and (d) are the mRNA expressions of APH-1a, HIF-1 α , PEN-2, and CREB. *P < 0.05, **P < 0.01.

5. Conclusion

HYD extract can obviously improve the spatial learning and memory ability of transgenic APP/PS1 mice. The mechanism was related to its protection of neurons and the structure of Nissl body, the reduction of APP cleavage and A β (especially A β 1-42), and the inhibition of γ -secretase activity which results from the inhibition of CREB activity and further downregulated expression of PEN-2.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

Conflicts of Interest

All the authors of this paper declare that they do not have a direct financial relation with the commercial identities mentioned in the paper.

Authors' Contributions

Hao Li and Jiangang Liu designed the experiments; Meixia Liu, Yun Wei, Yang Yang, Longtao Liu, Lin Liang, Jiangang Liu, and Hao Li performed the experiments, analyzed the data, and wrote the manuscript; Meixia Liu and Yun Wei equally contributed to the paper.

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Research Article

Quantitative Analysis and Biological Efficacies regarding the Neuroprotective and Antineuroinflammatory Actions of the Herbal Formula Jodeungsan in HT22 Hippocampal Cells and BV-2 Microglia

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Jodeungsan (JDS) is a traditional herbal formula that comprises seven medicinal herbs and is broadly utilized to treat hypertension, dementia, and headache. However, the effects of JDS and its herbal components on neurodegenerative diseases have not been reported. We examined the inhibitory effects of JDS and its seven components on neuronal cell death and inflammation using HT22 hippocampal cells and BV-2 microglia, respectively. Among its seven herbal components, *Uncaria sinensis* (US), *Chrysanthemum morifolium* (CM), *Zingiber officinale* (ZO), *Pinellia ternata* (PT), *Citrus unshiu* (CU), and *Poria cocos* (PC) exhibited significant neuroprotective effects in HT22 cells. In BV-2 cells, JDS significantly suppressed the production of tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6), indicating the antineuroinflammatory activity of JDS. In addition, the herbal extracts from ZO, *Panax ginseng* (PG), PT, CU, and PC exhibited inhibitory effects on the inflammatory response in microglia. These data imply that the JDS effect on neurodegeneration occurs via coordination among its seven components. To establish a quality control for JDS, a simultaneous analysis using five standard compounds identified hesperidin (37.892 \pm 1.228 mg/g) as the most abundant phytochemical of JDS. Further investigation of the combinatorial activities of two or more standard compounds will be necessary to verify their antineurodegenerative regulatory mechanisms.

1. Introduction

Neurodegenerative disease is a medical condition that is characterized by the progressive loss of neural tissues. Parkinson's, Alzheimer's, and Huntington's diseases and their related disorders are neurodegenerative diseases [1, 2]. Aging is one of the strongest risk factors for neurodegenerative diseases [3]. Senior populations are increasing in modern society, which leads to the increase in the prevalence of neurodegenerative diseases. Thus, these diseases are considered as significant challenges for both preclinical and clinical investigations. However, although abundant studies have tried to develop antineurodegeneration drugs, there are no powerful and effective agents; therefore, these diseases

are currently incurable. Because the pathogenesis of neurodegenerative diseases is complex and neuronal and nonneuronal cells in neurovascular cell units coordinately participate in disease progression [4, 5], a single molecule that targets these conditions is not a suitable approach in new drug development for neurodegenerative diseases. Recently, herbal medicines have been regarded as appropriate materials based on their positive aspect of having multiple components and multiple targets [6, 7]. Several articles have suggested the possibility that herbal medicines are useful therapeutic agents for neurodegenerative diseases [8–10].

Jodeungsan (JDS), also called Chotosan in Japan and Diaoteng San in China, is a traditional herbal formula that

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FIGURE 1: Chemical structures of the five standard compounds of JDS.

comprises seven medicinal herbs, Uncaria sinensis (US), Chrysanthemum morifolium (CM), Zingiber officinale (ZO), Panax ginseng (PG), Pinellia ternata (PT), Citrus unshiu (CU), and Poria cocos (PC). Clinically, JDS has been prescribed for age-related diseases such as hypertension [11], dementia and cognitive impairment [12, 13], and chronic headache [14]. Several clinical reports demonstrated the potential of JDS for treating dementia, one of the most common neurodegenerative diseases. Suzuki et al. reported that a double-blinded, randomized, and placebo-controlled study addressed the positive aspect of JDS on cognitive function and activities of daily living in patients with dementia [12]. Yamaguchi et al. reported that JDS improved the event-related brain potential in stroke patients with cognitive impairment [15]. Pharmacobiological evidence of the action of JDS against dementia has been described in in vitro or in vivo models [16–18]. However, most articles have described the anti-dementia effect of JDS itself, but not that of each of its herbal components.

Our study aimed to investigate the effect of JDS and its seven herbal components on neurodegenerative events using hippocampal and microglia cell lines. We also performed a simultaneous analysis of five standard compounds to improve the quality control of JDS.

2. Materials and Methods

2.1. Plant Materials. The seven crude herbal medicines that form JDS, Uncariae Ramulus et Uncus, Chrysanthemi Flos, Zingiberis Rhizoma, Ginseng Radix Alba, Pinelliae Tuber, Citri Unshii Pericarpium, and Poria Sclerotium, were purchased from the Kwangmyungdang herbal market (Ulsan,

South Korea). A voucher specimen has been deposited at the Herbal Medicine Research Division, Korea Institute of Oriental Medicine.

2.2. Chemicals and Reagents. The standard components, luteolin and apigenin, were purchased from ChemFaces Biochemical Co., Ltd. (Wuhan, China), and hesperidin, 6-gingerol, and narirutin were purchased from Biopurify Phytochemicals (Chengdu, China). The chemical structures of the standard components are shown in Figure 1. The purity of these standard components was ≥98.0%, as assessed using high-performance liquid chromatography (HPLC) analysis. The HPLC-grade solvents, acetonitrile and water, were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ, USA), and the analytical-grade reagent trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Apparatus and Chromatographic Conditions. The quantitative analysis was conducted using a Waters Alliance e2695 system (Waters Corp, Milford, MA, USA) equipped with a pump, degasser, column oven, autosampler, and photodiode array detector (#2998; Waters Corp). The data were acquired and processed using Empower software (version 3; Waters Corp). The chromatographic separation of the seven bioactive components was carried out at room temperature using Luna C_{18} analytical columns (250 × 4.6 mm, 5 μ m), supplied by Phenomenex (Torrance, CA, USA), with a gradient solvent system of 0.1% (v/v) aqueous TFA (A) and acetonitrile (B). The elution conditions were as follows: 15%–40% B for 0–30 min, 40%–100% B for 30–40 min, and 100% B for 40–47 min. The flow rate was 1.0 mL/min and the injection volume was 10 μ L. The ultraviolet (UV) wavelength for

Latin name	Scientific name	Amount (g)	Origin	
Uncariae Ramulus et Uncus	Uncaria sinensis (US)	9	China	
Chrysanthemi Flos	Chrysanthemum morifolium (CM)	6	Namwon, Korea	
Zingiberis Rhizoma	Zingiber officinale (ZO)	3	Andong, Korea	
Ginseng Radix Alba	Panax ginseng (PG)	6	Punggi, Korea	
Pinelliae Tuber	Pinellia ternata (PT)	9	China	
Citri Unshii Pericarpium	Citrus unshiu (CU)	9	Jeju, Korea	
Poria Sclerotium	Poria cocos (PC)	9	China	
Total amount		51		

TABLE 1: Herbal composition of JDS.

detecting components was 236 nm for narirutin, hesperidin, and 6-gingerol and 263 nm for luteolin and apigenin.

- 2.4. Preparation of Standard Solutions. The five reference standards were weighed accurately, dissolved in methanol at 1.0 mg/mL, and stored at <4°C. The stock solutions were diluted to yield a series of standard solutions with different concentrations for quantitative analysis.
- 2.5. Preparation of Sample Solutions. The seven dried crude herbal medicines, Uncariae Ramulus et Uncus, Chrysanthemi Flos, Zingiberis Rhizoma, Ginseng Radix Alba, Pinelliae Tuber, Citri Unshii Pericarpium, and Poria Sclerotium, were mixed as indicated in Table 1 (51 g) and extracted twice with 70% ethanol (306 mL) by refluxing for 2 h. The extracted solution was filtered through a filter paper (5 μ m) and evaporated using a rotary evaporator (EYELA N-1000, Rikakikai Co., Tokyo, Japan) under vacuum to dryness (8.164 g). The yield of JDS extract was 16%. For simultaneous determination of the powdered JDS extract, the 70% ethanol extract of the JDS was weighed accurately and dissolved in methanol at 20 mg/mL. The sample solution was filtered through a syringe filter (0.45 μ m) for HPLC analysis.
- 2.6. Calibration Curve and Determination of the Limits of Detection and Quantification. The calibration curves of all components were calculated from the peak areas of the standard solutions at different concentrations. The tested concentration ranges were as follows: narirutin $(12.5-400 \, \mu g/mL)$, hesperidin $(31.25-500 \, \mu g/mL)$, luteolin $(1.5625-25 \, \mu g/mL)$, apigenin $(0.78125-25 \, \mu g/mL)$, and 6-gingerol $(3.125-50 \, \mu g/mL)$. These solutions were measured in triplicate for the calibration curves. The limit of detection (LOD) and limit of quantification (LOQ) for the five standard components were calculated, using the slope of the calibration curve and the standard deviation (SD) of the intercept, as follows: LOD = $3.3 \times (SD \text{ of the response/slope})$ of the calibration curve); and LOQ = $10 \times (SD \text{ of the response/slope})$
- 2.7. Herbal Extract Treatment and Cytotoxicity Assay. BV-2 and HT22 cells were maintained in Dulbecco's modified Eagle's medium (Hyclone/Thermo, Rockford, IL, USA), supplemented with 10% fetal bovine serum (Hyclone/Thermo) and penicillin/streptomycin in a 5% CO₂ at 37°C. HT22 cells were cotreated with the herbal extract and hydrogen

peroxide (H_2O_2 , 250 μ M; Sigma-Aldrich) for 6 h. BV-2 cells were pretreated with various concentrations of each herbal extract for 2 h and treated with lipopolysaccharide (LPS, 1 μ g/mL; Sigma-Aldrich) for additional 22 h.

The cytotoxicity test was performed as described previously [19]. In brief, BV-2 and HT22 cells were plated on 96-well microplates at a density of 3×10^4 /well and 5×10^3 /well, respectively. Cells were treated with various concentrations of each herbal extract for 24 h. Cell counting kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added, and the cells were incubated for 4 h. The absorbance was read at 450 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The cell viability was calculated using the following equation:

Cell viability (%) =
$$\frac{\text{Mean OD in drug - treated cells}}{\text{Mean OD in untreated cells}}$$
 (1) × 100.

- 2.8. Measurement of Cytokine Production. Culture supernatants were collected from the cells treated with various concentrations of each herbal extract in the presence of LPS. An enzyme-linked immunosorbent assay (ELISA) kit from R&D systems (Minneapolis, MN, USA) was used in accordance with the manufacturer's instructions. The concentration of each sample was calculated according to the standards provided with the kits.
- 2.9. Statistical Analysis. The data are expressed as the mean \pm SEM. Data were analyzed using one-way analysis of variance and Dunnett's multiple comparisons test. P < 0.05 was considered significant.

3. Results

3.1. Optimization of HPLC Separation. An HPLC analytical method was established for the simultaneous separation of the five standard components from the 70% ethanol extract of JDS. As a result, a completely separated chromatogram was obtained within 40 min using two mobile phases consisting of 0.1% (v/v) aqueous TFA (a) and acetonitrile (b). The UV wavelength used for quantitative analysis was 236 nm for narirutin, hesperidin, and 6-gingerol and 263 nm for luteolin and apigenin. Under these established HPLC methods, the retention times of narirutin, hesperidin, luteolin,

Compound	Linear range (μg/mL)	Regression equation $(y = ax + b)^{(a)}$		Correlation coefficient (r^2)	LOD ^(b) (µg/mL)	LOQ ^(c) (µg/mL)
		Slope (a)	Intercept (b)	coefficient (7)	(prg/1112)	(1112)
Narirutin	12.5-400	18206	63887	0.9998	0.308	0.932
Hesperidin	31.25-500	20407	91283	0.9999	1.054	3.194
Luteolin	1.5625-25	40612	3770.9	1.0000	0.038	0.114
Apigenin	0.78125-25	81186	13562	0.9999	0.013	0.041
6-Gingerol	3.125-50	6274.2	2670.6	1.0000	0.175	0.531

Table 2: Linear range, regression equation, correlation coefficients, LODs, and LOQs for compounds.

⁽a) y = ax + b, y means peak area and x means concentration (μ g/mL); (b) LOD (Limit of detection): 3.3 × (SD of the response/slope of the calibration curve); (c) LOQ (Limit of quantitation): 10 × (SD of the response/slope of the calibration curve).

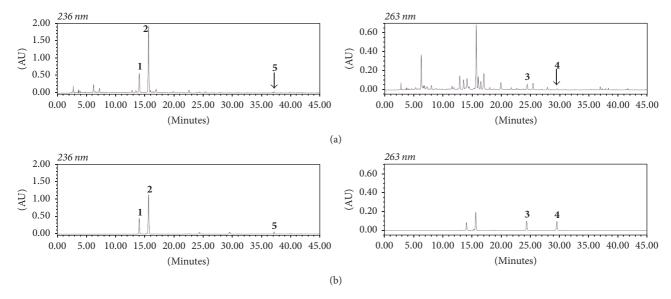


FIGURE 2: HPLC chromatograms of the 70% ethanol extract of JDS (a) and a standard mixture (b) at 236 nm and 263 nm. Narirutin (1), hesperidin (2), luteolin (3), apigenin (4), and 6-gingerol (5).

apigenin, and 6-gingerol were 14.09, 15.67, 24.37, 29.52, and 37.09 min, respectively. HPLC chromatograms of the 70% ethanol extract of JDS and the standard mixture are shown in Figure 2.

- 3.2. Linearity, LOD, and LOQ. The linear relationships between the peak area (y) and concentration $(x, \mu g/mL)$ of each component were expressed by the regression equations (y = ax + b), as shown in Table 2. The calibration curves for the five components showed good linearity $(r^2 \ge 0.9998)$. The LOD and LOQ for the tested components were 0.013–1.054 $\mu g/mL$ and 0.041–3.194 $\mu g/mL$, respectively.
- 3.3. Determination of the Five Standard Components of the JDS Extract. The HPLC analytical method established here was applied to the simultaneous quantification of the five components of the extracted JDS sample. The relative amount of the five standard components ranged from 0.061 to 37.892 mg/g and the results of the analysis are summarized in Table 3. Among the five components of JDS, hesperidin was the most abundant.

TABLE 3: The content of standard compounds in JDS.

Compound	Content (mg/g)		
Narirutin	12.289 ± 0.393		
Hesperidin	37.892 ± 1.228		
Luteolin	0.626 ± 0.026		
Apigenin	0.061 ± 0.003		
6-Gingerol	1.731 ± 0.038		

- 3.4. Cytotoxicity of JDS and Its Seven Herbal Components against HT22 and BV-2 Cells. To determine the cytotoxic effects of JDS and its seven components, a CCK assay was used. HT22 or BV-2 cells were treated with various concentrations of each herbal extract for 24 h and the cell viability was measured. The results are shown in Table 4. Cell treatment with each extract was performed in the nontoxic concentration range in all subsequent experiments.
- 3.5. Neuroprotective Effect of JDS and Its Seven Herbal Components in H_2O_2 -Damaged HT22 Hippocampal Cells. To

100 Cells $\mu g/mL$ SEM^(b) Mean Mean **SEM** Mean **SEM** Mean **SEM** JDS 102.20 1.77 103.15 3.38 100.16 4.42 91.96 5.34 US 95.87 1.85 92.96 1.36 95.87 2.52 85.72 2.34 CM 95.27 0.84 1.69 0.81 104.71 1.62 88.40 82.05 ZO 0.94 97.89 1.82 91.13 92.21 4.85 77.90 3.35 HT22 PG 103.40 1.20 105.66 1.90 103.74 2.34 100.03 2.30 PT 105.27 1.29 125.25 1.92 110.40 114.69 1.57 1.60 CU 96.42 1.29 97.49 0.96 100.73 1.53 109.72 2.90 PC 0.80 91.99 93.86 94.23 1.84 1.68 84.93 1.44 JDS 2.52 99.30 2.21 5.27 3.29 97.98 95.31 94.53 US 4.90 108.86 107.62 3.14 109.28 7.00 88.21 3.84 CM 94.64 6.92 89.48 2.78 77.09 4.26 75.22 2.66 ZO 97.21 94.25 3.63 8.35 60.73 3.72 88.73 1.63 BV-2 PG 120.32 7.98 108.30 4.20 106.74 5.41 93.96 3.13 PT 3.95 128.87 2.90 123.15 142.12 6.39 143.02 4.74 CU112.59 5.99 108.59 10.16 109.73 3.94 107.30 2.15 PC 132.52 10.35 111.75 6.64 111.81 13.52 100.52 10.66

TABLE 4: Cytotoxicity of JDS and its herbal components against HT22 and BV-2 cells (% of control)^(a).

examine the protective effects of JDS and its seven components in neuronal cells, the HT22 hippocampal cell line was used. Neuronal cell damage was induced by exposing cells to $\rm H_2O_2$, followed by treatment with various concentrations of the herbal extracts. Treatment with $\rm H_2O_2$ alone significantly reduced cell viability in comparison with the untreated control. The JDS extract significantly inhibited the $\rm H_2O_2$ -mediated cell damage (Figure 3(a)). Among the seven herbal components, US (b), CM (c), ZO (d), PT (f), CU (g), and PC (h) showed protective activity against $\rm H_2O_2$ -mediated cell damage.

3.6. Inhibitory Effect of JDS and Its Seven Herbal Components on Proinflammatory Cytokine Production in LPS-Stimulated BV-2 Microglia. Next, we investigated whether JDS and its seven herbal components suppress the production of proinflammatory cytokines, such as tumor necrosis factoralpha (TNF- α) and interleukin-6 (IL-6), in LPS-stimulated BV-2 cells. As shown in Figures 4 and 5, TNF- α and IL-6 levels increased in the culture media of LPS-stimulated BV-2 cells, and these increases were significantly reduced by the treatment with JDS (Figures 4(a) and 5(a)) and its components ZO (d), PG (e), PT (f), CU (g), and PC (h). PC had the most significant inhibitory effect on the LPS-stimulated TNF- α and IL-6 production (Figures 4(h) and 5(h)).

4. Discussion

Neuronal cell death plays a crucial role in neurodegeneration [20]. In general, the induction of neuronal cell death in neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's diseases, is controlled by pathways related to mitochondrial dysfunction and oxidative stress [21, 22].

Neuronal cell death is associated with neuroinflammation in the central nervous system [23]. Microglia are mainly involved in neuroinflammatory reaction mediated by environmental or microbial stimuli. Activated microglia promote the production of neurotoxin(s), thus leading to neuronal cell death [24].

In our study, we explored the effects of JDS and its seven herbal components on neurodegeneration. First, the neuroprotective effects of herbal extracts of JDS and its seven components were determined using the HT22 hippocampal cell line. To induce neuronal cell damage, HT22 cells were exposed to H₂O₂ according to the accumulating evidence of H2O2-mediated neuronal cell death and alternation of redox signaling [25-27]. In the presence of concurrent treatment with H₂O₂ and each of the herbal extracts, JDS had a significant effect of inhibition of neuronal cell death in H₂O₂-treated hippocampal cells. Its components US, CM, ZO, PT, CU, and PC also displayed neuroprotective effects that were concentration dependent. US treatment significantly reversed cell viability at lower concentrations $(90.91\% \pm 3.42\% \text{ and } 82.78\% \pm 4.97\% \text{ at } 12.5 \text{ and } 25 \,\mu\text{g/mL},$ resp.) compared with H_2O_2 -treated cells (53.00% \pm 3.92%). However, cotreatment with H_2O_2 and a higher concentration of US significantly increased the level of H₂O₂-induced cell death. ZO significantly blocked neuronal cell death at 2.5 and $5 \,\mu \text{g/mL}$ of the treatment (97.96% \pm 13.57% and 84.74% \pm 11.07%, resp.) compared with the H_2O_2 control (55.20% \pm 2.48%). The effect of PC was not strong compared to that of the other extracts. In cells that were cotreated with H₂O₂ and PC at 25 μ g/mL, a 1.58-fold increase was observed compared to that observed for the treatment with H_2O_2 alone (76.83% \pm 2.45% versus $48.53\% \pm 3.80\%$). CM, PT, and CU significantly protected against H₂O₂-induced cell death at a concentration ranging from 25 to $100 \,\mu\text{g/mL}$.

⁽a) The results are expressed as mean ± SEM of three independent experiments; (b) SEM, standard error of the mean.

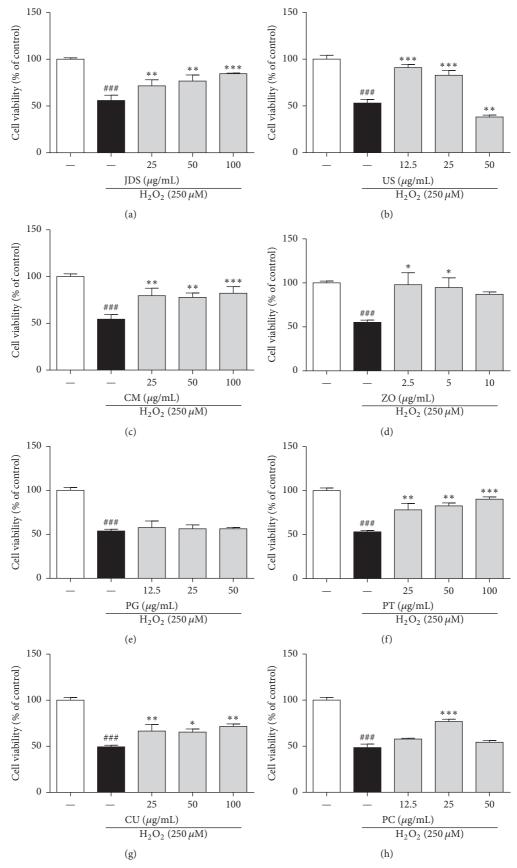


FIGURE 3: Neuroprotective effect of JDS and its herbal components in H_2O_2 -treated HT22 cells. Cells were seeded on 96-well plates and cotreated with various concentrations of JDS and its seven components, and H_2O_2 (250 μ M) for 6 h. Cell viability was assessed using the CCK-8 assay. Results are shown for JDS (a), US (b), CM (c), ZO (d), PG (e), PT (f), CU (g), and PC (h). The results are expressed as the mean \pm SEM of three independent experiments. *## P < 0.001 versus vehicle control cells, *P < 0.05, **P < 0.01, and ***P < 0.001 versus H_2O_2 -treated cells.

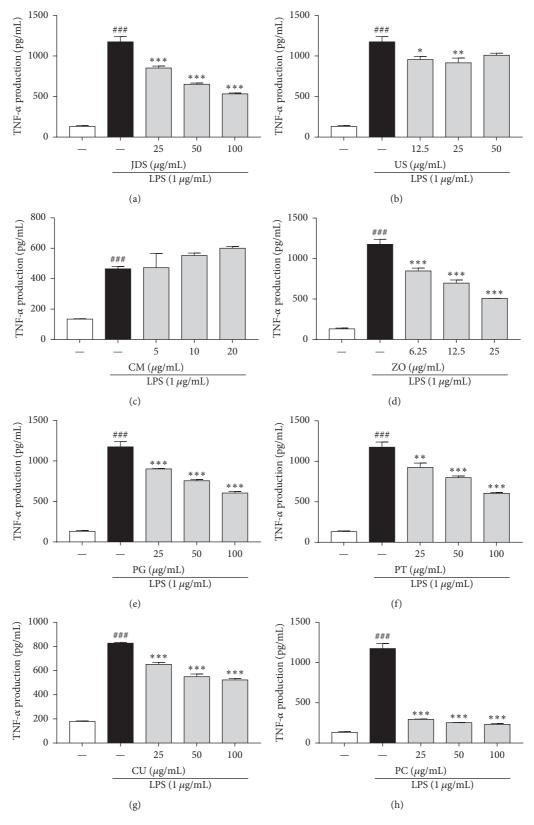


FIGURE 4: Effects of JDS and its herbal components on TNF- α production in LPS-stimulated BV-2 cells. Cells were pretreated with various concentrations of JDS and its seven components for 2 h and then stimulated with LPS (1 μ g/mL) for an additional 22 h. TNF- α production was determined using an ELISA kit. Results are shown for JDS (a), US (b), CM (c), ZO (d), PG (e), PT (f), CU (g), and PC (h). The results are expressed as the mean \pm SEM of three independent experiments. *## P < 0.001 versus vehicle control cells, *P < 0.05, **P < 0.01, and ***P < 0.001 versus LPS-treated cells.

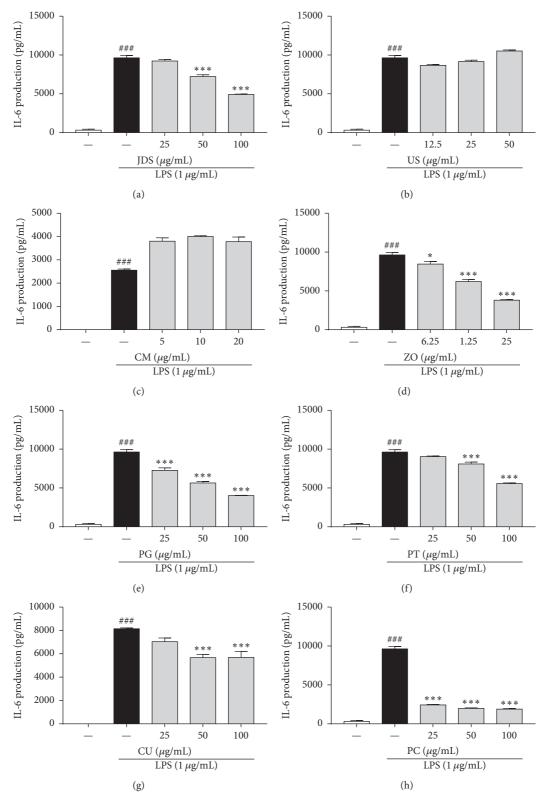


FIGURE 5: Effects of JDS and its herbal components on IL-6 production in LPS-stimulated BV-2 cells. Cells were pretreated with various concentrations of JDS and its seven components for 2 h and then stimulated with LPS (1 μ g/mL) for an additional 22 h. IL-6 production was determined using an ELISA kit. Results are shown for JDS (a), US (b), CM (c), ZO (d), PG (e), PT (f), CU (g), and PC (h). The results are expressed as the mean \pm SEM of three independent experiments. *## P < 0.001 versus vehicle control cells, * P < 0.05 and *** P < 0.001 versus LPS-treated cells.

Microglia that are activated by neuronal injury stimulate the release of inflammatory cytokines, such as TNF- α and IL-6 [24, 28]. Thus, we assessed the production of TNF- α and IL-6 to examine the antineuroinflammatory activities of JDS and its herbal components. The inflammatory reaction was stimulated by adding LPS to microglia. As expected, LPS stimulation significantly enhanced the production of the cytokines TNF- α and IL-6. The herbal mixture JDS significantly reduced the LPS-stimulated increase in cytokine generation observed in BV-2 cells. Among the seven components, ZO, PG, PT, CU, and PC had inhibitory effects on the production of TNF- α and IL-6, and PC had the most dramatic effect of all the drugs tested.

Overall, our data demonstrate that JDS has neuroprotective and antineuroinflammatory effects. Among its seven components, ZO, PT, CU, and PC were commonly effective regarding both neuroprotection and anti-inflammation. CM and US were effective regarding neuroprotection, but not antineuroinflammation, whereas PG inhibited the inflammatory reaction with no neuroprotective activity. These data suggest that the combination of each component in the JDS herbal formula can accelerate the pharmacological activity of the drugs against neurodegeneration via a synergistic interaction.

Moreover, a simultaneous analysis of the standard compounds of JDS was performed to build up a quality control for JDS. JDS consists of seven medicinal herbs: US, ZO, CM, PG, PT, CU, and PC [12, 13]. The main chemical constituents of each herbal medicine are as follows: corynoxeine, hirsutine, ursolic acid, and physcion in US [29, 30], 6-gingerol and 6-shogaol in ZO [31], luteolin, apigenin, camphene, and parthenolide in CM [32, 33], ginsenoside Rg1 and ginsenoside Rb1 in PG [34], 3,4-dihydroxybenzaldehyde, methyleugenol, and 5'-S-5'-thioadenosine from PT [35], hesperidin, esculetin, nomilin, and narirutin in CU [36], and pachymic acid, eburicol, and eburicoic acid in PC [37]. Among those compounds, we analyzed 6-gingerol, luteolin, apigenin, hesperidin, and narirutin using HPLC-PDA. The established HPLC-PDA method was successfully applied for the simultaneous analysis of the five compounds of the JDS extract. As a result, hesperidin (37.892 ± 1.228 mg/g) was determined as being the most abundant compound of JDS.

In conclusion, the *in vitro* or *in vivo* efficacies of each standard compound of JDS on neurodegenerative diseases, including Alzheimer's disease, have been reported [38–41]. However, the combinatorial effect of the standard chemicals has never been considered. Further studies will be necessary to address the advantages of using herbal formulas compared to the administration of single herbs. In addition, the investigation of the efficacy of JDS should be expanded over various neurodegenerative diseases, as, to date, it has focused on dementia or the cognitive disorders of neurodegenerative diseases. The molecular mechanisms that underlie their effects should also be studied to confirm their potential as therapeutic agents for neurodegenerative diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yu Jin Kim, Hye-Sun Lim, and Soo-Jin Jeong participated in the design of the study data analysis and manuscript preparation. Yu Jin Kim and Chang-Seob Seo conducted JDS extract and analysis of five constituents in JDS extract using HPLC. Hye-Sun Lim, Bu-Yeo Kim, and Soo-Jin Jeong conducted the biological examination *in vitro*. All authors read and approved the final manuscript. Yu Jin Kim and Hye-Sun Lim contributed equally to this study.

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Research Article

Amelioration of Scopolamine-Induced Learning and Memory Impairment by α -Pinene in C57BL/6 Mice

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Increasing evidence suggests that neurodegenerative disorders such as Alzheimer's disease (AD) are mediated via disruption of cholinergic neurons and enhanced oxidative stress. Therefore, attention has been focused on searching for antioxidant phytochemicals for the prevention and/or treatment of AD through their ability to fortify cholinergic function and antioxidant defense capacity. In this study, we have investigated the neuroprotective effect of α -pinene (APN) against learning and memory impairment induced by scopolamine (SCO, 1 mg/kg, i.p.), a muscarinic receptor antagonist in C57BL/6 mice. Administration of APN (10 mg/kg, i.p.) significantly improved SCO-induced cognitive dysfunction as assessed by Y-maze and passive avoidance tests. In Morris water-maze test, APN effectively shortened the mean escape latency to find the hidden platform during training days. To further elucidate the molecular mechanisms underlying the neuroprotective effect of APN, the expression of proteins involved in the acetylcholine metabolism and antioxidant system was examined. Particularly, APN treatment increased mRNA expression of choline acetyltransferase in the cortex and protein levels of antioxidant enzymes such as heme oxygenase-1 and manganese superoxide dismutase in the hippocampus via activation of NF-E2-related factor 2. These findings suggest the possible neuroprotective potentials of APN for the management of dementia with learning and memory loss.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by hyperphosphorylated microtubule-associated protein tau, neurofibrillary lesions composed of the β -amyloid peptide, aberrant oxidative and inflammatory processes, and disturbance in neurotransmission [1]. AD produces significant cognitive impairment that arises from damage to cholinergic neurons known to play a crucial role in learning and memory functions. Cholinergic deficit has been regarded as a marker of neurological pathology that is associated with memory dysfunction and consistently correlated with the severity of cognitive impairment in AD [2]. Therefore, the recuperation of cholinergic role remains a coherent marker for developmental programs targeting the remedy of AD symptoms.

Scopolamine (SCO) is a muscarinic acetylcholine receptor (mAChR) antagonist which disturbs learning and memory function in animals and humans similar to those conditions observed in the patients with AD [3]. SCO has been used in establishing experimental models to evaluate the effects of therapeutic potential and benefits of drug candidates in dementia. In addition, SCO is considered to elevate acetylcholinesterase (AChE) levels in the cortex and hippocampus and has been used to search for and evaluate antidementia drugs [4].

Prolongation of acetylcholine (ACh) release into the neuronal synaptic cleft has been targeted as a means of increasing cholinergic functions in AD, which can be achieved by blocking enzymatic hydrolysis of acetylcholine by AChE [5]. In particular, acetylcholinesterase inhibitors (AChEI) such as rivastigmine (Exelon®), galantamine (Reminyl®), and

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donepezil (Aricept®) and cholinergic agonists are approved pharmacological therapies for AD. However, AChEIs have limitations such as short half-lives and severe negative side effects including diarrhea, nausea, vomiting, and hepatotoxicity [6]. Therefore, complementary and alternative therapies utilizing nontoxic phytochemicals from medicinal plants and foods for the prevention and/or treatment of AD are highly desirable and promising.

Thuja orientalis Linn., which is widely distributed in Korea and China, has been traditionally used to treat diarrhea, gout, rheumatism, and chronic tracheitis [7]. Recently, laboratory studies have shown that the extracts from *Thuja* orientalis Linn. exhibit a variety of biological functions such as antioxidant, anti-inflammatory, and antielastase activities [8–10]. Furthermore, one of the active components, α -pinene (APN), has been reported to possess antioxidant, immunostimulant, and anti-inflammatory properties [11–15].

Besides disturbance of acetylcholine metabolism in memory loss, recent studies assert that neuropathology of AD is associated with oxidative and inflammatory processes [5]. Reactive oxidative species (ROS) are able to deficit cellular constituents and play an important role as a secondary messenger mediating inflammation [16]. Prior researches reported that oxidative stress is associated with memory dysfunction in the SCO-induced animal model of amnesia [17–21].

In this study, we have investigated the memory enhancing effect of APN on SCO-induced memory deficit in C57BL/6 mice using the Y-maze, Morris water-maze, and passive avoidance tests. Moreover, in order to elucidate the molecular mechanisms underlying the neuroprotective effect of APN, the expression of proteins involved in the acetylcholine metabolism and antioxidant enzymes including heme oxygenase-1 (HO-1) and superoxide dismutase (SOD) were additionally examined.

2. Materials and Methods

2.1. Chemicals and Reagents. APN, SCO, and other chemical reagents were obtained from Sigma-Aldrich (MO, USA). Anti- MnSOD, anti-CuZnSOD, anti-GAPDH, and anti-NF-E2-related factor 2 (Nrf2) antibodies were provided by Santa Cruz Biotechnology (TX, USA). Anti-HO-1 and anti-pphospho-Nrf2 (p-Nrf2) antibodies were supplied from Stressgen (MI, USA) and Epitomics (CA, USA), respectively.

2.2. Experimental Animals. Male C57BL/6 mice (20–25 g, 8 weeks old) were purchased from the Dae Han Bio-Link. Co., Ltd. (Chungcheongbuk-do, South Korea) and acclimated to the colony room for 3 days. The animals were maintained with constant temperature (21–23°C) and humidity (55–60%) under a 12 h light-dark cycle (light on 7:00–19:00). Mice were allowed free access to food and fresh water during environmental adaptation and behavior test periods. The experimental procedure was conducted in accordance with the Care and Use of Laboratory Animals of the Daegu Haany University and Keimyung University.

2.3. Y-Maze Test. The Y-maze is a three-arm horizontal maze with an angle of 120 degrees, which were of 28 cm length, 6 cm width, and 18 cm height. The maze floor and walls were constructed with white polyvinyl plastic. Mice were initially placed in one arm, and then the sequence and number of arm entries were monitored for an 8-min period. An actual alternation was defined when a mouse entered into all three arms on consecutive choices (i.e., ABC, BCA, or CAB, but not CAC, BAB, or ABA). The spontaneous alternation (%) was derived from the total number of alternations divided by the total number of arm entries minus two, which was multiplied by 100 as shown in the following equation: % Alternation = [(Number of alternations)/(Total number of arm entries – 2)] × 100. The number of arm entries also served as an indicator for movement and locomotor activity.

2.4. Morris Water-Maze Test. The Morris water maze was conducted in a circular pool (120 cm in diameter and 45 cm in height) with a featureless inner surface. The water-maze tank was placed with four external visual cues and filled with water containing a nontoxic white color. The temperature of the water was maintained by 23-25°C. A white platform (10 cm in diameter and 30 cm in height) was placed in one of the quadrants with equal area and submerged 2 cm below the water surface. During each trial session, the escape latency time spent to find the hidden platform was monitored by a video tracking system (Ethovision System, Noldus, Wageningen, Netherlands). During the four subsequent days of training the mice were given three trials per day with the submerged platform in the pool. When the mouse located on the plated platform, it was allowed to remain on it for an additional 10 sec. If the mouse did not find the hidden platform within 120 sec, the mouse was guided to the platform and permitted to remain on it for an additional 10 sec. On the last day, the hidden platform was removed from the watermaze tank and probe test was performed. Mice were allowed to swim for 90 sec and the staying time in the maze quadrant where the platform had previously been located was recorded.

2.5. Passive Avoidance Test. The step-through passive avoidance test apparatus (Gemini Avoidance System, San Diego, USA) consisted of illuminated $(25 \text{ cm} \times 20 \text{ cm} \times 20 \text{ cm})$ and nonilluminated chambers separated by a guillotine door $(5 \text{ cm} \times 5 \text{ cm})$. The floor of illuminated and nonilluminated compartments was composed of 2 mm stainless steel rods spaced 6 mm apart. During acquisition test session, mice were gently placed in the illuminated chamber and 20 sec later the guillotine door between the two chambers was opened. When mice entered the nonilluminated chamber, the door was automatically closed and electrical foot shock (1 mA, 5 sec) was delivered through the stainless steel rods. After 24 h, during the retention trial session, the mice were placed again in the illuminated chamber. Then the stepthrough latency time to enter the nonilluminated chamber was measured. If the mouse did not enter nonilluminated chamber within 300 sec, the retention trial was ended and the step-through latency time was scored 300 sec as the upper 2.5.1. Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from cortex of C57BL/6 mouse by TRI reagent (Molecular Research Center, OH, USA) according to the manufacturer's manual instruction. The sequences of primers were used in the polymerase chain reaction (PCR) using synthetic primers specific to choline acetyltransferase (ChAT), acetylcholine esterase (AChE), muscarinic acetylcholine receptors (M_1 mAChR and M_2 mAChR), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as follows:

ChAT

5'-AGG GTG ATC TGT TCAS CTC AG-3' (sense)

5'-TCT TGT TGC CTG TCA TCA TA-3' (antisense)

AChE

5'-AGA AAA TAT TGC AGC CTT TG-3' (sense)

5'-CTG CAG GTC TTG AAA ATC TC-3' (antisense)

M₁ mAChR

5'-CAG AAG TGG TGA TCA AGA TGC C-3' (sense)

5'-GAG CTT TTG GGA GGC TGC TT-3' (antisense)

M₂ mAChR

5'-TGC TGT GGC CTC CAA TAT GA-3' (sense)

5'-TGA CCC GAC GAC CCA ACT-3' (antisense)

GAPDH

5'-GCC AAG GTC ATC CAT GAC AAC-3' (sense)

5'-AGT GTA GCC CAG GAT GCC CTT-3' (antisense)

The specific region of a DNA strand was amplified by PCR mixture containing Taq polymerase (SolGent, Daejeon, Korea) under the following condition: denaturation step for 5 min at 95°C, then annealing step of 40 cycles for 60 sec at 51°C (ChAT), at 51°C (AChE), at 55°C ($\rm M_1$ mAChR), at 55°C ($\rm M_2$ mAChR), or at 57°C (GAPDH), and subsequent elongation step for 60 sec at 72°C. The amplified PCR products were analyzed by gel electrophoresis using 1.5% agarose gel prestained with ethidium bromide. Size separation images of the PCR products were evaluated by using QuantityOne Software of Image analysis Gel Doc XR System (Bio-Rad, CA, USA).

2.6. Western Blot Analysis. The hippocampus of C57BL/6 mouse were homogenized and incubated with RIPA buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 150 mM NaCl, 1% Triton X-100, and one complete protease inhibitor cocktail tablet (Roche diagnostics, IN, USA) on ice for 20 min. After incubation, the homogenates were centrifuged at 14,000 g for 30 min at 4°C and concentration of protein was determined by using bicinchoninic acid (BCA) protein assay kit (Pierce, IL, USA). The equal amount of protein samples (30 μ g) were separated on 10–12% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane (Pall., MI, USA) at 300 mA for 3 hr. After transferring, the membranes were blocked with 5% nonfat dried milk in phosphate-buffered saline (PBS) with 0.01% Tween-20 (PBST) for 1h at room temperature. The membranes were further incubated with primary antibodies at 4°C overnight. After three times washing with PBST, the membranes were reacted with horseradish peroxidase- (HRP-)conjugated anti-rabbit secondary antibody (Zymed, CA, USA) in 3% nonfat dried milk for 1h at room temperature. The membranes were visualized with the enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersharm Bioscience, NJ, USA) and images of specific target proteins were evaluated by using ImageQuant LAS 4000 Multi Gauge software (Fujifilm, Tokyo, Japan).

2.7. Statistical Analysis. The data were expressed as means \pm SEM and statistical analysis for multiple comparisons was conducted by using one-way ANOVA followed by the Tukey test for a series of behavior tests with SPSS software (PASW Statistics 18.0 KO for windows). The data values were defined to be statistically significant at p < 0.05.

3. Results

3.1. Effect of APN on Memory Impairment Induced by SCO in Y-Maze Test. The SCO-induced amnesia group exhibited significantly reduced spatial recognition memory compared with sham control group in Y-maze test (Figure 1(a)). However, SCO-decreased recognition memory performance was effectively restored by intraperitoneal administration of APN (10 mg/kg, i.p.) (Figure 1(a)). The APN-treated group showed a higher spontaneous alternation score as compared with SCO-injected amnesic group. However, under the same experimental condition, the total number of arm entries was not much different among the experimental groups (Figure 1(b)).

3.2. Effect of APN on Memory Loss Induced by SCO in Morris Water-Maze Test. We have conducted Morris water-maze test to confirm the memory enhancing effect of APN in C57/BL6 mice. During four consecutive training days, the mice in the sham group quickly found the location of the hidden platform than the SCO-induced amnesia group (Figure 2(a)). The SCO-injected group exhibited significantly delayed mean escape latency time compared with the sham control group from days 2 to 4. The group treated with APN (10 mg/kg, i.p.) significantly shortened the mean escape latency from days 2 and 4 (Figure 2(a)). At a dose of 10 mg/kg,

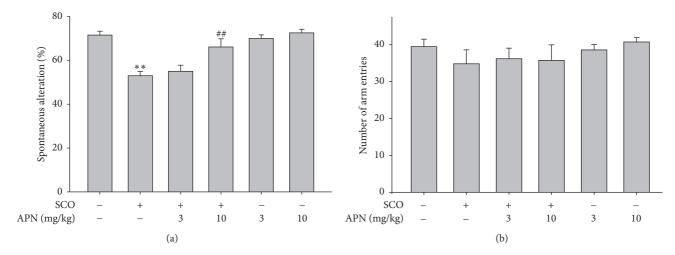


FIGURE 1: Memory enhancing effect of APN in Y-maze test. One hour before the test, mice were treated with vehicle or APN (3 or 10 mg/kg, i.p.) and, 30 min later, mice were injected with vehicle or SCO (1 mg/kg, i.p.). (a) Effects of APN on the SCO-induced spontaneous alternation. (b) Total number of arm entries in 8-min trials of Y-maze test. Data are represented as mean \pm SEM (n=7). Significant difference between the groups: **p < 0.05, vehicle-treated control versus SCO alone group; **p < 0.01, SCO alone group versus APN-treated group in combination with SCO.

the efficacy of APN was comparable to the sham group. As the higher doses of APN exhibited slightly lower efficacy than 10 mg/kg (data not shown), we have utilized up to 10 mg/kg as maximal effective dose of APN for subsequent behavior and molecular analysis. The representative pathways derived from each group during four training days were shown in Figure 2(b). In the probe trial, APN (10 mg/kg, i.p.)-treated group exhibited increased time spent in the quadrant where the platform has been placed (Figure 2(c)). Therefore, we demonstrated that APN enhanced spatial recognition in the Morris water-maze test.

3.3. Effect of APN on Cognitive Dysfunction Induced by SCO in the Passive Avoidance Test. During the acquisition trial of the step-through passive avoidance test, there were no significant differences among groups (Figure 3). However, during the retention trial which was performed 24 h after the acquisition trial, SCO-treated mice showed a significantly lower latency compared with sham control group (Figure 3). The decreased latency time indicates impaired retention of memory in the step-through type passive avoidance test. The SCO-induced memory impairment was effectively revered by administration of APN (10 mg/kg, i.p.) (Figure 3).

3.4. Effect of APN on the Expression of Enzymes and Receptors Involved in Acetylcholine Metabolism. To elucidate the possible neuroprotective molecular mechanisms of APN, the mRNA levels of enzymes involved in the acetylcholine metabolism and acetylcholine receptors were assessed by RT-PCR. In the cortex, SCO slightly decreased the mRNA expression of ChAT which is to join acetyl-CoA to choline resulting in the formation of the acetylcholine (Figure 4(a)). Conversely, intraperitoneal administration of APN markedly elevated mRNA levels of ChAT (Figure 4(a)). Under the same experimental condition, the mRNA expression of AChE, which degrades the neurotransmitter ACh, and muscarinic

acetylcholine receptors (M_1 mAChR and M_2 mAChR) was not significantly altered by SCO treatment in the presence or absence of APN (Figure 4(b)).

3.5. Effect of APN on the Expression of Antioxidant Enzymes. To further verify the underlying molecular mechanisms of APN against SCO-induced learning and memory impairment, the protein expression of antioxidant enzymes was measured by Western blot analysis. In the hippocampus, APN effectively increased protein levels of HO-1 and MnSOD (Figure 5(a)). Under the same experimental condition, the expression of the aforementioned antioxidant enzymes in the cortex was not much altered by administration of APN (data not shown). Furthermore, in the hippocampus, treatment of C57BL/6 mice with APN led to an activation of a redox-sensitive transcription factor Nrf2 via phosphorylation (Figure 5(b)), which may regulate the subsequent expression of antioxidant proteins.

4. Discussion

In this study, we have evaluated the effect of APN, one of the major compounds in *Thuja orientalis* Linn., to potentially prevent and treat learning and memory deficits in an animal model of amnesia. We have examined whether APN attenuates learning and memory impairments induced by SCO (1 mg/kg, i.p.), a muscarinic acetylcholine receptor antagonist, by conducting Y-maze, Morris water-maze, and passive avoidance tests. SCO interferes with learning and memory functions and subsequently causes impairment of learning acquisition and short-term as well as long-term memories. Moreover, cholinergic neurons in the CNS are involved in mediating reference (long-term) as well as working (short-term) memories of both humans and animals [22]. Therefore, disruption of cholinergic neurotransmission system plays a critical role in the early stage of AD [23].

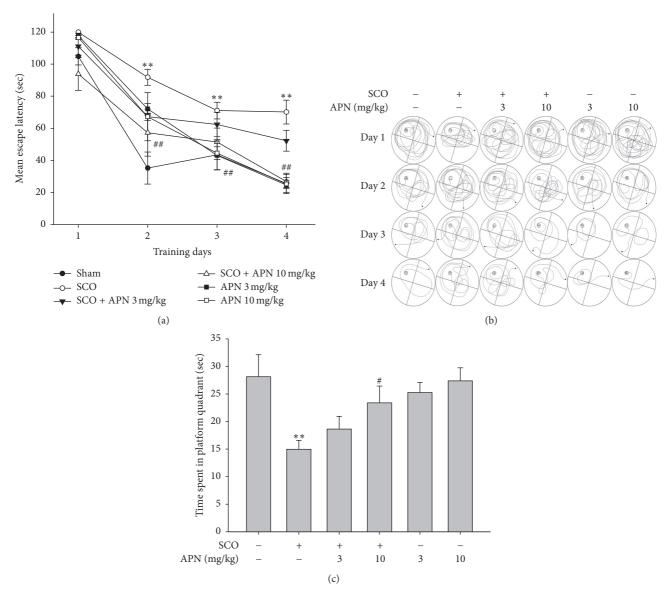


FIGURE 2: Protective effect of APN on the learning and memory deficit induced by SCO in Morris water-maze test. One hour before the test, mice were treated with vehicle or APN (3 or 10 mg/kg, i.p.) and then after 30 min injected with vehicle or SCO (1 mg/kg, i.p.). (a) Effect of APN on the SCO-induced spatial memory impairment was examined by mean escape latency. (b) The representative water-maze paths of each group were indicated during four training days. (c) On the last day of training trial, probe test was conducted in which the platform was removed from the pool and mice were allowed to swim and search it for 90 sec. Data are shown as mean \pm SEM (n = 7). Significant difference between the groups: **p < 0.01, vehicle-treated control versus SCO alone group; *p < 0.05 and **p < 0.01, SCO alone group versus APN-treated group in combination with SCO.

The spontaneous alternation score in the Y-maze test is an indication of working memory. SCO-induced reduction of spontaneous alternation score was increased by APN (10 mg/kg, i.p.) administration. The passive avoidance test is used as a useful tool for the estimation of working as well as reference memories. APN (10 mg/kg, i.p.) prolonged the step-through latency time and mitigated the memory impairments induced by SCO. The results suggest that APN may improve memory by rescuing the acetylcholine system from deficit caused by SCO.

The Morris water-maze test evaluates reference and spatial memories and detects changes in the central cholinergic

system. The APN (10 mg/kg) shortened this escape latency time on days 2 to 4. At the probe trial session, APN (3 mg/kg and 10 mg/kg, i.p.) dose-dependently increased the time spent within the target quadrant. The simultaneous analysis for a distinction between working and reference memories is well established through the Morris water-maze test. The reduction in escape latency from day to day in the first trial represents reference memory, while that from first trial to second trial indicates working memory [24, 25]. Considering the escape latency time markedly reduced during the training trials, it seems likely that APN improves long-term and spatial memories in SCO-induced amnesic mice.

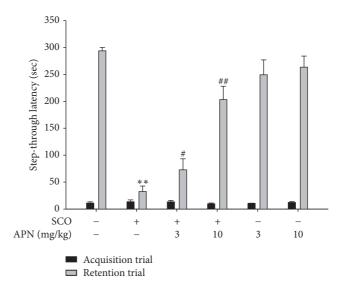


FIGURE 3: Protective effect of APN on the learning and memory deficit induced by SCO in passive avoidance test. During acquisition trial, one hour before this test, mice were treated with APN (3 or 10 mg/kg, i.p.) and, 30 min later, mice were injected with vehicle or SCO (1 mg/kg, i.p.). Effect of APN on the SCO-induced learning and memory deficit was monitored based on the step-through latency. Data are presented as mean \pm SEM (n=7). Significant difference between the groups: **p<0.01, vehicle-treated control versus SCO alone group; $\pm p<0.05$ and $\pm p<0.01$, SCO alone group versus APN-treated group in combination with SCO.

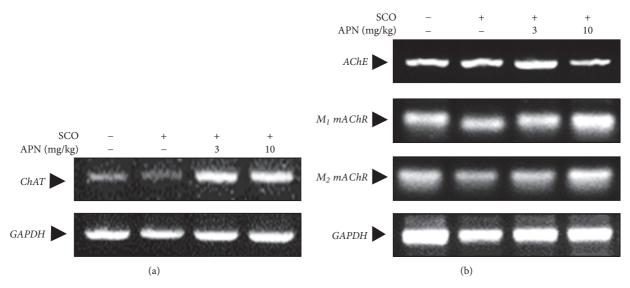


FIGURE 4: Effect of APN on the mRNA expression of enzymes and receptors related to acetylcholine. In the cortex of mice treated with SCO in the presence or absence of APN, mRNA levels of enzymes and receptors related to acetylcholine neurotransmission system such as (a) ChAT, (b) AChE, M_1 mAChR, and M_2 mAChR were determined by RT-PCR. GAPDH levels were compared to verify the equal amount of mRNA loading.

To further assess the neuroprotective mechanisms of APN, we have conducted a series of molecular analysis using cortical as well as hippocampal tissues. In both humans and animals these two brain areas particularly medial prefrontal cortex and hippocampus and their communications have been reported to play an essential role in encoding and retrieval of diverse memory processes [26]. In the cortex of SCO-induced amnesic animals, the mRNA levels of enzymes involved in the acetylcholine metabolism and muscarinic M_1 and M_2 AChRs were analyzed by RT-PCR. SCO lightly diminished the mRNA expression of ChAT in the cortical

region. Reversely, intraperitoneal administration of APN resulted in a dramatic increase in the mRNA levels of ChAT. However, the mRNA expression of AChE which reduces the availability of ACh in the synaptic cleft was not changed by SCO treatment in the presence or absence of APN.

A number of studies have provided experimental evidence on the cholinergic hypothesis of learning, memory, and cognition [27]. The brain lesions of cholinergic nuclei or acetylcholine receptors (postsynaptic muscarinic \mathbf{M}_1 receptors and presynaptic muscarinic \mathbf{M}_2 receptors) and use of cholinergic antagonists are often related to cause cognitive

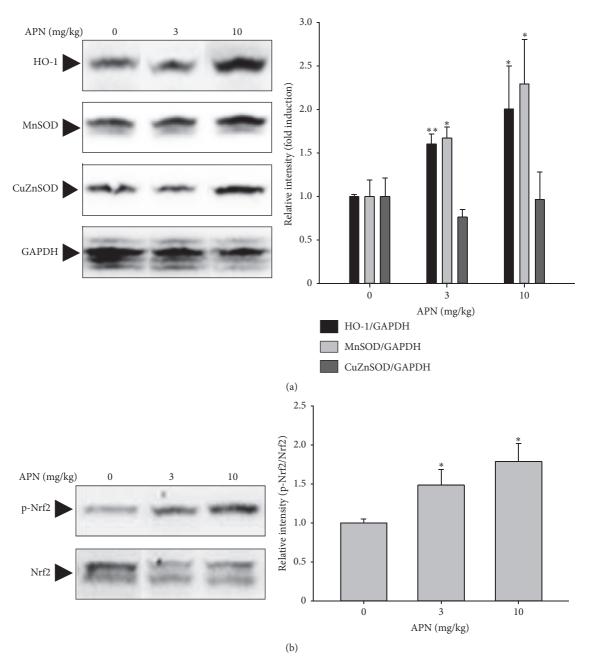


FIGURE 5: Effect of APN on the protein expression of antioxidant enzymes. (a) In the hippocampus of mice treated with SCO in the presence or absence of APN, protein levels of antioxidant enzymes such as HO-1, MnSOD, and CuZnSOD were determined by Western blot analysis. GAPDH levels were compared to normalize the equal amount of protein loading. (b) The protein expression of p-Nrf2 and Nrf2 was examined by Western blot analysis. Quantitative data of Western blot analysis was provided in the right panels. Significant difference between the groups: $^*P < 0.05$ and $^{**}P < 0.01$, vehicle-treated control versus APN-treated group.

dysfunctions similar to those observed in dementia [28–30]. Besides cholinergic hypothesis in learning and memory, many clinical and experimental researches reported that oxidative stress is involved in the pathological characteristics of neurodegenerative disorders including AD [31]. The activities and expression of antioxidant enzymes have been demonstrated to be altered in the pathology of neurodegenerative diseases [32]. According to recent studies, the dysfunction of

learning, memory, and cognition induced by administration of SCO in animal models is associated with changes in the expression of antioxidant enzymes [33].

In this study, to confirm the molecular basis for the antioxidant mechanisms of APN, the protein levels of diverse antioxidant defenses enzymes were evaluated by Western blot analysis. In the hippocampus, treatment of C57BL/6 mice with APN led to an increased protein expression of HO-1

and MnSOD, which seemed to be mediated by activation of Nrf2 via phosphorylation. HO-1 is a rate-limiting enzyme that catalyzes the degradation of heme. This process produces biliverdin and carbon monoxide, which can ameliorate oxidative stress-mediated neurodegenerative disorders [34, 35]. SOD is an enzyme catalyzing the dismutation of superoxide radical into molecular oxygen or hydrogen peroxide (H₂O₂). Three isoforms exist in human such as cytoplasmic SOD1 (CuZmSOD), extracellular SOD3 (CuZnSOD), and mitochondrial SOD2 (MnSOD). In other studies APN has been reported to protect U373-MG [36] and PC12 [37] cells from H₂O₂-induced cytotoxicity as well as oxidative stress by increasing the activity and protein expression of endogenous antioxidant enzymes such as SOD, HO-1, glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT).

Nrf2 is a redox-sensitive transcription factor which is induced in the brain following toxic and/or subtoxic levels of stress and mediates induction of antioxidant and phase II detoxification enzymes such as HO-1, SOD, γ-glutamylcysteine ligase (GCL), glutathione S-transferase (GST), GPx, NAD(P)H:quinone oxidoreductase 1 (NQO1), and CAT [38]. Nrf2 has been reported to play crucial roles in neuroprotection in acute CNS injuries as well as diverse neurodegenerative disorders [38]. In C6 cells SCO treatment decreased the protein levels of Nrf2 and subsequent expression of downstream target genes including SDO, GPx, and CAT, which ultimately led apoptotic cell death via oxidative stress [39]. Conversely, in another study compound K derived from red ginseng exerted memory enhancing effect against SCOinduced amnesia via induction of Nrf2 as the protective effect of compound K was abolished in Nrf2 knockout mice [40].

In conclusion, present results demonstrate that APN exhibits memory enhancing activity and regulates the expression of proteins related to synthesis of acetylcholine and antioxidant defense system. Therefore, APN might be one of the useful natural agents of cure and/or prevention for amnesia and neurodegenerative diseases with learning, memory, and cognitive dysfunctions.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Gil-Yong Lee and Chan Lee equally contributed to this work.

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Research Article

Extract of Fructus Cannabis Ameliorates Learning and Memory Impairment Induced by D-Galactose in an Aging Rats Model

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Hempseed (*Cannabis sativa* L.) has been used as a health food and folk medicine in China for centuries. In the present study, we sought to define the underlying mechanism by which the extract of Fructus Cannabis (EFC) protects against memory impairment induced by D-galactose in rats. To accelerate aging and induce memory impairment in rats, D-galactose (400 mg/kg) was injected intraperitoneally once daily for 14 weeks. EFC (200 and 400 mg/kg) was simultaneously administered intragastrically once daily in an attempt to slow the aging process. We found that EFC significantly increased the activity of superoxide dismutase, while lowering levels of malondialdehyde in the hippocampus. Moreover, EFC dramatically elevated the organ indices of some organs, including the heart, the liver, the thymus, and the spleen. In addition, EFC improved the behavioral performance of rats treated with D-galactose in the Morris water maze. Furthermore, EFC inhibited the activation of astrocytes and remarkably attenuated phosphorylated tau and suppressed the expression of presenilin 1 in the brain of D-galactose-treated rats. These findings suggested that EFC exhibits beneficial effects on the cognition of aging rats probably by enhancing antioxidant capacity and anti-neuroinflammation, improving immune function, and modulating tau phosphorylation and presenilin expression.

1. Introduction

In the past decades, the population birth rates are decreasing, and human lifespan is expanding. As a result, 11% of the world's population is over the age of 60, and this proportion is expected to double by 2050 [1]. As our populations are rapidly aging, there is increasing awareness of the age-related cognitive deficits caused by diseases associated with aging, such as Alzheimer's disease (AD). New therapies are urgently needed to treat or delay these diseases.

Among aging theories, free radical theory is one of the most widely accepted explanations for senescence [2]. This theory argues that aging leads to a disturbance of oxygen free radical metabolism, and the excess oxygen free radicals

attack cell membranes by peroxidating lipids and increase malondialdehyde (MDA) levels. At the same time, the activity of superoxide dismutase (SOD), a scavenger of superoxide anion, is reduced [3]. High doses of D-galactose (D-gal) result in accumulation of galactitol, osmotic stress, and reactive oxygen species (ROS) that aggravate aging [4]. Meanwhile, D-gal overload in the brain subsequently causes neurotoxicity such as oxidative stress and neuroinflammation [5, 6] and eventually leads to cognitive deficits. Hence, the administration of D-gal has been widely used for establishing animal aging model [7] and to study age-related neurodegenerative diseases [8].

Hempseed is the fructus of *Cannabis sativa*, which has been commonly used as a Chinese traditional medicine and

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nonstaple food for centuries. It contains a variety of essential fatty acids, such as linoleic acid (ω -6) and alpha-linolenic acid (ω -3) [9]. Thus, extract of Fructus Cannabis (EFC) may have great utility as a health food with the potential to prevent several lipid-associated diseases [10]. Hempseed also contains antioxidant peptides that reduce oxidative stress in spontaneously hypertensive rats [11]. In addition, dietary hempseed reduces platelet aggregation because it is enriched in polyunsaturated fatty acids [12]. Hempseed meal protein hydrolysate prevents hypertension and lowers blood pressure in spontaneously hypertensive rats [13]. However, whether EFC protects against the cognitive deficits associated with aging is unknown.

Residents dwelling in Hongshuihe River basin, Guangxi Province, have used hempseed as a popular health food for centuries. In previous studies, we evaluated the cognitive function of longevity population in Hongshuihe River basin by using the psychometric tests of the mini-mental state examination (MMSE) [14]. The results revealed that long-lived individuals with good cognition might be associated with the benefit of dietary hempseed. It is thus reasonable to assume that EFC may have potential neuroprotective effect against brain aging and memory impairments. In the current study, we aim to determine if EFC improves learning and memory in rats treated with D-gal and define the mechanism underlying its protective effects on brain aging.

2. Materials and Methods

- 2.1. Chemicals and Reagents. Hempseed was purchased in farm product market in Bama County, Guangxi Province, China. D-Galactose was purchased from Sigma-Aldrich (Hamburg, Germany). SOD and MDA kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Primary antibodies for total tau, phosphorylated tau, and presenilin-1 were purchased from Abcam (Cambridge, MA, US), and anti-glial fibrillary acidic protein (GFAP) antibody was from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor 555 antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA). GAPDH was from Good Here Biotechnology (Hang Zhou, China). Goat anti-rabbit IgG and goat anti-mouse IgG were purchased from LI-COR Biosciences (Lincoln, NE, US).
- 2.2. Preparation of EFC. Hempseed (Cannabis sativa L.) was identified by Dr. Zhenguo Zhong, a professor of Guangxi University of Traditional Chinese Medicine. EFC was prepared as described previously [15]. Briefly, hempseed was airdried and was dehulled using a laboratory huller machine (HT80-20, Shan Dong Instrument, China). Then the seeds were extracted with 90% ethanol for 24 hours and filtrated. The filtrate was concentrated to dry and the total yield was 5 g (1%) in terms of starting materials. EFC was dissolved in saline prior to administration at doses of 200 mg/kg and 400 mg/kg.
- 2.3. HPLC-MS Analysis of Components in EFC. The ethanol extract was subjected to column chromatography under

reduced pressure on silica gel, eluted with a chloroform-methanol solvent system. Its components were analyzed by high performance liquid chromatography (HPLC) with a Lichrospher 100 RP chromatographic column and identified according to mass spectrometry (MS). The mobile phase consisted of acetonitrile and ammonium acetate (12:88) at a flow rate of 0.8 ml/min during a run time of 34 minutes and the column temperature was 35°C.

2.4. Animals and Drug Administration. Three-month-old Sprague-Dawley (SD) male rats (SPF grade, wt 259–278 g) were obtained from the Experimental Animal Center, Guangxi Medical University (SYXK 2009-0002). The rats were housed in ventilated cages at $24 \pm 2^{\circ}$ C, with a humidity of $50 \pm 10\%$, under a 12 h light/dark cycle. Standard diet and water were available ad libitum. The experimental protocols were approved by the Institutional Ethical Committee of Guangxi Medical University (approval number 20110501202).

After 1 week of acclimatization to vivarium conditions, the animals were screened for the Morris water maze test. Rats that reached the hidden platform within 90 seconds were selected. Forty eligible rats were randomly divided into five groups with 8 rats for each: (1) control group; (2) EFC group (400 mg/kg); (3) D-gal group (D-gal-induced aging group); (4) D-gal + EFC (200 mg/kg) group; and (5) D-gal + EFC (400 mg/kg) group. To establish the animal model of aging, rats in the D-gal group were injected intraperitoneally with D-gal at a dose of 400 mg/kg/day for 14 weeks, and the rats in control and EFC (400 mg/kg) groups were injected with an equal volume of normal saline. At the same time, EFC was administered intragastrically once per day for 14 weeks.

- 2.5. Morris Water Maze Test. After 14 weeks of drug administration, rats (n = 8 each group) were tested in Morris water maze as described previously [16]. The experimental apparatus consisted of a circular pool filled with water (24 \pm 2°C) which was rendered opaque by adding ink. The hidden platform was submerged below the water surface in the center of target quadrant. During 5 days of navigation training, the rats underwent 4 trials per day with four quadrants of rotational starting points and allowed a maximum of 90 s to search for the hidden platform (escape latency). On the sixth day, the probe trial was performed by removing the platform and allowing each rat to swim freely for 60 s. The escape latency and the number of platform crossings as well as time spent in the target quadrant were recorded by a video camera mounted on the ceiling, and data were analyzed by using SMART Behavior Analysis System.
- 2.6. Observation of the General Appearance, Body Weight, and Organ Index. The general appearance of the rats was observed daily throughout the experiment, and body weight was recorded weekly. Rats were sacrificed after behavioral tests and brain tissues were collected for biochemical analysis or western blotting analysis. Hippocampus tissues were immediately collected and assayed as described below. The hearts and livers, the spleens, and the thymus glands were gathered and weighed. The organ index was calculated using

the following equation: organ index = organ weight (g)/body weight (g) \times 100%.

2.7. SOD Activity and MDA Level Measurements in the Hippocampus. Hippocampus tissues were prepared in ice-cold physiological saline and centrifuged at 1.2×10^4 rpm for 15 minutes at 4°C, and supernatants were collected for analysis. SOD activities and MDA levels in the hippocampus were analyzed according to the manufacturer's instructions.

2.8. Immunofluorescent Staining. It is well known that the activation of astrocytes is indicated as increased glial fibrillary acidic protein (GFAP) level. The protocol for immunofluorescent staining has been described previously [17]. Sections were incubated at 4°C overnight with a mouse monoclonal anti-GFAP antibody (1:400, Sigma-Aldrich, MO, USA). Following incubation with primary antibody, the sections were rinsed in TBS and then incubated with goat anti-mouse IgG Alexa Fluor 555 antibody (1:1000, Waltham, MA, USA) for 1h at room temperature. Images from the CA1 region of hippocampus in each section were observed with a microscope (ECLIPSE Ti-s, Nikon, Japan) and digital sight camera (ECLIPSE TS100, Nicon, Japan). The staining of GFAPpositive cells was measured using computerized planimetry (Image-Pro Plus Media Cybernetics, Inc., Rockville, USA). Results of 4 separate measurements for each section were expressed as mean \pm SE; statistical differences were assessed by one-way ANOVA tests.

2.9. Western Blot Analysis. The proteins of hippocampus tissue were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated overnight at 4°C with the primary antibodies appropriately diluted. The secondary antibody was diluted 1:3000 in Tris-buffered saline with Tween 20 (TBST). LI-COR Odyssey platform (infrared laser) was used to detect the immune-reactive bands. GAPDH was used as an internal control, and the intensity of the bands was quantified using Odyssey V3.0 software.

2.10. Statistical Analysis. Results were analyzed by one-way analysis of variance (ANOVA) with Dunnett's test for multiple post hoc comparisons, and data are expressed as means \pm SE (SPSS19.0). Differences were considered statistically significant for P < 0.05.

3. Results

3.1. HPLC-MS Analysis of Components in EFC. It is well known that hempseed is rich in essential fatty acids (EFAs). Therefore, EFC was analyzed by HPLC-MS to identify the components of EFAs. The results show that EFC contains mainly polyunsaturated fatty acids with omega-6 polyunsaturated fatty acids (ω -6 PUFAs) and omega-3 polysaturated fatty acids (ω -3 PUFAs) in a ratio of 3:1. HPLC chromatogram of EFC is shown in Figure 1 and the graph of mass spectrometry is given in Figure 2.

3.2. EFC Treatment Improved the General Appearance and Increased the Body Weights and Organ Indices. After 14 weeks

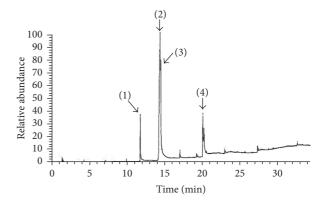


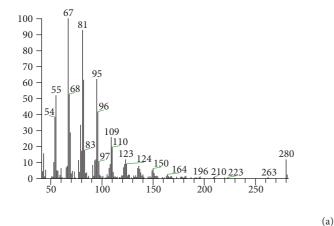
FIGURE 1: *HPLC chromatogram of EFC.* (1) *n*-Hexadecanoic acid, (2) 9,12-octadecadienoic acid (ω 6), (3) 9,12,15-octadecatrienoic acid (ω 3), and (4) butyl 9,12-octadecadienoate.

of D-gal exposure, rats in the D-gal model group exhibited signs similar to natural aging, such as grayed hair, hair loss, being spiritless, and physical inactivity. Treatment with Dgal plus EFC (400 mg/kg) or EFC treatment (400 mg/kg) improved the general appearance of the rats. The mean body weight in D-gal group notably decreased after 10 weeks, but those of the other groups increased steadily throughout the experiment (Figure 3(a)). Moreover, the mean body weight gain of the D-gal model group was significantly less than that of the control group (P < 0.05, Figure 3(b)). However, the increases of body weight in D-gal plus EFC (400 mg/kg) and EFC treatment (400 mg/kg) group were significantly higher than that in the D-gal group (P < 0.05). The organ indices, including heart, liver, thymus, and spleen, in the D-gal model group were significantly lower than those of the control group (P < 0.05) (Figure 4). However, these parameters turned toward normal after EFC administration.

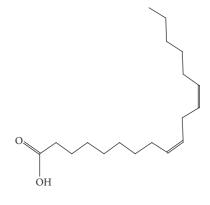
3.3. EFC Protects against the Memory Impairment Caused by D-Gal. The Morris water maze test is widely used to evaluate the spatial learning and memory. The navigation training showed a decrease in escape latency and distance to target platform in all groups, which demonstrated that rats in all groups have the ability to learn and to navigate to the goal. There was no difference in escape latencies and distances to hidden platform between groups on Day 1 of training. However, from Day 2 to Day 5, the post hoc comparisons revealed that D-gal model group showed longer escape latencies and distances to find the target platform than the control group (P < 0.05, Figures 5(a) and 5(b)), whereas rats in D-gal plus EFC (400 mg/kg) and EFC treatment (400 mg/kg) group had shorter latencies and distances to find the hidden platform than the D-gal group (P < 0.05).

At the end of the acquisition training, the hidden platform was removed, and a probe trial was used to assess the reference memory. Rats in D-gal plus EFC ($400 \, \text{mg/kg}$) and EFC treatment ($400 \, \text{mg/kg}$) group spent more time in the target quadrant than those in D-gal group (P < 0.05, Figure 5(c)) and crossed over the location of the previous platform more often than D-gal-treated rats (Figure 5(d))

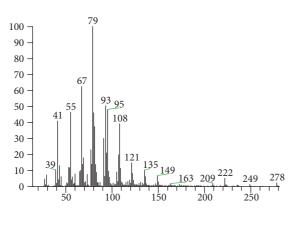
SI 923, RSI 941, replib, Entry# 7681, CAS# 60-33-3, 9,12-Octadecadienoic acid (Z, Z)-

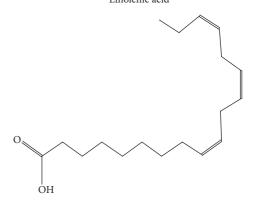


9,12-Octadecadienoic acid (Z, Z)-Formula C18H32O2, MW 280, CAS# 60-33-3, Entry# 7681 cis-9, cis-12-Octadecadienoic acid



9,12,15-Octadecatrienoic acid, (Z, Z, Z)-Formula C18H30O2, MW 278, CAS# 463-40-1, Entry# 44302 Linolenic acid





(b)
FIGURE 2: Graph of mass spectrometry. (a) 9,12-Octadecadienoic acid (ω6) and (b) 9,12,15-octadecatrienoic acid (ω3).

(P < 0.05). These results indicated that EFC treatment prevented the age-related memory deficits induced by D-gal.

Figure 5(e) illustrates representative path-length traces from the last day of navigation training. The rats in D-gal model group took much more time and traveled greater distances to reach the target platform and searched for the goal aimlessly. However, the rats in D-gal plus EFC (400 mg/kg) and EFC treatment (400 mg/kg) group reached the goal in less time and traveled a shorter distance than the D-gal-treated rats.

3.4. Effect of EFC on the SOD Activity and MDA Level in Hippocampus Tissue. To assess the effects of EFC on oxidative stress induced by D-gal, we examined SOD activities and MDA levels in the hippocampus. The activities of SOD in the D-gal model group were significantly lower than those of the control group (P < 0.05, Figure 6). However, the activities of SOD in D-gal plus EFC (400 mg/kg) and EFC treatment (400 mg/kg) group were significantly higher than that of the D-gal model group (P < 0.05). The levels of MDA in the D-gal group in the hippocampus were significantly higher (P < 0.05) than those of the normal control group.

Additionally, compared with D-gal group with elevated MDA level, the MDA levels of D-gal plus EFC (400 mg/kg) and EFC treatment (400 mg/kg) group notably reduced (P < 0.05).

3.5. Effect of EFC on the Activation of Astrocytes in Hippocampus Tissue. Astrocytes play critical roles in regulating synaptic transmission and synaptic plasticity which are associated with learning and memory. GFAP is a specific marker for activated astrocytes; thus the expression of GFAP in hippocampal dentate gyrus was examined in the current study. Immunofluorescent analysis showed an increased number of GFAP-stained cells in the dentate gyrus of D-gal treated group compared with control group (P < 0.05). However, treatment with EFC significantly reduced the number of GFAP-positive cells in EFC (400 mg/kg) group and D-gal plus EFC (400 mg/kg) group compared with D-gal treated rats (P < 0.05, Figure 7).

3.6. Effect of EFC on the Expression of the Aging-Related Protein. To investigate whether EFC has potential neuroprotective effect against brain aging, we examined the levels of presenilin 1 (PS1), total tau, and phosphorylated tau. Western

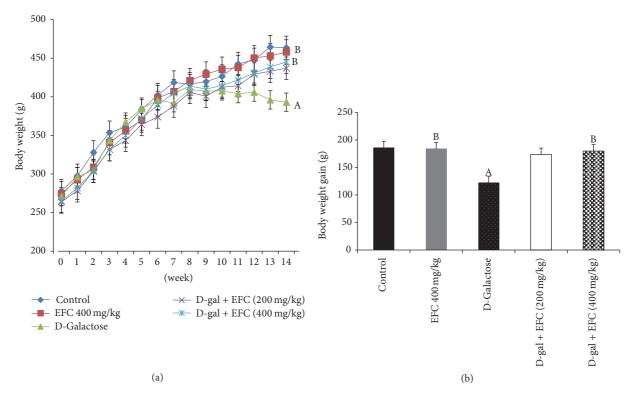


FIGURE 3: Effect of EFC on body weight and mean body weight gain in aging rats induced by D-gal. (a) Effect of EFC on body weight. (b) Mean body weight gain at the end of week 14. The results are presented as mean \pm SE (n = 8). $^{A}P < 0.05$ as compared to the normal control group. $^{B}P < 0.05$ as compared to the D-gal model group.

blot analysis indicated that the levels of PS1 and the phosphorylation of tau in the hippocampus of the D-gal administration group were significantly higher than those in the control group (P < 0.05, Figure 8). However, in D-gal plus EFC (400 mg/kg) and EFC treatment (400 mg/kg) group, expressions of PS1 and phosphorylated tau were significantly lower than those in the D-gal model group (P < 0.05), but there was no significant difference in total tau level between groups.

4. Discussion

In the current study, we accelerated aging and induced memory impairment by D-galactose in rats model for 14 weeks which mimics the natural aging process in humans [18]. D-gal-injected rats showed increased MDA levels and decreased SOD activities, compared with control group. Additionally, D-gal treatment resulted in significant decrease in organ indices, including heart, liver, thymus, and spleen. Furthermore, notably stimulated astrocytes reactivity indicated as increased GFAP level, as well as hyperphosphorylated tau and overexpression of presenilin 1, were found in the hippocampus of D-gal treated rats. These findings showed that brain oxidative stress, neuroinflammation, and aggregation of neurotoxic proteins were stimulated by D-gal. However, treatment with EFC could reverse these changes and attenuate the effect of aging on learning and memory in D-gal treated rats.

Successively subcutaneous injections of D-gal (100-500 mg/kg) for 8–12 weeks have been widely used to generate a senescence model for oxidative stress and antiaging research [19, 20]. The mechanism of D-gal-induced aging involves an accumulation of galactitol, the abnormal product of D-gal metabolism in cells which causes the generation of ROS [21]. Excessive ROS exposure results in the impairment or loss of enzyme activity and altered cell membrane permeability [22]. Thus, the accumulating galactitol could increase the osmotic stress in cell, which would result in impairment of cellular functions, increased metabolic disorders, and accelerated aging [23, 24]. The present study clearly demonstrates that administration of D-gal at 400 mg/kg per day for 14 weeks caused severe age-related appearance changes and a significant decrease in body weight gain, as well as consequent cognitive impairment associated with aging. However, long-term treatment with EFC partially alleviated these adverse effects.

We investigated the learning and memory ability of rats in a Morris water maze. D-gal-treated rats showed significantly longer escape latencies and distances to the hidden platform and less staying time, as well as more times crossing over the location of the previous platform. However, EFC treatment (400 mg/kg) could attenuate these outcomes. These data are in agreement with previous reports which suggested that the improvement of cognition might be the beneficial effect of polyunsaturated fatty acids enriched in the hempseeds [25, 26].

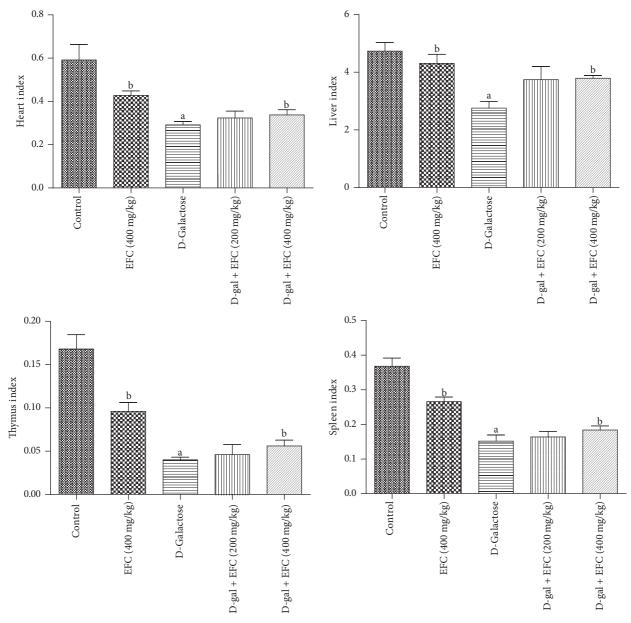


FIGURE 4: Effect of EFC on organ indices in aging rats induced by D-gal. The results are presented as mean \pm SE (n = 8). $^aP < 0.05$ as compared to the control group. $^bP < 0.05$ as compared to the D-gal model group.

We found that SOD activity was significantly decreased in D-gal treated rats, and MDA content was dramatically increased. However, EFC reversed these changes in the D-gal-treated rats by enhancing the antioxidization capacity. Senescence is the result of oxidative stress, according to the free radical theory of aging [27]. D-gal induces oxidative stress and generates ROS. D-gal overload may induce changes that mimic the normal aging process in rodents [28–30], and oxidative stress caused by D-gal has an important role in the age-associated cognitive decline [5, 31]. There is a growing consensus that SOD is an important antioxidant enzyme that acts as the first line of defense [32], and increased MDA is an important marker for lipid peroxidation, which is an indicator of oxidative damage of membranes. Hence,

increasing SOD viability and decreasing levels of MDA are beneficial for preventing oxidative damage [33].

After 14 weeks of D-gal injection, the heart and liver indices of rats were notably decreased, which implying that aging was accelerated. To the best of our knowledge, the heart and the liver are both important metabolic organs and the function of heart is to provide the pumping pressure that allows blood flow deliver adequate oxygen and nutrients to organs and tissues. The liver is the main part of biological oxidation, participating in glucose, lipid, and protein metabolism as well as immune defense. Meanwhile, the thymus and the spleen indices of rats were significantly decreased, indicating that D-gal can also cause the atrophy of immune organs, as well as the declined immune function,

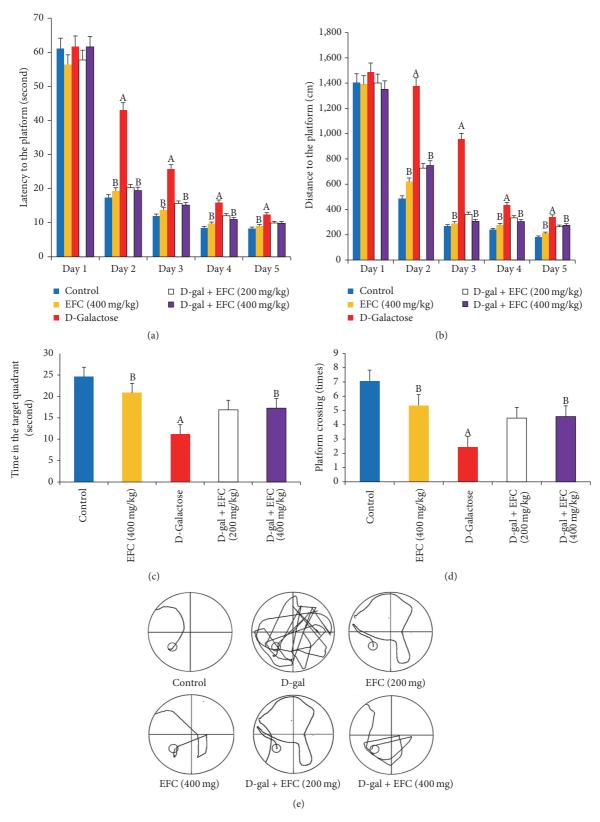


FIGURE 5: Effect of EFC on memory impairment induced by D-gal administration (n = 8 for each group). The Morris water maze test was carried out to access the spatial learning and memory ability of rats. (a) Latencies to find a hidden platform in the water maze during the 5 training days. (b) Mean distance to reach the hidden platform during the 5 training days. (c) Time spent in the target quadrant where the platform was located during training days in the probe test. (d) Numbers of crossings of the exact location of the previous platform during the probe test. (e) The representative traces during the probe test. The results are presented as mean \pm SE (n = 8). n = 80. As compared to the normal control group. Brown of the probability of

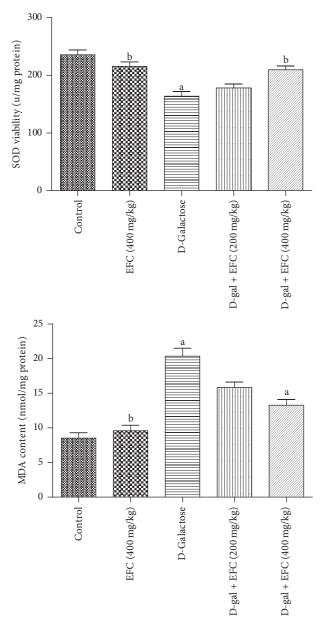


FIGURE 6: Effect of EFC on SOD activity and MDA levels in the brain of aging rats induced by D-galactose. The results are presented as mean \pm SE (n=4). $^aP<0.05$ as compared to the control group. $^bP<0.05$ as compared to the D-gal model group.

which may be one of the reasons of immune aging. These results support the concept that thymus is the first organ to be sensitive to aging in the body, and thymus atrophy is the most obvious immunologic change in aging [34]. Thus, the thymus and spleen indices could be used to evaluate aging [35]. Herein, as in D-gal-treated rats, EFC significantly increased the function of heart and liver as well as the immune system as Luo and colleagues observed in amnesic mice [36].

Astrocytes are the main components of glial cells in the nervous system, mounting evidence indicated that neuroinflammation is a result of activation of astrocytes which leads to the memory impairment in the D-gal aging mouse model [5]. Our result in the present study clearly demonstrated that 14 weeks of D-gal administration significantly stimulated astrocytes reactivity indicated as increased GFAP level. However, EFC treatment could reverse these changes, indicating that activation of astrocytes may trigger memory impairment and the reversal of activation of astrocytes by EFC treatment might be an outcome of the antineuroinflammation. Therefore, EFC improved the cognitive deficits induced by D-gal administration partly through the inhibition of inflammatory responses in brain.

To further investigate the possible mechanism of EFC in ameliorating the memory decline in D-gal administration rats, we examined the levels of total tau, phosphorylated tau, and PS1. Tau is a microtubule-associated protein; its main functions are to facilitate the assembly and maintenance of microtubules and to maintain axonal transport in neurons [37]. To the best of our knowledge, senile plaques and neurofibrillary tangle (NFT) are two main pathological features of AD and hyperphosphorylated tau is the main component of NFT [38, 39]. When tau is hyperphosphorylated and aggregates in the neuron, it becomes defective and no longer stabilizes microtubules properly [40]. Moreover, hyperphosphorylated tau forms paired helical filaments (PHF) that weaken its binding to microtubules [41]. The death of neurons and cognitive dysfunction are related to the excessive phosphorylation of tau protein. Furthermore, hyperphosphorylated tau is the main component of NFTs, and NFT formation is considered to be an early pathological change in AD [42]. In addition, the number of NFTs in the CA1 of hippocampus seems to be closely related to cognitive status [43]. By western blot analysis, we found tau phosphorylation at Ser396 in D-gal model group was significantly higher than that in the control group. However, EFC (400 mg/kg) significantly attenuated tau phosphorylation. These results showed EFC could reduce the phosphorylation level of tau to ameliorate memory impairment induced by D-gal.

The present study demonstrated that PS1 expression was markedly higher in the aging model than the control group, and that EFC (400 mg/kg) significantly decreased PS1 expression. The results indicated that treatment with EFC could improve spatial learning and memory in D-gal-treated rats by attenuating tau phosphorylation and by suppressing expression of PS1, which are related to brain aging. Mutations in PS1 are the major cause of the most aggressive form of familial Alzheimer's disease (FAD) which leads to defective learning and impaired memory [44, 45]. In addition, PS1 represents the catalytic components of protease complexes that directly cleave the amyloid precursor protein (APP) and is responsible for most amyloid β -peptide (A β) production. Alzheimer-associated mutations in PS1 increase production of A β 42 (the neurotoxic form of A β), resulting in deposition of A β and senile plaques which is a hallmark of AD [46]. Furthermore, PS1 mutant knockin mice have spatial memory deficits in the Morris water maze and PS1 mutations show age-dependent effects on synaptic plasticity and learning and memory [47]. On the other hand, the overexpression of PS1 can also affect the process of tau phosphorylation. The underlying mechanism lies in the interaction of tau and glycogen synthase kinase 3 beta (GSK-3 β), PS1 could directly bind

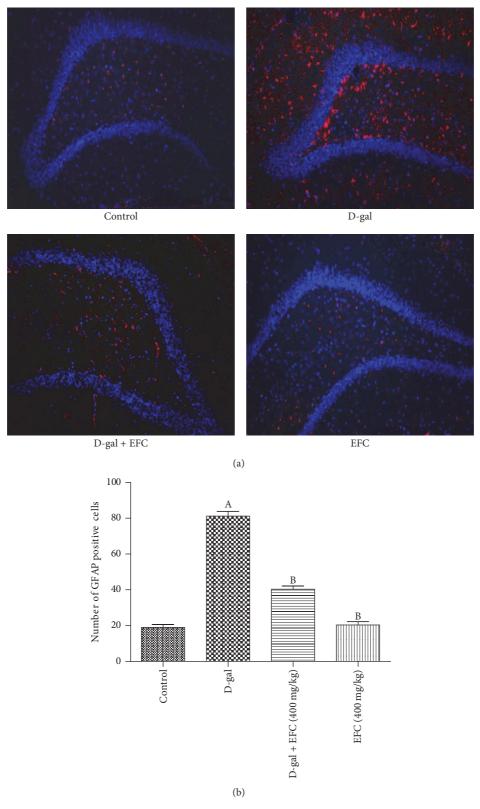


FIGURE 7: Effect of EFC on GFAP expression in the dentate gyrus of hippocampus in D-gal-treated rats measured by immunofluorescence (×100). (a) The images of immunofluorescence staining of GFAP (red), the scale bar represents 100 μ m. (b) The bar graph summarizing the number of GFAP-positive cells. All values are expressed as mean \pm SE (n=4), $^AP<0.05$ as compared to the normal control group. $^BP<0.05$ as compared to the D-gal model group.

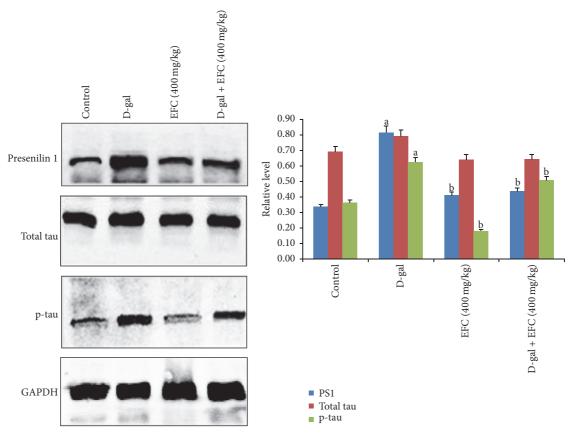


FIGURE 8: Effect of EFC on the expression of presenilin 1, total tau, and p-tau in the hippocampus of aging rats induced by D-gal. The relative protein levels between the tested target protein and internal standard GAPDH were calculated and labeled on y-axis. Data are expressed as the mean \pm SE (n = 4). $^aP < 0.05$ as compared to the normal control group. $^bP < 0.05$ as compared to the D-gal model group. The bands are from a representative blot. Lane 1: normal control group; lane 2: D-gal-treated group; lane 3: EFC treatment group; lane 4: D-gal plus EFC (400 mg/kg) treatment group.

tau and GSK-3 β to the same region of PS1, which regulates phosphorylation of tau [48]. Moreover, PS1 overexpression inhibited neurite growth and promoted neuritic dystrophy and tangle formation by interfering with Notch 1 signaling and enhancing pathological changes in tau [49].

5. Conclusion

In summary, we found that EFC mitigated the features of aging induced by D-gal in rats and improved behavioral performance in Morris water maze. The antiaging effects of EFC include reducing oxidative stress, improving immune function, and inhibiting the activation of astrocytes as well as attenuating tau phosphorylation and suppressing PSI expression (Figure 9). Therefore, our findings suggest that EFC would be useful for relieving age-related memory impairments.

Abbreviations

EFC: Extract of Fructus Cannabis

D-gal: D-Galactose
AD: Alzheimer's disease
HPLC-MS: High performance liquid

chromatography-mass spectrometry

MWM: Morris water maze SOD: Superoxide dismutase MDA: Malondialdehyde PUFA: Polyunsaturated fatty acid

EFAs: Essential fatty acids BDNF: Brain-derived neurotrophic factor GFAP: Glial fibrillary acidic protein

PS1: Presenilin-1

NFT: Neurofibrillary tangle.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Ning-Yuan Chen and Cheng-Wu Liu are equal contributors to this paper.

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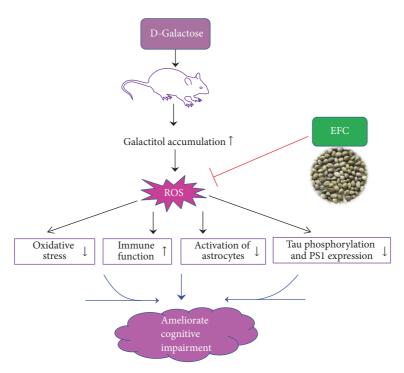


FIGURE 9: Proposed mechanism of the effect of EFC on ameliorating cognitive impairment induced by D-gal. EFC could relieve age-related cognitive impairment by attenuating oxidative stress, improving immune function, and inhibiting the activation of astrocytes as well as attenuating tau phosphorylation and suppressing PS1 expression in the brain of D-gal treated rats.

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Research Article

Salidroside Protects against MPP+-Induced Neuronal Injury through DJ-1-Nrf2 Antioxidant Pathway

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Parkinson's disease (PD) is the second most common neurodegenerative disorder. We have found that salidroside (Sal) exhibited neuroprotective effects against MPP+ toxicity. However, the molecular mechanism is not fully understood. In this study, we found that Sal significantly prevented MPP+-induced decrease of mRNA and protein expression of Nrf2, GCLc, SOD1, and SOD2 in SH-SY5Y cells. Moreover, silencing of Nrf2 significantly inhibited Sal-induced increase in mRNA and protein expression of GCLc, SOD1, and SOD2. But Nrf2 silence did not significantly impact Sal-exhibited effects on DJ-1 expression. Silencing of Nrf2 significantly suppressed the decrease of apoptosis induced by Sal in MPP+-treated SH-SY5Y cells. Sal significantly prevented MPP+-induced decrease of the mRNA and protein expression of DJ-1 in SH-SY5Y cells. Moreover, silencing of DJ-1 significantly inhibited Sal-induced increase in mRNA and protein expression of Nrf2, GCLc, SOD1, and SOD2 in MPP+-treated SH-SY5Y cells. These results indicated that DJ-1 was an upstream regulator of Nrf2 in the neuroprotective effects of Sal. Furthermore, silencing of DJ-1 significantly suppressed the decrease of apoptosis induced by Sal in MPP+-treated SH-SY5Y cells. In conclusion, Sal prevented MPP+-induced neurotoxicity through upregulation of DJ-1-Nrf2-antioxidant pathway. Our findings provide novel insights into the neuroprotective effects of Sal against PD.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting more than 1% of humans over 60 years of age [1, 2]. PD is attributed to loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and characterized by the presence of ubiquitinated alpha-synuclein- (α -syn-) containing cytoplasmic inclusions called Lewy bodies in surviving SNpc neurons [3, 4]. Although the accurate mechanism of PD is not known, oxidative injury and associated apoptotic cell death are believed to play an important role [2, 5, 6]. Large quantity of studies has shown that antioxidant intervention is effective for the treatment of PD and other neurodegenerative diseases [6]. However, clinical therapy of PD is limited by shortage of validated drugs [7, 8].

Salidroside (p-hydroxyphenethyl-b-D-glucoside; C14H20O7) (Sal) is a major component extracted from *Rhodiola rosea*

L., which is a plant popular in traditional medicine in Asian and Eastern European countries (Figure 1(a)). It is shown that Sal possesses a variety of pharmacological activities, including antioxidant, antiaging, antitumor, antihypoxic and neuroprotective effects [9–13]. Our previous studies have shown that Sal protects against apoptosis in SH-SY5Y cells by inhibiting the NO pathway [14]. We also show that Sal protects against PD in mouse model through ROS-NO-related mitochondrion pathway [15]. These results suggest that the antioxidant activity of Sal plays a pivotal role in the neuroprotective effects against PD. However, the mechanism underlying the neuroprotective effects of Sal is still far from being completely understood.

MPTP- (1-methyl-4-phenyl-1,2,5,6- tetrahydropyridine-) induced neuronal injury in neuron cells and neurodegenerative disorder in animals are usually used for the study

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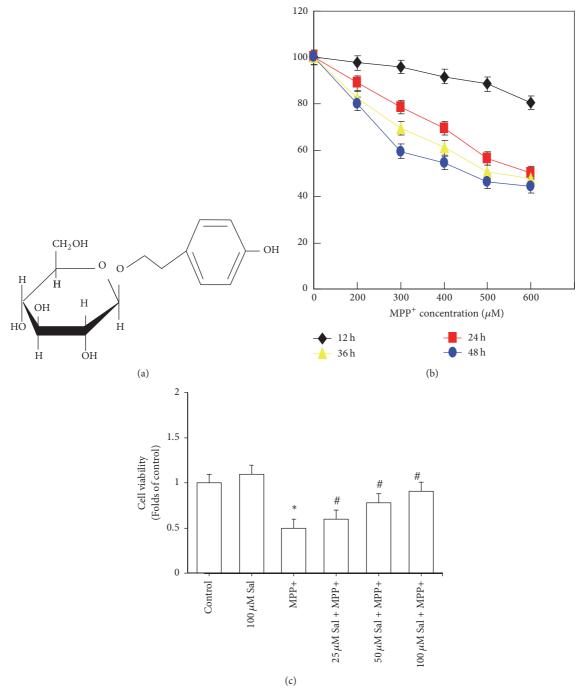


FIGURE 1: Effect of Sal on cell viability in MPP+-treated SH-SY5Y cells. SH-SY5Y cells were pretreated with 25–100 μ M Sal for 24 h and then exposed to 500 μ M MPP+ for an additional 24 h. At the end, cell viability was determined by MTT assay. * p < 0.05, compared with control. # p < 0.05, compared with MPP+ treated cells.

of the pathogenesis and treatment of PD [16–18]. The neurotoxin MPTP causes neurotoxicity through the active metabolite MPP+ (1-methyl-4-phenylpyridinium). MPP+ could be selectively taken up by dopaminergic neurons via the dopamine transporter of the plasma membrane [19]. MPP+ results in neuronal loss in substantia nigra and striatal dopamine (DA) depletion, leading to PD-like behavioral impairments [20, 21]. Moreover, oxidative stress is involved in MPP+-associated PD-like neuronal injury [22, 23].

DJ-1 is a novel antioxidant protein and abnormal regulation of DJ-1 is closely related to the development of PD [24–27]. In the present study, we aimed to investigate whether the neuroprotective activities of Sal against PD involve the regulation of DJ-1, using SH-SY5Y cells, a clonal human neuroblastoma cell line. We found that Sal protects against MPP+-induced apoptotic cell death in SH-SY5Y cells through regulation of DJ-1-nuclear factor- (erythroid-derived 2-) like 2 (Nrf2) pathway.

2. Materials and Methods

- 2.1. Chemicals and Materials. β -actin and Nrf2 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz). DJ-1, GCLc, SOD1, and SOD2 antibodies were obtained from Cell Signaling Technology (CST, USA). Hoechst 33258 and MPP⁺ were obtained from Sigma (St. Louis, MO). Sal was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Xi'an, China.
- 2.2. Cell Culture and Transfection. SH-SY5Y cells were purchased from American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagle medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated horse serum (Gibco), 5% heat-inactivated fetal calf serum (Gibco), 100 IU/ml penicillin, and 100 mg/ml streptomycin in a humidified incubator containing 5% CO₂ at 37°C. SH-SY5Y cells were treated with 50 ng/ml 2.5S nerve growth factor (Promega, Madison, WI, USA) for 9 days to induce differentiation. Cells were plated in 6-well plates and passaged at 60-70% confluence. In the experiments, cells were pretreated with 25–100 μ M Sal for 24 h and then exposed to 500 µM MPP+ for an additional 24 h. In some experiments, cells were transfected with siNrf2 or siDJ-1 (Santa Cruz Biotechnology) using lipofectamine 2000 transfection reagent 2-4 days before the treatment of Sal and MPP+.
- 2.3. Cell Viability. SH-SY5Y cells were seeded in 96-well plates at 1×10^4 cells per well. After the treatment with Sal and MPP+, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Aldrich) assay. Briefly, cells were incubated with 500 μ g/ml MTT at 37°C for 4 h. After that, the medium was removed and 150 μ l dimethyl sulfoxide (DMSO) was added and shaking was conducted for 10 min. Absorbance was measured at 570 nm in a microplate reader (BioRad, USA) and the results were expressed as folds of control.
- 2.4. Determination of Cell Death. Cell death was evaluated using an FITC-labeled Annexin V and propidium iodide (PI) assay kit (Sigma). In brief, after the treatment, cells were harvested and centrifuged and then resuspended in binding buffer at a concentration of 1×10^6 cells/ml. Then, the mixture was added with 5 μ l of 20 μ g/ml Annexin V and 50 μ g/ml PI and incubated in a humidified incubator for 15 min. After the binding, cell death was analyzed by a flow cytometer (BD Biosciences, CA, USA).
- 2.5. Evaluation of Cell Morphology. SH-SY5Y cells were plated on coverslips precoated with poly-L-lysine in 24-well plates and treated by Sal and MPP+ as mentioned above. After the treatment, cells were fixed with 4% paraformaldehyde for 30 min, washed with PBS, and then incubated with Hoechst 33258 (3 μ g/ml) for 30 min at room temperature in the dark. After rinsing with PBS, fluorescence was assessed using a fluorescence microscope (Olympus, Japan).
- 2.6. Reverse Transcriptase-Polymerase Chain Reaction Analysis (RT-PCR). mRNA was isolated from the cells using trizol-reagent (Invitrogen, USA) and the quality of isolated RNA was checked by 1.2% formaldehyde. The RT-PCR reaction

- used a template cDNA followed by PCR amplification with Taq DNA polymerase in the same tube. PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.
- 2.7. Western Blot Analysis. Cells were lysed in lysis buffer [50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5 mM MgCl₂, 10% glycerol, 1% Triton X-100, and 0.1% SDS] with protease inhibitor cocktail (Roche Diagnostics, Switzerland) on ice for 20 min. The lysates were centrifuged for 20 min at 20,000 g. Protein concentration was determined using BCA method (Thermo Fisher Scientific, USA) and lysates were mixed with loading buffer. The protein mixtures were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies and then incubated with HRP-conjugated secondary antibody (Thermo Fisher Scientific, USA). Bands were visualized by chemiluminescence reaction using an ECL detection system (Thermo Fisher Scientific, USA), followed by capture using BioRad Imaging Systems (BIORAD, USA).
- 2.8. Evaluation of Oxidative Stress. For the determination of ROS, cells were incubated with $1\,\mu\rm M$ 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate and acetyl ester (Thermo Fisher) prepared in Hank's Balanced Salt Solution for 20 min at 37°C and then washed for three times and scanned using a microplate fluorometer (Tecan) with Ex at 510 nm/Em at 580 nm and Ex at 495 nm/Em at 520 nm, respectively. Glutathione was measured using a luminescence-based system (GSH Assay, Promega, Madison, WI, USA). Cells were washed with Hank's Balanced Salt Solution and lysed with lysis regents. The GSH level was expressed as in relative light units (RLU)/mg protein.
- 2.9. Statistical Analysis. All assays were carried out in three independent experiments and the results were expressed as the mean \pm SEM and analyzed by GraphPad Prism software. The statistical significance of differences among more than two groups was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons. The data were deemed to be statistically significant when the p value was less than 0.05.

3. Results

3.1. Sal Protects against MPP+-Induced Cytotoxicity in SH-SY5Y Cells. SH-SY5Y cells are usually used as a neuron model for studies of MPP+ neurotoxicity and PD [28]. In the present study, we evaluated the neuroprotective effect of Sal using SH-SY5Y cells. Cells were exposed to $0-600 \,\mu\mathrm{M}$ MPP+ for 12-48 h and the results showed that MPP+ resulted in a significant decrease of cell viability in a concentration and time-dependent manner (Figure 1(b)). Cells were pretreated with $25-100 \,\mu\mathrm{M}$ Sal for 24 h and then exposed to $500 \,\mu\text{M}$ MPP+ for an additional 24 h. We showed that Sal concentration-dependently prevented MPP+-induced decrease of cell viability (Figure 1(c)). Annexin V/PI staining is a common method for the detection of apoptotic cell. We found that Sal significantly decreased the number of Annexin V/PI-stained cells treated by MPP+ which was in a concentration-dependent manner (Figure 2(a)). Apoptotic

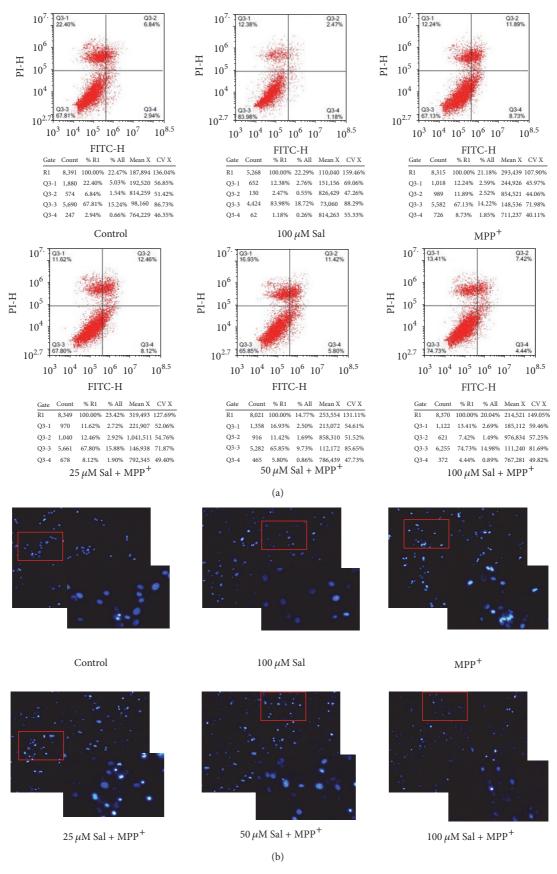


FIGURE 2: Effect of Sal on cell death in MPP+-treated SH-SY5Y cells. SH-SY5Y cells were pretreated with 25–100 μ M Sal for 24 h and then exposed to 500 μ M MPP+ for an additional 24 h. At the end, cell death was determined using Annexin V/PI assay kit and analyzed by flow cytometry (a). Cells were also stained with Hoechst to observe the morphological changes of nuclei (b).

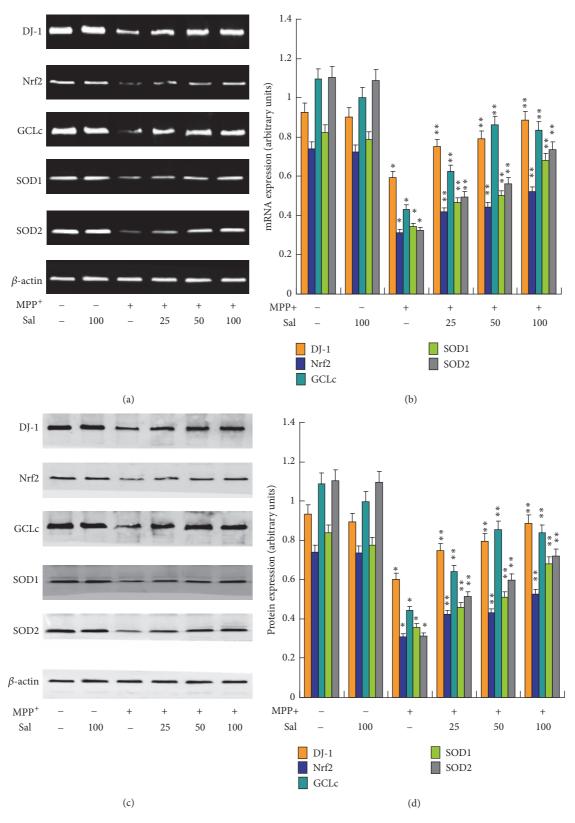


FIGURE 3: Effect of Sal on key antioxidant regulator expression in MPP+-treated SH-SY5Y cells. SH-SY5Y cells were pretreated with 25–100 μ M Sal for 24 h and then exposed to 500 μ M MPP+ for an additional 24 h. ((a) and (b)) mRNA expression of DJ-1, Nrf2, GCLc, SOD1, and SOD2 was determined and statistical analysis was showed. ((c) and (d)) Protein expression of DJ-1, Nrf2, GCLc, SOD1, and SOD2 was determined and statistical analysis was showed. *p < 0.05, compared with control. **p < 0.05, compared with MPP+.

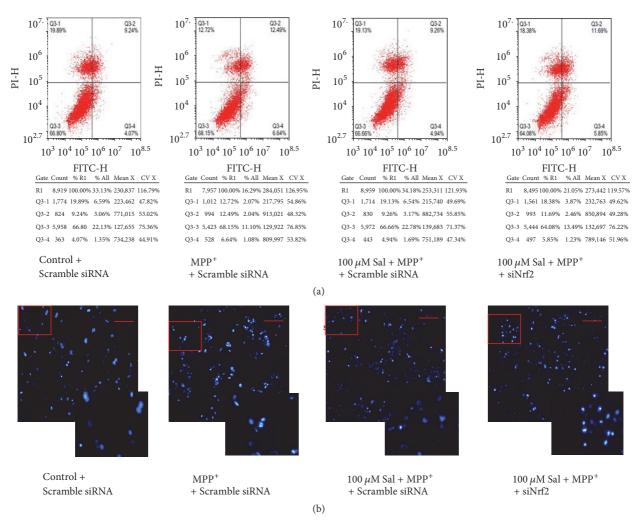


FIGURE 4: Role of Nrf2 in Sal-induced effects on cell death in MPP+-treated SH-SY5Y cells. SH-SY5Y cells were transfected with siNrf2, pretreated with 25–100 μ M Sal for 24 h, and then exposed to 500 μ M MPP+ for an additional 24 h. At the end, cell death was determined using Annexin V/PI assay kit and analyzed by flow cytometry (a). Cells were also stained with Hoechst to observe the morphological changes of nuclei (b).

cell could also be morphologically evaluated by Hoechst staining. In Hoechst staining, apoptotic cells are characterized by reduced nuclear size, chromatin condensation, intense fluorescence, and nuclear fragmentation. We showed that Sal notably inhibited MPP+-induced increase of chromatin condensation, intense fluorescence, and nuclear fragmentation in SH-SY5Y cells (Figure 2(b)). These results indicated that Sal protected against MPP+-induced cytotoxicity in SH-SY5Y cells.

3.2. Sal Protects against MPP+-Induced Changes of Antioxidant Protein Expression in SH-SY5Y Cells. To further examine the mechanism of Sal-exhibited neuroprotective effects, we examined the effect of Sal on the expression of key antioxidant proteins, including DJ-1, Nrf2, glutamate-cysteine ligase catalytic subunit (GCLc), superoxide dismutase 1 (SOD1), and SOD2. The results showed that MPP+ induced a significant decrease of the mRNA (Figures 3(a) and 3(b)) and protein (Figures 3(c) and 3(d)) expression of DJ-1, Nrf2, GCLc, SOD1, and SOD2. The treatment of Sal significantly

prevented MPP+-induced decrease of the mRNA (Figures 3(a) and 3(b)) and protein (Figures 3(c) and 3(d)) expression of DJ-1, Nrf2, GCLc, SOD1, and SOD2 in SH-SY5Y cells. The results indicated that Sal exhibited protective effects against MPP+-induced imbalance of redox state through regulation of key antioxidant proteins.

3.3. Upregulation of Nrf2 Is Involved in the Neuroprotective Effects of Sal against MPP+ Cytotoxicity. To examine whether regulation of Nrf2 was involved in the neuroprotective effects of Sal against MPP+ cytotoxicity, SH-SY5Y cells were transfected with siNrf2. We showed that silencing of Nrf2 significantly suppressed the decrease of Annexin V/PI-stained cells induced by Sal in MPP+-treated SH-SY5Y cells (Figure 4(a)). Sal-induced decrease of chromatin condensation, intense fluorescence, and nuclear fragmentation in MPP+-treated cells were inhibited by siNrf2 (Figure 4(b)). Moreover, silencing of Nrf2 significantly inhibited Sal-induced increase in mRNA (Figures 5(a) and 5(b)) and protein (Figures 5(c) and 5(d)) expression of Nrf2, GCLc, SOD1, and SOD2 in MPP+-treated

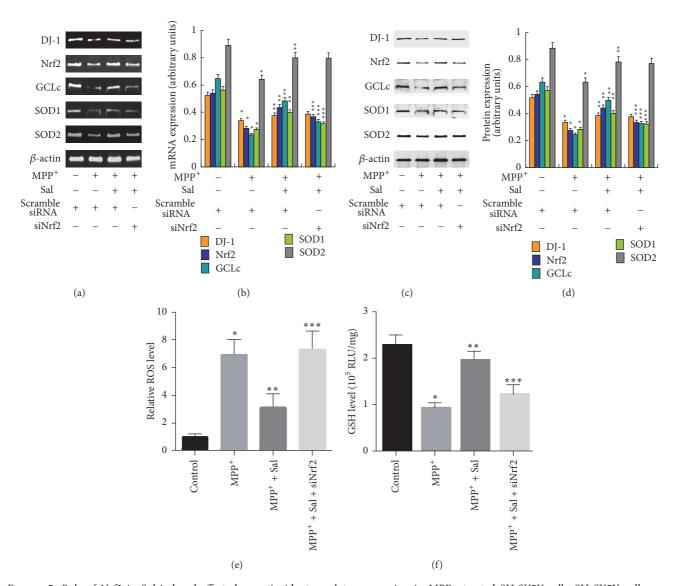


FIGURE 5: Role of Nrf2 in Sal-induced effects key antioxidant regulator expression in MPP+-treated SH-SY5Y cells. SH-SY5Y cells were transfected with siNrf2, pretreated with 25–100 μ M Sal for 24 h, and then exposed to 500 μ M MPP+ for an additional 24 h. ((a) and (b)) mRNA expression of DJ-1, Nrf2, GCLc, SOD1, and SOD2 was determined and statistical analysis was showed. ((c) and (d)) Protein expression of DJ-1, Nrf2, GCLc, SOD1, and SOD2 was determined and statistical analysis was showed. (e) ROS level was determined using DCFH-DA staining. (f) GSH level was determined by an assay kit. *p < 0.05, compared with control. **p < 0.05, compared with MPP⁺. ***p < 0.05, compared with Sal.

SH-SY5Y cells. However, Nrf2 silence did not significantly affect Sal-exhibited effects on DJ-1 expression (Figures 5(a), 5(b), 5(c), and 5(d)). Moreover, Sal inhibited MPP+-induced increase of reactive oxygen species (ROS) (Figure 5(e)) and decrease of glutathione (GSH) level (Figure 5(f)). Silencing of Nrf2 significantly inhibited Sal-induced decrease ROS level and increase of GSH level in MPP+-treated cells (Figures 5(e) and 5(f)). The results indicated that Nrf2-regulated antioxidant enzymes were involved in the neuroprotective effects of Sal against MPP+ toxicity.

3.4. Upregulation of DJ-1 Is Involved in the Neuroprotective Effects of Sal against MPP+ Cytotoxicity. SH-SY5Y cells were further transfected with siDJ-1 to examine whether regulation

of DJ-1 was involved in the neuroprotective effects of Sal against MPP+ cytotoxicity. The results showed that silencing of DJ-1 significantly suppressed the decrease of Annexin V/PI-stained cells induced by Sal in MPP+-treated SH-SY5Y cells (Figure 6(a)). Moreover, Sal-induced decrease of chromatin condensation, intense fluorescence, and nuclear fragmentation in MPP+-treated cells were inhibited by siDJ-1 (Figure 6(b)). Furthermore, silencing of DJ-1 significantly inhibited Sal-induced increase in mRNA (Figures 7(a) and 7(b)) and protein (Figures 7(c) and 7(d)) expression of DJ-1, Nrf2, GCLc, SOD1, and SOD2 in MPP+-treated SH-SY5Y cells. Moreover, silencing of DJ-1 significantly inhibited Sal-induced decrease of ROS level and increase of GSH level in MPP+-treated cells (Figures 7(e) and 7(f)). The results

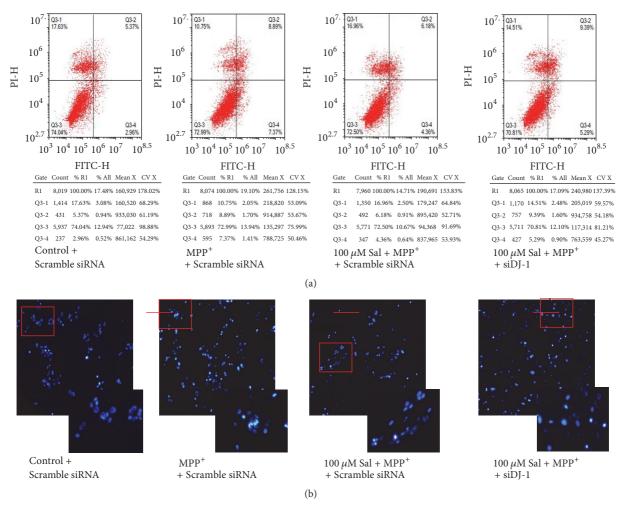


FIGURE 6: Role of Nrf2 in Sal-induced effects on cell death in MPP+-treated SH-SY5Y cells. SH-SY5Y cells were transfected with siDJ-1, pretreated with 25–100 μ M Sal for 24 h, and then exposed to 500 μ M MPP+ for an additional 24 h. At the end, cell death was determined using Annexin V/PI assay kit and analyzed by flow cytometry (a). Cells were also stained with Hoechst to observe the morphological changes of nuclei (b).

indicated that DJ-1 was an upstream regulator of Nrf2-antioxidant enzymes, which were involved in the neuroprotective effects of Sal against MPP+ toxicity.

4. Discussion

Our previous studies have found that Sal exhibited neuroprotective effects against MPP+ toxicity in vitro and in vivo [14, 15, 29]. However, the molecular mechanism underlying this beneficial effect is far from being completely understood. We explored the antioxidant mechanism of Sal-possessed neuroprotective effects in SH-SY5Y cells.

Increase of ROS generation and oxidative stress is considered to be a common pathway in the development of PD and its associated diseases [30–32]. It is well known that Nrf2 is central regulator of redox status in various biological processes. Nrf2 can bind with antioxidant response element, controlling the transcription of a battery antioxidant enzymes, including GCL and SOD [33]. SOD functions to catalyze the transition of O^{2-} to oxygen and H_2O_2 . SOD1 (cytoplasmic copper and zinc SOD, Cu/ZnSOD) and SOD2

(mitochondrial manganese SOD, MnSOD) are the main types of SOD in organisms. GCLc is the catalytic subunit of GCL, which is the rate-limiting enzyme responsible for catalyzing de novo synthesis of glutathione (GSH) from the precursor amino acids cysteine, glutamate, and glycine [34]. GSH is the most important hydrophilic antioxidant and plays a critical role in various cellular processes through direct detoxification or acting as a cofactor. Abnormal expression of Nrf2, GCLc, SOD1, and SOD2 has been reported to be associated with PD and other neurodegenerative diseases [35–40]. In the present study, we tested the possible role of Nrf2 and related antioxidant enzymes in the protective effects of Sal. We found that Sal significantly prevented MPP+induced decrease of the mRNA and protein expression of Nrf2, GCLc, SOD1, and SOD2 in SH-SY5Y cells. Moreover, silencing of Nrf2 significantly inhibited Sal-induced increase in mRNA and protein expression of GCLc, SOD1, and SOD2 in MPP+-treated SH-SY5Y cells. But Nrf2 silence did not significantly affect Sal-exhibited impacts on DJ-1 expression, indicating that DJ-1 was not a downstream regulator of

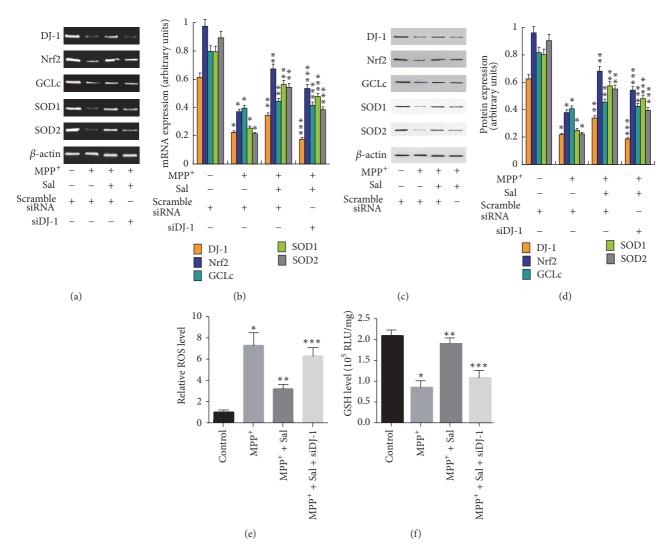


FIGURE 7: Role of Nrf2 in Sal-induced effects on key antioxidant regulator expression in MPP+-treated SH-SY5Y cells. H-SY5Y cells were transfected with siDJ-1, pretreated with 25–100 μ M Sal for 24 h, and then exposed to 500 μ M MPP+ for an additional 24 h. ((a) and (b)) mRNA expression of DJ-1, Nrf2, GCLc, SOD1, and SOD2 was determined and statistical analysis was showed. ((c) and (d)) Protein expression of DJ-1, Nrf2, GCLc, SOD1, and SOD2 was determined and statistical analysis was showed. (e) ROS level was determined using DCFH-DA staining. (f) GSH level was determined by an assay kit. *p < 0.05, compared with control. **p < 0.05, compared with MPP⁺. ***p < 0.05, compared with Sal.

Nrf2 signaling. Silencing of Nrf2 significantly suppressed the decrease of apoptosis induced by Sal in MPP+-treated SH-SY5Y cells. The results demonstrated that Nrf2-regulated antioxidant enzymes were involved in the neuroprotective effects of Sal.

DJ-1 is a novel antioxidant regulator that could protect cells from oxidative stress and induce Nrf2 expression [41]. Recent evidence has also suggested that abnormal function of DJ-1 plays a role in the pathogenesis of PD [42–44]. In this study, we also examined the role of DJ-1 in neuroprotective effects of Sal. We found that Sal significantly prevented MPP+-induced decrease of the mRNA and protein expression of DJ-1 in SH-SY5Y cells. Moreover, silencing of DJ-1 significantly inhibited Sal-induced increase in mRNA and protein expression of Nrf2, GCLc, SOD1, and SOD2 in

MPP+-treated SH-SY5Y cells. These results indicated that DJ-1 was an upstream regulator of Nrf2 in neuroprotective effects of Sal. Furthermore, silencing of DJ-1 significantly suppressed the decrease of apoptosis induced by Sal in MPP+-treated SH-SY5Y cells. The results demonstrated that DJ-1-regulated Nrf2 antioxidant pathway was involved in the neuroprotective effects of Sal.

In conclusion, we found that Sal prevented MPP+induced neurotoxicity through upregulation of DJ-1-Nrf2-antioxidant pathway. Further studies are needed to investigate whether DJ-1 exhibits a transcriptional regulation of Nrf2 and those antioxidant enzymes or affects Nrf2-antioxidant enzymes through indirect regulation. Overall, our findings provide novel insights into the neuroprotective effects of Sal against PD and associated neurodegenerative diseases.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Research Article

Passiflora cincinnata Extract Delays the Development of Motor Signs and Prevents Dopaminergic Loss in a Mice Model of Parkinson's Disease

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Passiflora cincinnata Masters is a Brazilian native species of passionflower. This genus is known in the American continent folk medicine for its diuretic and analgesic properties. Nevertheless, few studies investigated possible biological effects of P. cincinnata extracts. Further, evidence of antioxidant actions encourages the investigation of possible neuroprotective effects in animal models of neurodegenerative diseases. This study investigates the effect of the P. cincinnata ethanolic extract (PAS) on mice submitted to a progressive model of Parkinson's disease (PD) induced by reserpine. Male (6-month-old) mice received reserpine (0.1 mg/kg, s.c.), every other day, for 40 days, with or without a concomitant treatment with daily injections of PAS (25 mg/kg, i.p.). Catalepsy, open field, oral movements, and plus-maze discriminative avoidance evaluations were performed across treatment, and immunohistochemistry for tyrosine hydroxylase was conducted at the end. The results showed that PAS treatment delayed the onset of motor impairments and prevented the occurrence of increased catalepsy behavior in the premotor phase. However, PAS administration did not modify reserpine-induced cognitive impairments. Moreover, PAS prevented the decrease in tyrosine hydroxylase immunostaining in the substantia nigra pars compacta (SNpc) induced by reserpine. Taken together, our results suggested that PAS exerted a neuroprotective effect in a progressive model of PD.

1. Introduction

The genus *Passiflora* (Passifloraceae) comprises species noted by their edible fruits, exotic flowers, and use in folk medicine for sedative, anxiolytic, diuretic, and analgesic effects [1–5]. The phytochemical profile of the species of this genus is complex. Phenols, cyanogenic glycosides, alkaloids, and flavonoids can be found in their composition and can be

responsible for their pharmacological effects [2]. These compounds could also be related to biological activities of these plants such as anti-inflammatory [6], sedative [7], antihyperglycemic [8], antiulcer [9], anxiolytic [10–13], and antioxidant [9, 14–16] actions. David and colleagues [17] reported higher antioxidant action and lower toxicity of the *Passiflora cincinnata* methanolic extract when compared to other plants from Brazilian *Caatinga*. In addition, Wondracek

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and colleagues [18] detected carotenoids compounds such as neoxanthin, trans-violaxanthin, antheraxanthin, lutein, zeaxanthin, and trans- β -carotene in the *P. cincinnata* fruit pulp. In this respect, carotenoids can modulate intracellular signaling cascades associated with inflammatory cytokines and antioxidant enzymes production. These modulatory activities lead to antioxidant, antiapoptotic, and anti-inflammatory effects that may represent an important improvement in the treatment of neurodegenerative disorders [19].

Neurodegenerative disorders such as Parkinson's and Alzheimer's diseases show severe social and economic challenges, with high impact on public health systems around the world. PD is a progressive and degenerative neurological pathology, characterized by neuronal loss in multiple brain regions, but mostly dopaminergic neurons in the substantia nigra pars compacta (SNpc) [20, 21]. Additionally, the neuropathology of this disease is characterized by the formation of intraneuronal protein clusters of α -synuclein, referred to as Lewy's bodies [22-24]. The degeneration in SNpc cells and consequent dopaminergic depletion in the striatum result in the classic motor symptoms of PD: resting tremor, rigidity, postural instability, and bradykinesia [20, 25, 26]. This depletion may be result of an imbalance between the production of prooxidants (e.g., reactive oxygen species) and endogenous antioxidant agents (e.g., catalase and glutathione), which generates cellular machinery damage, leading to events such as endoplasmic reticulum or mitochondrial dysfunctions, protein degradation, and apoptosis [27].

Dopaminergic medications used in the treatment of patients with Parkinson's disease are associated with motor and nonmotor behavioral side effects. The dopamine precursor 3,4-dihydroxyphenylalanine (levodopa or L-dopa) is the most efficient treatment to control motor deficits of PD patients [28, 29]. However, patients treated with levodopa (up to 80%) develop side effects such as dyskinesia and motor fluctuations due to the on-off effect [29–31]. In addition, current available treatments do not reduce or preclude neurodegeneration [32, 33].

Over the years, the use of animal models to evaluate neurochemical and neuropathological aspects of PD has been critical to the understanding of PD's etiology, as well as the validation of potential treatments. The chronic administration of a low dose of reserpine (0.1 mg/kg, s.c.) has been proposed as a progressive pharmacological model of PD in rats [34] and mice [35], mainly because this protocol gradually provokes motor and nonmotor impairments mimicking the progressive nature of the PD symptoms [36]. The alkaloid reserpine induces monoamine depletion, oxidative stress, inflammation, proapoptotic commitment, reduction in tyrosine hydroxylase levels, increase in α -synuclein immunostaining, and upregulation of DA receptors [36]. In addition, this protocol induces progressive motor impairments preceded by cognitive deficits [35, 37], which is consistent with the general development of the disease in humans. The aim of the present study was to evaluate the effects of the ethanolic extract of Passiflora cincinnata on motor, cognitive, and neuronal parameters in mice submitted to repeated treatment with reserpine.

2. Material and Methods

2.1. Ethanolic Extract Preparation. Leaves of Passiflora cincinnata were collected in Moita Bonita city, Sergipe state, Brazil. Professionals from the University of Sergipe Herbarium identified the specimen. A voucher specimen (ASE 11,112) has been deposited in the herbarium of the institution for reference. After identification, samples were dried at 37°C in an oven with air renewal and airflow for 48 h until complete dehydration. The material was crushed with a knife mill and subsequently powdered (293.3 g). Afterwards, it was extracted by maceration at room temperature in 90% ethanol for 5 days. The extract was filtered in vacuum, and the solvent was removed using a rotary evaporator (45°C) under reduced pressure and freeze-dried, yielding the ethanolic extract of the PAS (EEPc). The percentage of EEPc yield was 35.2%.

2.2. Animals. Six-month-old male Swiss mice were housed in groups of 6–8 per cage $(30 \times 37 \times 16 \, \mathrm{cm})$ under conditions of acoustic isolation, controlled airflow and temperature $(25 \pm 1^{\circ}\mathrm{C})$, and a 12 h light/dark cycle (lights on 6:30 a.m.) with food and water available ad libitum. Animals used in this study were handled in accordance with Brazilian law for the use of animals in research (Law Number 11.794) and the local ethics committee for animal usage approved all the procedures (Protocol CEUA/UFRN number 003/2013). All efforts were made to minimize animal pain, suffering, or discomfort during treatment.

2.3. Drugs Treatment and General Procedures. Reserpine (RES, Sigma Chemical Co., USA) was dissolved in glacial acetic acid (1%) and then diluted to the correct concentration with distilled water. Vehicle consisted of the same amount of acetic acid and water as in the reserpine solution. Both reserpine and vehicle were injected subcutaneously (s.c.) in a volume of 10 mL/kg.

Before the beginning of experimental procedures, animals were gently handled for 10 min for 5 consecutive days. The apparatuses were cleaned with 5% alcohol solution after each behavioral session and all behavioral data were registered and analyzed by the video-tracking software Anymaze (Stoelting, USA), except for the catalepsy test and the oral movement's evaluation that were manually registered by researchers blind to treatment.

2.4. Experimental Design. Mice were randomly assigned to one of four groups: CTR/CTR (n=13), CTR/PAS (n=15), RES/CTR (n=17), and RES/PAS (n=16). Animals received subcutaneous injections of vehicle (CTR) or 0.1 mg/kg of reserpine (RES) at a volume of 10 mL/kg body weight, every 48 h for 40 days of treatment. Moreover, mice received daily intraperitoneal injections of PAS vehicle (CTR) or 25 mg/kg of extract at a volume of 10 mL/kg body weight for 40 days. Animals did not show signs of intoxication, weight lost, pain, or discomfort during the treatment, showing good biological tolerability of the PAS ethanolic extract.

Animals were submitted to the following procedures before the daily injections (between 8:00 a.m. and 4:00 p.m.): (1) catalepsy test; (2) assessment of oral movements 48 h after

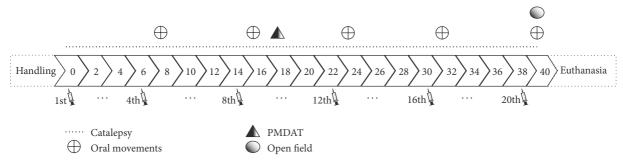


FIGURE 1: Schematic representation of the neuroprotective evaluation of PAS administration in mice.

the 4th, 8th, 12th, 16th, and 20th injections; (3) discriminative avoidance task 24 h before (training) and after (testing) the 10th injection; (4) evaluation of open field behavior 48 h after the 20th injection. Experimental design is shown in Figure 1.

2.5. Behavioral Tests

2.5.1. Catalepsy Test. The catalepsy behavior was assessed by placing the animal's forepaws on a horizontal bar positioned 5 cm above the bench surface. Catalepsy was defined as an immobile posture (keeping both forepaws on the bar) and was measured up to a maximum of 180 s. Three trials per animal in each observation day were carried out, and the results were analyzed considering the mean value of these trials.

2.5.2. Oral Movements. Mice were individually placed in a transparent glass box $(20 \times 20 \times 15 \, \text{cm})$ with mirrors positioned under and behind it to allow behavioral quantification when the animal faced away from the observer. The frequency of vacuous chewing movement (mouth openings in the vertical plane not directed toward physical material) and duration (s) of twitching of the facial musculature were measured continuously for 10 min.

2.5.3. Plus-Maze Discriminative Avoidance Task (PMDAT). The apparatus employed is a modified elevated plus-maze, made of wood, containing two open arms $(27.5 \times 6.5 \text{ cm})$ opposite to two enclosed arms (27.5 \times 6.5 \times 18 cm). One lamp and one loudspeaker were placed over one of the enclosed arms (aversive enclosed arm). In each side of the apparatus, there were different extramaze visual cues that animals could use to distinguish the location of different arms of the maze. Two behavioral sessions were performed in each experiment. During the training session, mice were placed individually in the center of the apparatus facing the open arms intercept and, over 10 min, every time animals entered in the aversive enclosed arm they received aversive stimuli until leaving the arm. These aversive stimuli were noise (80 dB) and light (100 W). The test session was carried out 24 h after the training session. In this test, mice were again placed in the apparatus for 5 min, without receiving any aversive stimulation. Animals were considered to be in a certain arm when the four paws passed over its entrance [38].

In both sessions, the amount of time spent in each enclosed arm (aversive and nonaversive) was registered and compared in each group for learning and memory evaluation. Moreover, locomotor activity and anxiety-like behavior were evaluated by total distance travelled in the apparatus and percentage of time spent on open arms (% TOA, time spent in open arms divided by the summation of time spent in open and enclosed arms), respectively. Further, in the training session, ethological components of risk assessment were evaluated by the following parameters: head dipping (frequency of movements of the head toward the floor), categorized into protected head dipping (PHD, when performed from the center of apparatus) and unprotected head dipping (UHD, when performed from the open arms), and stretched attend postures (SAP, defined by stretching and contraction of body to its original position without locomotion).

2.5.4. Open Field. At the final day of the protocol animals were submitted to the open field in order to evaluate locomotor and exploratory activities. The apparatus was a circular arena (50 cm in diameter) with 40 cm high walls, made of wood and painted black. Animals were placed in the center of the apparatus for free exploration during 10 min. Distance travelled in the whole arena (m), average speed (m/s), and time spent in the center of the open field (s) were evaluated.

2.5.5. Tissue Processing. Upon completion of the behavioral procedures, animals were deeply anesthetized with intraperitoneal injection of thiopental sodium (100 mg/kg) and perfused transcardially with 100–150 mL phosphate-buffered saline (PBS), pH 7.4, containing 0.2% heparin, followed by 150 mL PBS with 4% paraformaldehyde 0.1 M. The brains were removed from the skull and postfixed in the same fixative solution previously described and stored at 4°C. After 24 hours, we transferred the brains to a solution containing sucrose 30% 0.1 M PBS, at 4°C. Each brain was fixed in Tissue-Tek® (Sakura, Japan) at -20° C. Then we serially sliced the brains in the coronal plane into 30 μ m thick sections with a cryostat microtome (Leica, Germany) at a temperature of -20° C.

2.5.6. Tyrosine Hydroxylase (TH) Immunohistochemistry. Following tissue processing, we performed immunohistochemistry for TH, using a free-floating protocol. Sections

were washed out 4 times with PBS (pH 7.4) for 5 min each and consecutively washed with 0.3% H₂O₂ solution for 20 min to reduce endogenous peroxidase activity. For the detection of TH, sections were incubated with rabbit anti-tyrosine hydroxylase polyclonal antibody (cat # AB152 Chemicon, USA, 1: 10,000). The antibody was diluted in triton x-100 0.4% and PBS with 2% albumin serum, for 18-24 h at room temperature. Afterwards, sections were incubated with goat biotinylated anti-rabbit IgG (Vector Labs, USA, 1:5,000) diluted with triton x-100, 0.4% NaCl, and PBS for 2h at room temperature. Then, a new washout process was carried out followed by an incubation with avidin-biotin-peroxidase solution (ABC Elite kit, Vector Labs, Burlingame, USA). The reaction was developed by adding of 3,3-diaminobenzidine (DAB, Sigma-Aldrich, USA) and 0.01% H₂O₂ 0.1 M phosphate buffer solution for 1-2 min. Then, we left sections to dry, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped with Entellan (Merck). All sections were immunostained concomitantly, to minimize possible background differences between samples. Sections were examined under brightfield illumination with an optical microscope (Nikon Eclipse Ni-E), attached with a digital camera (Motic 5.0) to record images.

In order to estimate the number of TH+ cells in SNpc and TH levels in striatum, four sections of each animal were selected for each region evaluated (SNpc and striatum, n =4–7 per group): one at the rostral level, two at medium level, and one at caudal level, representative of the rostrocaudal extension of each area of interest. The exact location of the regions was determined on the basis of the Paxinos and Franklin [39] mice brain atlas. These sections were chosen by a systematic sampling and all measurements were performed in a blind manner. All TH+ cells of SNpc on each section were counted and the mean of the four measures was registered. Additionally, TH+ levels in striatum fibers were assessed by analysis of relative optical densitometry (ROD), using ImageJ software (version 1.48, NIH, USA). For this purpose, we transform our images in 8-bit color grade (i.e., grayscale), and four random fields were chosen in the target area (dorsal striatum). The mean values of gray level in the target areas were subtracted from the mean value of a control region (used to assess "noise" or nonspecific staining, i.e., cortex or corpus calosum). Finally, all values were normalized considering the control group mean value, in order to evaluate proportional alterations.

2.6. Statistical Analysis. Data normality and the homogeneity of variances were, respectively, tested by the Shapiro-Wilk and Levene's tests. All comparisons among groups for locomotor parameters and anxiety-like behaviors from PMDAT were performed by one-way ANOVA followed by Dunnett's test, whereas learning and memory parameters were analyzed by the paired-samples t-test. Catalepsy behavior and oral movements were compared between groups across treatment period using ANOVA with repeated measures followed by Tukey's test. In the open field test, parameters were compared between groups using one-way ANOVA followed by Tukey's test. Results were expressed as mean \pm SEM and p < 0.05 was considered to reflect significant differences.

3. Results

3.1. Catalepsy Behavior. ANOVA with repeated measures revealed time versus treatment interaction $[F_{(20.60)} = 6.365,$ p < 0.001]. Post hoc analysis showed that repeated treatment with reserpine induced progressive increase in the duration of catalepsy behavior, with RES-treated animals being significantly different from control group from the 26th (RES/CTR) and the 30th (RES/PAS) days onwards (see Figure 2(a)). No differences were found considering chronic administration of PAS per se (CTR/PAS). To clarify the differences between the groups we subdivided the treatment length in three phases: basal (beginning of procedures to 12th day), premotor (14th to 26th day), and motor (28th to 40th day) phases. This new analysis, now subdivided by phases, revealed time × treatment interactions for premotor $[F_{(6,18)} = 4,942, p <$ 0.001] and motor phases $[F_{(6,18)} = 3,534, p < 0.001]$. Post hoc analysis revealed significant increased catalepsy in RES/ CTR compared to CTR/CTR and RES/PAS groups in the premotor phase (Figure 2(c)). In the motor phase both RES/ CTR and RES/PAS groups showed increased catalepsy time when compared to CTR/CTR group (Figure 2(d)).

3.2. Oral Movements. ANOVA with repeated measures revealed effect of treatment $[F_{(3,55)}=14.112,\ p<0.001]$ for duration of oral twitching. We found a significant increase in RES/CTR group when compared to CTR on the 16th (48 h after the 8th injection, p<0.01) and 40th (48 h after the 20th injection, p<0.01) days, and RES/PAS group showed increase in 16th, 24th, and 40th days (p<0.01) (Figure 3(a)).

For number of vacuous chewing movements, ANOVA with repeated measures revealed time \times treatment interaction [$F_{(4,12)} = 2.247$, p < 0.05]. Indeed, animals receiving reserpine (RES/CTR and RES/PAS) showed a significant increase when compared to control groups in all assessment days (Figure 3(b)).

3.3. Plus-Maze Discriminative Avoidance Task (PMDAT). No differences were found in total distance travelled in the training $[F_{(3,26)} = 1.708, p = 0.193]$ and test $[F_{(3,26)} = 1.672, p = 0.201]$ sessions (Table 1).

No differences were found in % TOA [$F_{(3,26)} = 0.407$, p = 0.749], UHD [$F_{(3,26)} = 1.291$, p = 0.301], and PHD [$F_{(3,26)} = 2.171$, p = 0.119]. However, one-way ANOVA revealed an increase in CTR/PAS values of SAP [$F_{(3,26)} = 3.175$, p < 0.05] when compared to the CTR/CTR group (Table 1).

In the training session, paired-samples t-test showed that all groups spent more time in the nonaversive enclosed arm indicating that all animals learned the task [CTR/CTR: $t_{(3)} = 4.501$, p < 0.05, CTR/PAS: $t_{(5)} = 3.090$, p < 0.05, RES/CTR: $t_{(7)} = 6.404$, p < 0.001, and RES/PAS: $t_{(8)} = 7.903$, p < 0.001] (Figure 4(a)). However, in the test session, only CTR/CTR and CTR/PAS groups remembered the learned task [CTR/CTR $t_{(3)} = 4.107$, p < 0.05, CTR/PAS $t_{(5)} = 2.302$, p = 0.07] (Figure 4(b)).

3.4. Open Field Test. In this test, no differences were found for total distance travelled $[F_{(3,55)}=2.670,\ p=0.057]$ and time spent in the center of open field $[F_{(3,55)}=0.322,\ p=0.809]$.

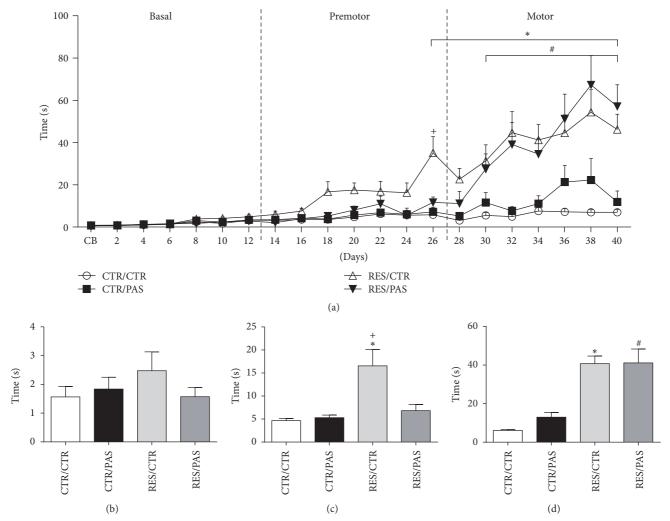


FIGURE 2: Effects of repeated administration of *Passiflora cincinnata* extract (25 mg/kg) and reserpine (0.1 mg/kg) on catalepsy behavior of mice. (a) Entire treatment analysis, (b) basal phase, (c) premotor phase, and (d) motor phases. Data are expressed as mean \pm SEM. *p < 0.05 RES/CTR compared to CTR/CTR; *p < 0.05 RES/PAS compared to CTR/CTR; and *p < 0.05 RES/CTR compared to RES/PAS (repeated measures ANOVA followed by Tukey's post hoc test).

Table 1: Effects of repeated administration of *Passiflora cincinnata* (25 mg/kg) and reserpine (0.1 mg/kg) on total distance travelled (training and test sessions) and anxiety-like parameters (training session) in plus-maze discriminative avoidance task.

Treatment	Total distance travelled (meters)		Anxiety-like parameters (frequency)			
	Training	Test	% TOA	SAP	PHD	UHD
CTR/CTR	11.82 ± 2.99	$9.41 \pm 4,04$	7.27 ± 2.74	26.25 ± 2.50	5.75 ± 1.38	8.00 ± 3.70
CTR/PAS	14.23 ± 2.50	9.56 ± 3.24	19.58 ± 5.63	$42.50 \pm 4.06^*$	14.17 ± 4.44	24.00 ± 9.47
RES/CTR	9.64 ± 1.20	4.96 ± 1.68	18.92 ± 10.80	38.38 ± 3.02	6.63 ± 1.66	10.38 ± 3.22
RES/PAS	9.41 ± 1.09	4.18 ± 0.86	14.13 ± 4.52	35.44 ± 2.76	8.22 ± 1.28	17.78 ± 5.27

Data expressed as mean ± SEM. * P < 0.05 compared to CTR/CTR (one-way ANOVA followed by Tukey's post hoc test). % TOA: percentage of time spent on open arms, SAP: stretched attend postures, PHD: protected head dipping, UHD: unprotect head dipping.

However, reserpine groups (RES/CTR and RES/PAS) showed a decrease in the average speed $[F_{(3,55)} = 7.152, p < 0.001]$ (Figure 5).

3.5. Tyrosine Hydroxylase Immunohistochemistry. For the number of TH+ cells in SNpc, one-way ANOVA revealed

significant differences between groups $[F_{(3,21)} = 7.329, p < 0.005]$. Post hoc analysis revealed a decrease in the number of TH+ cells on RES/CTR when compared to CTR/CTR group as well as an increase on RES/PAS when compared to RES/CTR group (Figure 6(a)). No differences were found in relative optical density of dorsal striatum $[F_{(3,21)} = 0.268, p = 0.847]$ (Figure 6(b)).

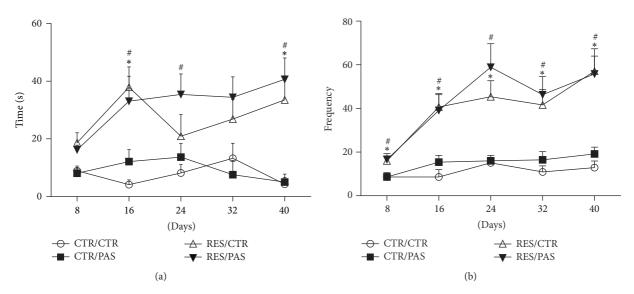


FIGURE 3: Effects of repeated administration of *Passiflora cincinnata* extract (25 mg/kg) and reserpine (0.1 mg/kg) on oral movements of mice. (a) Twitching and (b) vacuous chewing. Data are expressed as mean \pm SEM. *p < 0.05 RES/CTR compared to CTR/CTR; *p < 0.05 RES/PAS compared to CTR/CTR (repeated measures ANOVA followed by Tukey's post hoc test).

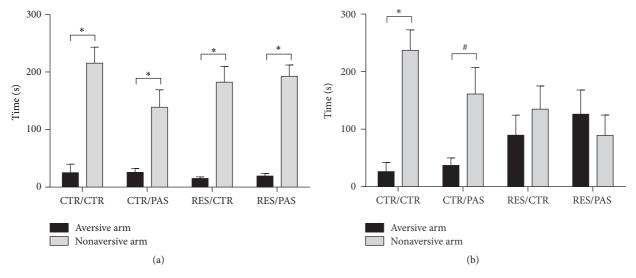


FIGURE 4: Effects of repeated administration of *Passiflora cincinnata* extract (25 mg/kg) and reserpine (0.1 mg/kg) on mice exploration of the aversive and nonaversive arms in plus-maze discriminative avoidance task. (a) Training session and (b) test session. Data are expressed as mean \pm SEM. *p < 0.05 and *p = 0.07 compared to aversive arm (paired-samples t-test).

4. Discussion

In this study, we investigated the effects of the administration of the ethanolic extract of *P. cincinnata* on reserpine-induced parkinsonism. Our main results showed that mice chronically treated with PAS displayed a delayed onset of motor impairments induced by reserpine, but the treatment did not modify reserpine-induced cognitive impairment. In addition, concomitant PAS treatment prevented the depletion of TH+ SNpc cells caused by the chronic administration of reserpine.

Reserpine administration induces depletion of monoamines by blocking vesicular monoamines transporters (VMATs), which results in motor disturbances like tremor, rigidity, and hypokinesia [40–42]. This blockage of VMATs generates a cytoplasmic accumulation and further decrease of neurotransmitters release. Moreover, monoamines left in cytoplasm are metabolized, generating reactive metabolites, which leads to oxidative stress [43–46]. Thus, it seems to be an appropriate animal model for development of new drugs for treatment of PD [47, 48].

The majority of studies using reserpine as a PD model focus on a single high dose administration [49–52]. In an attempt to mimic the PD's progressive profile, a recent study from our group demonstrated that chronic administration of a low dose of reserpine was able to induce progressive motor impairment, accompanied by lipid peroxidation due

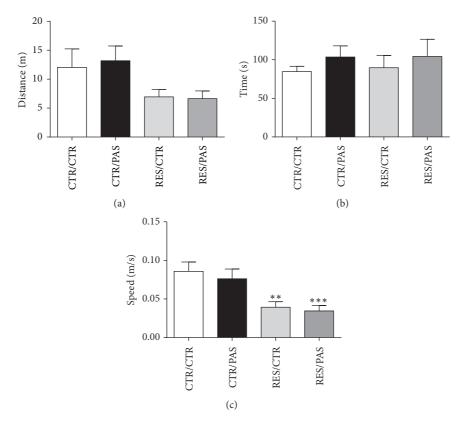


FIGURE 5: Effects of repeated administration of *Passiflora cincinnata* extract (25 mg/kg) and reserpine (0.1 mg/kg) on (a) total distance travelled, (b) time spent in central zone, and (c) average speed of mice in open field. Data are expressed as mean \pm SEM. ** p < 0.01 and *** p < 0.005 compared to control (one-way ANOVA followed by Tukey's post hoc test).

to oxidative stress [34] and tyrosine hydroxylase depletion in dorsal striatum and SNpc [37]. In the present study, we used an adaptation of this protocol to mice, as described by Campêlo and colleagues [35]. Specifically, there is an increase in the treatment duration; that is, the number of injections was altered from 10 to 20 (during 40 days). This adaptation is necessary because mice are more resilient to reserpine than rats. A possible explanation to this resilience is the fact that mice have less monoamine oxidase (MAO) activity in the brain compared to rats [53], which could contribute to a lower formation of oxidative metabolites from dopamine degradation (e.g., hydrogen peroxide). Furthermore, these physiologic differences may also be responsible for the minor decrease in TH+ SNpc cells (this reduction is more expressive in rats), and consequently no reduction of striatal densitometry, which might have mitigate possible PAS effects (Figure 6(b)). We suggest that this result may be related to a compensatory mechanism. In this respect, previous studies have reported that remaining dopaminergic neurons in SNpc could sustain a more intense expression of TH to replace the new dopamine demand in their terminals, converting more tyrosine into dopamine [54]. This new dopamine demand would occur in response to reserpine action on VMAT, leading to the sustained expression of TH densitometry levels reported in dorsal striatum of reserpine groups.

Interestingly, the administration of the ethanolic extract of *P. cincinnata* delayed the onset of the motor impairment

(increased catalepsy behavior) induced by reserpine treatment. Indeed, while RES/CTR group showed motor deficits from the 26th day after the beginning of treatment, the impairment was present in the RES/PAS group only from the 30th day onwards (Figure 2). Furthermore, the coadministration with PAS also prevented the tyrosine hydroxylase depletion in the SNpc cells, which occurred in the RES/CTR group (Figure 6(a)). Nevertheless, reserpine-treated animals also showed reduction in average speed in open field locomotion (Figure 5(c)) and increased oral twitching and vacuous chewing movements (Figure 3), which were not prevented by PAS cotreatment.

Some studies have demonstrated the neuroprotective activity of antioxidant compounds in animals treated with reserpine. The antioxidant substances (e.g., ebselen and vitamins E and C) reduced oxidative stress parameters such as thiobarbituric acid reactive substances (TBARS) and catalase levels [55–57]. In this context, an earlier research showed that *P. cincinnata* has an antioxidant activity [17]. Based on this report, we speculate another mechanism underlying this antioxidant effect. It is known that flavonoids may trigger an internal cellular response through the activation of the PKC/ARE/Nrf2 pathway [58]. This signaling promotes transcription of NAD(P)H:quinone oxidoreductase-1 (NQO1) and other detoxifying genes [59], which is impaired by reserpine treatment because it decreases PKC activity [60]. Consequently, we could infer that this characteristic is

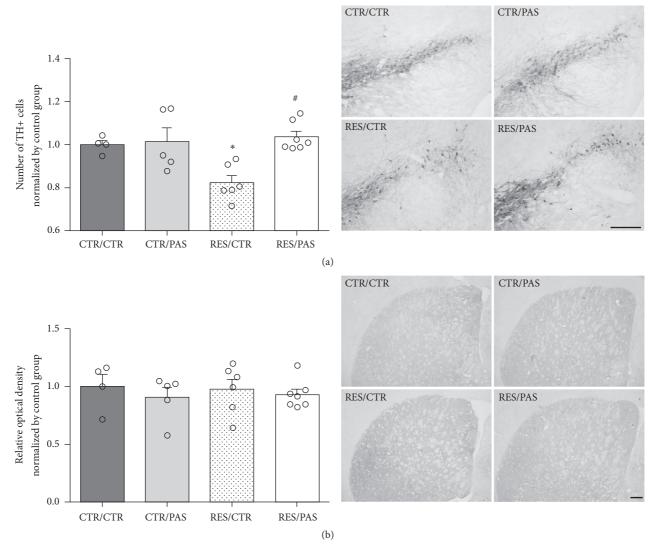


FIGURE 6: Effects of repeated administration of *Passiflora cincinnata* extract (25 mg/kg) and reserpine (0.1 mg/kg) on (a) TH+ cells of SNpc and (b) relative optical density (ROD) of dorsal striatum, both normalized by CTR values. Data are expressed as mean \pm SEM. * p < 0.05 compared to CTR/CTR. * p < 0.05 compared to RES/CTR. (one-way ANOVA followed Tukey's post hoc test). Magnification 100x (a) and 40x (b), black bold lines are scale bars, corresponding to 200 μ m.

responsible for the delay in the onset of motor deficits in the catalepsy test, as well as the decrease in tyrosine hydroxylase depletion in SNpc cells.

As mentioned above, in this PD model a more reduction in TH+ SNpc cells is observed in rats than in mice. Therefore, the PAS effects in the striatum would be better detectable if mice showed an expressive reduction of TH+ cells. In other words, it is possible that different metabolism rates between species may have overshadowed the results regarding TH levels in the striatal dopaminergic projections. Nevertheless, it the TH+ cell count in the SNpc did reduce after reserpine treatment, and PAS was able to prevent it.

Regarding the oral movements evaluation, it is important to highlight the differences found in the sensitivity of both parameters to the effects of reserpine treatment. Both parameters were able to detect motor deficits in the beginning of the treatment, even before the appearance of catalepsy increment. However, the effect of time and the interaction between time and treatment were only observed for vacuous chewing, a motor alteration well established in the literature as a consequence of reserpine treatment [34, 55, 61–63]. Regardless, development of those impairments in our animals were not prevented or delayed by PAS treatment.

Regarding cognitive evaluation in the PMDAT, we did not observe any changes in learning, since all groups were able to discriminate the aversive and the nonaversive arms during the training session (Figure 4(a)). On the other hand, in the test session, reserpine groups had a retrieval deficit, which was not affected by cotreatment with PAS (Figure 4(b)). The memory impairment in animals that received reserpine corroborates previous data from our group [64, 65] and other reports [66, 67]. This impairment might be compared to recognition or evaluation deficits present in patients with PD. Studies proposed that these changes are linked to an

imbalance in basal ganglia dopamine availability. This imbalance affects circuit connections to regions related to cognitive and emotional functions, like prefrontal cortex, amygdala, hippocampus, and ventral tegmental area, among other regions [68–71]. Importantly, similar to the evaluation of oral movements, cotreatment with PAS did not alter reserpine-induced memory deficit. Taken together, these two findings suggest that the protocol of PAS treatment used here was not fully effective in preventing all alterations related to reserpine-induced parkinsonism.

In addition, although PAS treatment did not improve other parameters, the positive effects observed in catalepsy behavior and tyrosine hydroxylase expression are relevant because they suggest a potential delay in the neurodegeneration caused by PD. The use of this chronic treatment with a low dose of reserpine, a well established model for PD [36], positively contributed to demonstrating the subtle effect of PAS that might not be evidenced in acute treatment with neurotoxins (the usual method of PD inducement in rodent models). We believe that a preventive (i.e., before reserpine injections) treatment with PAS, increased doses of the extract, or longer treatments could have a more widespread effect. Alternatively, the application of fractions as well as isolated *P. cincinnata* compounds could also be more effective.

Regarding possible effects on anxiety-like behavior, no differences were found in relation to percentage of time in open arms (Table 1), but studies have demonstrated that the evaluation of risk assessment behavior (stretched attend posture and head dipping) may be useful in anxiety evaluation [72, 73]. In the present study, we observed an increase in the anxiety level (stretched attend postures frequency, Table 1) in animals treated with PAS extract (training session), and this effect could be responsible for the slight performance reduction presented in memory retrieval by this group, although it was not significantly different from control (Figure 4(b)).

5. Conclusions

In summary, this research suggests that the ethanolic extract of *P. cincinnata* has neuroprotective properties that may have therapeutic potential for PD. Based on the literature, this effect is probably related to an antioxidant-related action (see above). However, further studies are required to assess the range of these effects regarding parkinsonian symptoms, as well as to determine the structure of active compounds and their mechanisms of action.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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Review Article

A Review of Dietary Ziziphus jujuba Fruit (Jujube): Developing Health Food Supplements for Brain Protection

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The fruits of *Ziziphus jujuba*, known as jujube or Chinese date, are being consumed all around the world because of their health benefits, as both food and herbal medicine. Traditionally, one of the main functions of jujube, as described in herbal medicine, is to benefit our brain by calming down the mind and improving quality of sleep. Here, the activities of jujubes on nervous system are summarized and discussed. Jujube possesses neuroprotective activities, including protecting neuronal cells against neurotoxin stress, stimulating neuronal differentiation, increasing expression of neurotrophic factors, and promoting memory and learning. Flavonoid, cAMP, and jujuboside could be the potential bioactive ingredients to account for the aforesaid biological activities. These findings imply that jujube is a potential candidate for development of health supplements for prevention and/or treatment of neurological diseases.

1. Introduction

Jujubae Fructus, the fruit of Ziziphus jujuba Mill. (Rhamnaceae), also known as jujube, or Chinese date, or red date, has been widely used as food and Chinese herbal medicine for over 3,000 years. Jujube is indigenous to Chinese culture (Figure 1). The description of jujube was first recorded in Classic of Poetry (1046-771 BC). Today, there is a wide distribution of jujube-related products in the world. The consideration of jujube as a vital food and/or medicine has a long history of record in China. In ancient Chinese book on herbal medicine Huangdi Neijing (475-221 BC), jujube was described as one of the five most valuable fruits in China. In Shennong Bencao Jing (300 BC-200 AD), an earlier book recoding medicinal herbs, jujube was considered as one of the superior herbal medicines that prolonged our life-span by nourishing blood, improving quality of sleep, and regulating digestive system.

Jujube has been consumed for thousands of years, which is still gaining influence on our daily life. Recent phytochemical and pharmacological results have revealed that flavonoid, polysaccharide, and triterpenic acid are the main active ingredients within jujube. Based on the literatures, both flavonoid and polysaccharide could account for antioxidative effect of jujube [1–3]. Moreover, jujube polysaccharides were also proposed to be main active ingredients contributing to its immune-modulating and hematopoietic functions [4, 5]. Triterpenic acids were considered as active ingredients for the effect on anti-inflammatory and anticancer activities [6, 7]. In addition, betulinic acid and jujuboside B could be the active components showing beneficial effects on cardiovascular system [8, 9].

The study on biological activities has supported the health benefits of jujube as both food and medicinal herb. According to Chinese medicinal theory, jujube is considered as a medicinal herb that calms the mind and relieves

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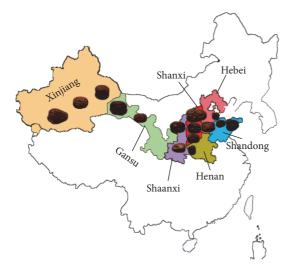


FIGURE 1: The cultivation areas and major production areas of jujube in China. The cultivation areas of jujube in China were highlighted and shown. The insert shows the location of major production areas of China, including Xinjiang, Gansu, Shaanxi, Shanxi, Hebei, Henan, and Shandong provinces. Shaanxi and Shanxi are considered as the original cultivation areas of jujube.

mental tension. Clinically, jujube is commonly prescribed, either as single herb or in tranquillizing formulae combined with other herbal medicines, for the treatment of insomnia and forgetfulness. Previous reviews have summarized the fruit composition and its health benefits [10, 11]. However, the studies focused on neuroprotective activities are rather limited. Therefore, the neurobeneficial roles of jujube will be introduced in this review. In addition, the potential bioactive compounds of jujube related to brain benefits are further discussed.

2. Jujube on Neuron Differentiation

According to historical usage in China, one of the main functions of jujube was considered to benefit our brain by calming down the mind and improving quality of sleep. In modern science, benefiting our brain is usually related to neurobeneficial effects, for example, neuroprotection effect and neurotrophic action. In neurological disorders, for example, neurodegenerative diseases, insomnia, and depression, several common pathological conditions among them are found, that is, neurogenesis impairment, neurotrophic factor deficiency, and oxidative stress. Hence, the traditional function of jujube in benefiting the brain may be closely related to its neurobeneficial effects (Figure 2). Here, the neurobeneficial effects of jujube are summarized (Table 1).

Neurogenesis is a well-orchestrated process consisting of neuronal differentiation, synapse formation, and cell proliferation. Some studies suggested that, in pathological condition of neurodegenerative diseases, various types of neurogenesis could be observed, indicating that neurogenesis might be a compensatory mechanism in neurodegenerative processes [12]. Thus, the promotion of neuronal differentiation could be a means to prevent these diseases. Cultured pheochromocytoma PC12 cells are a pertinent model system for the study of neuronal differentiation [13]. The characters of PC12 cells are very similar to sympathetic neuron system, that is, the characters of neurofilament (NF) expression and neurite outgrowth in responding to challenge of nerve growth factor (NGF). The findings on jujube indicated that application of jujube water extract on cultured PC12 cells for 72 hours could induce cell differentiation: the induced differentiated cell accounted for ~25% of the total cells in cultures. The results also showed that application of jujube water extract in cultured PC12 cells for 72 hours could dose-dependently stimulate the expressions of NF68, NF160, and NF200 [13].

Astrocytes are the most abundant cell in the nervous system. One of its main functions is to synthesize and release neurotrophic factors, that is, NGF, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), neurotrophin 3 (NT3), and NT4/5 [14]. These factors are vital for neuronal survival, growth, and differentiation, and the deficiency of which can cause neurological impairments [15]. Thus, the upregulation of neurotrophic factors can play a positive role in treating neurological diseases. The effect of jujube on neurotrophic factor expression was investigated in cultured astrocytes [16]. The treatment with jujube water extract stimulated the expression of neurotrophic factors in a dose-dependent manner, having the highest induction of ~100% for NGF, 100% for BDNF, 100% for GDNF, and 50% for NT3. For NT4 and NT5, no obvious morphological change was observed in jujube-treated astrocytes.

The signaling of cAMP-PKA-CREB is well known to play a role in neuronal differentiation of PC12 cells [17]. Hence, the involvement of cAMP pathway in jujube-induced neurite outgrowth and neurofilament expression was revealed (Figure 2). The findings showed that jujube-induced neurite outgrowth and neurofilament expressions were attenuated by application of H89 (a cyclic AMP-dependent PKA inhibitor) [13]. CREB, the nuclear transcription factor, has been known to play a role in neuronal differentiation [18]. It was reported that jujube was able to stimulate the phosphorylation of CREB, and its inductive effect could be fully blocked by H89 [13]. Besides, the application of jujube water extract could stimulate the transcriptional activity of CRE. Further studies also showed that pretreatment with H89 significantly blocked jujube-induced neurotrophic factors including NGF, BDNF, and GDNF, indicating the possible involvement of PKA signaling in jujube-induced neurotrophic factor expression [16].

3. Neuroprotection against Oxidation Insult

In the process of neurodegeneration, the functions of neuron are markedly decreased. In Parkinson and Alzheimer diseases, oxidative stress, as the main considerable factor, is believed to cause neuronal damage in progress of diseases [29]. In cultured cells, jujube water extract was reported to protect neuronal cells against *tert*-butyl hydroperoxide-(tBHP-) induced oxidative injury. In addition, it was found that jujube water extract could inhibit tBHP-induced

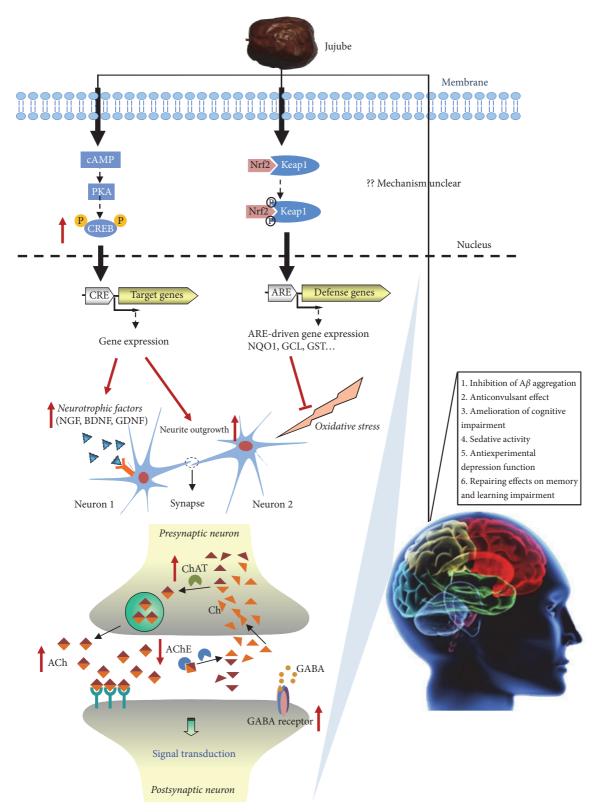


FIGURE 2: The neuroprotection effects of jujube. Jujube induces neurite outgrowth and neurothrophic factor expression via cAMP-dependent PKA signaling. Jujube possessed neuroprotection against oxidative stress via enhancing cellular Nrf2-dependent ARE-driven gene expressions. Jujube improves the choline acetyltransferase (ChAT) activity and shows inhibitory activity against acetylcholinesterase (AChE). Jujube increases the level of acetylcholine (ACh) in the brain. Jujube stimulates the transcriptional expression of GABA receptor subunits in rat hippocampal neurons.

TABLE 1: Neuronal beneficial properties of jujube.

Findings	Model	Treatment	Reference
Jujube induced neurite outgrowth and neurofilament expression via cAMP-PKA-CREB signaling	Cultured PC12 cells; neuronal differentiation	Jujube extract compared with forskolin and control	[13]
The mature jujube possessed better effect in inducing neurofilament expression than that of the immature on	Cultured PC12 cells; e neurofilament expression	Mature jujube extract compared with immature jujube extract	[19]
Jujube stimulated the expressions of neurotrophic factors and antioxidant enzymes	Cultured astrocytes; mRNA expression	Jujube at various concentrations (0–3 mg/ml)	[16]
Jujube protected neuronal cells against oxidation injury via activation of transcriptional activity of ARE	Cultured PC12 cells; tBHP induced oxidative stress	Jujube extract compared with Vit.C, tBHQ ^b , and negative control	[20]
Jujube protected neurons from ischemic damage	Ischemic damage in gerbil hippocampus	Oral administration of jujube extract for 10 days	[21]
Jujube increased pentobarbital-induced sleep time and reduce free movement on mice	Kunming mice, behavior and sleeping studies	Jujube at 8 g/kg was administered orally	[22]
Jujube promoted learning and memory in ovariectomized rat model	SD rats, Morris water maze test	Ovariectomized rats in 6 groups: jujube groups, positive, model, and sham surgery groups	[23]
Hydroalcoholic extract of jujube ameliorates seizures, oxidative stress, and cognitive impairment in epilepsy rat model	Rat, experimental seizure models	Hydroalcoholic extract of jujube (100, 250, 500, and 1000 mg/kg) was administered orally	[24]
Jujube had repairing effects on memory and behavioral disorders produced by NBM lesion in rats	Wistar male rats; Morris water maze test	Rats in 7 groups: normal, AD, AD/normal + jujubes at two doses, and sham	[25]
Methanolic jujube extract activated ChAT. Oleamide from jujube reversed the memory and/or cognitive impairment in mice model	Cultured MC-IXC cells, mice	Oleamide at 0.4–2.4 mM on ChAT ^a activity; mice were treated with oleamide for 4 weeks	[26]
Jujube increased the cAMP content in plasma and hippocampus of animals	ICR male mice, cAMP level in hippocampus and serum	i.g. administration of jujube at 0.35 g/kg	[27]
A polypeptide Snakin-Z from jujube possessed cholinesterase inhibitory activity	Cholinesterase inhibitory activity	Snakin-Z at 1.5 mg/mL has 80% inhibitory activity	[28]

^aChAT, choline acetyltransferase. ^btBHQ, *tert*-butyl hydroquinone.

ROS (reactive oxygen species) formation in cultured PC12 cells [20]. The Nrf2 (nuclear factor (erythroid-derived 2-) like 2-) dependent ARE (antioxidant response element-) driven genes, including glutamate-cysteine ligase (GCL), glutathione S-transferase (GST), and NAD(P)H quinone oxidoreductase (NQO1), have been demonstrated to play an important role in protecting cells against oxidative stress [30, 31]. Jujube water extracts stimulated the ARE-mediated transcriptional activity, indicating the activation of Nrf2 pathway (Figure 3). Besides, the application of jujube induced the amounts of NQO1, GCLC (catalytic subunit of GCL), GCLM (modifier subunit of GCL), and GST mRNA levels in cultured astrocytes [16]. Jujube was also revealed to protect ischemic damage in gerbil hippocampus via its antioxidant effect, that is, the upregulation of superoxide dismutase (SOD) 1 and reduction of lipid peroxidation [21].

4. Jujube on Insomnia, Learning, and Memory

In Chinese herbal medicine, jujube was prepared as a tea that was used against insomnia [32, 33]. Indeed, jujube was found to increase pentobarbital-induced sleep time and to reduce free movement on mice [22]. In support of this, Peng et al. (2000) reported that the seed of *Z. jujuba* prolonged the hexobarbital-induced sleeping time in mice and decreased the locomotor activity in rats. Moreover, jujube was shown

to possess anxiolytic effect by increasing the first time entry, as well as the total change and time spent in the white chamber of black and white test [34]. Besides, flavonoids and saponins from seed of Z. jujuba showed sedative and hypnotic effects, which caused a significant reduction of walking time and coordinated movement ability of mouse, significantly prolonging its sleeping time [35]. Jujuboside A, one of saponins from seed of Z. jujuba, stimulated the expression of GABA receptor subunits in rat hippocampal neurons [36] and ameliorated behavioral disorders of the dementia mouse model induced by A β 1–42 [37]. Methanolic extract and oleamide from jujube showed activation effect on choline acetyltransferase (ChAT). Meanwhile, oleamide was found to attenuate the scopolamine-induced amnesia in mice, a useful in vivo model for Alzheimer disease [26]. Jujube was reported to improve learning and memory in ovariectomized rat model, and the effect of which might be due to an increase of estrogen in the blood, as well as the levels of nitric oxide and acetylcholine in the brain [23]. Moreover, hydroalcoholic extract of jujube was also demonstrated to possess anticonvulsant effect and amelioration of cognitive impairment induced by seizures in rats [24]. The seed extract of Z. jujuba showed a promising effect in ameliorating memory in mice with alcohol-induced memory retrieval disorders and might help to improve learning capacity to some extent [38]. In line with the aforementioned reports, jujube

FIGURE 3: Structures for chemical compounds in jujube having potential neuroprotection effect. Seven chemical markers found in jujube including kaempferol 3-O-rutinoside, quercetin 3-O-rutinoside, (–)-catechin, (–)-epicatechin, swertish, spinosin, and cAMP were reported to possess effect on neuroprotection.

was reported to possess repairing effects on memory and learning impairment induced by bilateral electric lesion of the nucleus basalis of Meynert in rats [25]. In addition, jujube extract having 1% of cAMP was found to have antidepression function in animal model [39].

5. Two Developmental Stages of Jujube in Neuroprotection

Two types of jujubes are commonly found in China market: fresh immature jujubes were consumed as fruits, and dried mature jujubes were used as Chinese medicinal herb. Chemically, a sharp decrease of flavonoidic compounds was revealed during maturity [20, 40], while the amounts of selected compounds were significantly lower in immature jujubes than that of mature ones, including xanthine, hypoxanthine, adenine, uridine, guanosine, cGMP, and cAMP [20]. Moreover, the metabolite variations between mature and immature jujubes were also studied. Levels of isoleucine, threonine, acetate, creatine, and glucose were lower in immature jujube than those of mature one, while levels of alanine, sucrose, and formate were lower in mature jujube than those of immature one [19]. For biological assessments, the antioxidative functions of jujube extracts were first compared [20]. The antioxidative effects were varied amongst different maturity jujubes. The fresh immature jujube had better effect on antioxidation activity than that of mature one. However, mature jujube showed better effect in inhibition of ROS formation and activation of Nrf2-antioxidant response element signaling [20]. Furthermore, in terms of neuronal differentiation, mature jujube possessed better effect, about 60% higher than that of immature one [19]. These findings suggested the specific usage of different maturity of jujubes.

6. Jujube-Containing Herbal Decoctions on Neuroprotection

In Chinese herbal medicine, jujube is commonly included in herbal mixture, called Fu Fang, that is having specific combination of different herbs. These specific requirements of herbal preparation have not been changed. Among thousands of different herbal decoctions, Ganmai Dazao Tang and Chaihu Guizhi Tang are commonly used today for the treatment of depressive disorders [41]: both of them contain iuiube as a key herb within the formulation. Taking Ganmai Dazao Tang as an example, this prescription having licorice, wheat, and jujube was recorded in a book Jingui Yaolue (Jingui Collection of Prescriptions), written by a well-known Chinese medicine scholar Zhang Zhongjing (150 BC to 219 AD) in Han Dynasty of China. This herbal decoction was traditionally prescribed for women suffering from anxiety. Ganmai Dazao Tang was reported to be one of top 10 Chinese herbal formulae prescribed for insomnia in Taiwan [42]. Although the molecular mechanisms for these herbal formulae are unclear, these antidepressive formulae may possess effect on neurotrophic factor expression, as it is well believed that reduction of BDNF expression is observed in depression patients [43, 44]. Indeed, Ganmai Dazao Tang was reported to induce BDNF expression in depressive animal model [45]. Besides, the jujube-containing herbal decoctions, including Guizhi Tang, Neibu Dangguijianzhong Tang, and ZaoTang, induced neuronal differentiation and expressions of antioxidant enzymes in cultured PC12 cells [46], for example, GCLC, GCLM, GST, and NQO1 via the activation of ARE. Together with the result of jujube as single herb alone, these findings could illustrate the functions of jujube within a multiherbal decoction.

7. Potential Bioactive Ingredients on Neuroprotection

Phenolic compounds are widely found in plants, which comprise a large group of bioactive ingredients. Flavonoids are commonly found in fruits, and some of which have been reported to possess neuroprotective effect (Figure 3) [47, 48]. Among different types of flavonoids in jujube, Zhu et al. (2007) reported that kaempferol 3-O-rutinoside possessed neuroprotective activity against oxidation insult and prevention of A β aggregation [47]. Quercetin 3-O-rutinoside was shown to have anti-A β aggregation effect. Both (–)-catechin and (-)-epicatechin showed inhibition of A β aggregation and $A\beta$ -induced toxicity [47]. In addition, kaempferol 3-O-rutinoside showed protective effect on permanent focal cerebral ischemia and on neuronal cultures [49], as well as in reducing memory dysfunction, energy metabolism failure, and oxidative stress in multi-infarct dementia model rats [50]. Spinosin possessed potentiating effect on pentobarbitalinduced sleep, and the effect of which was related to the postsynaptic 5-HT receptor [51, 52]. Indeed, spinosin and swertish, isolated from jujube seeds, were reported to possess significant sedative activity [53]. Yet, the flavonoids within jujube, including (-)-catechin, (-)-epicatechin, kaempferol 3-O-rutinoside, and quercetin 3-O-rutinoside, were tested for their neurotrophic action, yet no significant effect was found among them [48].

Cyclic nucleotides and their derivatives participate in the regulation and modulation of lots of physiological processes in body. These chemicals exhibit multiple bioactivities, that is, neuroprotective effects [54, 55]. Among them, cAMP was reported to be a relative large amount in jujube, and interestingly its content was much higher than other horticultural fruits (Figure 3) [56-58]. The amount of cAMP, at approximately the amount containing within 2 mg/mL of jujube water extract, showed the activity on neuronal differentiation in cultured PC12 cells. This firmly indicated that jujube cAMP exerted a role in neuronal differentiation, as reported for jujube extract [13]. Besides, a report showed that jujube was able to increase the cAMP content in plasma and hippocampus of animal model [27], and the authors hypothesized that the exogenous cAMP in jujube water extract might induce the aforesaid results. In parallel, cAMP is well believed to be involved in formation of depression. Indeed, cAMP isolated from jujube was found to possess antimelancholic property in animal model of depression [39]. In addition, Jujubosides, found in Semen Ziziphi Spinosae and jujube [59], were reported to possess hypnotic effects [35, 60]. To support this, jujuboside A could adjust GABA receptor subunit mRNAs expression in hippocampal neurons [61]. Oleamide from jujube reversed the scopolamine-induced memory and/or cognitive impairment in mice model [26]. In contrast, a polypeptide snakin-Z, isolated from jujube, possessed cholinesterase inhibitory effect [28].

8. Development and Perspective

Having over 3,000 years of history, jujube is still a popular fruit in our daily life for its health benefits [62]. Based on

the aforementioned cellular and animal findings, jujube has a great potential in developing further as food and medicinal supplements for brain health. Jujube water extract is the most common usage form. It can be prepared into decoction and drunk daily, or it can be combined with other foods for the preparation of a delicious soup. Clinical studies showed that no adverse and drug interactions have been reported for the consumption of jujube [63–66]. In addition, the recent studies also revealed that jujube water extract contained higher amount of ingredients as compared to that of ethanol extract [20]. Hence, jujube water extract could be a good choice as an alternative to be prescribed for neurological disorder. Moreover, the quality of herbal medicine, as jujube here, could be varied from each other due to numerous factors, for example, harvest season, geographic region, and postharvest treatment. Thus, it is essential to establish different parameters in receiving chemical standardized jujube water extract. In order to chemically standardize jujube water extract in terms of its HPLC profile and chemical contents, the HPLC fingerprint and quantification methods were employed to reveal its HPLC profile and quantify the main ingredients [20]. The standardization parameters can be employed to ensure the consistent quality of jujube water extract and to further make sure of its repeatability of experimental results.

The active ingredients of jujube include nucleotide and flavonoid, which may show benefit effects in brain functions. In consideration of the benefit of cAMP in jujube, numerous studies are targeting on the processes in extracting and separating cAMP from jujube [39, 67, 68]. Hence, the enrichment of jujube nucleotide may be developed into a food or medicinal product for the treatment of diseases, such as depression or neurodegeneration. During the production of beverages and other kinds of food with jujube, such as cake, the peel is usually discarded. Jujube peel extract was found to contain higher amount of phenolic compounds than that of pulp [69]. These findings might imply that jujube peel could serve as an inexpensive source of natural antioxidant to protect neuronal cells against oxidation insult. The seed of jujube is also discarded in either food products or TCM practices. The seed from Z. jujuba var. spinosa, the sour jujube, possessed hypnotic effect [35, 60], indicating that jujube seed might also be a selection for insomnia as either raw material or source for active ingredient.

The usage of jujube in China is not restricted as a single fruit; it is commonly prescribed in multiherbal decoctions for various purposes. Jujube was commonly employed as the main ingredient within all the herbal formulae written by Zhang Zhongjing (150 BC to 219 AD). According to Zhang's theory of Chinese medicine, jujube was included in herbal decoction as to adjust the taste of other herbs, for example, to harmonize the spleen and stomach, to harmonize the nutrient and defense, and to calm the mind. Having jujube as the active ingredient, the major function of jujube-containing decoctions for the treatment of brain related diseases should be expected.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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