Evidence-Based Complementary and Alternative Medicine

Potential Therapeutic of Medicinal Plants in Mood Disorders

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Contents

Ethnobotany, Phytochemistry, Biological Activities, and Health-Promoting Effects of the Genus *Bulbophyllum*
Javad Sharifi-Rad, Cristina Quispe, Abdelhakim Bouyahya, Naoual El Menyiy, Nasreddine El Omari, Md Shahinozzaman, Mim Ara Haque Ovey, Niranjan Koirala, Mamata Panthi, Andrea Ertani, Silvana Nicola, Natallia Lapava, Jesús Herrera-Bravo, Luis A. Salazar, Sushil Changan, Manoj Kumar, and Daniela Calina
Review Article (15 pages), Article ID 6727609, Volume 2022 (2022)

***Baccharis dracunculifolia* DC Hydroalcoholic Extract Improves Intestinal and Hippocampal Inflammation and Decreases Behavioral Changes of Colitis Mice**
Tauani Caroline Santos França, Ana Julia Ribeiro, Luísa Natália Bolda Mariano, Ana Caroline dos Santos, Larissa Venzon, Lincon Bordignon Somensi, Ruan Kaio Silva Nunes, Camila André Cazarin, Karen Luz Okubo, Helenita Priscila Poerner, Jairo Kneupp Bastos, Márcia Maria de Souza, and Luísa Mota da Silva
Research Article (14 pages), Article ID 5833840, Volume 2022 (2022)

An Insight into Phytochemical, Pharmacological, and Nutritional Properties of *Arbutus unedo* L. from Morocco
Mohammed El Haouari, Najat Assem, Sushil Changan, Manoj Kumar, Sevgi Durna Daştan, Jovana Rajkovic, Yasaman Taheri, and Javad Sharifi-Rad
Review Article (19 pages), Article ID 1794621, Volume 2021 (2021)

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2023, Article ID 1183809, 15 pages
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Research Article

Neuropharmacology Potential of the Hydroalcoholic Extract from the Leaves of *Piper cernuum*: Anxiolytic, Hypnotic, and Antidepressant-Like Effects

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Aim. The use of medicinal plants in the treatment of mental illnesses is a reality that accompanies the history of civilizations, and the Piper genus exhibits many species with pharmacologically proven central effects. Then, this study evaluated the neuropharmacological effects of the hydroalcoholic extract from *Piper cernuum* (HEPC) leaves to validate its uses in folk medicine. *Materials and Methods*. Primarily Swiss mice (female, 25–30 g) were pretreated with HEPC (50–150 mg/kg, p.o.), vehicle, or the positive control, and submitted to open-field test (OFT), inhibitory avoidance test (IAT), tail suspension test (TST), and forced swim test (FST). Also, mice were exposed to pentylenetetrazol- and strychnine-induced seizure assay, pentobarbital-induced hypnosis test, and elevated plus-maze (EPM). The GABA levels and MAO-A activity were measured in the animal's brain after 15 days of HEPC administration (150 mg/kg, p.o.). *Results*. Mice pretreated with HEPC (100 and 150 mg/kg) and exposed to pentobarbital presented decreased sleep latency and increased sleep duration (HEPC 150 mg/kg). In EPM, the HEPC (150 mg/kg) increased the frequency of entry and the time of exploration of mice in the open arms. The antidepressant-like properties of HEPC were demonstrated by the decrease in the mice's immobility time when tested in FST and TST. The extract did not show anticonvulsant activity, in addition to not improving the memory parameters of animals (IAT) or interfering with their locomotor activity (OFT). Besides, HEPC administration decreased the MAO-A activity and increased the GABA levels in the animal's brain. *Conclusion*. HEPC induces sedative-hypnotic, anxiolytic-, and antidepressant-like effects. These neuropharmacological effects of HEPC could be, at least in part, related to the modulation of the GABAergic system and/or MAO-A activity.

1. Introduction

Anxiety, depression, and epilepsy are neurological disorders with significant social impacts especially nowadays [1, 2]. Associated with these diseases are also sleep disturbances that constitute not only a symptom of such pathologies but can also be characterized as a pathology other than [3]. Moreover, epilepsy is a severe neurological disorder with a prevalence of 6.38 per 1000 persons worldwide [4].

Recent data estimate that about 4.4% of the world's population, approximately 322 million people live with depression, while the estimated number of patients with anxiety in the world is about 264 million [5]. For such diseases, current treatments vary in effectiveness considering individual differences, which can lead to cases of refractoriness [6].

It is known that from ancient times, people have used different methods and procedures to treat psychiatric disorders and have often used medicinal plants as one of these

resources [7–9]. Indeed, medicinal plants are important sources of biologically active substances, providing the basis for pharmacological research and discovering new molecules with antidepressant, anxiolytic, hypnotic, and anticonvulsant effects [10, 11]. Considering this background, it is necessary to provide scientific information about the pharmacological profile of medicinal plants to identify their safety and efficacy [12], collaborating to validate their uses.

One widely distributed plant in pantropical regions is the *Piper* genus. *Piper* plants are also known under the common name "pepper." The presence of oil cells in the structures of almost all *Piper* sp. places them in the group of aromatic plants [13]. Besides their well-known uses as culinary spices, the secondary metabolites isolated from these plants, among which are terpenoids, lignans, and alkaloids, among other classes, show wide-ranging human health effects [10]. Regarding psychopharmacological properties, the effects of Kava (*Piper methysticum*) are already known, which has been explored as a potential phytotherapy option for generalized anxiety disorder [14]. Furthermore, the pharmacological potential of *Piper nigrum* as a neuroprotector against epilepsy and biochemical alterations arising from Alzheimer's Disease is also reported in the literature [15, 16].

Other *Piper* species are underexplored for their pharmacological properties despite widespread uses, and an example is *Piper cernuum* Vell, popularly known as "Pariparoba," "João-guarandi-do-grado," and "Pimenta-de-Morcego." This species is considered a medicinal plant and is used by rural communities to treat pain conditions, such as bellyache and muscle pain (topically), besides hepatic and renal complications [17].

There is little scientific information about the *P. cernuum* leaves extract effects but are data showing its antileishmanial [18] and cytotoxicity effects against oral squamous cell carcinoma effects [19]. In addition, from leaves extracts the following cinnamic acid derivatives were isolated: methyl 3,4-dimethoxy-dihydrocinnamate, 3,4-dimethoxy-dihydrocinnamic acid, methyl 3,5-dimethoxy-4-hydroxy-dihydrocinnamate (methyl dihydro sinapate), methyl 3,5-dimethoxy-4-hydroxy-cinnamate (methyl sinapate), and cubebin [20]. (−)-Bornyl p-coumarate too isolated from leaves exhibited antitrypanosomal activity and effect on plasma membrane permeability [21]. However, the essential oils of this species have already been studied in relationship to antimicrobial activity and chemical composition [22–28]. Some compounds present in oil exhibit antituberculosis [29] and antitumor [30] effects too.

Recent results obtained in our laboratories indicated that the hydroalcoholic extract of *P. cernuum* leaves showed no toxicity when administered to male or female rats, lower cytotoxicity, and mutagenicity [31]. Therefore, these previous reports about its safety and the neuropharmacological potential of species of this genus led us to experimentally investigate whether the hydroalcoholic extract of *P. cernuum* leaves has psychopharmacological effects like other species of the genus currently identified as potent natural sources and alternatives for the treatment of human illness, especially mental illness.

2. Material and Methods

2.1. Plant Material and Extract Procedure. *P. cernuum* Vell leaves were collected (a cultivated specie) in Blumenau, a city in the state of Santa Catarina, Brazil (latitude: 26°58′43.8″S, longitude: 49°03′43.0″W, and altitude: 200 m) in March in the years of 2015. Prof. Andre Luis de Gasper, Curator of the Dr. Roberto Miguel Klein Herbarium from the Regional University of Blumenau, classified the material. Samples of the specimen were deposited in this Herbarium with the number 41606. This specie was registered on National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under registration number A862470.

The extracting procedure was described by Wolff et al. [31]. The *Piper cernuum* leaves were dried at 40°C for seven days after the material was triturated and submitted to agitation at Bertel agitator. The particles with diameters between 0.31 e 0. and 57 mm were used. The procedure of extraction was conducted under dynamic maceration at room temperature for 4 hours. Plant material (250 g) was successively extracted with ethanol 90°GL, plant: solvent ratio of 1 : 10 (w/v). The extractive solution was filtered under vacuum filtration after it was concentrated under reduced pressure and finally the extract was dried under a current of air at 50°C giving a yield of 5.7% in relation to leaves. The phytochemical profile of the hydroalcoholic extract from *Piper cernuum* (HEPC) was already described by Wolff et al. [31].

2.2. Pharmacological Tests

2.2.1. Animals and Ethics. Female Swiss mice (25–35 g) were obtained from Central Animal Facility/UNIVALI and maintained at 22–24°C with water and food *ad libitum*, under a 12 : 12 h light/dark cycle (lights on at 07 : 00 h). Female mice were employed in this study considering the higher prevalence of depression in women, as well as the greater availability in the vivarium. In addition, females with a controlled estrous cycle were used to avoid interference in the results. All procedures were conducted between 9:00 a.m. and 04:00 p.m. and each animal was tested only once. Behavioral tests were performed in real-time by trained researchers, taking care to minimally stress the animals. All efforts were made to minimize animal suffering and to reduce the number of animals used in our experiments, and the procedures were performed according to the Brazilian Society of Science of Laboratory Animals' guidelines for the proper care and use of experimental animals. The experimental procedures were approved by the Ethics Committee of the University of Vale do Itajaí, Brazil (protocol no. 037/2019).

2.2.2. Drugs and Treatments. Several drugs used in the research were obtained from Sigma Chemical Co. (St. Louis, USA): pentylenetetrazol, strychnine, sodium pentobarbital, and imipramine. Diazepam was obtained from Cristália (SP, Brazil). Each drug was dissolved in saline (0.9% NaCl) and

administered by intraperitoneal (i.p.) route in a volume of 10 mL/kg body weight. HEPC (50, 100, 150 mg/kg), solutions were prepared daily with distilled water plus 2% DMSO before the use and administered orally (p.o.) by gavage in a volume of 10 mL/kg. The vehicle (negative control) consisted of distilled water plus 2% DMSO and was administered orally (p.o.) by gavage in a volume of 10 mL/kg. All treatments with HEPC and vehicle were performed 1 hour before the experiments. The experimental N was 06–08 individuals per experimental group.

2.2.3. Open-Field Test (OFT). The first behavioral test used in this study was the OFT. Depending on the protocol adopted, many studies on drug effects usually employ the OFT to evaluate locomotor and exploratory parameters of animals before submitting them to tests to verify anxiolytic-like and antidepressant effects [32]. The open-field apparatus consisted of a 40×60 cm dark plastic box with 50 cm high boundary walls, and the box was divided into 12 equal squares. After treatments, vehicle, and HEPC (50, 100, and 150 mg/kg, p.o.), the animals were individually placed in the center of the box for 30 s for adaptation and then allowed to freely explore the area for 6 min. The animal's locomotor activity was registered as the total number of times (counts) that the animal crossed a square during the test. A count was considered when the animal crossed from one square to the next. The open-field test was performed at 30 (for intraperitoneal treatments) or 60 (for oral treatments) min after drugs or extract treatments. Locomotor activity was defined as a horizontal activity with displacement and was expressed in terms of the total number of quadrantes crossed. In this test, the number of rearing performed by each animal was also recorded. Rearing was characterized by the behavior of the animal to lift the body and keep it upright, leaning on its hind legs to explore the apparatus. After each trial, the apparatus was cleaned with 70% ethanol to remove the odor clues left by the previous animal.

2.2.4. Pentylenetetrazol (PTZ) and Strychnine (STR) Induced Seizure Test. The anticonvulsive activity of the plant extract was evaluated through the pentylenetetrazol (PTZ) and strychnine (STR) tests. These tests were based on the method described by Holzmann et al. [33] with slight modifications. In the first experiment, 60 minutes after the oral administration of vehicle or HEPC (50, 100, and 150 mg/kg, p.o.), PTZ (100 mg/kg, i.p.) was administered individually in mice, and immediately the animals were transferred to the observation box. The documented parameters in this experiment were latency to seizure-onset and the number of mice not exhibiting seizures. The seizure was defined as jerky movements of the whole body or convulsion. Each mouse was observed for one whole hour for the occurrence of seizure. The same procedure was performed in another set of experiments; however, the convulsant agent utilized was STR (4 mg/kg). Phenobarbital (30 mg/kg, i,p), the positive control used in both experiments, was administered 30 min before testing.

2.2.5. Pentobarbital-Induced Hypnosis. To evaluate the hypnosis-potentiating activity of the extract, sodium pentobarbital (50 mg/kg, i.p.) was injected into female mice 60 min after oral administration of HEPC (50, 100, and 150 mg/kg). The latency and hypnosis time were recorded. Hypnosis time was measured by the loss of the righting reflex, with the recovery of this reflex being considered the end of the hypnosis time [33]. Diazepam (2.0 mg/kg, i.p.) was administered 30 min before the test as a reference drug.

2.2.6. Elevated Plus-Maze Test (EPM). The procedure used to evaluate the possible anxiolytic effect was like that described previously [32]. The wooden EPM apparatus was shaped as a cross, with two open arms ($50 \times 10\ \text{cm}^2$) with sides of 1 cm in height, and two closed arms ($50 \times 10 \times 15$ cm). The central area of the maze measured $10 \times 10\ \text{cm}^2$. The apparatus was elevated to a height of 70 cm. During the test, each animal was placed at the center of the maze facing the closed arm 60 min after administration of VP extract (50–150 mg/kg, p.o.) or vehicle. All entries to the open or closed arms were scored for 5 min, and the total time spent in each arm was recorded. An entry was defined as placing the mouse's four paws into an arm, and no time was recorded when the animal was in the center of the maze. Diazepam (0.75 mg/kg, i.p., was administered to mice 30 min before the test) was used as a reference drug.

2.2.7. Inhibitory Avoidance Test (IA). Mice were trained in a one-trial, stepping-down inhibitory avoidance paradigm (IA), a highly validated learning task in which stepping down from a platform when a context is associated with a foot shock increases step-down latency [34]. The IA training apparatus was a $50\ \text{cm} \times 25\ \text{cm} \times 25\ \text{cm}$ Plexiglas box with a 5 cm high, 8 cm wide, and 25 cm long platform on the left end of a series of bronze bars that constitutes the floor of the box. During training, the animals were gently placed on the platform facing the left rear corner of the training box. When they stepped down and placed their four paws on the grid, they received a 2 s, 0.4 mA scrambled foot shock. They were then immediately withdrawn from the training box. Memory retention was evaluated in a test session carried out 24 h after training. In the test, trained animals were placed back in the training box platform until they eventually stepped down to the grid. The latency to step-down during the test session was taken as an indicator of memory retention. A ceiling of 180 s was imposed for the step-down latencies during the retention test. To evaluate the possible effect of the extract on the animal's memory consolidation, the treatment with HEPC (50, 100, or 150 mg/kg) or vehicle was given 30 min after the IA training session.

2.2.8. Tail Suspension Test (TST) and Forced Swim Test (FST). The TST and FST were used to assess the possible antidepressant-like effect of HEPC. The forced swim test (FST) is used to assess learned helplessness in rodents and has often been used to examine the effect of antidepressants in animal models of depression. The test was performed

according to Porsolt et al. [35] with minor modifications [36]. On the day of the test, the mice were individually introduced into a glass cylinder (height: 45 cm; diameter: 20 cm) containing water (25 ± 2°C; depth: 25 cm) from which they could not escape and had to swim or float. The fluctuation time (in seconds) in the last 4 minutes of the test session was recorded to reflect the depressive-like behavior of the mice. The mice's floating status means no movement except the movement necessary to keep the mice's heads above the water to breathe. The tail suspension test (TST) is also a useful behavioral tool for examining the effects of antidepressant drugs [37]. During the test, the mice were placed in a wooden apparatus divided into 4 compartments to be acoustically and visually isolated. A metallic bar suspended from the base 50 cm above the floor was adapted to the apparatus. On this bar, the animals were suspended with adhesive tape and kept approximately 1 cm from the tip of the tail. As with the FST, immobility time was recorded during the last 4 min of a 6 min session. Imipramine (30 mg/kg, p.o), HEPC (50, 100, or 150 mg/kg, p.o.), or vehicle was administered 1 h before both tests. TST was also used to evaluate the antidepressant-like effect of HEPC when administered chronically. Imipramine (30 mg/kg, p.o.), HEPC (150 mg/kg, p.o.), or vehicle was administered daily for 15 days and, on the last day of treatment, 1 h before the test. After the experiments, the animals used in the subchronic treatment to evaluate the antidepressant-like effects of EHPC were euthanized and their brains were collected for biochemical tests.

2.2.9. Time Course of the HEPC Antidepressant-Like Effect. In another set of experiments, different groups of mice were administered with HEPC 150 mg/kg (p.o.) or vehicle and subjected to the TST after 1 h, 2 h, 3 h, and 4 h from the administration. For each hour tested, a different group of animals was used, and we were careful to avoid mice learning [38].

2.2.10. Euthanasia and Animal Disposal. The animals were euthanized with anesthetic overdose as decided by the UNIVALI Animal Ethics Committee (CEUA/UNIVALI), except for the group used for biochemical tests, which were euthanized by cervical dislocation to avoid possible interference in the results. The carcasses were collected and sent for disposal and incineration by responsible technicians, following laboratory and institution procedures.

2.3. Biochemical Analysis. The levels of gamma-aminobutyric acid (GABA) and monoamine oxidase (MAO)- A activity in the animal's brain subchronically treated for 15 days with HEPC (150 mg/kg, p.o.), imipramine (30 mg/kg, p.o.), diazepam (2 mg/kg, i.p.), or vehicle were measured. The group that received diazepam was incorporated into the protocol separately. The mice were decapitated, and the brains were quickly transferred to a cold glass plate for dissection. The brain was quickly isolated, and homogenized being stored at −80°C until further analysis, as described by Zimath et al. [38], except for samples intended for measurement of GABA levels.

2.3.1. Quantification of MAO-A Enzyme Activity Levels. First, the brain homogenate was centrifuged for 10 minutes at 4000 rpm at 4°C. The supernatant was removed and centrifuged for 120 minutes at 10000 rpm at 4°C. The pellets were resuspended with phosphate buffer. Protein concentration was adjusted to 1 mg/ml and measured using bovine albumin as a standard. To evaluate the MAO-A activity, 50 μL of the sample plus 30 μL of 4 mM 5-HT (as a specific substrate for MAO-A) and 100 μL of phosphate buffer were incubated for 20 minutes at 37°C. After 20 minutes, the reaction was stopped with 50 μL of 1 M HCl. The reaction product was extracted with 2200 μL of butyl acetate. The organic phase was used, and the absorbance was measured at 280 nm. To perform the blank 50 μL of the sample, plus 50 μL of 1 M HCl, and 100 μL of phosphate buffer was incubated for 20 minutes at 37°C. Subsequently, 30 μL of 4 mM 5-HT and 2200 μL of butyl acetate were added. The organic phase was used and measured in a spectrophotometer at 280 nm. MAO-A enzyme activity was expressed as nmol/mg of protein [38, 39].

2.3.2. Estimation of GABA by Spectrophotometry. The GABA content was determined from the whole brain homogenized in a tube containing 5 mL of 0.01 M HCl. Brain homogenate was transferred to a bottle containing 8 mL of ice-cold absolute alcohol and kept for 1 h at 0°C. The content was centrifuged for 10 min at 16,000 rpm, the supernatant was collected in a Petri dish. The precipitate was washed with 5 mL of 75% alcohol three times and washes were combined with supernatant. Next, samples were evaporated to dryness at 70°C in a water bath. To the dry mass, 1 mL water, and 2 mL chloroform were added and centrifuged at 2000 rpm. The upper phase containing GABA (2.0 mL) was separated and 10 mL of it was applied as spot on Whatman paper (N° 41). The mobile phase consisted of n-butanol (50 mL), acetic acid (12 mL), and water (60 mL). The paper chromatogram was developed with ascending technique. The paper was dried in hot air and then spread with 0.5% ninhydrin solution in 95% ethanol. The paper was dried for 1 h at 90°C. The blue color spot developed on the paper was cut and heated with 2 mL of ninhydrin solution in a water bath for 5 min. Water (5.0 mL) was added to the solution and kept for 1 h. The supernatant (2.0 mL) was decanted, and absorbance was measured at 570 nm by using spectrophotometry. GABA standard was used to extrapolate the absorbances of the samples [40].

2.4. Statistical Analysis. Data were evaluated for normality by the Shapiro–Wilk test and Grubbs' test was used to detect outliers. For parametric tests, the results were presented as means ± standard error of means (S.E.M). Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA), when applicable, followed by the Bonferroni post-test. The data obtained in the step-down IA task

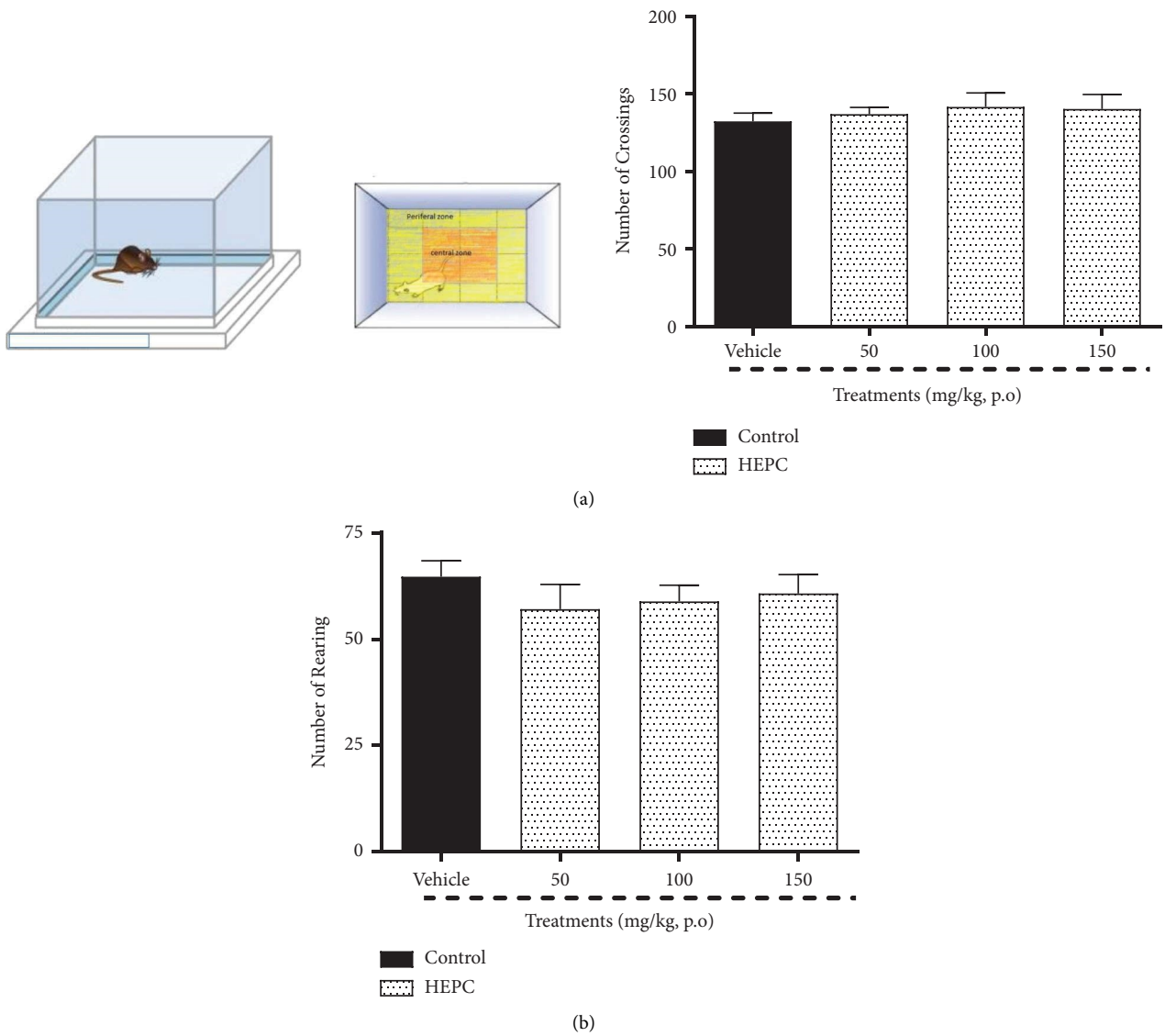

Figure 1: HEPC effects in animal motor performance of mice exposed to open-field test (OFT). HEPC does not change the number of mice (a) crossing or (b) rearing when submitted to the open-field test. Results are presented as the means ± S.E.M. ($n = 8$–10). ANOVA followed by Bonferroni's multiple comparisons test when compared to the vehicle group (treated with distilled water plus 2% DMSO).

were reported as median ± interquartile ranges (25 and 75). The analysis of IA data was nonparametric because this procedure involved a cutoff score, and Kruskal–Wallis's test was performed followed by Dunn's test. All analyses were performed using the GraphPad Prism 7.0 program (San Diego, USA). Values of $p < 0.05$ were considered significant.

3. Results

3.1. HEPC Effects in Animal Motor Performance of Mice Exposed to Open-Field Test (OFT). No statistical difference among the experimental groups was obtained in the behavioral parameters recorded by OFT. Our data show that the administration of treatments does not change the number of animal passages (crossings), ($F(3.36) = 0.3370$, $p = 0.7986$), as well as did not change the number of exploratory activities (rearing), ($F(3.36) = 0.5570$, $p = 0.6468$), indicating that it probably does not change the mobility of animals in the other behavioral tests used in this study (Figures 1(a) and 1(b)).

3.2. HEPC Effects on Pentylenetetrazol (PTZ) and Strychnine (STR) Induced Seizure Test in Mice. The effect of HEPC treatment on PTZ and strychnine-induced seizure models is shown in Figure 2. In the PTZ-induced seizure (Figure 2(a)), HEPC at doses of 50, 100, or 150 mg/kg were not able to increase latency time for the onset of seizures compared with the vehicle group. As expected, phenobarbital increased the latency time for the onset of seizures ($F(4.43) = 8.554$, $p < 0.0001$). In strychnine-induced seizures (Figure 2(b)), the treatment of animals with HEPC did not increase the latency time for the onset of seizures compared to the vehicle

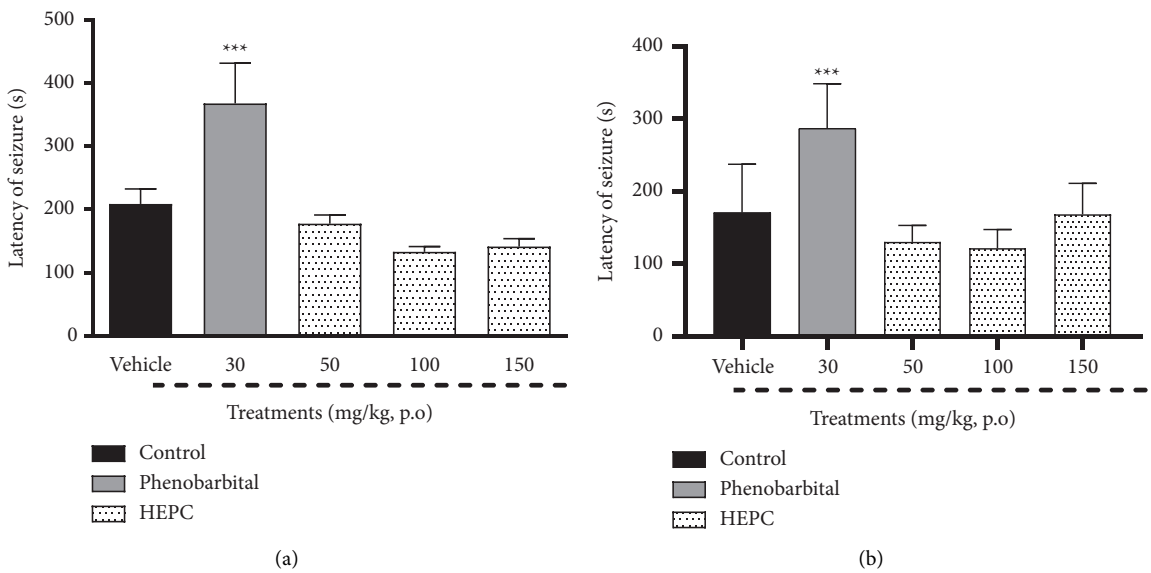

Figure 2: HEPC effects on Pentylenetetrazol (PTZ) and strychnine (STR) induced seizure test in mice. Phenobarbital, but not HEPC, alters latency for seizures induced by pentylenetetrazol (PTZ) (a) or strychnine (STR) (b) in mice. Results are presented as the means ± S.E.M. ($n = 8$–10). One–-way ANOVA followed by Bonferroni's multiple comparisons tests. $^{***}p < 0.0001$ compared to the group treated with vehicle (treated with distilled water plus 2% DMSO).

group. Nevertheless, in this test, the typical anticonvulsant effect of phenobarbital was verified ($F(4.42) = 17.94$, $p < 0.0001$).

3.3. HEPC Effects on Pentobarbital-Induced Hypnosis (PIH) in Mice. The effect of HEPC treatment on pentobarbital-induced hypnosis is shown in Figure 3. The results obtained demonstrate that the treatment of animals with HEPC at 100 or 150 mg/kg decreased the sleep latency time when compared with the vehicle group ($F(4.43) = 9.501$, $p < 0.0001$). Diazepam, used as a positive control, also promoted a decrease in sleep latency time when compared to the vehicle group ($p < 0.001$), (Figure 3(a)).

In Figure 3(b), the total sleep time was shown, which was changed only with the treatment of HEPC at 150 mg/kg, when compared with the vehicle group ($F(4.44) = 42.14$, $p < 0.001$). The treatment of animals with diazepam showed similar results, promoting an increase in total sleep time, and reproducing its characteristic hypnotic effect ($p < 0.001$).

3.4. HEPC Effects on Anxious Behavior of Mice Exposed to Elevated Plus-Maze Test (EPM). The results presented in Figure 4 demonstrate that the treatment of the animals with HEPC only at 150 mg/kg increased the frequency of entry into open arms (Figure 4(a)), and the time of mice exploration in these arms (Figure 4(b)) of the apparatus, when compared with the vehicle group (($F(4.43) = 23.64$, $p < 0.0001$), ($F(4.43) = 74.78$, $p < 0.0001$), respectively). As expected, during the experiment, the classic anxiolytic-like effect of diazepam was observed, increasing such behavioral parameters in the open arms of the EPM when compared to the vehicle group ($p < 0.001$).

3.5. HEPC Effects on Mice Submitted to Inhibitory Avoidance Test (IAT). Figure 5 shows the effects of HEPC on memory retention in animals submitted to IAT. It was observed that the animals learned the inhibitory avoidance task, comparing the test and training sections for each treatment group, with an increase in the latency of descent from the apparatus platform. However, when the test session is evaluated (only the dark bars in Figure 5) and compared with the vehicle (represented by the 0 doses of HEPC), it is verified that there was no statistically significant effect of the treatment on the animals' memory, demonstrating thus, that HEPC did not exhibit effects on the memory of the animals at the doses tested.

3.6. HEPC Antidepressant-Like Effect in Mice Submitted to Tail Suspension Test (TST) and Forced Swim Test (FST). The antidepressant-like property of HEPC is demonstrated in Figures 6(a) and 6(b). In Figure 6(a), it is observed that the acute treatment of the animals with HEPC at doses of 50, 100, and 150 mg/kg promoted a significant decrease in the immobility time of the animals when submitted to FST when compared with the control group treated with vehicle (($F(4.38) = 31.71$, $p < 0.0001$)). In the TST (Figure 6(b)), only HEPC at 50 and 100 mg/kg were effective in reducing the animals' immobility time ($p < 0.001$) when compared to the vehicle-treated control group (($F(4.38) = 4.671$, $p = 0.0036$)). In both tests, respectively, the classic antidepressant-like effect of imipramine was observed ($p < 0.001$) compared to the vehicle-treated control group. The results observed in Figures 7(a) and 7(b), respectively, demonstrate that after 7 and 14 days of treatment, HEPC (150 mg/kg) continues to promote an antidepressant-like effect, evaluated by the decrease in the immobility time of mice when submitted to FST

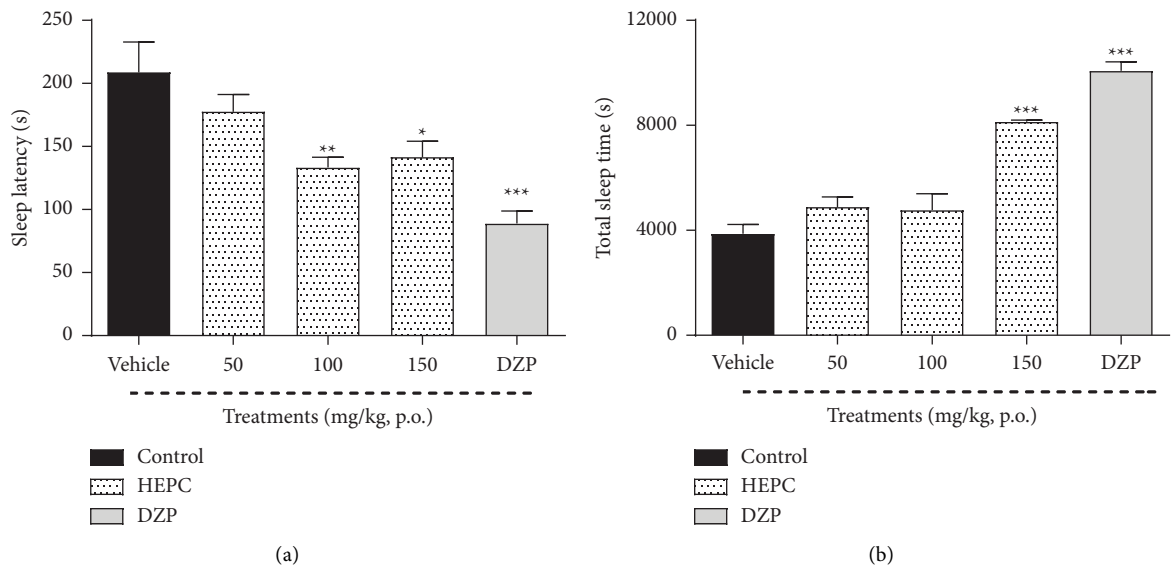

Figure 3: HEPC effects on pentobarbital-induced hypnosis (PIH) in mice. HEPC increases sleep latency (a) and total sleep time (b) in pentobarbital sleep induction test in mice. Results are presented as the means ± S.E.M. ($n = 6$–8), one-way ANOVA followed by Bonferroni post-test. $^{***}p < 0.0001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ compared to the group treated with vehicle (treated with distilled water plus 2% DMSO). DZP-diazepam.

when compared to the vehicle-treated control group ($p < 0.0001$). In this experiment, the antidepressant-like effect of imipramine (30 mg/kg) was observed only on the 14th day when compared to the vehicle-treated control group (($F(2.23) = 24.84$, $p < 0.0001$); ($F(2.23) = 4.585$, $p = 0.0122$), respectively). Regarding the duration of the antidepressant-like effect of HEPC, it was observed (Figure 7(c)) that the treatment with HEPC (150 mg/kg, p.o.) produced a significant effect by reducing the immobility time of the animals after 1, 2, 3, and 4 hours ($p < 0.05$, $p < 0.001$, $p < 0.05$, respectively) of its administration when compared with the vehicle-treated control group. The peak of the antidepressant-like effect was observed 2 h after applying HEPC (($F(6.51) = 10.64$, $p < 0.0001$)). In the experiments related to the subchronic treatment with HEPC, as well as the time of action of the same, the animals were also submitted to the open-field test without any change in their motor performance (results not shown).

3.7. HEPC Effects on MAO-A Enzyme Activity and the Estimation of GABA by Spectrophotometry Measured in Mice's Brain. To relate a possible antidepressant mechanism of action elicited by HEPC, the effects of treatments on the activity of the MAO-A enzyme were analyzed. Figure 8(a) shows that HEPC promoted a decrease in MAO-A activity ($p < 0.01$), when compared to the vehicle group (($F(3.20) = 6.388$, $p = 0.0068$)). On the other hand, the effects obtained with HEPC on the estimation of GABA revels that the treatment with HEPC and DZP promoted a significant increase in the levels of GABA when compared with the vehicle ($p < 0.05$), as demonstrated in the Figure 8(b) (($F(3.20) = 16.10$, $p < 0.0001$)).

4. Discussion

The investigation of new pharmacological strategies for the treatment of mood disorders, sleep disorders, or anxiety disorders is essential for the discovery of drugs with efficacy, fewer side effects, and low toxicity which could be used in longer treatments, and medicinal plants play an important role in this context [11, 12, 41]. Humanity has explored plants' psychoactive effects for millennia, using them in their most natural form [42, 43]. However, the pharmaceutical market has been demanding the pharmacological validation of extracts and/or active principles, their efficacy, and above all, security regardless of whether the use is for nervous system pathologies [44]. Therefore, to contribute to this field, this research investigated the psychoactive effect of the hydroalcoholic extract of *Piper cernuum* (HEPC), using experimental tools employed to screen psychotropic drugs.

Based on the literature on the pharmacological potential of the *Piper* genus on epilepsies, we investigated the effect of HEPC in two chemical models of seizures. The pentylenetetrazol- and the strychnine-induced seizure model [45]. Moreover, piperine, a marker of the genus, exhibits an antiepileptic effect detected in these models and in others [46, 47]. Essential oils obtained from *Piper*'s species also exhibit anti-convulsant properties [48]. However, neither the treatment with the essential oil of the plant (results not shown) nor the treatment with HEPC, at the doses used, administered acutely, did not demonstrate an anticonvulsant effect in both seizure tests. To observe an effect with subchronic treatment (15 days) at the highest dose, animals were subjected to a pentylenetetrazol-induced seizure model (results not shown), and even then, the effect was not detected.

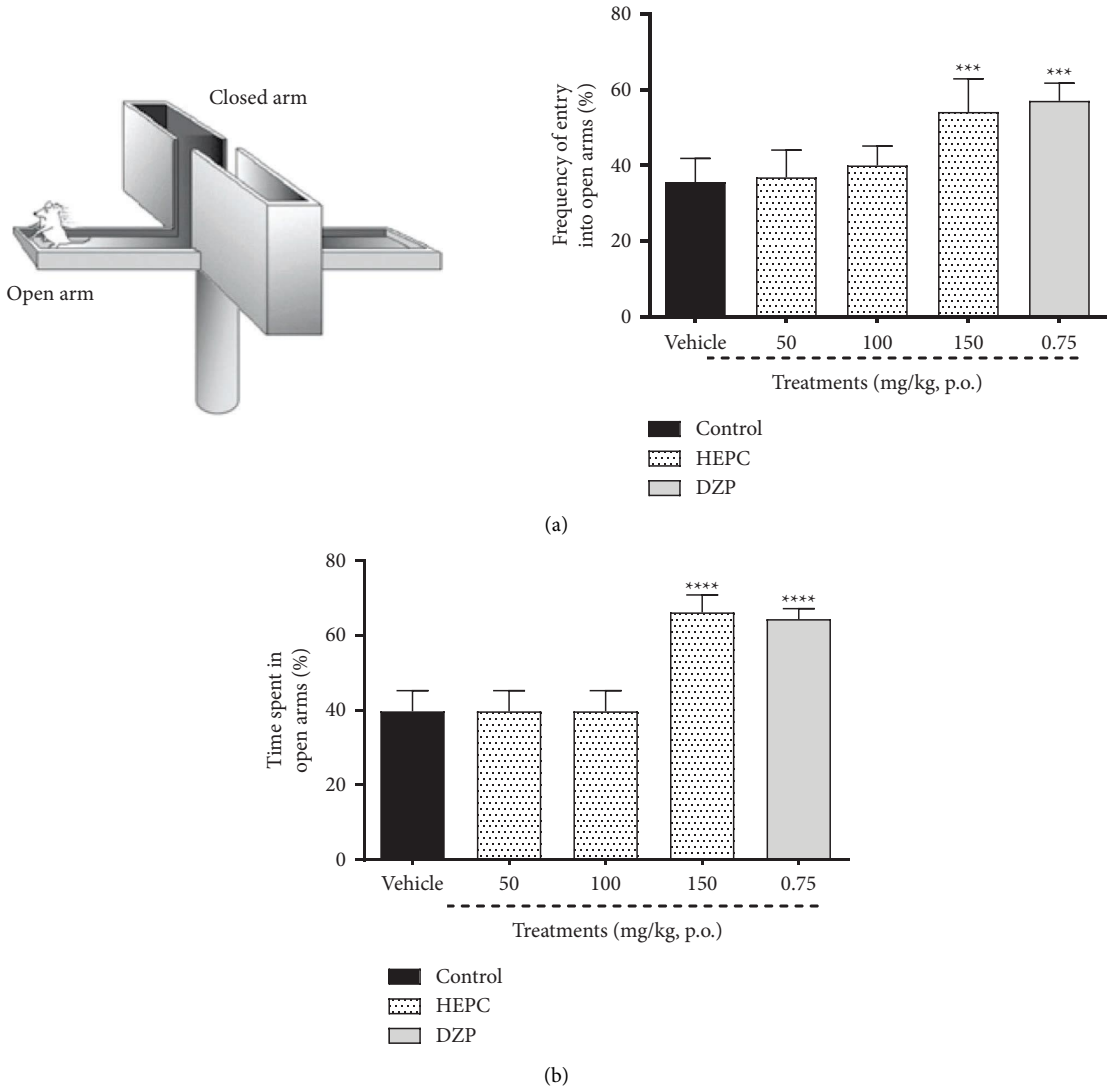

Figure 4: HEPC effects on anxious behavior of mice exposed to elevated plus-maze test (EPM). HEPC administration increased the mice frequency of entries (a) and time spent (b) in the open arms of the apparatus. Results are presented as the means ± S.E.M. ($n = 8$–10). One-way ANOVA was followed by Bonferroni's post-test. *** $p < 0.001$ and **** $p < 0.0001$ compared to the group treated with vehicle (VEH). DZP- diazepam.

Sleep disorders are frequent in neuropsychiatric and/or neurodegenerative diseases. The use of herbs with hypnotic characteristics in the management of insomnia is common among people and, herbal formulations with renowned hypnotic effects are easy to find [49, 50]. A plant with multiple effects can be advantageous in treating neuropsychiatric diseases whose patients exhibit sleep disorders [51]. In the present study, we evaluated the hypnotic effect of HEPC, and mice treated with the extract were subjected to the barbiturate-induced sleep test.

Substances that depress the CNS, in general, increase the duration of sleep produced by pentobarbital, and there may also be a reduction in the latency for the effect of the barbiturate used, seen by the reduction in the induction time, that is, from the application of the barbiturate until the loss of the postural reflex by the animal [52]. Our results showed that HEPC effectively decreased sleep latency and increased the total sleep time of animals submitted to the barbiturate-induced sleep test. It is expected that an agent with allosteric effects on the GABAA receptor promotes pentobarbital potentiation and, therefore, a depressant effect on the CNS [53] and that hypnotic substance reduce sleep latency and increase the total sleep time of the animals submitted to barbiturate-induced sleep test [32]. Therefore, our results corroborate those that evidenced the hypnotic effects of *Piper* sp. on sleep-related disorders, mainly regarding their maintenance [54].

The most widespread psychoactive effect of the *Piper* genus in the literature is, without a doubt, the anxiolytic effect. Many species such as *Piper nigrum* and *Piper sylvaticum* Roxb. have this pharmacological property very well documented and studied [55]. However, undoubtedly, the most studied species of the genus regarding this pharmacological property is *Piper methysticum* or simply Kava Kava [14]. There are several articles evidencing the use of Kava for generalized anxiety disorders [56].

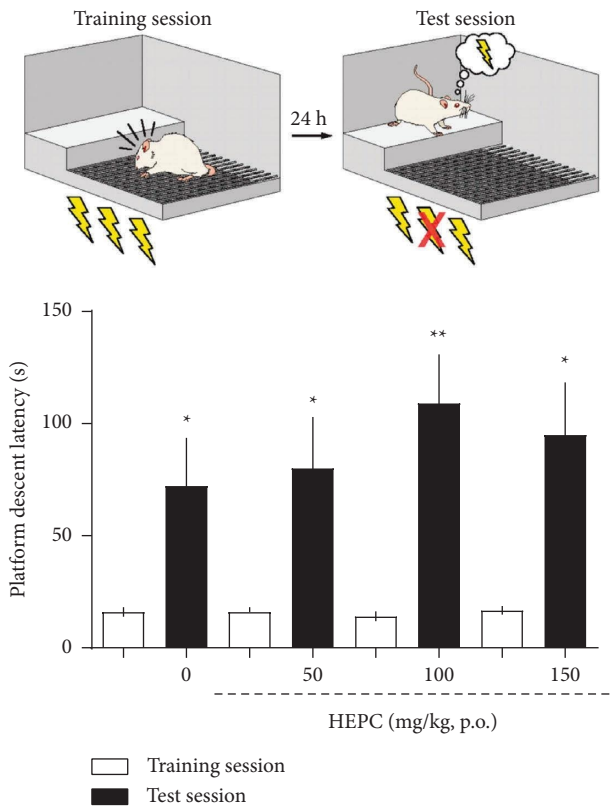

Figure 5: HEPC effects on mice submitted to inhibitory avoidance test (IAT). HEPC does not alter the memory of animals submitted to the inhibitory avoidance test. The white bars refer to the results obtained in the training sessions, and the dark bars refer to the results of the test session. The treatments were given immediately after training in a single trial, on memory retention of inhibitory avoidance measured 24 h later. Each bar represents the median (interquartile range) for 8 to 10 animals per group. The data obtained in the step-down IA task were reported as median ± interquartile ranges (25 and 75). The analysis of IA data was nonparametric because this procedure involved a cutoff score, and the Kruskal–Wallis test was performed followed by Mann–Whitney's U test. $^{*}p < 0.05$ and $^{**}p < 0.01$.

Based on it, the anxiolytic effect of HEPC was tested in animals subjected to the elevated plus-maze test (EPM), an experimental tool frequently used to detect and evaluate the anxiolytic/anxiogenic properties of drugs. The entries and time spent in the open arms is the main indicator of fear in the EPM, given that an open area is extremely aversive to rodents. In addition, no thigmotaxis enhances the state of fear and aversion in the animals in open arms [33]. Our results with HEPC showed that only the treatment with a dose of 150 mg/kg was effective in increasing the frequency of entry into the open arms of the apparatus, as well as increasing the time spent by the animals in the same arm, suggesting a type-anxiolytic effect of this extract, making *Piper cernuum* an interesting source for research to new therapeutic option in the management of anxiety.

The effect of HEPC on memory consolidation was also examined, and effects on the animals' memory were not observed in the inhibitory avoidance test. Anxiolytic and hypnotic substances that do not promote cognitive impairment are welcome from a pharmacological point of view. BDZs are still the most used pharmacological class to produce sedative and hypnotic activities, but they have a propensity effect to cause cognitive impairment [57].

Mice acutely administered with HEPC presented a reduction in the immobility time when exposed to the FST and TST, both predictive behavioral paradigms to evaluate the antidepressant-like effect of substances in rodents [35, 37, 58]. Additionally, we verified that the antidepressant-like effect of HEPC also occurs with subchronic treatment since the immobility time of the animals in the FST at 7 and 14 days of treatment was reduced. Noteworthy, HEPC exerted an antidepressant-like effect for 4 hours from the administration, without compromising the locomotor activity of mice when exposed to the OFT. Crossings number in the OFT was registered as a parameter to investigate possible effects triggered by the treatments on the motor activity of mice, to discard a false positive or negative result from TST and FST, as well as in other behavioral tests performed here that require the integrity of the animals' motor system [33, 38].

The relationship between *Piper* and depression is little explored in the literature, compared to insomnia [59], epilepsy [54], and anxiety [41]. The articles are more related to piperine and its role as an antidepressant agent [60]. In recent times we have studied the pharmacological potential of *Piper amplum*, *Piper aduncum* L, and *Piper molluscum* as psychotherapeutic agents and we are having positive results regarding antidepressant-like effects (work in progress). Thus, the focus of the present study was to investigate the antidepressant-like effect of HEPC.

The mechanisms by which HEPC could be acting were partially investigated in this study. However, further studies are needed to clarify better the pharmacodynamic mechanism of action of the plant extract, which may be one of the limitations of the present study that can be remedied in later studies. On the other hand, we tried through the biochemical assays available in our laboratories to study two systems related to anxiety and depression.

The antidepressant and anxiolytic-like effects of the extract were observed in behavioral trials with acute and subchronic treatments (mainly regarding the antidepressant effect). Thus, animals treated with repeated doses of the extract had decreased depressive-like compounds and they were used in biochemical tests. So, the participation of the GABAergic system in the extract's mechanism of action was investigated in the animals' brains. The mechanisms underlying the pathophysiology and treatment of depression and stress-related disorders remain unclear, but studies in depressed patients and rodent models are beginning to yield promising insights [61]. The implication of GABA is based mainly on animal models, whereas clinical studies in depressed patients show alterations in GABA levels in plasma and cerebrospinal fluid. Neuroimaging studies using spectroscopy also indicate decreased GABA levels in different brain areas, which may normalize after antidepressant therapy, and these findings translate into clinical response [62, 63]. On the other hand, the literature is rich on the

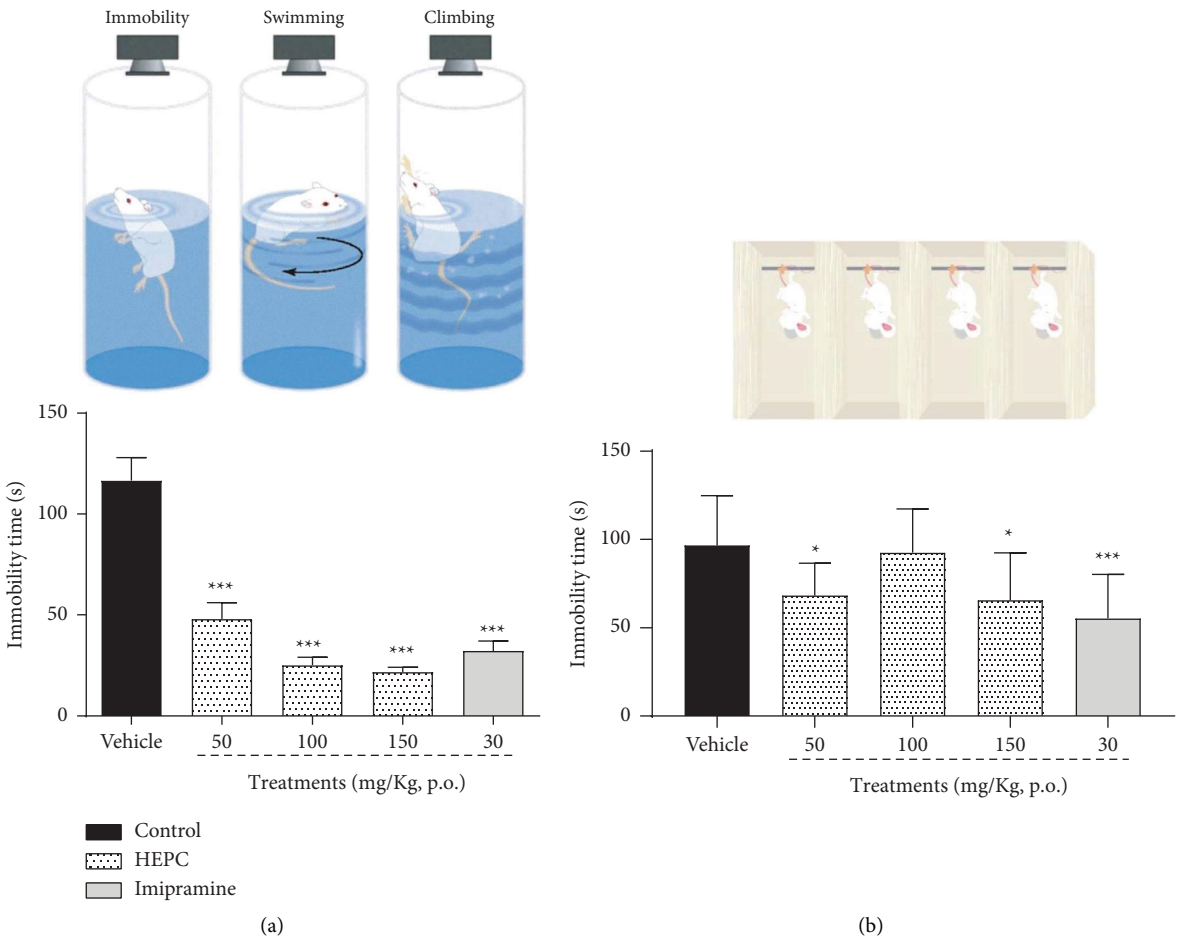

Figure 6: HEPC antidepressant-like effect in mice submitted to tail suspension test (TST) and forced swim test (FST). HEPC elicited an antidepressant-like effect in mice submitted to the forced (a) swimming test and in the tail (b) suspension test. Results are presented as the means ± S.E.M. ($n = 8$–10). One-way ANOVA was followed by Bonferroni's post-test. $^{***}p < 0.0001$ and $^{*}p < 0.05$ compared to the group treated with vehicle (VEH).

relationship between stress and depression [64], showing this relationship in rodents and humans [65]. The period of stress can result in the chronicity of depression, and the literature has shown that even in rodents, subchronic stress induces similar behavioral effects in male and female mice despite sex-specific molecular adaptations in the *nucleus accumbens* [66]. In addition, some studies have shown that the use of the depression model induced by chronic or subchronic stress increases oxidative stress or changes neurotransmitter levels in important mesolimbic structures in the brains of animals treated with the vehicle when compared to the naive group and that the treatment of animals with antidepressants, especially inhibitors selective serotonin and norepinephrine reuptake or substances of natural origin, ameliorates stress-induced behavioral as well as oxidative alterations [67, 68]. Our results demonstrated that the stress generated by the subchronic administrations and daily handling of animals can promote in the groups treated with vehicle an increase in MAO activity and a decrease in the levels of GABA in the brain of the animals, different from that observed in naive animals. The results showed that there is a significant increase in GABA levels in the groups treated with HEPC, supporting the idea that HEPC interacts with the GABAergic system in terms of increasing its neurotransmission. GABA is the most abundant inhibitory neurotransmitter in the CNS [69], exerting its effects on GABAA, GABAB, and GABAC receptors [70–73]. Given that the GABAergic system is closely linked with the regulation of sleep [74], anxiety [75], and epilepsy [76], it is possible to infer that, at least in part, the anxiolytic and hypnotic properties of the extract involve this system.

The relationship between the GABAergic system with depression is not yet well investigated in the literature, although it exists [61], perhaps because there are no antidepressant drugs that act directly on the GABAergic system. However, it is known that the glutamatergic system, which is controlled by the GABAergic system, is involved with the pathogenesis of depression. The glutamatergic hypothesis of depression is an exacerbation of the glutamatergic system that is observed in depressive patients [77], suggesting an indirect involvement of this system with depressive episodes [78].

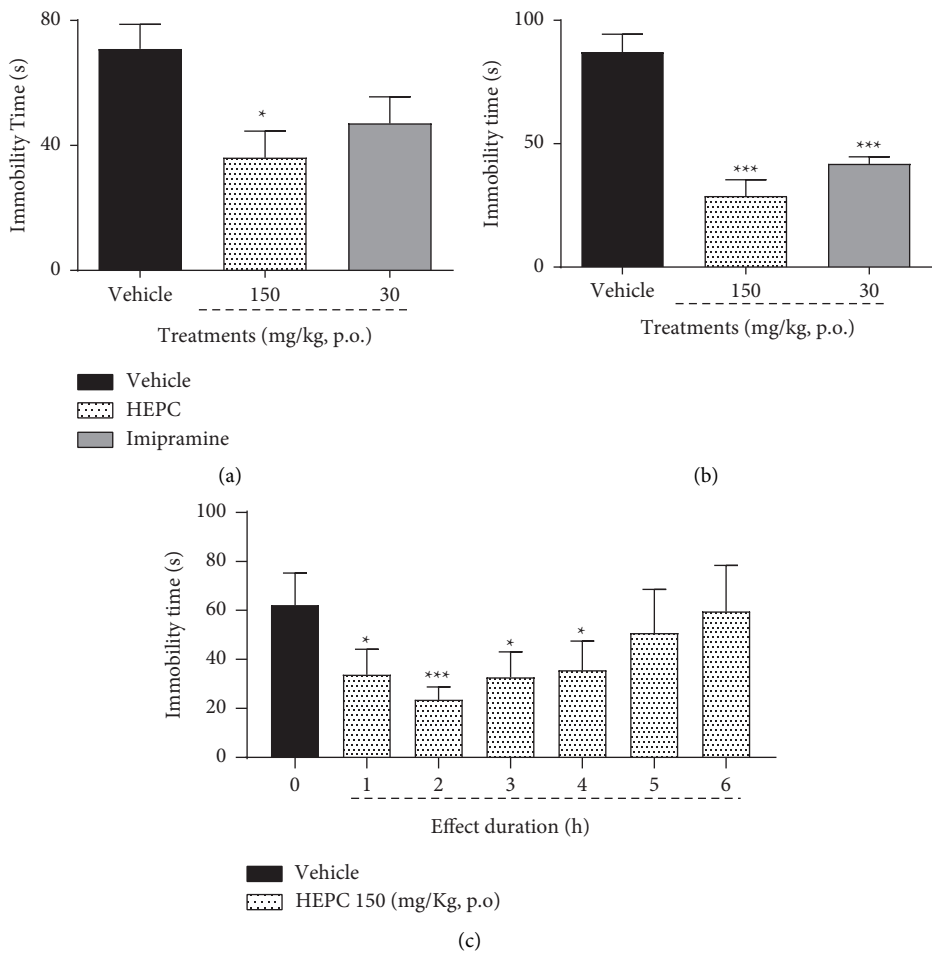

Figure 7: Time course of the HEPC antidepressant-like effect in mice subchronically treated. Effect of HEPC in the immobility time of mice submitted to the tail suspension test (a) 7 days and (b) 14 days after treatments. Panel (c) represents the time course of the HEPC antidepressant-like effect at 150 mg/kg (p.o.). Results are expressed as mean ± S.E.M ($n = 8$–10). * $p < 0.05$ and *** $p < 0.0001$ compared with the vehicle-treated group; one-way ANOVA followed by Bonferroni's post-test.

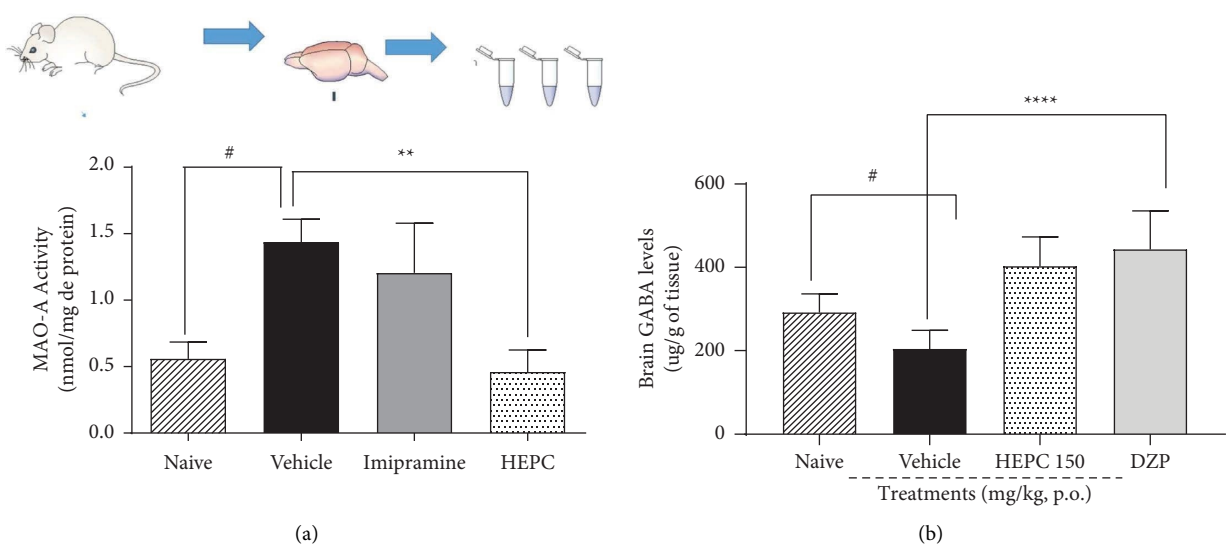

Figure 8: HEPC effects on MAO-A enzyme activity and the estimation of GABA by spectrophotometry measured in mice's brain. HEPC decreased MAO-A activity (a) and increased GABA brain levels (b). ** $p < 0.001$ and **** $p < 0.0001$ compared to vehicle. # $p < 0.05$, compared to Naive. Results are presented as the means ± S.E.M. ($n = 5$–10). One-way ANOVA was followed by Bonferroni's post-test. Naïve = animals without treatment.

In addition, studies have been demonstrating that depression and chronic stress exposure cause atrophy of neurons in cortical and limbic brain regions implicated in depression, and brain imaging studies have been demonstrating altered connectivity and network function in the brains of depressed patients. Studies of the neurobiological basis of these alterations have focused on an excitatory glutamate neuron together with an inhibitory GABA interneuron. They demonstrate structural, functional, and neurochemical deficits in both major neuronal types that could lead to signal integrity degradation in cortical and hippocampal regions [61]. Therefore, our results regarding the extract's antidepressant-like properties may also be related to the increase in GABA levels in the brain of the treated animals.

Antidepressants may act by modulating the MAO enzyme, and this mechanism has already been detected in secondary metabolites from plants [79]. The MAO enzyme metabolizes xenobiotics and endogenous amines and neurotransmitters, including 5-hydroxytryptamine (5-HT, serotonin), dopamine (DA), noradrenaline (NA), tyramine, and tryptamine [80]. It occurs as two isoenzymes, MAO-B is involved in neurodegenerative diseases and MAO-A in psychiatric conditions such as MDD [81]. Here, the pretreatment with HEPC promoted the MAO-A inhibition in the brain, maybe in structures that are closely related to the limbic system and mood modulation. Together, these results indicate that MAO-A inhibition may be involved in the HEPC antidepressant and anxiolytic effects. Inhibition of MAO-A by the extract would consequently promote an increase in the brain concentration of important neurotransmitters, such as serotonin, whose alterations in its levels are involved in both diseases, depression, and anxiety.

The relationship between the pharmacological effects detected in this study and the phytochemical profile of the extract used can be analyzed. As reported previously, *P. cernuum* leaves are used in folk medicine as an infusion or a macerate with alcohol, and therefore the alcoholic extract was chosen to access the psychopharmacology properties of this plant in this study. Wolff and collaborators [31] described that this same extract did not present significant toxicity when administered to male or female rats and presented a high-performance liquid chromatography profile for this extract. In the leaves of *P. cernuum* were found cinnamic acids derivatives, lignans as cubebin and hinokinin [18, 20], which are extracted more efficiently with ethanol and can be present in HEPC. Some of these phytoconstituents exhibit psychopharmacological properties already reported in the literature and may be contributing to the psychoactive effects found in *P. cernuum*. For cinnamic acid, antidepressant effects have been recently reported [82, 83], as well as the anticonvulsant potential of this compound and its derivatives [84]. GABA transporters serve as a target for anxiolytic, antidepressant, and antiepileptic therapies. Interaction with important neurotransmitter transporters has been characterized for a lignan derivative (−)-cubebin, and (−)-hinokinin, and the results obtained to date suggest that hinokinin can serve as a tool to develop new therapeutic anxiety drugs that target dopamine, norepinephrine, and GABA transporters [85]. Another interesting class in Piperaceae was alkaloids. This class of compounds has psychoactive characteristics that are also interesting and may be directly or indirectly involved in many of its effects. In the extract used, the alkaloids were not individually characterized, however, these phytoconstituents have already been identified in several species of pipers being responsible for their various effects [86, 87].

5. Conclusion

Together, the results suggest that HEPC has therapeutic potential as an antidepressant, anxiolytic, and sedative-hypnotic agent. Preliminarily, is possible to suggest that the neuropharmacological effects of HEPC could be, at least in part, related to the modulation of the GABAergic system and/or MAO-A activity. However, studies are needed to evaluate the chronic effects of HEPC on anxiety to verify if the increase in GABA levels could also be related to its anxiolytic effect. We also suggest that further experiments be conducted with male mice to verify possible variations in the pharmacological effects of the extract according to sex.

Data Availability

The data that support the findings of this study are available from the corresponding author, MMS, upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

MMS conceived and designed the research; AM and JDJ were responsible for collecting the plant material and preparing the extracts; MAM, CAC, CES, and NS contributed to the data collection of the experiment; LMS, MMS, and MB analyzed and interpreted the data; MMS and LMS wrote the article; APD, MMS, and LMS modified the manuscript. All authors have read and approved the final manuscript.

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Research Article

Involvement of Anti-Inflammatory and Stress Oxidative Markers in the Antidepressant-like Activity of *Aloysia citriodora* and Verbascoside on Mice with Bacterial Lipopolysaccharide-(LPS-) Induced Depression

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Aloysia citriodora Palau is popularly used to treat nervous disorders. Experimental evidence has indicated that verbascoside (VBS) isolated from *A. citriodora* has pharmacological potential. In this study, we evaluated the antidepressant-like effects of a hydroalcoholic extract of *A. citriodora* (HEAc) and VBS against lipopolysaccharide- (LPS-) induced depressive-like behavior in mice. In the pretreatment protocol (performed to evaluate the preventive potential), mice were pretreated with HEAc (3, 30, or 300 mg/kg) or VBS (30 mg/kg) before the administration of LPS. In the posttreatment protocol (performed to evaluate the therapeutic potential), mice were initially administered LPS and were subsequently given HEAc (3, 30, or 300 mg/kg) or VBS (30 mg/kg). In both treatments, the mice were submitted to an open-field test and tail suspension test (TST) at 6 and 24 h after LPS administration. The posttreatment evaluation revealed that HEAc (30 or 300 mg/kg) and VBS produced an antidepressant-like effect, as indicated by a reduction in the time spent with no movement in the TST. Moreover, HEAc (30 or 300 mg/kg) was found to reduce interleukin-6 (IL-6) levels and N-acetyl-glycosaminidase activity in the hippocampus, increase glutathione (GSH) levels in the hippocampus and cortex, and enhance IL-10 in the cortex and, at a dose of 300 mg/kg, reduced myeloperoxidase activity in the cortex. Contrastingly, no comparable effects were detected in mice subjected to the pretreatment protocol. Administration of VBS similarly reduced the levels of IL-6 in the hippocampus and increased GSH levels in the cortex. Our observations indicate that both HEAc and VBS show promising antidepressant-like potential, which could be attributed to their beneficial effects in reducing neuroinflammatory processes and antioxidant effects in the central nervous system.

1. Introduction

Despite clinical advances in the treatment of neuropsychiatric disorders, mood disorders such as major depressive disorder (MDD) are still considered an important public health problem. MDD is characterized by depressed mood and/or anhedonia, which may be related to other behavioral changes and vegetative symptoms that compromise physical

and mental health [1]. Furthermore, depression is a leading cause of disability and affects approximately 300 million people worldwide [2].

Although considerable attention has been focused on the multifactorial and heterogeneous disorder of depression, its etiology remains poorly understood [3]. Furthermore, it is well established that depressive conditions often occur in the absence of a psychobiological triggering factor, and there is evidence to indicate the involvement of somatic, genetic, and environmental factors in its genesis, particularly the disturbance of hypothalamic functions and neurotransmission [4,5]. The monoaminergic hypothesis is currently the most widely accepted molecular mechanism proposed to explain the etiology of depression. According to this pathophysiological proposal, depression may arise because of depletion and disturbance of the activity of neurotransmitters, mainly serotonin, norepinephrine, or dopamine, in the central nervous system (CNS). Thus, several pharmacological agents used to treat MDD target this pathway, such as tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), serotonin and noradrenaline reuptake inhibitors (SNRIs), and selective serotonin reuptake inhibitors (SSRIs) [4]. However, this therapy is not effective in all patients, which is in part attributable to the heterogeneity of the etiological factors of MDD which remain little explored. Moreover, these medications can have significant side effects, including nausea, drowsiness, vertigo, tremors, and fatigue [6–8]. In addition, it is estimated that 50% of patients are refractory to the initial antidepressant treatment and approximately 30% fail to reach remission after several pharmacological attempts [9].

In recent decades, advances have, nevertheless, been made regarding our understanding of the psychological bases underlying MDD, with observations of brain circuits revealing abnormal information processing as well as the characterization of the cellular and molecular alterations associated with this disorder. These advances have been made possible, at least in part, using rodent models, which have facilitated the investigation of molecular targets, including those stress axis abnormalities and elevated neuroinflammation [10].

Neuroinflammation is an important immune defense mechanism induced in response to infectious agents or damaged cells within the CNS. However, excessive neuroinflammatory responses may result in neuronal damage that is observed in several neurodegenerative diseases and disorders, including depression [11]. In this context, accumulating evidence indicates that inflammation may play a pivotal role in the pathophysiology of depression [12–14]. It has also been suggested that depression is an inflammatory disorder characterized by increased levels of proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), and IL-6 in several brain regions [15], and similar findings have been reported in experimental studies [16]. Consistent with these observations, it has been demonstrated that the blockade of IL-1 or the inhibition of transcription factor nuclear factor kappa B (NF-κB) shows promise in preventing depression-like behavior in rodents [17, 18].

The findings of other investigations have indicated that depressive patients generally show evidence of oxidative stress [19], which may arise from the continued disruption of the antioxidant defense system and an enhanced redox imbalance that culminates in an increase in the activity of reactive oxygen species (ROS) generation [20]. This perturbation of the balance between the system of oxidizing agents and antioxidants may lead to irreversible changes and promote tissue damage in different organs. Among the various organs, the brain is particularly susceptible to oxidative damage. This susceptibility can be attributed to the fact that the brain utilizes large amounts of oxygen and the fact that neural cells contain high levels of lipids, including nonsaturated fatty acids, with which free radicals readily react [21]. In addition, the oxidative stress induced by excess ROS can in turn exacerbate the expression of inflammatory cytokines and aggravate the pathogenesis of various mood disorders. Indeed, the findings of previous studies have indicated that exposure to persistent oxidative stress contributes to enhancing the susceptibility to depression and that these effects can be mitigated by treatment with antioxidants [22].

In animal models, lipopolysaccharide (LPS, a bacterial endotoxin) is used to create a neuroinflammation-related model of MDD that is characterized by behavioral changes, including a reduced sucrose preference and an increase in despair-like behavior. These symptoms are associated with the increased brain expression of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α [23–26]. Notably, the levels of these inflammatory mediators can be reduced by the administration of certain natural compounds that exert modulatory effects in several brain areas [27].

In this latter context, human populations have for long selected and used plants according to their therapeutic and preventive significance; this has been documented in various ethnopharmacological records [28, 29]. These phytochemical compounds have been shown to exercise anti-neuroinflammatory effects against LPS-activated microglia via different mechanisms [27], and, in recent years, there has been a growth in the number of pharmacological studies that have examined the properties of plant-based extracts, substances isolated from plants, and synthetic derivatives based on promising natural products [30].

Aloysia citriodora Palau (Verbenaceae) is a medicinal plant native to South America, with global distribution, and is considered to have a high bioactive potential. This shrub, which can grow to heights of up to 3 m, has leaves with an aromatic lemon-like odor; hence it is commonly referred to as lemon verbena [31]. Depending upon the country in which it is found, the plant is also variously known as cidró, cidrão, erva-luisa, or dulce-lima. Reports describing the traditional use of this species by the Inca culture dating back to the 17th century exist, indicating its ethnopharmacological importance [32]. The plant is popularly used in South America, North Africa, and south of Europe to treat fever, insomnia, and anxiety [33], as well as digestive disorders, and as a sedative and antispasmodic agent [34]. Moreover, traditional Mexican medicine has used this plant for sleep and stomach disorders, depression, and anxiety

[35]. Its biological effects can probably be ascribed to its essential oil, which is comprised primarily of monoterpenes (geranial, neral, and limonene) and sesquiterpenes, and flavonoids and phenylpropanoids are present in the plant in large amounts [36]. These compounds have also been demonstrated to have antioxidant, anxiolytic, anticancer, anesthetic, antimicrobial, and sedative effects, both *in vitro* and *in vivo* [37].

However, despite its widespread popular use, there have, to the best of our knowledge, been no studies that have examined the neuroprotective and antidepressant potential of the extracts and compounds isolated from *A. citriodora*. Given this deficiency, we sought to evaluate the antidepressant-like effects of the hydroalcoholic extract of the plant and its major constituent, verbascoside, using a mouse model of depression and investigated the underlying modes of action.

2. Methods

2.1. Plant Material. The plant material of *A. citriodora* was collected in Chapecó (SC) (27° 05′ 37″ S and 52° 39′ 58″ W) in October 2018. The botanical identification was performed by Professor Adriano Dias de Oliveira, curator of the Herbarium of the Community University of the Region of Chapecó (Unochapecó), where a voucher was deposited (#3777).

2.2. Extract Production of A. citriodora. The samples (leaves) from *A. citriodora* were dried at 25 ± 5°C and pounded in a knife mill (CiemLab®, CE430), passed through a sieve (32 Mesh/Tyler, 500 μm), identified, and stored with protection from light. The hydroalcoholic extract (HEAc) was produced through extractive method of maceration (5 days) using dry-milled leaves of the plant (400 g) and ethanol 70% (4 L). After filtration through Büchner funnel, the HEAc was concentrated using evaporation under reduced pressure (40°C), lyophilized, weighed (149.48 g; 37.37% yielding), and stored at −20°C.

2.3. Chemical Analysis

2.3.1. Estimation of Total Flavonoid Content. The total flavonoid content was analyzed according to Woisky and Salatino [38] with modifications. Briefly, 1 mL of the HEAc (1000 μg/mL, in MeOH) was added to 1 mL of AlCl3 2% and after 60 min the spectrophotometer readings were performed at 425 nm. The calibration curve was produced using quercetin (in methanol) as standard (10, 15, 20, 25, and 30 μg/mL), and the readings were performed in triplicate. The quantification of flavonoids was determined in milligrams per gram of extract.

2.3.2. Obtainment of Verbascoside. A sample of HEAc (50 g) was diluted with water (500 mL) followed by mechanical agitation (20 min). Subsequently, the resulting solution was transferred to a separating funnel and submitted to liquid/liquid partition successively with hexane and EtOAc (ten times per solvent, 500 mL each). After solvent evaporation, an aliquot (2 g) of the EtOAc fraction was submitted to liquid column chromatography using silica gel (0.063–0.200 mm; Merck®, Darmstadt, Germany) as the stationary phase, as well as eluents hexane and EtOAc (10 : 100 v/v, respectively) in increasing polarity up to 100% EtOAc as mobile phase. The nine subfractions obtained were similarly pooled by using thin-layer chromatography (TLC) with EtOAc : MeOH : H_2O (100 : 13.5 : 10 v/v) as the mobile phase and analyzed using a UV/Vis spectrometer at 366 nm and revealed with H_2SO_4 (10% in methanol) followed by heating at 110°C (10 min). Subfraction 8 (0.289 g) was observed as a spot by the TLC analysis, yielding verbascoside (VBS), which was identified by spectroscopic techniques (^{1}H NMR, ^{13}C NMR, and ESI-MS).

2.3.3. Mass Spectrometry Analysis (ESI-IT-MSn). The direct flow infusion of the samples was performed on the Thermo LTQ-XL apparatus (IT-MS) linear ion trap analyzer equipped with electrospray ionization (ESI) source in negative mode (Thermo, San Jose, CA, USA). Stainless steel capillary tube at 280°C, spray voltage of 5.00 kV, a capillary voltage of −35 V, tube lens of−100 V, and a 10 μL/min flow were used. Multiple-stage fragmentations (ESI-MSn) were performed using the collision-induced decomposition (CID) method against Argon for ion activation. The first event was a full scan mass spectrum to acquire data on ions in the range of m/z 154–2000. The second scan event was an MS/MS experiment performed using a data-dependent scan on the $[M\text{-}H]^-$ molecules from the compounds of interest at a collision gas flow rate of 30%.

2.4. Free Radical Scavenging In Vitro Activity. The free radical scavenging potential was evaluated by the decrease of 2,2-diphenyl-1-picrylhydrazyl (DPPH) absorbance, according to Xie and Schaich [39]. The aliquots of HEAc, VBS, ascorbic acid and gallic acids, and quercetin (15–250 μg/mL), or distilled water (negative control group), were mixed with DPPH methanolic solution (400 μg/mL). Subsequently, the samples were incubated (5 min at 25°C), and the absorbance was read at 517 nm in triplicate. The values were interpolated into a standard curve of DPPH (0–60 μM) and expressed as IC_{50} (the concentration of the sample required to inhibit 50% of radical).

2.5. Animals. Adult female Swiss mice weighing 25–30 g (8 weeks) were provided by Central Animal House of Unochapecó and maintained in propylene cages under standard laboratory conditions (12 h light/dark cycle, the temperature of 22 ± 2°C), with free access to food and water. Food was withdrawn 12 h before the experiments and water was provided ad libitum. Several studies performed in female rodents at different stages of the estrous cycle have shown no interference in the results [40, 41]. The experiments were conducted following ARRIVE guidelines and the National Council for Animal Experimentation (CONCEA) after approval by the Institutional Animal Ethics Committee of Unochapecó (protocol number 014/18).

2.6. Dose-Response Study. According to popular usage, *A. citriodora* preparations are usually made with 15 g of plant material (approximately 2 g of dry leaves) and 150 mL of water, administered orally 2 to 3 times a day [31]. The extractive solution was dried (in an oven, 100°C), and the dry residue obtained was weighed and expressed as a percentage of yield about the dehydrated plant (0.49%). Thus, there is 2.238 mg of dry residue in 450 mL. For an 80 kg person, this administration represents an intake of approximately 27.9 mg/kg/day. Consequently, HEAc was tested at 3, 30, and 300 mg/kg, ensuring appropriate doses on a logarithmic scale to verify possible dose-response efficacy adequately and achieve a higher dose and a lower dose.

2.7. Experimental Design and Drug Treatment. The assays conducted in this study were performed following procedures described by Müller et al. [42], with minor modifications. Initially, the animals (Swiss mice) were randomly divided into two treatment groups. One group was subjected to a pretreatment, which was performed to evaluate the preventive potential of the *A. citriodora* extracts, and the other was subjected to a posttreatment, designed to evaluate the therapeutic potential of these extracts. The animals within these two groups were further subdivided to receive one of the following treatments ($n = 8$ per group, gavage). The naive group (*N*) was orally administered distilled water plus Tween 80 and not exposed to LPS, whereas the vehicle group (Veh) was treated orally with distilled water plus Tween 80 and exposed to LPS. Among the four treatment groups exposed to LPS, mice in three groups were administered HEAc (3, 30, or 300 mg/kg, p. o), whereas those in the fourth group were administered verbascoside (VBS, 30 mg/kg; p. o). As a positive control group, we treated mice with fluoxetine (Flu, 30 mg/kg). In the posttreatment protocol, we also assessed a group that received HEAc (30 mg/kg, p. o) and was not exposed to LPS, which was named per se.

In the preventive approach (pretreatment protocol, Figure 1(a)), which aimed to analyze the possible pretreatment effect of HEAc and VBS, the mice were acclimatized to the experimental room for 2 h, after which they were administered vehicle, HEAc, VBS, or fluoxetine (each (10 mL/kg) by oral gavage. After 1 h, all animals (except for those in the N and per se groups) received LPS (600 μg/kg, i.p) [18], and, at 6 and 24 h after the administration of LPS, the mice were subjected to an open-field test (OFT) for 6 min. At 24 h after LPS administration, mice were subjected to a tail suspension test (TST) for 6 min.

In the posttreatment protocol (Figure 1(b)), except the N and per se groups, all groups received LPS (600 μg/kg, i.p). At 5 h after the administration of LPS, the mice received the vehicle, HEAc, VBS, or fluoxetine by oral gavage, and, at 6 and 24 h after LPS administration, all animals were subjected to the OFT (6 min). At 24 h after LPS administration, the mice were evaluated in the TST (6 min).

At 1 h after the TST in both pre- and posttreatment protocols, all mice were anesthetized (thiopental sodium, 50 mg/kg; i.p) and subsequently euthanized by exsanguination. Thereafter the cortex and hippocampus were removed for subsequent analyses.

2.8. Behavioral Tests

2.8.1. Evaluation of Sickness Behavior. Sickness behavior was characterized by the intensity of symptoms monitored at 15-minute intervals during the one hour before the OFT. Symptoms were assessed on a 4-point scale, as described by Gandhi et al. [43]. Mice were evaluated for lethargy (as indicated by reduced locomotion and a curled body posture), ptosis (drooping eyelids), and piloerection (ruffled, greasy fur), with each symptom being considered equivalent to 1 point, resulting in a scale ranging from 0 to 3, with zero being indicative of no symptoms and 3 indicating that all symptoms were present. Scoring was performed by three independent observers, and, after confirming the scores, the values were calculated as averages for subsequent statistical analysis.

2.8.2. Open-Field Test (OFT). The administration of LPS (600 μg/kg, i.p) to animals is assumed to mimic the central and peripheral infectious processes in humans, resulting in adaptive responses. To determine the behavioral responses to the modulation promoted by the treatments, mice were evaluated for locomotor activity in the OFT at 6 and 24 h after LPS administration. The animals were placed individually in acrylic boxes ($40 \times 30 \times 30$ cm), the floor of which was divided into 24 equal squares. The crossing (number of squares crossed with the four paws), rearing (the number of times a mouse raised on its hind legs), grooming episodes (washing of the coat), and amount of fecal bolus were recorded in 6-min sessions.

2.8.3. Tail Suspension Test (TST). The activities of the HEAc, VBS, and fluoxetine on depressive-like behavior in mice before and after the LPS treatment were evaluated using the TST as previously described by Steru et al. [44], with minor modifications. Immediately after the OFT (24 h after LPS administration), the animals were suspended by the tail 70 cm above the floor using adhesive tape (1 cm from the tip of the end). During the final 4 min of each 6-min session, the time (in seconds) during which each mouse remained immobile was recorded.

2.9. Obtaining Homogenate of the Cortex and Hippocampus. This preparation was applied only for groups of animals that demonstrated an antidepressant-like effect on TSC in the pretreatment and/or posttreatment protocols. To analyze the markers in the total cortex and hippocampus, the tissues were homogenized in 200 mM phosphate buffer (pH 6.5). This homogenate was used to determine reduced glutathione (GSH), lipid hydroperoxides (LOOH), and N-acetyl-β-D-glucosaminidase (NAG). The supernatant was used to determine the activity of myeloperoxidase (MPO), superoxide dismutase (SOD), and catalase (CAT) enzymes, in addition to measuring the levels of cytokines (IL 6 and IL-10).

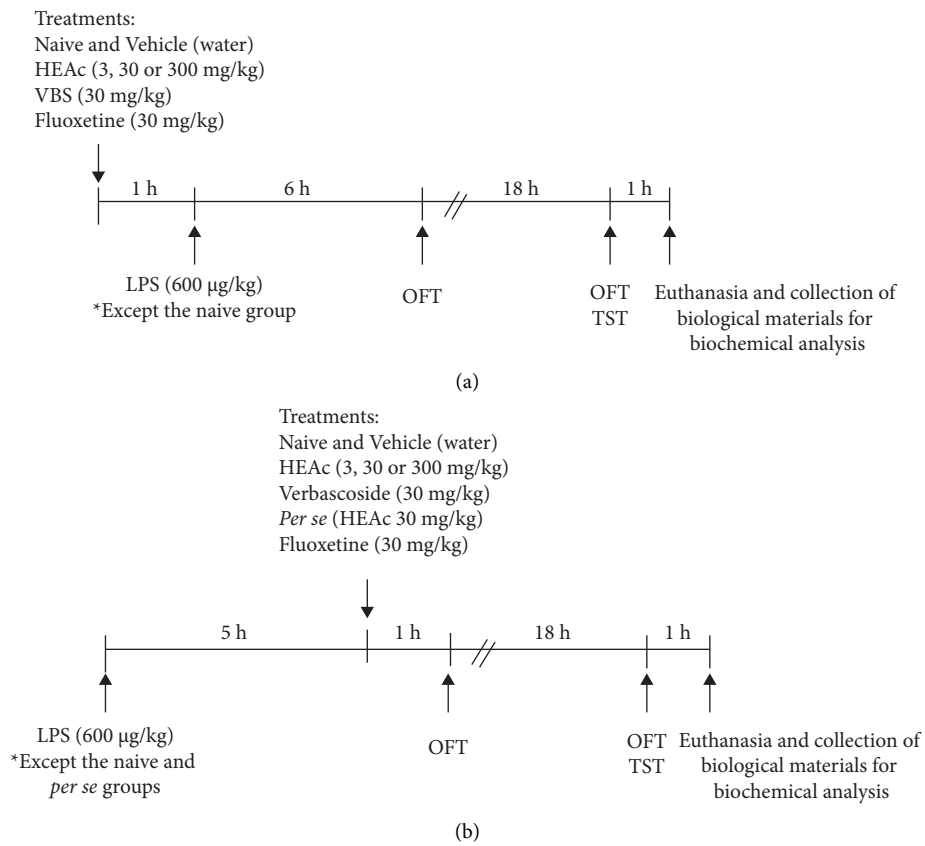

Figure 1: The timeline of the experimental design of the pretreatment (a) and posttreatment (b) stages in the evaluation of the antidepressant-like activity of the hydroalcoholic extract of *A citriodora* (HEAc) and verbascoside. Note: lipopolysaccharide (LPS; 600 μg/kg, i.p). N (naive); Veh (vehicle, water); HEAc (*Aloysia citriodora* hydroalcoholic extract: 3, 30, or 300 mg/kg); VBS (verbascoside, 30 mg/kg); Flu (fluoxetine 30 mg/kg). OFT: open-field test; TST: tail suspension test.

2.10. Evaluation of Inflammatory Parameters

2.10.1. Determination of Myeloperoxidase (MPO) Activity. To quantify MPO activity, the pellets were resuspended in 80 mM potassium phosphate buffer (500 μL, pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). Subsequently, the solution was centrifuged at $11000 \times g$ for 20 min at 4°C. The supernatant was analyzed at 620 nm in the presence of 7.5% H_2O_2 and 3,3′,5,5′-tetramethyl-benzidine (TMB, 18.4 nM) and expressed as millimeter optical density units (mOD)/mg of protein [45, 46].

2.10.2. Quantification of Cytokine Levels. Cortex and hippocampal supernatants were used to estimate cytokine levels by enzyme-linked immunosorbent assay (ELISA). The number of aliquots was equalized by protein concentration. Aliquots (100 μL at 0.20 mg/mL of protein) were used to measure interleukin-6 (IL-6) and IL-10 using R&D Systems® (Minneapolis, MN) mouse cytokine ELISA kits according to the manufacturer's instructions. Cytokine absorbance was measured by a microplate reader at 450 and 550 nm.

2.10.3. Determination of N-Acetyl-β-D-Glucosaminidase (NAG) Activity. The activity of NAG is based on the hydrolysis of p-nitrophenyl-N-acetyl-β-D-glucosamine (substrate) by N-acetyl-β-D-glucosaminidase, releasing p-nitrophenol [47]. Samples containing 25 μL of the supernatant or 25 μL of distilled water (blank) were incubated with 100 μL of citrate buffer (5 mM, pH 4.5) in the presence of the substrate (2.24 mM). The plates were incubated for 60 minutes at 37°C and the reaction was interrupted with glycine buffer (200 mM, pH 10.4) and measured in a spectrophotometer at 405 nm. Results were expressed as optical density unit (O.D.)/mg protein/hour.

2.11. Evaluation of Oxidative Stress Markers

2.11.1. Determination of Free GSH Levels. Aliquots of homogenate of the cortex and hippocampus were mixed with trichloroacetic acid (12.5%) using vortex (10 min) and centrifuged (15 min at $4000 \times g$) [48]. TRIS buffer (0.4 M, pH 8.9) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, 0.01 M) were added to the supernatant. Subsequently, the absorbance was measured at 415 nm in a microplate reader.

Results were compared to a standard GSH curve and expressed in μg/g tissue.

2.11.2. Determination of Lipid Hydroperoxide (LOOH) Levels. Lipid hydroperoxides are highly reactive peroxidation products, thus being determined by Ferrous Oxidation-Xylenol Orange (FOX2) [49]. For these analyses, the cortex homogenate was mixed with 90% methanol by vertexing and centrifuged (20 min, 9000 ×g). The supernatant was added to the FOX2 reagent and incubated for 30 min at room temperature. Absorbance was evaluated at 560 nm and results were expressed in mmol/mg tissue.

2.11.3. Determination of the SOD Activity. The analysis of SOD activity was performed by using a method to evaluate the ability of a solution to inhibit pyrogallol autooxidation [50]. For this, supernatant aliquot of 1 mM pyrogallol was added to 200 mM Tris HCl-EDTA (pH 8.5) and incubated for 20 min. After the reaction time, the absorbance was measured at 405 nm and the amount of SOD capable of inhibiting the autooxidation of pyrogallol by 50%, relative to the control, was defined as a unit (U) of SOD activity. The results were expressed as U/mg of protein.

2.11.4. Determination of the CAT Activity. To measure CAT activity, the reactions were performed in the presence of cortex supernatant aliquots, 5 mM TRIS/EDTA buffer (pH 8.0), 30% H_2O_2, and ultrapure water. The decrease in absorbance was measured at 240 nm for 1 min. The results were expressed in μmol of H_2O_2 consumed/min/mg of protein [51].

2.11.5. Measurement of Protein Concentration. Protein concentration was measured in a spectrophotometer at 590 nm using the Bradford reagent (Amresco®), and bovine albumin (0.012–0.100 mg/mL) was used as a standard curve.

2.12. Statistical Analysis. Data were evaluated as normality by the Shapiro-Wilk test and Grubbs' test was used to detect outliers. Statistically significant differences between groups were calculated by the application of an analysis of variance (ANOVA) followed by the Tukey test using the software GraphPad version 8.00 for Windows (GraphPad Software, La Jolla, CA, USA). The values were represented by means ± standard error of the means (S.E.M) and p values less than 0.05 ($p < 0.05$) were used as the significance level.

3. Results

3.1. Phytochemical Analysis

3.1.1. Quantification of Flavonoids. Analysis though of spectrometric UV/Vis using calibration curve the quercetin (10–30 μg/mL; $y = 0.0614x - 0.0384$; $R^2 = 0.9973$) showed high amount flavonoids in the hydroalcoholic extract of *A. citriodora* (11.6 mg/g; 1.2%).

3.1.2. Isolation and Identification of Verbascoside. Verbascoside was isolated in subfraction 9 of fraction EtOAc of extract of *A. citriodora* (Figure 2). This compound was identified by comparison of their experimental spectra (^{1}H NMR, ^{13}C NMR, and ESI-MS) with those previously described [52–54]. Verbascoside or actoside: this compound was obtained as a crystalline powder, with a melting point of 142–145°C (water): ($C_{29}H_{36}O_{15}$); ESI-MS: 623.08 $[M+H]^-$; ^{1}H NMR (400 MHz, CD_3OD, δ, ppm): δ 1.09 (3H, *d*, $J = 6.10$ Hz, H-6″), δ 2.79 (2H, *m*, H-7), δ 3.28 (1H, *t*, $J = 9.5$ Hz, H-4″), δ 3.40 (1H, *t*, $J = 9.0$ Hz, H-2′), δ 3.55 (1H, *m*, H-5′, H-3″, H-5″), δ 3.52–3.72 (2H, *m*, H-6′), δ 3.73–4.05 (2H, *m*, H-8a, H-8b), δ 3.83 (1H, *t*, $J = 9.0$ Hz, H-3′), δ 3.93 (1H, dd, $J = 3.3$ Hz, $J = 1.83$ Hz, H-2″), δ 4.37 (1H, *d*, $J = 7.89$ Hz, H-1′), δ 4.93 (1H, *m*, H-4′), δ 5.19 (1H, *d*, $J = 1.47$ Hz, H-1″), δ 6.28 (1H, *d*, $J = 15.97$ Hz, H-8‴), δ 6.57 (1H, dd, $J = 8.0$ Hz, $J = 2.00$ Hz, H-6), δ 6.69 (1H, *d*, $J = 8.00$ Hz, H-5), δ 6.71 (1H, *d*, $J = 2.02$ Hz, H-2), δ 6.79 (1H, *d*, $J = 8.00$ Hz, H-5‴), δ 6.95 (1H, dd, $J = 8.20$ Hz, $J = 2.0$ Hz, H-6‴), δ 7.06 (1H, *d*, $J = 2.02$ Hz, H-2‴), δ 7.60 (1H, *d*, $J = 15.90$ Hz, H-7‴). 13C NMR (100 MHz, CD3OD, δ, ppm): δ 17,0 (C-6″), δ 36.5 (C-7), δ 62,4 (C-6′), δ 70.9 (C-4′), δ 71.0 (C-3″; C-5′; C-5″), δ 72 (C-2″), δ 72.4 (C-8), δ 73.9 (C-4″), δ 76.2 (C-2′), δ 81.7 (C-3′), δ 103.2 (C-1″), δ 104.4 (C-1′), δ 113.4 (C-8‴), δ 114.8 (C-2‴), δ 116.2 (C-5, C-5‴), δ 117.2 (C-2), δ 120.9 (C-6), δ 121 (C-6‴), δ 125.8 (C-1‴), δ 129.7 (C-1), δ 143.1 (C-4), δ 144.7 (C-3), δ 145.5 (C-3‴), δ 148.0 (C-7‴), δ 148.4 (C-4‴), δ 166.5 (C-9‴).

The negative mode mass spectrum (ESI-MS) of verbascoside (VBS) provided an m/z ratio of 623.08 revealing the deprotonated molecular ion $[M–H]^-$, which confirmed the possible molecular mass as 624.51 compatible with the proposed molecule. Additional experiments on MS2 of the ion m/z 623 produced another ion from the main fragment at m/z 461. The ion at m/z 461 is considered to result from the loss of the caffeoyl moiety $[M–H–162]^-$ of the parental ion m/z 623. The MS3 spectrum of the ion at m/z 461 yielded a very weak ion at m/z 315 upon losing a unit of rhamnose.

3.1.3. In Vitro Assay of Free Radical Scavenging Activity. The antioxidant activities of HEAc, VBS (15–250 μg/mL), and the positive controls (gallic acid, ascorbic acid, and quercetin) were determined based on the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) elimination assay. HEAc and VBS presented IC_{50} of 25.59 and 11.9 μg/mL, respectively, confirming its strong antioxidant capacity *in vitro*. In line with expectations, gallic and ascorbic acids and quercetin also showed significant effects in reducing DPPH, with IC_{50} values of 3.27, 8.9, and 12.2 μg/mL, respectively.

3.2. Behavioral Effects of the HEAc and VBS in the Pretreatment Experiment. In the pretreatment experiment, we found that the administration of LPS 1 h after treatments was effective in promoting disordered behavior, mediated by inflammatory cytokines (sickness behavior). Accordingly, for the mice in all groups, we recorded maximum values of sickness behavior (4 points) characterized by lethargy

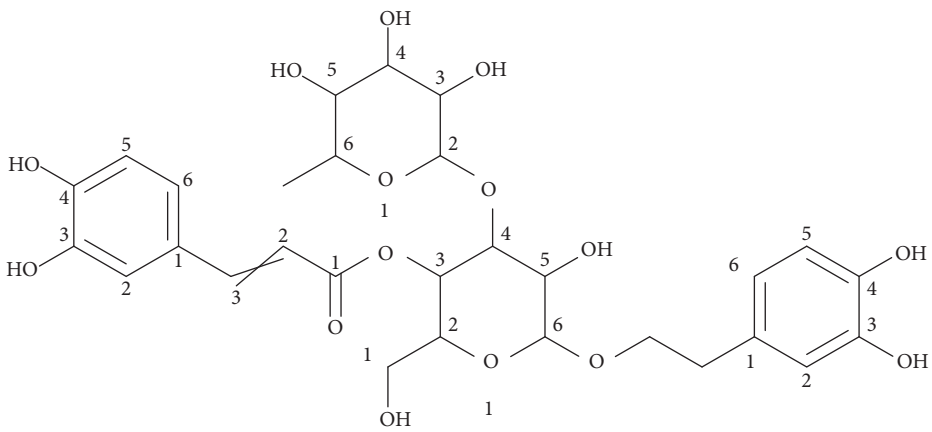

Figure 2: Chemical structure of verbascoside.

(indicated by reduced locomotion and exploratory activity), hunched posture, ptosis, and piloerection. Subsequently, at 6 h after LPS exposure, mice in the vehicle group (exposed to LPS and treated with water, Veh) showed significantly reduced locomotor activity in the OFT compared with the mice in the N group ($p < 0.05$). Similarly, compared with the mice in the N group, we detected reduced locomotor activity in the mice administered HEAc (3, 30, or 300 mg/kg) or verbascoside (VBS; 30 mg/kg) when these were subjected to the OFT ($p < 0.001$). In contrast, mice treated with fluoxetine (Flu, 30 mg/kg) showed no significant difference compared with mice in both N and Veh groups (Figure 3(a)). Compared with the N group, we also observed a reduction in rearing and grooming among animals in the Veh, HEAc, VBS, and Flu groups which is probably due to the intensity of the sickness symptoms ($p < 0.001$) (Figures 3(c) and 3(e)). It is worth noting here that, at this stage of the experiment, except for the HEAc 30 mg/kg treatment group, all treatment groups showed a significant reduction in the number of feces compared with those produced by the N group mice ($p < 0.01$) (Figure 3(g)).

In line with expectations, we observed that, 24 h after exposure to LPS, all mice had recovered from the sickness behavior. Consequently, we observed no behavioral differences between mice in the different treatment groups and those in the N group ($p > 0.05$) concerning our evaluations of locomotor activity, rearing, grooming, and excretory behavior (Figures 3(b), 3(d), 3(f), and 3(h)). At this stage of the experiment, in the TST, the immobility times of the Veh group mice were longer than those of mice in the N group ($p < 0.01$), thereby confirming the depressive-like behavior in this neuroinflammation model. However, we detected no significant differences in this regard between mice in the treatment and Veh groups (Figure 4).

3.3. Behavioral Effects of the HEAc and VBS in the Posttreatment Experiment. At 5 h after exposure to LPS in the posttreatment experiment, the mice in all groups were observed to show sickness behavior. Compared with the N group mice, those in the Veh group were found to be characterized by an increase in the intensity of symptoms (3.14 ± 0.34) ($p < 0.001$). However, compared with the Veh group mice, those in the HEAc (30 or 300 mg/kg) and Flu groups were observed to have lower intensities of sickness behavior ($p < 0.001$ and $p < 0.01$, respectively), thereby indicating a certain degree of protection against LPS (Figure 5).

After exposure to LPS for 6 h, we detected no significant differences between mice in the N and per se groups for the number of crossings in the OFT (245.30 ± 7.28 and 264.80 ± 13.61, respectively). However, compared with the N group mice, Veh group mice were characterized by a marked reduction in locomotor activity ($p < 0.001$), whereas mice receiving HEAc (3, 30, or 300 mg/kg) and Flu were observed to complete a significantly higher number of crossings compared with the Veh-treated mice ($p < 0.01$ and $p < 0.001$, respectively). Furthermore, mice administered VBS showed no significant difference in locomotor activity compared with the Veh group mice (Figure 6(a)). Concerning rearing, we observed that mice subjected to all treatments performed fewer rearing compared with the N group mice ($p < 0.001$), although we detected no significant difference between mice in the treatment and Veh groups. Moreover, with regard to the grooming and feces evaluations, we detected no significant difference among the N, Veh, and other treatment groups (Figures 6(c), 6(e), and 6(g)).

We observed that, 24 h after exposure to LPS, the mice in all experimental groups showed evidence of recovery from sickness behavior, as indicated by similar values in the numbers of crossings in the OFT. The treatment groups showed no significant difference from the N group in the amount of rearing ($p > 0.05$). However, compared with the Veh group, mice in the VBS and Flu groups showed less grooming behavior ($p < 0.001$ and $p < 0.01$, respectively), and the number of feces produced by mice in all treatments was lower than that produced by mice in the N group ($p < 0.05$) (Figures 6(b), 6(d), 6(f), and 6(h)).

In the TST, the longest period of immobility (depressive-like effect) was recorded for mice in the Veh group (66.29 ± 6.31 s), which was significantly longer than that of the N group mice ($p < 0.01$) (37.01 ± 3.10 s). Results obtained for the per se group were found to be broadly comparable to those recorded for N group (45.17 ± 7.32 s), whereas, for

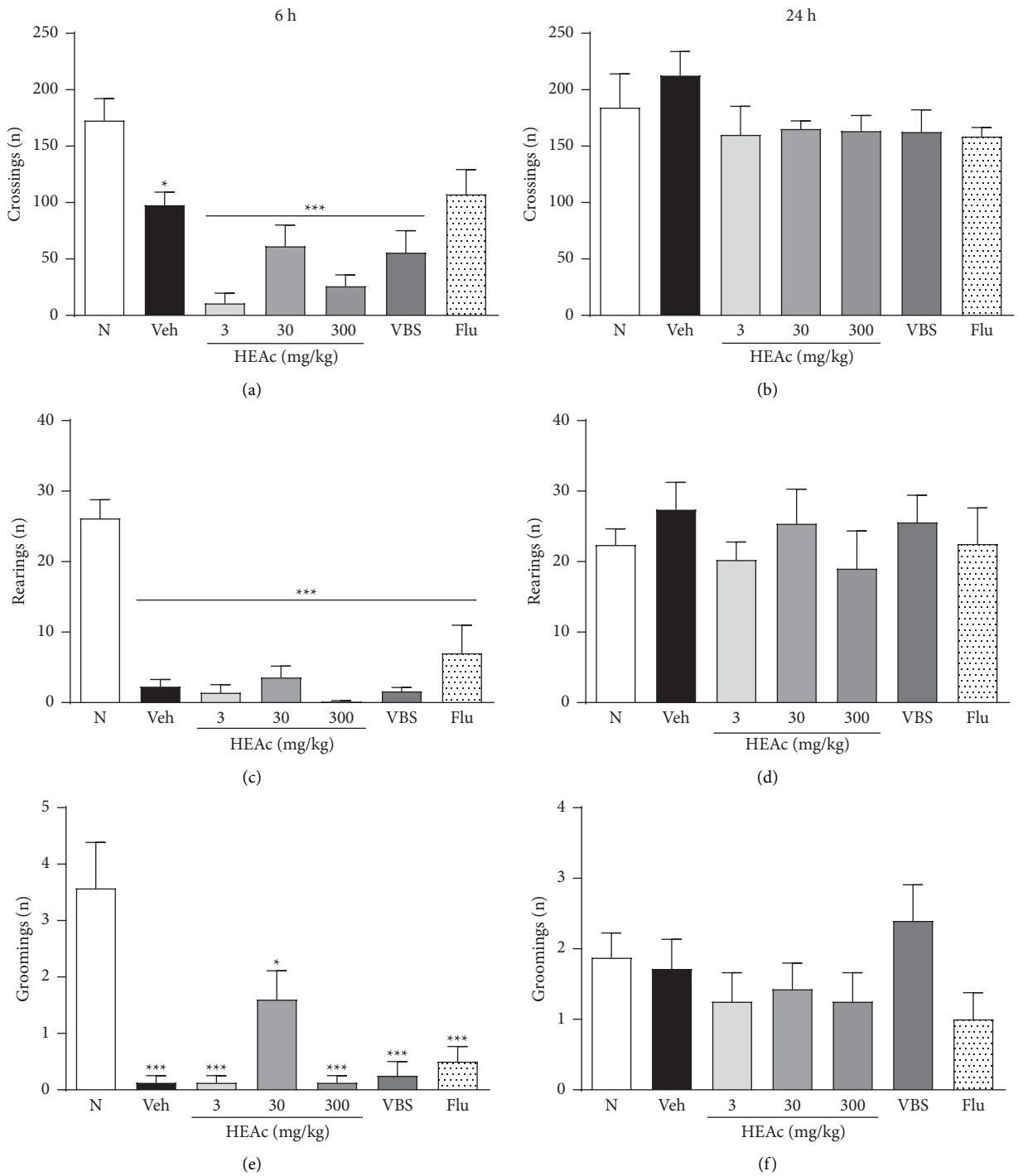

Figure 3: Continued.

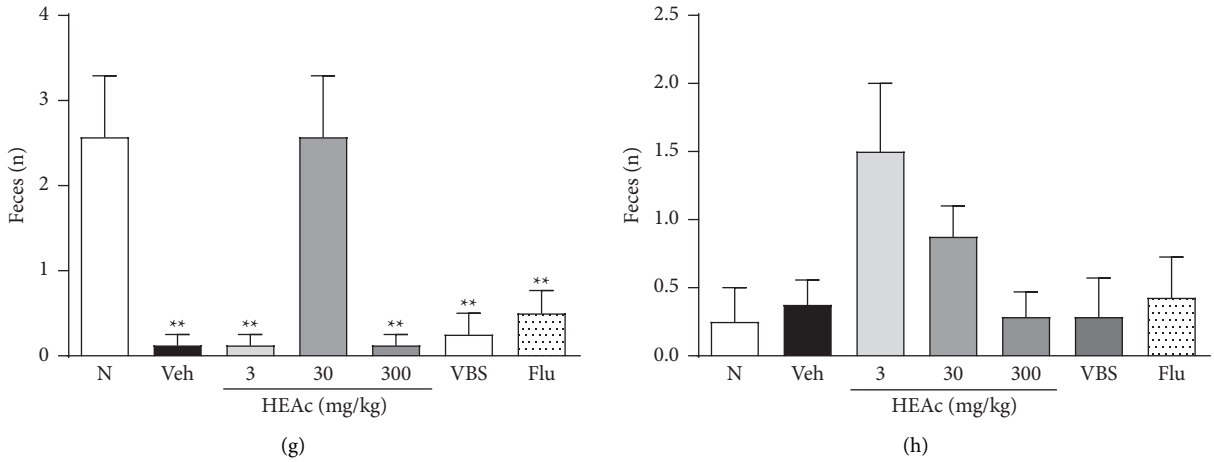

Figure 3: Behavioral effects of HEAc and verbascoside in mice in the pretreatment experiment. Note: pretreatment experiment: treatments administered (gavage) 1 h before exposure to bacterial lipopolysaccharide (LPS; 600 μg/kg, i.p). N (naive); Veh (vehicle, water); HEAc (*Aloysia citriodora* hydroalcoholic extract 3, 30, or 300 mg/kg); VBS (verbascoside, 30 mg/kg); Flu (fluoxetine 30 mg/kg). A–H: values are expressed as mean ± SEM ($n = 8$). ANOVA (one way), post hoc Tukey. $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.001$ compared to the naive group.

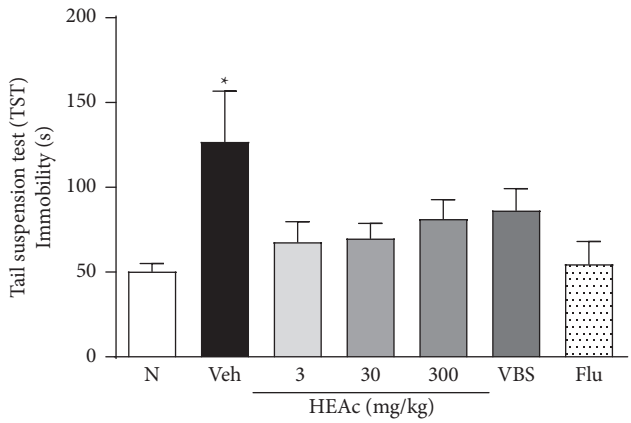

Figure 4: Antidepressant-like effects of HEAc and verbascoside (VBS) in the pretreatment experiment. Tail suspension test (TST) at 24 h after exposure to LPS. Note: pretreatment experiment: treatments administered (gavage) 1 h before exposure to bacterial lipopolysaccharide (LPS; 600 μg/kg, i.p). N (naive); Veh (vehicle, water); HEAc (*Aloysia citriodora* hydroalcoholic extract 3, 30, or 300 mg/kg); VBS (verbascoside, 30 mg/kg); Flu (fluoxetine 30 mg/kg). Values are expressed as mean ± SEM ($n = 8$). ANOVA (one way), post hoc Tukey. $^{*}p < 0.05$ compared to the naive group.

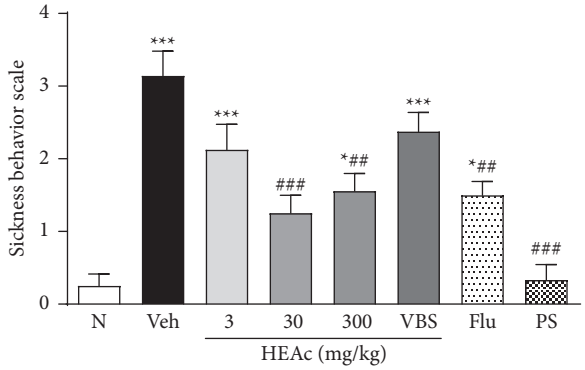

Figure 5: Intensity of sickness behavior of HEAc and verbascoside (VBS) in the therapeutic experiment. Note: N (naive); Veh (vehicle, water), hydroalcoholic extract of *Aloysia citriodora* (HEAc: 3, 30, or 300 mg/kg, po), VBS (verbascoside 30 mg/kg, po), Flu (fluoxetine 30 mg/kg), PS (Per se group, HEAc). Values are expressed as mean ± SEM ($n = 8$ mice/group). ANOVA (one way), post hoc Tukey. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to the naive group. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to the Veh group.

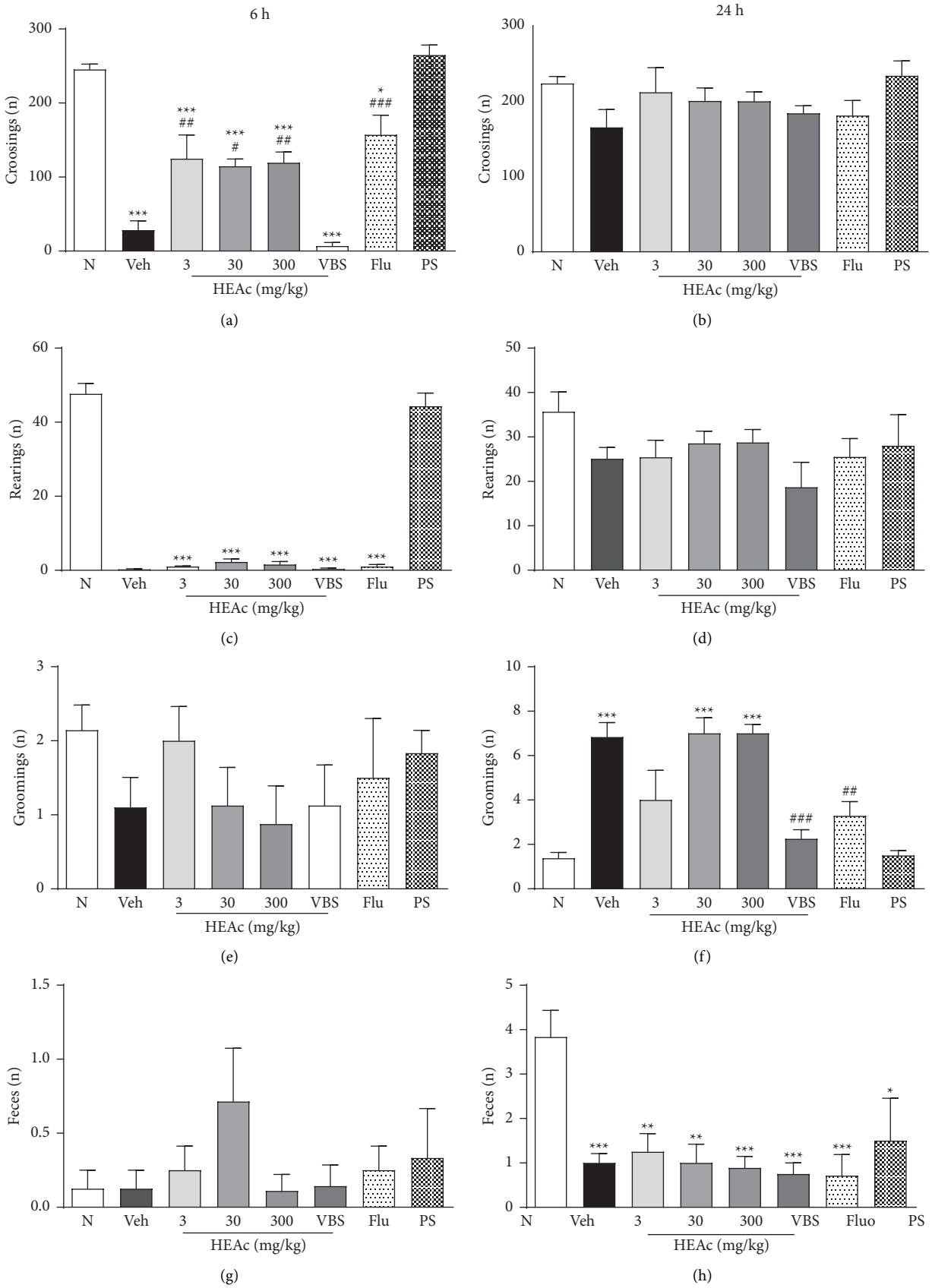

Figure 6: Behavioral effects of HEAc and verbascoside in mice in the posttreatment experiment. Note: posttreatment experiment: treatments administered (gavage) 5 h after exposure to bacterial lipopolysaccharide (LPS; 600 μg/kg, i.p). N (naive); Veh (vehicle, water); HEAc (*Aloysia citriodora* hydroalcoholic extract 3, 30, or 300 mg/kg); VBS (verbascoside, 30 mg/kg); Flu (fluoxetine 30 mg/kg), PS (per se group, HEAc). A–H: values are expressed as mean ± SEM ($n = 8$). ANOVA (one way), post hoc Tukey. $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.001$ compared to the naive group. $^{\#}p < 0.05$, $^{\#\#}p < 0.001$, and $^{\#\#\#}p < 0.001$ compared to the Veh group.

mice administered HEAc at doses of 30 and 300 mg/kg and VBS, we detected significant reductions in the period of immobility compared with that of Veh group mice ($p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively). As anticipated, mice receiving Flu showed the most pronounced treatment effect, with a marked reduction in the time spent with no movement compared with the Veh group mice ($p < 0.001$) (Figure 7).

3.4. Effect of HEAc and VBS on Inflammatory Markers in the Hippocampus and Cortex. Due to the promising results obtained in the posttreatment experiment, the effects of HEAc (30 and 300 mg/kg) and VBS (30 mg/kg) (more effective doses in TST) on inflammatory markers in the hippocampus and cortex were investigated. In the hippocampus, treatments with HEAc (30 and 300 mg/kg), VBS, and Flu were not able to decrease MPO levels (Figure 8(a)). Elevated NAG levels in the Veh group (155.80 ± 15.75) compared ($p < 0.05$) to the N group (83.97 ± 5.09) were decreased by HEAc 300 mg/kg (42, 58%) compared to the Veh group ($p < 0.01$) (Figure 8(b)). The Veh group also increased the values of interleukin-6 (IL-6) (316.30 ± 28.16); however, HEAc (300 mg/kg) and VBS demonstrated a strong anti-inflammatory effect, decreasing IL-6 compared to the Veh group (47.13% and 47.94%; $p < 0.001$ and $p < 0.01$, respectively). Similar results to the fluoxetine (30 mg/kg) group were found (Figure 8(c)).

In the cortex, the Veh group increased MPO levels (0.24 ± 0.02) in comparison ($p < 0.01$) with the N group (0.08 ± 0.02) and these levels were decreased by HEAc 300 mg/kg (77.91%; $p < 0.001$) and VBS (59.16%; $p < 0.01$), when compared to the Veh group (Figure 9(a)). No differences were observed between the groups tested in the levels of NAG and IL-6 (Figures 9(b) and 9(c)). In addition, the level of IL-10, an anti-inflammatory cytokine, was elevated in treatments with HEAc (30 and 300 mg/kg), with similar values in the Flu and N groups (Figure 9(d)).

3.5. Effect of HEAc and VBS on Markers of Oxidative Stress in the Hippocampus and Cortex of Mice Evaluated in the Posttreatment Experiment of Antidepressant-like Activity. LPS in the mouse hippocampus was able to decrease free GSH levels. In this evaluation, the N group had higher free GSH levels (24.18 ± 3.28) compared ($p < 0.01$) to the Veh group (11.52 ± 1.29). On the other hand, HEAc (30 and 300 mg/kg) prevented GSH depletion (85% and 213%, respectively) compared to the Veh group ($p < 0.05$ and $p < 0.001$). In the evaluations of SOD activity, no differences were observed between treatments and groups N and Veh (Table 1).

In the cortex, reduced levels of GSH (21.94 ± 0.84) were again observed in the Veh group, compared to the N group (36.97 ± 3.62) ($p < 0.001$), evidencing oxidative stress. However, HEAc at doses tested (30 and 300 mg/kg) as well as VBS restored GSH levels compared to group N. SOD and CAT activities as well as LPO levels were not significantly altered by HEAc and VBS, when compared with the N group (Table 2).

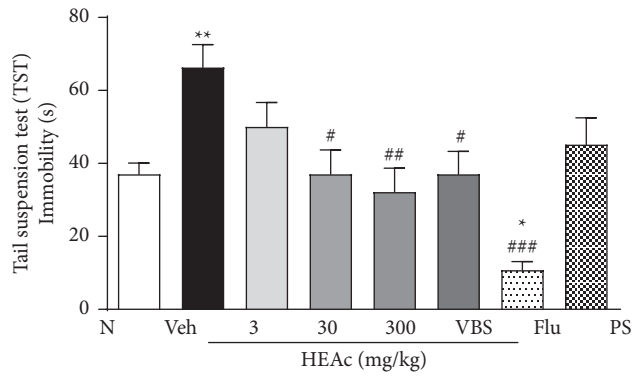

Figure 7: Antidepressant-like effects of HEAc and verbascoside (VBS) in the posttreatment experiment. Tail suspension test (TST) at 24 h after exposure to LPS. Note: posttreatment experiment: treatments administered (gavage) 5 h after exposure to bacterial lipopolysaccharide (LPS; 600 μg/kg, i.p). N (naive); Veh (vehicle, water); HEAc (*Aloysia citriodora* hydroalcoholic extract 3, 30, or 300 mg/kg); VBS (verbascoside, 30 mg/kg); Flu (fluoxetine 30 mg/kg), PS (per se group, HEAc). Values are expressed as mean ± SEM ($n = 8$). ANOVA (one way), post hoc Tukey. $^{*}p < 0.05$ and $^{**}p < 0.05$ compared to the naive group. $^{\#}p < 0.05$, $^{\#\#}p < 0.001$, and $^{\#\#\#}p < 0.001$ compared to the Veh group.

4. Discussion

Given that *Aloysia citriodora* Palau is widely used for its well-established culinary and ethnopharmacological properties [55], in this study, we sought to examine the purported antidepressant potential of this plant. Based on phytochemical analysis of the hydroalcoholic extract (HEAc) of the leaves of this plant, we were able to isolate the major constituent, verbascoside (VBS), and we subsequently evaluated the antidepressant-like activities of HEAc and VBS *in vivo*. Depressive behavior in mice was evaluated based on pre- and posttreatment administration of these agents in mice exposed to lipopolysaccharide (LPS), a bacterial endotoxin known to promote neuroinflammation. Our findings indicate that the pharmacological effects observed in mice administered HEAc (30 and 300 mg/kg) and VBS (30 mg/kg) may involve a reduction in inflammatory and oxidative parameters.

Our initial chemical analysis of HEAc revealed representative amounts of flavonoids consistent with the findings of Tammar et al. [56] who determined the presence of these compounds in the leaves of *A. citriodora* collected from different locations. Similarly, Skaltsa and Shammas [57] isolated several flavones from the plant extract, and, subsequently, Carnat et al. [58], Ragone et al. [59], and Quirantes-Piné et al. [52] detected the presence of flavone glucosides such as vitexin and isovitexin, in addition to apigenin-7-diglucuronide and chrysoeriol-7-diglucuronide. In more recent studies, further flavonoids, including jaceosidin, nepetin, and nepitrin, have been newly identified in this plant, all of which have a flavone structure [60]. Flavones are particularly active in the human body and can act in the CNS. More specifically, these compounds have been demonstrated to act on the benzodiazepine site in

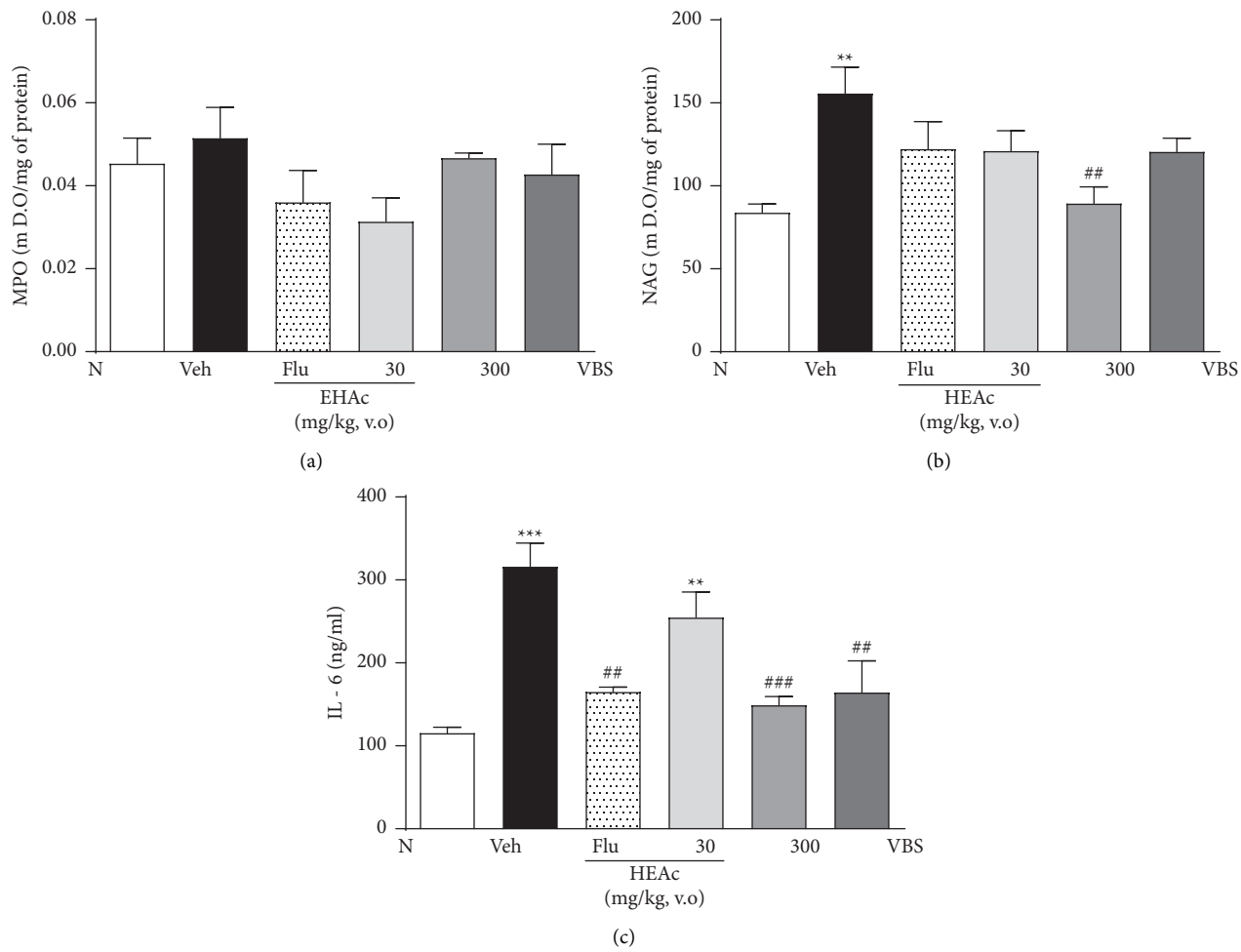

Figure 8: Effects of HEAc and VBS on the inflammatory markers MPO, NAG, and IL-6 in the hippocampus of mice evaluated in the posttreatment experiment of antidepressant-like activity. Note: N (naive); Veh (vehicle, water); Flu (fluoxetine 30 mg/kg); HEAc (*Aloysia citriodora* hydroalcoholic extract 30 or 300 mg/kg); VBS (verbascoside, 30 mg/kg). Values are expressed as mean ± SEM ($n = 8$). ANOVA (one way), post hoc Tukey. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the naive group. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to the Veh group.

GABAA receptors [61, 62] and MAOA and MAOB enzymes [63], thereby providing clues as to their anxiolytic and anticonvulsant effects, as well as antidepressant activities [64]. In addition, HEAc fractionation used in this study provided isolation of VBS. Plants with known high concentrations of VBS have traditionally been used to treat inflammation and microbial infections [65]. These biological effects, along with the antioxidant, immunomodulatory, and neuroprotective activities of this compound, have previously been confirmed both in vivo and in vitro [66, 67]. This provides substantial evidence to indicate that the biological effects of *A. citriodora* can be attributed, at least in part, to the presence of flavonoids and VBS.

In recent years, numerous advances have been made in our efforts to gain a better understanding of the genetic, biochemical, and immunological changes involved in MDD. Recent investigations have, for example, demonstrated the interaction between proinflammatory cytokines and the hypothalamic-pituitary-adrenal (HPA) axis in patients with depression or experimental animals. Among the cytokines believed to be implicated in the association between stress and depression are IL-1β and IL-6, known to be potent activators of the HPA axis, which, consequently, can modulate noradrenergic and serotonergic mechanisms [68–70]. Furthermore, in animal models, cytokines have been shown to promote anhedonia, a key feature of depression [71]. They may also trigger disease-associated behavior (e.g., piloerection, ptosis, anorexia, and reduced social and exploratory behaviors) reflecting neurovegetative features of depression. Similar to cytokines, LPS is a potent activator of microglia and HPA, promoting neuroinflammation and depressive symptoms, respectively. In animal experiments, it has been established that the systemic administration of LPS promotes the activation of Toll-like-4 receptors (TLR4), thereby inducing the release of IL-1β, along with a cascade of other cytokines, including IL-6 and TNF. It is believed that these cytokines influence central neurons, resulting in certain behavioral changes [27, 72, 73].

In this study, we aimed to mimic the action of cytokines in neuroinflammation as well as the consequent behavioral responses. To induce a neuroinflammatory response, we administered LPS to mice according to two protocols that

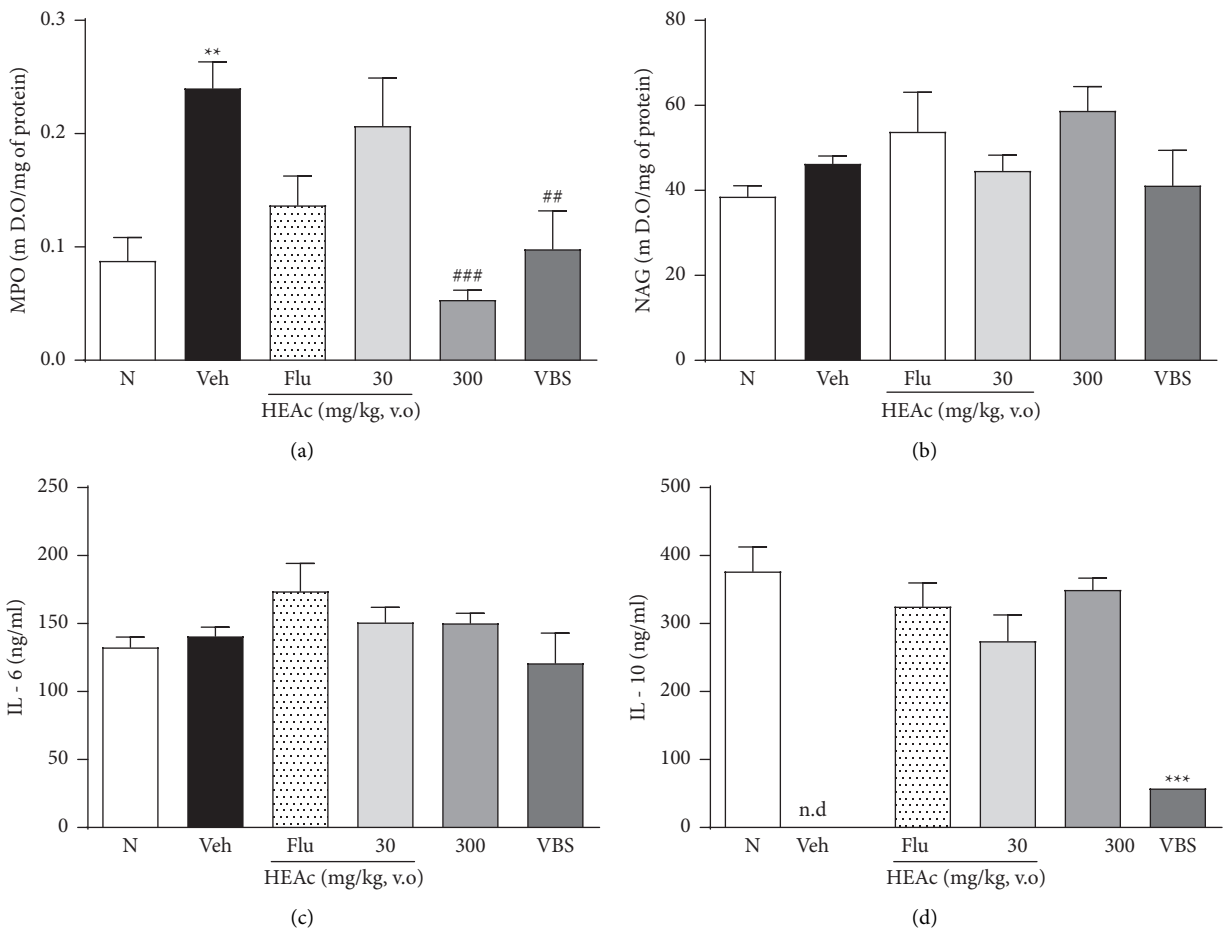

Figure 9: Effects of HEAc and VBS on the markers MPO, NAG, IL-6, and IL-10 in the cortex of mice evaluated in the posttreatment experiment of antidepressant-like activity.

Table 1: Effect of HEAc and VBS on the levels of GSH and SOD activity in the hippocampus of mice evaluated in the posttreatment experiment of antidepressant-like activity.

Treatments	GSH	SOD
N	24.18 ± 3.28	22.24 ± 2.08
Veh	$11.52 \pm 1.29^{**}$	18.10 ± 1.57
Flu	13.98 ± 0.64	25.30 ± 1.25
HEAc 30	$21.34 \pm 3.21^{\#}$	20.09 ± 1.63
HEAc 300	$36.06 \pm 1.35^{\#\#\#*}$	19.32 ± 1.93
VBS	13.12 ± 1.00	20.24 ± 1.45

Note. N, naive; Veh, (vehicle, water); Flu, fluoxetine (30 mg/kg) hydroalcoholic extract of *Aloysia citriodora* (HEAc, 30 or 300 mg/kg); VBS, verbascoside (30 mg/kg). ANOVA (one way) ($n = 8$), post hoc Tuckey. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the N group. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared to the Veh group. Reduced glutathione (GSH; μg/mg tissue); SOD (superoxide dismutase, U/mg protein).

Table 2: Effect of HEAc and VBS on oxidative parameters in the cortex of mice evaluated in the posttreatment experiment of antidepressant-like activity.

Treatments	GSH	LPO	SOD	CAT
N	36.97 ± 3.62	1.85 ± 0.03	43.23 ± 2.93	3.71 ± 2.69
Veh	21.94 ± 0.84^{a}	1.85 ± 0.08	68.22 ± 2.18	6.89 ± 1.38
Flu	26.33 ± 2.84^{a}	2.00 ± 0.06	41.75 ± 3.28^{a}	3.91 ± 1.71
HEAc 30	36.39 ± 1.87^{b}	2.02 ± 0.03	38.87 ± 3.05^{a}	2.70 ± 1.02
HEAc 300	30.90 ± 1.24^{b}	1.75 ± 0.08	64.40 ± 3.57	6.74 ± 0.29
VBS	30.81 ± 2.38^{b}	1.94 ± 0.02	53.52 ± 2.43^{a}	8.31 ± 1.58

Note. N, naive; Veh, (vehicle, water); Flu, fluoxetine (30 mg/kg) hydroalcoholic extract of *Aloysia citriodora* (HEAc, 30 or 300 mg/kg); VBS, verbascoside (30 mg/kg). ANOVA (one way) ($n = 8$), post hoc Tuckey. $^{a}p < 0.05$ compared to the N group (naive group). $^{b}p < 0.05$compared to the Veh group (vehicle group). Reduced glutathione (GSH; μg/mg tissue); lipid hydroperoxide (LPO, μm/MDA/g); SOD (superoxide dismutase, U/mg protein); CAT (catalase, μmol H_2O_2/min).

differed in terms of the temporal application of the treatments (either before or after treatment). In the pretreatment experiment, LPS administered to mice 1 h after treatments were observed to be highly effective in promoting sickness behavior. In line with expectations, 6 h after LPS exposure, all treatment groups showed a significant reduction in locomotor activity, as evaluated by OFT. We also observed reductions in rearing and grooming behavior and the production of feces, which we speculate could be attributable to an accentuation of the sickness behavior. However, 24 h after exposure to LPS, we found that all mice had recovered from the sickness behavior, and, in TST, only mice in the Veh group showed prolonged immobility, thereby corroborating the validity of this test. Based on these findings, we can thus conclude that, at the assessed doses, HEAc and VBS do not seem to have a protective effect against the adverse effects of LPS.

Although the findings of several studies have indicated the preventive effects of antidepressants or natural products on LPS-induced behavioral changes, similar therapeutic effects could not be demonstrated [74, 75]. In contrast, other authors have demonstrated that posttreatment administration of synthetic or natural compounds can mitigate LPS-induced depressive behavior in mice [18, 76, 77]. These seemingly contradictory observations serve to highlight the differences in the pharmacological effects between compounds as well as biological outcomes when using distinct experimental protocols [42].

In the posttreatment experiment, 5 h after exposure to LPS, the mice in all groups showed sickness behavior. However, we found that the intensity of depressive symptoms was lower in those mice administered HEAc (30 and 300 mg/kg) or fluoxetine (Flu), thereby providing evidence for a certain protective effect of these treatments against cell damage. At 6 h after LPS administration, mice in the Veh group showed a marked reduction in locomotor activity in the OFT. In contrast, mice receiving HEAc (3, 30, or 300 mg/kg) or Flu treatments showed a significant increase in the number of crossings compared with those made by Veh group mice. Thus, it appears probable that, apart from the lowest dose assessed, HEAc may interfere with the immune response of mice triggered by LPS. We suspect this could be attributable to the modulation of receptor activation pathways, such as TLR4, or the transcription factors of genes that are activated and regulated via NF-κB, which regulates the genes associated with the responses of the innate and adaptive immune systems [78]. In addition, flavonoids and phenylpropanoids such as VBS are known to preferentially bind to benzodiazepine GABAA receptors and act as partial agonists promoting anxiolytic effects [79]. These observations may be consistent with the greater locomotion of mice treated with HEAc in the OFT, as mice in the per se group showed no significant differences from the N group mice, thus ruling out a stimulation effect.

A notable observation was that, at 24 h after exposure to LPS, the mice began to show evidence of recovery, as indicated by the similar values obtained in the OFT for the mice in different groups. Among the mice subjected to TST, we found that, compared with Veh group mice, those administered HEAc (30 and 300 mg/kg) or VBS were characterized by reductions in the period of immobility. In this test, the time an animal remains immobile is taken to be indicative of its state of despair and is assumed to reproduce a condition corresponding to human depression. Consequently, a reduction in the period of immobility is considered indicative of an antidepressant effect [80]. This finding is of particular interest, given the significant correlation between the efficacy of antidepressants and the data obtained using this biological model [81].

It is well established that the administration of LPS can promote the development of depressive-like behavior, and it has been proposed that this effect is associated with the induction of neuroinflammation in the cortex and hippocampus. Accordingly, preventing or ameliorating the inflammatory process could serve as an alternative approach to obtaining an antidepressant effect [27, 82–85].

Our findings provide evidence to indicate a certain effect of HEAc against neuroinflammation, including a reduction in N-acetyl-glucosaminidase activity in the hippocampus of mice administered the 300 mg/kg dose of this extract. This indicates that the treatment might protect cells against inflammation, as macrophages are also involved at the beginning of the inflammatory process [86]. Further, in the hippocampus, we observed that HEAc (300 mg/kg), VBS, and Flu showed an anti-inflammatory effect by decreasing IL-6 levels. IL-6 is among the main inflammation-associated cytokines and has also been shown to induce depressive-like symptoms in animal models of inflammation [87]. We detected a significant reduction in myeloperoxidase (MPO) activity in the cortex of mice treated with HEAc (300 mg/kg) or VBS. The activity of MPO is routinely monitored to evaluate the migration of neutrophils to the inflamed tissue, as a considerable amount of this enzyme is released into the extracellular medium following cellular activation [88]. In addition, we also detected elevated levels of the anti-inflammatory cytokine IL-10 in mice administered HEAc (30 and 300 mg/kg). Collectively, these observations provide persuasive evidence corroborating the anti-inflammatory effects of HEAc and its major constituent, VBS, in different regions of the brain. This is consistent with the proposition that a reduction in the inflammatory process is responsible, at least partially, for the observed antidepressant-like effects.

An additional mechanism that contributes to the anti-inflammatory activity of natural compounds is their ability to regulate the cellular redox state. This could be attributed to direct antioxidant action involving the capture of free radicals or by inducing the production of enzymes that comprise the endogenous antioxidant defense system (superoxide dismutase, catalase, and glutathione peroxidase) [89, 90]. In our oxidative stress evaluation tests carried out in the hippocampus, we established that administration of HEAc (30 and 300 mg/kg) prevented the depletion of glutathione (GSH), considered a first-line agent of antioxidant defense that plays a role in the capture of ROS [91]. This antioxidant effect of HEAc, mediated via the maintenance of basal levels of GSH, was similarly detected in the cortex of mice administered VBS. These observations are consistent

with the intense antioxidant activities of HEAc and VBS observed in vitro, as indicated by the findings of DPPH radical scavenging assays.

5. Conclusion

In this study, we demonstrate that the hydroalcoholic extract of the leaves of *Aloysia citriodora*, along with its major constituent, VBS, has anti-inflammatory and antidepressant-like activities and propose that the pharmacological effects are mediated, at least in part, via a reduction in proinflammatory markers and oxidative stress. We accordingly believe that the findings of this study make an important contribution to the current understanding of the pharmacological mechanisms of this medicinal plant. In addition, our observations validate the widespread medicinal use of *A. citriodora* and offer the prospect of developing novel antidepressant agents.

Abbreviations

MDD: Major depressive disorder
5-HT: Serotonin
NA: Norepinephrine
DA: Dopamine
ADT: Tricyclic antidepressants
MAOIs: Monoamine oxidase inhibitors
SSRIs: Selective serotonin reuptake inhibitors
LPS: Lipopolysaccharide from *Escherichia coli*
HEAc: Hydroalcoholic extract from *A. citriodora*
N: Naive
Veh: Vehicle
VBS: Verbascoside
PS: Per se
Flu: Fluoxetine
OFT: Open-field test
TST: Tail suspension test
GSH: Glutathione
LOOH: Lipid hydroperoxides
MPO: Myeloperoxidase
NAG: N-acetyl-β-D-glucosaminidase
SOD: Superoxide dismutase
GST: Glutathione transferase
CAT: Catalase
TNF-α: Tumor necrosis factor-alpha
IL-6: Interleukin-6
DTNB: 5,5′-Dithiobis (2-nitrobenzoic acid)
CDNB: 1-Chloro-2,4-dinitrobenzene
FOX2: Ferrous Oxidation-Xylenol Orange
CNS: Central nervous system
NF-KB: Nuclear factor Kappa β transcription
NOS-2: Nitric oxide synthase
COX-2: Cyclooxygenase-2
MAPK: Mitogen-activated protein
TLR: Toll-like receptors
STAT: Signal transducer and activator of transcription.

Data Availability

The articles, images, and analysis tables used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

The Regulation of miR-206 on BDNF: A Motor Function Restoration Mechanism Research on Cerebral Ischemia Rats by Meridian Massage

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As a frequent disease affecting the nervous system, cerebral infarction has emerged as a major cause of disability and elicits disorders in motor, sensation, and cognition as sequelae. No clear mechanism has been known in meridian massage despite it having been proved to be an effective therapeutic option. The study was carried out to explore the treatment of meridian massage on cerebral ischemia in rats and its effects on motor function restoration and nerve cell's ultrastructure in the ischemic territory. The alleviated nerve damages and recovered injured brain tissues were found in the cerebral infarction model of SD rats after meridian massage. Expressions of miR-206 and the brain-derived neurotrophic factor (BDNF) in the gastrocnemius muscle were all well observed. The effects of miR-206 on BDNF were testified by overexpressed and interfered miR-206 in the C2C12 myoblast. Moreover, at the molecular level, meridian massage downregulated miR-206 expression at an elevated level of BDNF. Consequently, meridian massage exerts a vital role in promoting cerebral ischemia restoration, which is expected to provide an addition to the application of traditional Chinese medicine (TCM) in the reconstruction and treatment of cerebral ischemia.

1. Introduction

Cerebral infarction, a common disease affecting the nervous system, has evolved into the top three lethal diseases causing mortality [1]. Despite the risk of stroke dropping by 25% till 2010 as medical technologies and methods advanced, the rate of disability remains on the rise even over 80%, struck by sequelae, namely, motor, sensory, and cognitive disorders, which become a heavy burden carried upon the shoulders of patients, their families, and society [2, 3]. Early formal training on functional rehabilitation has been proved effective in the restoration and improvement of the motor function after episodes of cerebral infarction living a better quality of life for patients. Of all the therapies, meridian massage manifests a satisfactory efficacy in clinical application, while little has been revealed on its working mechanisms. In light of the meridian-collateral theory in traditional Chinese medicine (TCM), meridian massage in combination with the naprapathy principle treats diseases employing conducting meridians and relieving muscle spasms, thus stimulating muscles to produce a passive movement and regulating Qi and blood in viscera [4–6]. Our team has made clinical achievements with the application of meridian massage which ameliorates the motor function of patients suffering from cerebral infarction. An interference of rehabilitation training alters the expression patterns of angiopoietin, Nogo-A, and brain-derived neurotrophic factor (BDNF) and facilitates axonal bud synaptic reconstruction for the recovery of functions in the brain and loops [7]. In addition, the therapy of rehabilitation training promotes the proliferation of glial cells, vascular endothelial cells, and macrophages around necroses and accelerates necrosis reconstructions, thereby advancing compensatory effects of normal tissues and facilitating the recovery of the

motor function [8, 9]. Clinically, meridian massage in an early stage of rehabilitation training produces an efficient outcome in improving the cerebral blood flow of acute cerebral infarction patients through strengthening motor function recovery and enhancing the paralyzed limb functions. In addition, early training on functional rehabilitation contributes to a prominent result. Most studies on the mechanism of meridian massage on motor function reconstruction after cerebral infarction give priority to cerebral microangiogenesis, neurotrophic factors, and various brain-protective proteins, while the mechanism of meridian massage in promoting skeletal muscle recovery remains to be explored.

Neurotrophic factors present as proteins that maintain functions, support the survival of the neurons, and promote cell growth as well as differentiation [10]. Myogenic neurotrophic factors are possibly uptaken by the motor nerve axon terminal and retrograde transported to neuronal somas, which affects the survival and differentiation of motor neurons and exerts vital importance in the development and function maintenance of neuromuscular junction (NMJ) [11, 12]. The neurotrophic factors related to motor neurons include neurotrophins and BDNF, among which BDNF functions as one of the crucial members in the neurotrophic family and produces an essential role in neuron survival, growth, differentiation, neurotransmitter release, and synaptic plasticity [13–15].

The miRNA regulation of genes is speculated to contribute to the performance of highly complex functions for vertebrate muscles, and the specific expression of miR-206 has been reported in skeletal vertebrate muscles and high expression in slow muscles [16]. Accordingly, it may regulate gene expression as muscle satellite cells develop and muscle fiber types converse in adult animals. Most miRNAs inhibit the translation of target genes in the cytoplasm, while the expression of miR-206 is noticed in the cytoplasm and nucleus of myoblasts. The unique expression pattern indicates a significant role of miR-206 in skeletal muscle pathophysiology, which requires further study in pursuit of the significance of miR-206 and other molecules during the course.

More investigations suggest that miR-206 regulates the expression of the BDNF which means the inhibition of miR-206 increases the level of BDNF [16]. By mediating multiple functions of synaptic plasticity, the BDNF plays a role in improving cognitive function, neurogenesis, as well as neuronal differentiation. Studies in Alzheimer's disease (AD) rats have demonstrated that increased levels of brain-derived neurotrophic factors and the administration of AM206 and miR-206 blocker, both may enhance the expression of hippocampal synaptophysin and neurogenesis and cognitive function [17]. The expression of miR-206 indicates an upregulation in the frontal cortex during cerebral ischemia. In normal circumstances, the level of miR-206 is undetectable or too low to be detected in the brain, while it is highly expressed in neurological-related diseases, suggesting that miR-206 might participate in the pathogenesis of AD by inhibiting the BDNF expression, thereby affecting motor function [18–20]. So, the maintenance of miR-206 at an appropriate physiological level is proved to be of great significance. Exercise experiments have revealed that the miR-206 expression is high in a quiet state but the level is reduced through a chronic endurance exercise. After the training, the miR-206 expression falls back to the former level, implying that miR-206 may directly alter the physiological effects of human endurance sports, that is why miR-206 is known as a skeletal muscle function regulator. As mentioned in the previous study, meridian massage may act as an external force on muscle tissues, facilitating expressions of BDNF and inhibiting miR-206 in muscles, thereby improving the synaptic function of neuromuscular junctions and easing the dysfunction by recovering the impaired motor function. Based on the previous research, this study has been established to dig out the effects of meridian massage on the reconstruction of the motor function, the ultrastructure of nerve cells in the ischemic territory, and the expressions of both miR-206 and BDNF in gastrocnemius muscles. We also aim to investigate the mechanism of meridian massage on motor function reconstruction after cerebral infarction to treat the rat models of cerebral infarction utilizing meridian massage therapy.

2. Methods

2.1. Animals. A total of 72 male SD rats weighing 250~280 g were provided by the Chongqing Enswil Biotechnology Co., Ltd., 12 h–12 h day and night alternated. The animals were supplied with food and water at will at a temperature of 23–25°C, and the experiment was performed after adaptive feeding for one week. The experiment was approved by the ethics committee of Guizhou University of traditional Chinese medicine.

2.2. Animal Modeling and Sampling. The SD rats were anesthetized with 1% pentobarbital sodium (50 mg/kg) and fixed on the operation board with a medical tape in a supine position. The skin of the neck of the rats was shaved and disinfected with an alcohol swab. A 2~3 cm cut was made in the skin in the middle of the neck, and the submandibular gland and right muscle were bluntly separated exposing the common carotid artery, which was carefully separated, and so did the vagus nerve. Internal and external carotid arteries were dissected with care, and the external carotid artery was ligatured. With the internal carotid artery being temporarily clamped by an arterial clamp, the distal end of the common carotid artery was ligatured. The 4–0 surgical suture was utilized for ligation with the common carotid artery elevated. A V-shaped cut was opened with a spring scissors and a special suture was inserted, slightly tightening the prepared suture to avoid blood reflux. No resistance caused by the insertion of the tethered wire was felt until the mark of the tethered wire reached the bifurcation of the common carotid artery and internal carotid artery if the wire entry was smooth. Then, a slight resistance could be felt at the tip of the tethered wire; then, we stopped plugging and ligated the prepared line. Muscles and skins were sutured layer by layer, disinfected with cotton balls containing alcohol, and the rats

were put back into the cage. The rats were anesthetized 1.5 h after surgery the neck was disinfected and then cut with an open. Ophthalmic tweezers were employed to clamp the sites to be sutured. The suturing line was pulled out to the head and cut the excess before being sutured layer by layer and sterilized. As for the sham operation group, only the common carotid artery was dissected and sutured immediately. The rats were placed back in their cages after the operation supplied with sufficient feed and water.

The rats were randomly divided into the model group, meridian massage group, and sham operation group, with 24 in each group. Twenty-four hours after the successful establishment of the middle cerebral artery occlusion (MCAO) rat model, the rats from the model group and the sham operation group were kept in ordinary cages, no rehabilitation training was given but food, water, and movement were at their will. Conversely, the model group + meridian massage group were given meridian massage for 21 days. According to the positioning standards of Experimental Acupuncture Science and Chinese Veterinary Acupuncture Science, the rats were massaged at the Biguan (ST31), Taichong (LV3), Fenglung (ST40), and Zusanli (ST36) acupoints, with the emphasis on the gastrocnemius muscle and the back of the thigh [21]. The rats' limbs were slightly hot and able to bear for 10 min, twice a day, for 21 consecutive days. The SD rats in each group were anesthetized with 7% chloral hydrate (0.5 mL/100 g) after 3, 7, 14, and 21 days, respectively, before samples were collected as below after successful anesthesia.

The whole blood: The abdominal cavity of the rats was opened and the abdominal aorta was located. One sample of whole blood was collected with an anticoagulation vacuum blood collection tube for the subsequent extraction of leukocytes. Another whole blood sample was collected with a non-anticoagulation blood collection tube and centrifugated. The supernatant was collected and stored at −80°C. The gastrocnemius muscle: Following the blood collection, the gastrocnemius muscle was dissected, washed with PBS, and then stored at −80°C for later use. The brain: The chest cavity was opened and the apex of the heart was connected to the injection needle of the infusion bag. The right atrial appendage was cut off to perform heart perfusion. PBS perfusion was initiated until the lungs turned white, then switched to 4% of paraformaldehyde and continued the perfusion. Dura mater and the whole brain were removed. The hippocampus and hemiplegic lateral frontal lobe were isolated and kept for 48 h which were scheduled for section staining. The electron microscopy sample was switched with 2.5% glutaraldehyde perfusion after the PBS perfusion and the rest surgery parts were identical.

2.3. HE Staining. All washed samples were fixed with 4% paraformaldehyde, and these tissues were immersed in 75% ethanol, 85% ethanol, 95% ethanol I, 95% ethanol II, 100% ethanol I, and 100% ethanol II in an orderly fashion, each for 2 hr. The dehydrated tissues were then soaked in 1/2 xylene, xylene I, and xylene II, in turn, each for 10 min. After transparent treatment, tissues were immersed in melted paraffin for 3 h to make paraffin sections. Tissues made from paraffin blocks were cut into slices at a thickness of 5 μm with a microtome, laid flat on the fall-proof glass, and placed in a baking machine at 55°C to ensure the tissue pieces were tightly attached to the sliding glass. The paraffin sections were initially soaked in xylene I, xylene II, and 1/2 xylene for 10 min each for dewaxation and then in 100% ethanol I, 100% ethanol II, 95% ethanol I, 95% ethanol II, 85% ethanol, and 75% ethanol for 5 min each. All samples were washed twice with distilled water for 2 min each time for a total of 3 times. Staining was performed with hematoxylin for 10 min. After that, tap water was utilized to rinse the excessive staining solution for about 5 min and then washed with distilled water. Of 1% of hydrochloric acid, alcohol was used to decolorize excessive hematoxylin staining solution in the cytoplasm. Eosin staining lasted 30 s. Running water was lasted for 3 min to remove the excessive staining solution, and it was washed with distilled water again and dehydrated with 95% ethanol twice, 2 min each time. Then, xylene was utilized for hyalinization twice, 5 min each time. The neutral resin was used for blocking. Microscopic examination displayed that the nucleus turned blue and the cytoplasm was either red or pink.

2.4. Transmission Electron Microscopy Observation. The samples were prefixed with 3% glutaraldehyde, re-fixed with 1% osmium tetroxide, and then dehydrated with acetone at concentrations of 30% $\longrightarrow$ 50% $\longrightarrow$ 70% $\longrightarrow$ 80% $\longrightarrow$ 90% $\longrightarrow$ 95% $\longrightarrow$ 100% (three times at 100%) in an orderly fashion. Permeation was conducted with a dehydrating agent and epoxy resin (model Epon812) successively at ratios of 3 : 1, 1 : 1, and 1 : 3, respectively. Each step lasted 30~60 min. The infiltrated sample blocks were placed in an appropriate mold filled with embedding solution and embedded into a solid matrix (embedded blocks) for heating and polymerization. This preparation was performed for later use. About 50 nm thickness ultrathin slices were prepared by using an ultrathin slicer, which was available in floating on the liquid surface in the groove, which was transferred to a copper mesh, stained with uranium acetate first, followed by lead citrate, at room temperature for 15~20 min, and observed with a transmission electron microscope (JEM-1400PLUS).

2.5. RNA Extraction and RT-qPCR. The total RNA was extracted with the TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized through the TaqMan® MicroRNA Reverse Transcription kit (Thermo Fisher Scientific) in line with the manufacturer's instructions of use. qPCR assay was performed to quantify amounts of miRNA and mRNA with iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) through the iCycler iQ™ qPCR detection system (Bio-Rad Laboratories). Relative levels of miR-206 and BDNF were calculated as the inverse log of $\Delta\Delta$Ct and normalized to the reference genes. Thermocycling was set at the following conditions: 95°C for 10 min; followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min; annealing at 55°C for 30 sec; and elongation at 72°C for 3 min. GAPDH was regarded as an internal control for the analysis of BDNF mRNA expression and U6 was deemed as the internal control for the identification of miR-206

expression. Primers included U6–F, CCTGCTTCGGCAGCACATAT; U6-R, GAACGCTTCACGAATTTGCGT; GAPDH-F, GCAAGTTCAACGGCACAG; GAPDH-R, GCCAGTAGACTCCACGACATA; mir-206-F, GCGGAGTGGAATGTAAGGAA; mir-206-R, GTGGAGTCGGCAATTCAGTT; BDNF-F, GTCCACGGACAAGGCAACT; and BDNF-R, CAGCAGCTCTTCGATCACG.

2.6. ELISA. The ELISA kit was utilized to detect the expression level of BDNF (RD, DY248). Blank wells, standard wells, and sample wells were set for determination. 100 μL of the sample diluent was added to the blank wells Another 100 μL of standard or test samples was added to the remaining wells. The reaction processes were performed at 37°C for 120 min. The liquid was discarded and spun dry. A 100 μL working solution of biotinylated antibody was added to each well, incubated at 37°C for 60 min, with the supernatant being discarded and spun dry. The plates were washed 3 times after being soaked for 1–2 min each time, 350 μL/per well, spinning dry. 100 μL of horseradish peroxidase-labeled avidin working solution was added to each well and incubated at 37°C for 60 min. After that, the supernatant was discarded, spun-dried. The plates were rinsed 5 times after being soaked for 1–2 min each time, 350 μL/per well, spun-dried. 90 μL of the substrate solution was added to each well in an orderly manner and incubated at 37°C in the dark for 5–10 min. 50 μL of the stop solution was added to each well in sequence to terminate the reaction. Successive measurement of the optical density of each well was carried out with an enzyme-linked instrument (Thermo Fisher Scientific) at a wavelength of 450 nm. Procedures were performed within 15 min after the addition of the stop solution.

2.7. Western Blot. Tissue samples were lysed with a radioimmunoprecipitation assay buffer (Beyotime, China). Protein concentrations were examined by using the BCA Protein Assay kit (Pierce; Thermo Fisher Scientific). A number of protein extracts (30 μg total protein/lane) were resolved with 10% SDS-PAGE, transferred onto PVDF membranes, and blocked with 5% dried skim milk at room temperature for 1h. The PVDF membranes were incubated with the BDNF primary antibody (cat. no. BM1307; 1 : 500; IGEE) and β-actin antibody (cat. no. BM026; 1 : 1,000; IGEE) at 4°C overnight with gentle agitation, and then treated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1 : 1,000; cat. no. BM003; IGEE) at room temperature for 2 h. β-Actin was served as a loading control. The protein bands were visualized by using an enhanced chemiluminescence system (Beyotime, China).

2.8. C2C12 Myoblast Culture. C2C12 myoblasts were cultured in a DMEM high-glucose medium (Sigma, China) containing 10% fetal bovine serum (Hyclone, USA) and incubated in a incubator with 37°C, 5% CO_2, at a constant temperature, and the medium was changed every other day. When the cells are confluent and reach about 80% of the growth density, a negative pressure is formed through the cell flexible substrate-loading device to generate a tensile strain on the culture membrane, so that the adherent and growing cells are subjected to tension.

2.9. Luciferase Reporter Assay. Wild-type (WT) or mutant (MUT) BDNF-3′UTR containing miR-206 binding sites were inserted into the psiCHECK2 vector (Promega, USA). The 293 T cells (1 x 10^5 cells/well) were co-transfected with 0.1 mg of psiCHECK2-WT BDNF-3′-UTR or 0.1 mg psiCHECK2-MUT BDNF-3′-UTR and 10 nM of miR-206 mimics or 10 nM of miR-206 inhibitors in a way of Lipofectamine® 2000 reagent (Invitrogen, USA) as per the manufacturer's instructions of use. Cells were cultured at 37°C for 48 h, whose luciferase activities were analyzed by using a dual-luciferase kit (Promega, USA) in terms of the manufacturer's instructions of use. The activity of firefly luciferase was normalized to corresponding Renilla luciferase activity.

2.10. Statistical Analysis. Results were presented as the mean ± SEM. Significance analysis was established by the SPSS 13.0 software. Data were analyzed according to Student's *t*-test or one-way analysis of variance followed by Tukey's honest significant difference test. $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1. Meridian Massage Restored the Neuronal Cell Morphology and Structure of Cerebral Infarction in Rats. The cerebral infarction model of SD rats was established (Figures 1(a) and 1(b)). Observations were made on the morphology of the cerebral cortex and hippocampus by HE staining. No obvious changes were visible over time at the injured parts in cerebral cortex models, while nerve cell edema was displayed severely affected with an increasing perinuclear space, and inflammatory cell infiltration was also revealed in some interstitium. The degeneration of nerve cell edema at injured parts was significantly relieved over time in the treatment group (repeated massage) compared with the model group. Results of HE staining in the hippocampus revealed that nerve cells of the hippocampus took on a scattering distribution in the model group. Blurred contours of nuclear shrinkage cells were presented and various edemas were observed in the interstitium (Figure 1(c)). The alignment of nerve cells in the treatment group (repeated massage) turned regular gradually and arranged tightly as time passed by in comparison with the model group.

3.2. Meridian Massage Restored the Damaged Nerve Cells and Reduced Apoptosis and Autophagy. Structures of nerve cells in rats were observed in the sham operation group, the cerebral infarction model group, and the meridian massage treatment group via transmission electron microscopy. In the brain tissue samples of the sham operation group, nuclei of nerve cells presented round, chromatin was evenly distributed, the nuclear membrane was clear and complete, and the

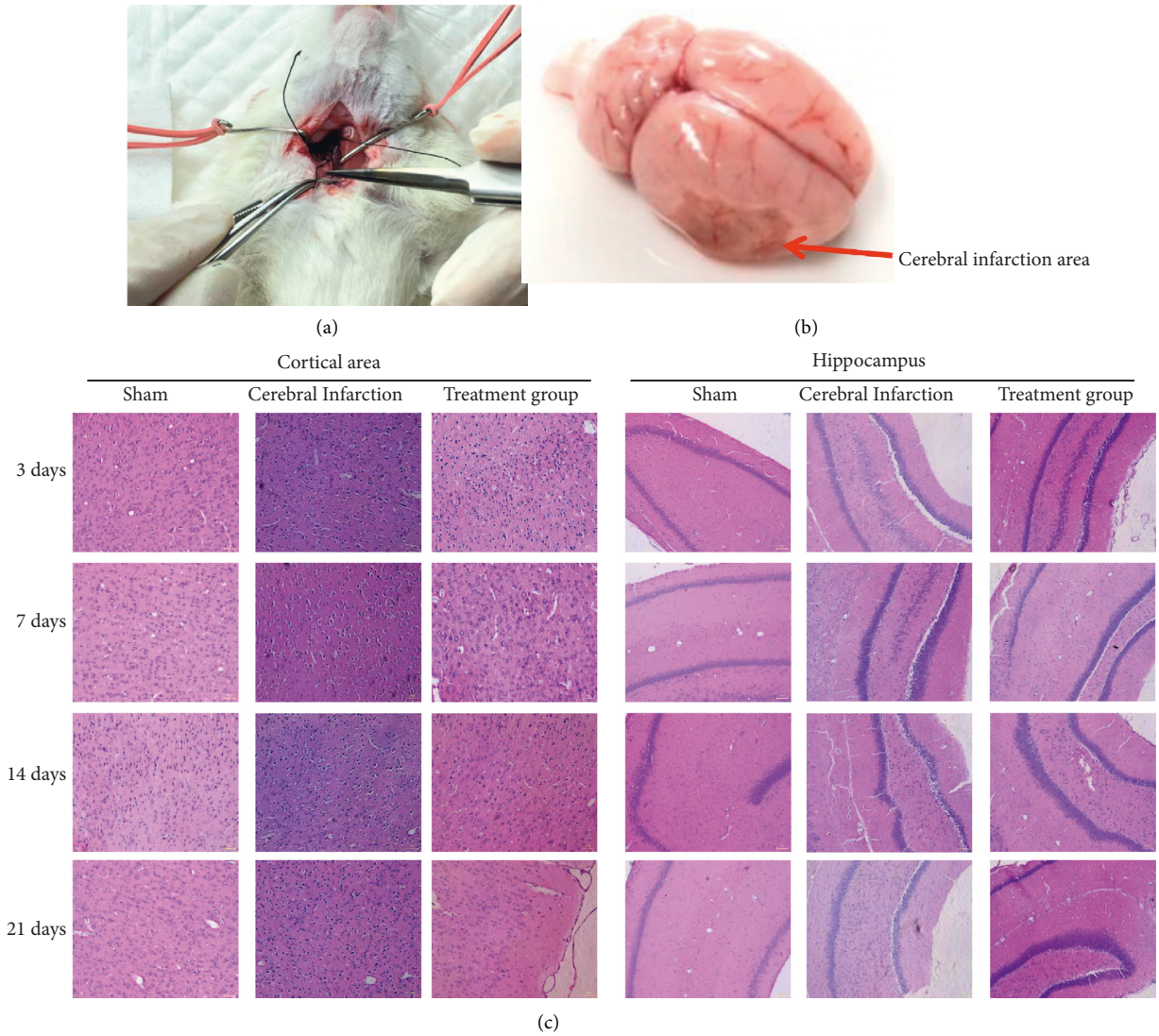


Figure 1: Cerebral infarction model in rats and HE staining. (a) Schematic diagram of cerebral infarction model operation; (b) cerebral infarction area; (c) HE staining to detect the hippocampus area and cortical area.

cytoplasm was rich in complete and clear ribosomes, myelin sheaths, microfilaments, and mitochondria, among which the nerve myelin sheaths were relatively compact (Figure 2). The model group featured characteristics of apoptotic cells: blurred brain samples, some necrotic nerve cells with loose structures, rupture of cell membranes, massive mitochondria swelling in the cytoplasm, disappearing cristae forming into vacuoles, aggregation of nucleus chromatin, integral nuclear membranes but with shrinkage cell volumes, denatured myelin sheaths of myelin nerve fibers and obvious autophagy. Results of the treatment group presented round nuclei of the nerve cells, evenly distributed chromatin, clear and integral nuclear membranes, mitochondria in the cytoplasm, rough endoplasmic reticulum, and ribosomes. Swelling occurrence and autophagy and apoptosis were distinctly reduced in the treatment group compared with the model group. The abovementioned findings attested to the therapy of meridian massage that restored nerve cell damage and reduced apoptosis and autophagy.

3.3. Meridian Massage Reduced the miR-206 Expression and Enhanced the BDNF Expression after Cerebral Infarction. Subsequently, we determined expressions of miR-206 and BDNF in separate samples of gastrocnemius muscles and whole blood in rats by qPCR on days 3, 7, 14, and 21; results indicated that the expressions of miR-206 in whole blood and gastrocnemius muscles ranked the top in the model group (Figures 3(a) and 3(c)), the second was the treatment group and the lowest was the sham operation group. Such findings provided opposite data against the expression patterns of BDNF (Figures 3(b) and 3(d)).

The expression of BDNF in gastrocnemius muscles was testified by western blot (Figure 4(a)). From the results of the grayscale analysis, we know that the BDNF was highly expressed in the sham operation group, followed by the massage group, and the last one was the model group (Figure 4(c)). The expression of BDNF in gastrocnemius muscles was subjected to ELISA. BDNF expression results exhibited 108.6 pg/mL, 116.4 pg/mL, 156.8 pg/mL, and

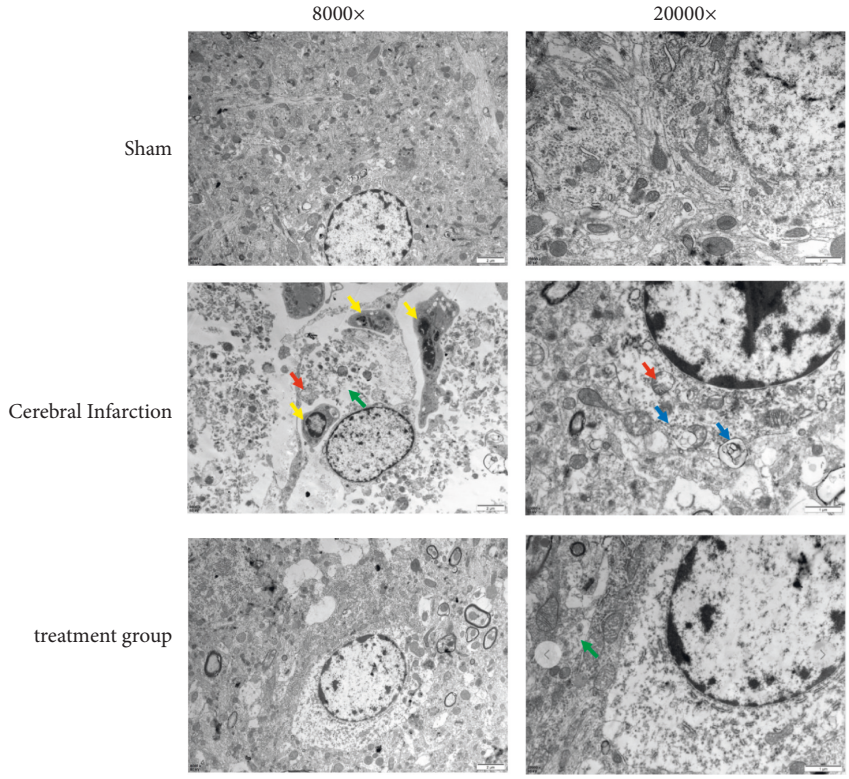
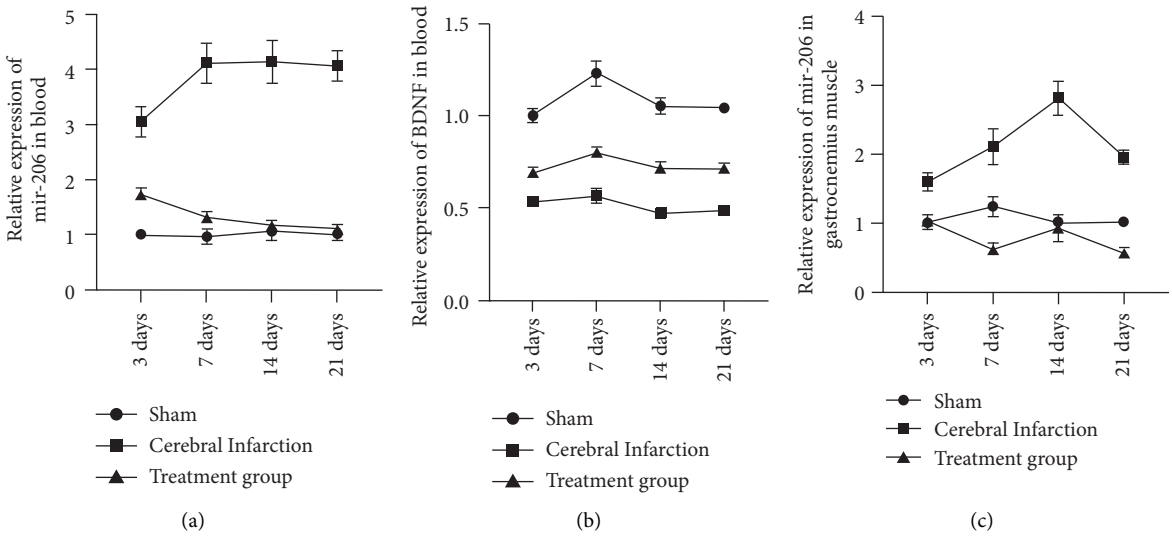


Figure 2: Transmission electron microscopy detection of rat nerve cells. Yellow arrows represent apoptotic cells; red arrows represent swollen mitochondria; green arrows represent intracytoplasmic vacuole; blue arrows represent autophagy.



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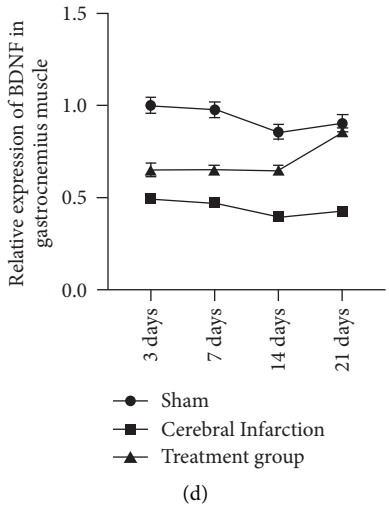


Figure 3: qPCR detection of the miR-206 and BDNF expression in gastrocnemius muscle and whole blood. (a) miR-206 expression in whole blood; (b) BDNF expression in whole blood; (c) miR-206 expression in gastrocnemius muscle; (d) BDNF expression in gastrocnemius muscle.

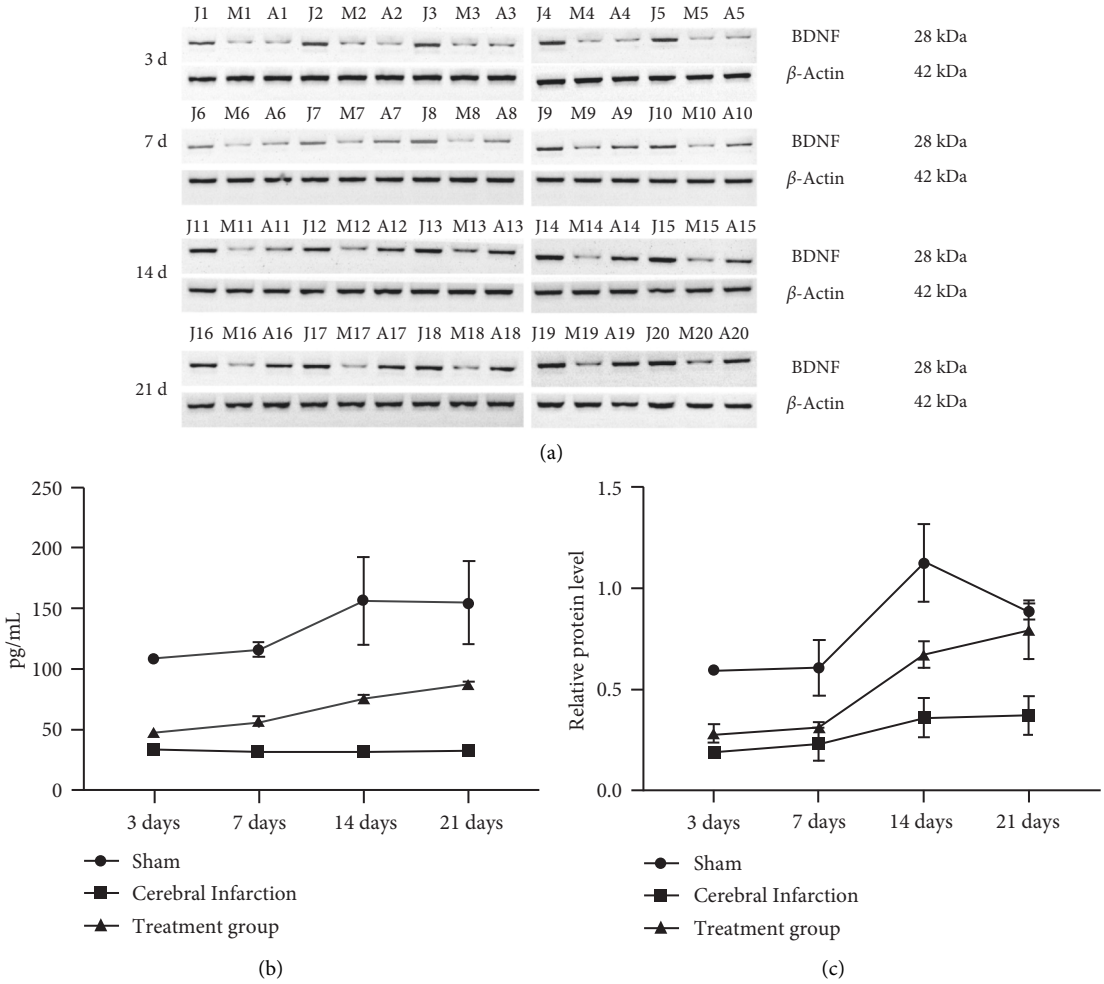


Figure 4: The Western blot and ELISA results of the BDNF expression in gastrocnemius muscle. a: WB detection; j: Sham; m: cerebral infarction. (a) Treatment group; (b) ELISA detection of BDNF in gastrocnemius muscles; (c) Western blot statistical results.

154.7 pg/mL on days 3, 7, 14, and 21, respectively, in the sham operation group. The expressions of the BDNF were 33.62 pg/mL, 32.28 pg/mL, 32.28 pg/mL, and 33.03 pg/mL on days 3, 7, 14, and 21, respectively, in the model group. Moreover, those in the meridian massage group were 47.87 pg/mL, 56.70 pg/mL, 75.70 pg/mL, and 87.70 pg/mL on days 3, 7, 14 and 21 (Figure 4(b)). The above results indicated that meridian massage reduced the expression of miR-206 and increased the expression of BDNF after cerebral infarction.

3.4. BDNF Acted as a Target Gene of miR-206. Detection by a dual-luciferase reporter system revealed that the ratio of Renilla luciferase/firefly luciferase was reduced in the miR-206 and BDNF-WT-3′UTR co-transfection group, whereas the ratio of Renilla luciferase/firefly luciferase exhibited a remarkable change in the BDNF-MUT-3′UTR group indicating BDNF served as a target gene of miR-206 (Figure 5(a)). To further verify the effect of miR-206 on the BDNF, we overexpressed or silenced miR-206 in C2C12 myoblast cells (Figure 5(b)). Results of qPCR showed that miR-206 was downregulated in traction tension-treated C2C12 myoblast cells (Figure 5(c)). Overexpression of miR-206 significantly reduced the mRNA level of BDNF, and its level was restored after traction tension treatment, contrary to miR-206 inhibition (Figures 5(d) and 5(e)). Immunofluorescence and western blot at the protein level showed the same expression trend as mRNA (Figures 6(a)–6(d)).

4. Discussion

Meridian massage functions as a benign physical stimulus based on scientific research. An external force is provided by massage manipulations working at specific parts and acupuncture acupoints on the body surface. The massage technique is employed to stimulate the body and adjust the functions of corresponding organs [22, 23]. Hemiplegic limb movements are difficult to coordinate mostly due to a unified balance that cannot be achieved in antagonistic activities of the nervous system between excitement and inhibition after cerebral infarction. The abnormal antagonistic functions can be improved through massage manipulations, thereby coordinating the movement of the body extremities. Timely meridian massage and limb function training can speed up motor function reconstruction in the affected limbs, reduce sequelae occurrence, and shorten hospital stay for patients with cerebral infarction. Acupoint massage for these patients with cerebral infarction and hemiplegia can promote the recovery of the affected limbs and facilitate their living ability for the rest of their lives. Rehabilitation training after cerebral ischemia promotes nerve regeneration and the recovery of motor function. The recovery of brain functions can be achieved for a large number of patients because of plasticity changes after brain injury, which presents a theoretical basis for rehabilitation treatment of cerebral infarction [24, 25]. Muscles and joints are moved by rehabilitation training, through which many sensation impulses on the body and skin were input into the central nervous system, stimulating cerebral blood circulation, enhancing excitability of nerve cells in the semidark region around lesions, facilitating recovery and compensation of neuron functions and maximizing the function of reconstruction, thereby promoting the formation of normal functional patterns [26, 27]. By manipulating meridian massage in rats after cerebral infarction, we found in this study that meridian massage effectively improved the morphology and structure of brain tissues and reduced autophagy and apoptosis of brain tissue cells in rats after cerebral infarction, through which an obvious improvement was achieved offering an insight of research guidance for meridian massage application in clinical rehabilitation physiotherapy.

This study testified target relationship and differential expressions between miR-206 and BDNF in the cerebral infarction model group and the meridian massage group by qPCR and WB. As a neurotrophic factor, the BDNF exerts essential roles in the in vivo development of motor neurons, the survival of adult motor neurons, survival of pathological motor neurons, and even the regeneration of axons and protection of brain tissues after ischemia. The BDNF serves notably as the most effective bioactive substance to maintain and protect the survival and growth of motor neurons and is the only one continuously expressed in the central nervous system and peripheral nerve tissues. Furthermore, the successive expression of the BDNF in gastrocnemius muscles after birth indicates a long-term role in promoting the development and maintenance of NMJ function [28, 29]. It is assumed the role in refining the synapse since one was given to birth, which is of great significance for the survival and vitality of nerves. Previous experiments have revealed that appropriate exercise increases the expression of BDNF-promoted functional changes in synapses and assisted the NMJ synaptic function in NTs through presynaptic depolarization [18, 19]. Meridian massage is identical to an exercise that produces constant stimulation on affected areas and induced BDNF expression.

The expression of miR-206 is significantly upregulated with the prolongation of denervation time, whose precursor molecules are associated with the formation of the terminal motor synapses. Hence, miR-206 molecules may be involved in the pathophysiological process of skeletal muscle atrophy [30, 31]. After denervation of skeletal muscle atrophy, some expression changes occur in such muscle-specific miRNAs, miR-206 in particular exhibits a dramatically upregulated expression, thus we speculate that these skeletal muscle-specific miRNAs might act as regulators in the process of denervated skeletal muscle atrophy. A load experiment of skeletal muscle hypertrophy in rats revealed that no change was noticed in the expression of mature miR-206 despite the level of the original miR-206 transcript being highly upregulated. Among rats with muscular dystrophy, the expression of miR-206 in septum presented 4.5 folds higher than that of normal rats, while no change of miR-206 was observed in other skeletal muscles [32]. A high level of miR-206 was seen in newly formed muscle fibers indicating that it may be associated with myogenesis and maturation processes of muscle atrophy.

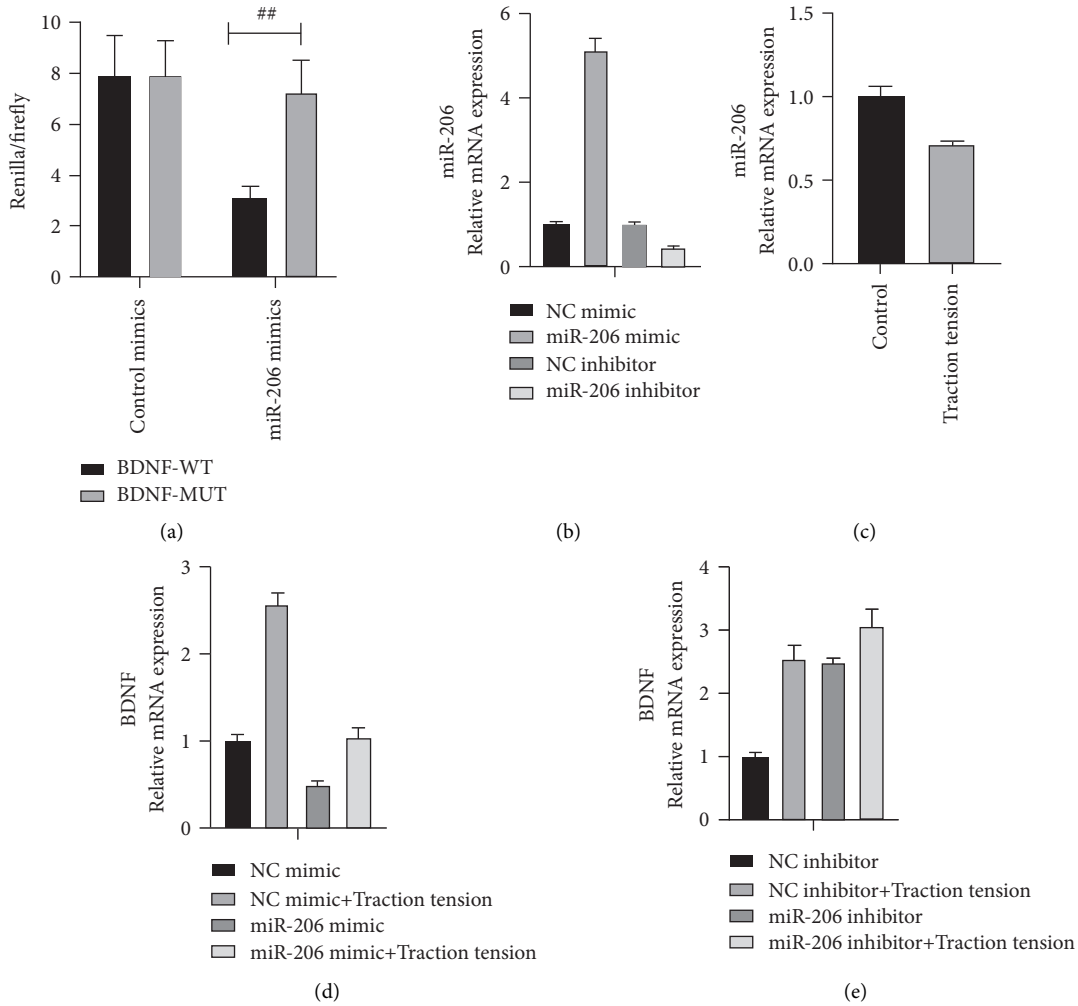
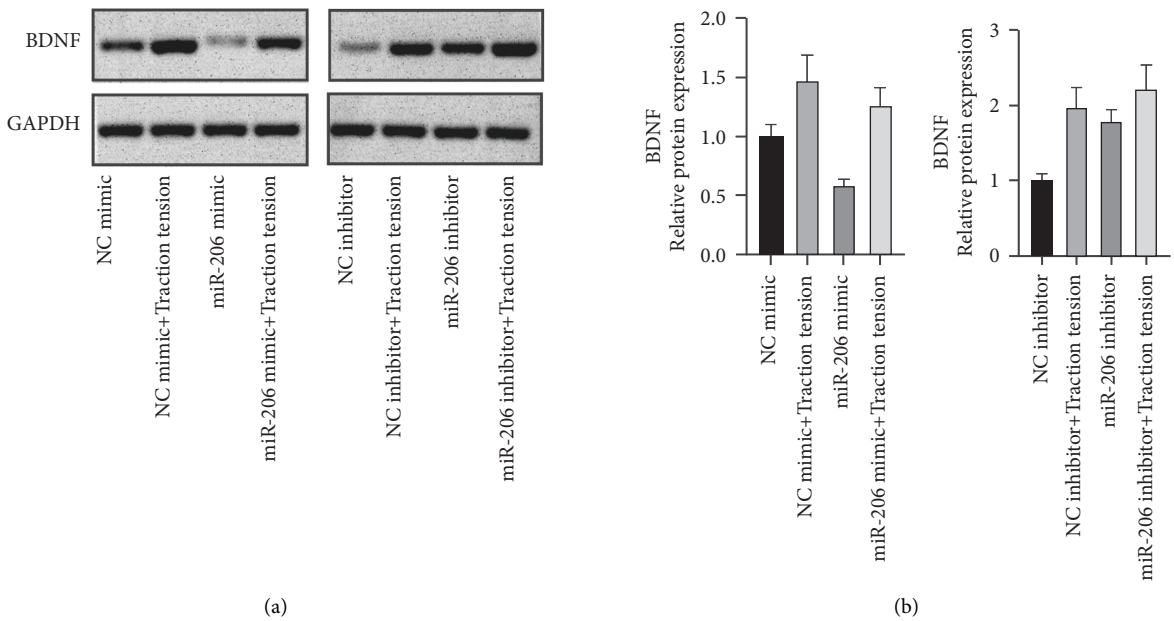


Figure 5: miR-206 acts on the BDNF and downregulates its expression at the mRNA level. (a) Dual-luciferase reporter system to detect the targeting relationship between miR-206 and BDNF. (b) The expression of miR-206 in traction tension-treated C2C12 cells was detected by qPCR. (c, d) Regulation of BDNF expression in C2C12 cells by overexpression or silencing of miR-206. $^{##}P < 0.01$ between two compared groups.



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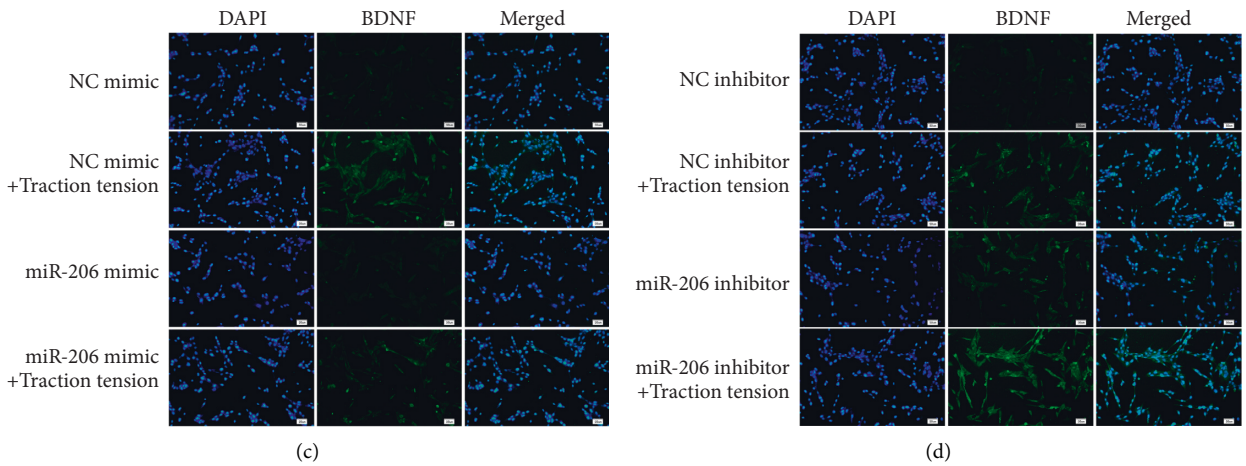

Figure 6: miR-206 downregulated BDNF at the protein level. (a, b) BDNF protein level in C2C12 cells was detected by western blot. Relative protein expression was measured by Image J. (c, d) The expression of BDNF was detected by immunofluorescence in C2C12 cells.

5. Conclusions

This study demonstrates that meridian massage assists in reducing nerve damage and repairing brain tissues to inhibit the expression of miR-206, thereby enhancing the expression of the BDNF. Our findings further reveal that the restoration of brain structures and functions after cerebral infarction can be promoted through successive meridian massage, which is expected to provide a certain research insight for TCM application in cerebral infarction recovery and treatment. It provides a theoretical basis for the clinical application of meridian massage, but the mechanism of action is not yet fully understood, which will be the goal of further research.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Guofeng Shi and Ping Zeng contributed equally to this work.

Acknowledgments

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Research Article

Myricetin Inhibited Fear and Anxiety-Like Behaviors by HPA Axis Regulation and Activation of the BDNF-ERK Signaling Pathway in Posttraumatic Stress Disorder Rats

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Posttraumatic stress disorder (PTSD) is a stress-related psychiatric or mental disorder characterized by experiencing a traumatic stress. The cause of such PTSD is dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and imbalance of monoamines. Myricetin (MYR) is a common natural flavonoid that has various pharmacological activities. We investigated the effects of MYR on fear, depression, and anxiety following monoamine imbalance and hyperactivation of HPA axis in rats exposed to a single prolonged stress (SPS). Male rats were dosed with MYR (10 and 20 mg/kg, i.p.) once daily for 14 days after exposure to SPS. Administration of MYR reduced freezing responses to extinction recall, depression, and anxiety-like behaviors and decreased increase of plasma corticosterone and adrenocorticotropic hormone levels. Also, administration of MYR restored decreased serotonin and increased norepinephrine in the fear circuit regions, medial prefrontal cortex, and hippocampus. It also increased the reduction in the brain-derived neurotrophic factor (BDNF) and tropomyosin-related kinase B mRNA expression and the ratio of p-ERK/extracellular signal-regulated kinase (ERK) in the hippocampus. Thus, MYR exerted antidepressant and anxiolytic effects by regulation of HPA axis and activation of the BDNF-ERK signaling pathway. Finally, we suggest that MYR could be a useful therapeutic agent to prevent traumatic stress such as PTSD.

1. Introduction

Post-traumatic stress disorder (PTSD) is a serious psychological disorder that affects people who have experienced unexpected or traumatic events, such as sexual assault, a natural disaster, or war, or even exposure to disaster-related news [1, 2]. Furthermore, more than 70% of the world's population will be exposed to at least one traumatic event in their lifetime and more than 17% will be diagnosed with PTSD [3]. PTSD is characterized by re-experience, avoidance, and hyperarousal [4, 5]. Chronic PTSD is also associated with other psychiatric disorders, such as anxiety, depression, drug addiction, sleep disturbances, and memory impairment [6, 7]. Given its associated psychological and occupational impairments, PTSD is emerging as a social problem [4].

Neurobiological factors implicated in PTSD include activation of the monoamine system, changes in the neuroendocrine system, and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis [8–10]. In other words, it is caused by traumatic stress-induced emotional fear memory and anxiety-like symptoms, arising through an imbalance among neurotransmitters, such as serotonin (5-HT), dopamine, and norepinephrine (NE), in the fear circuit regions (e.g., the medial prefrontal cortex (PFC), hippocampus (HIP), and amygdala (AMY)) [11]. Decreased 5-HT and increased NE were observed in the PFC and HIP of PTSD patients [9]. PTSD patients also maintain high concentrations of glucocorticoids (GCs) because of HPA axis, dysregulation and stress amplification caused by increased adrenocorticotropic hormone (ACTH), corticotropin-releasing hormone (CRH), and NE activity [12]. Human and animal studies have demonstrated increased circulating GC levels as a result of exposure to excessive stress, leading to overactivation of the AMY and impaired extinction of fear learning [13, 14].

Several studies have also reported that stress-induced elevation of corticosterone (CORT) increased the fear response by decreasing the expression of brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin-related kinase B (TrkB) [15, 16].

Selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs), which are currently used as therapeutic agents for PTSD, have limited effectiveness and cause various side effects, such as withdrawal, sedation, dependence, and cognitive dysfunction [17, 18]. Therefore, there is a need for new safe and effective drugs.

Myricetin (MYR; 3,3′,4′5,5′,7-hexahydroxylflavone) is a natural flavonoid found in berries, vegetables, teas, and the fruits of various plants [19]. MYR has various pharmacological effects, including antioxidant, antiapoptotic, antiphotoaging, anticancer, antidiabetic, and anti-inflammatory effects [20–24]. In particular, MYR showed neuroprotective effects in the HIP of stressed mice and reduced neuronal damage in animal models of neurodegenerative diseases (e.g., ischemic stroke, epilepsy, Parkinson's disease, and Alzheimer's disease) [25–27]. MYR attenuated brain damage in a rat model of cerebral ischemia by activating the nuclear factor (erythroid-derived 2)-like 2 pathway and reducing oxidative stress [28]. A growing body of evidence suggests the potential of MYR as a therapeutic option for preventing stress- and trauma-related psychiatric conditions, including PTSD.

In this study, a single prolonged stress (SPS) was used to induce PTSD-like symptoms in an animal model, through recall of fear extinction [29]. This model is based on the finding that people with multiple or early traumas are more likely to develop PTSD after a traumatic event [30].

We evaluated the effects of MYR on depression- and anxiety-like behaviors in rats exposed to SPS using the fear conditioning test (FCT), forced swim test (FST), and elevated plus maze (EPM) test. Moreover, we investigated regulation of the HPA axis and neurotransmitters and activation of the BDNF-extracellular signal-regulated kinase (ERK) signaling pathway, which leads to stress-induced behavioral alterations.

2. Methods

2.1. Animals and MYR Administration. As experimental animals, 6- to 7-week-old male Sprague-Dawley rats (200~220 g; Samtaco Animal Co., Seoul, Korea) were used. All animal care and experimental procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP(SE)-21-045).

The rats were divided into five groups: saline-treated normal control (SAL) group, SPS only group, MYR treatment groups, and positive control (PAX) group ($n = 8$/groups). For the experiment, the standard doses of MYR (10 and 20 mg/kg, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and paroxetine hydrochloride (15 mg/kg, PAX, positive control; Sigma-Aldrich) were determined based on a previous study [25]. MYR and PAX were dissolved in 0.9% saline before use and were injected intraperitoneally (i.p.) once daily for 14 days. The experimental schedule is shown in Figure 1.

2.2. SPS. In the SPS animal model, rats were immobilized for 2 hours, forced to swim for 20 min, and then rested for 15 min. After rest, rats were exposed to isoflurane (2–3%) until losing consciousness. For sensitization, rats were left alone in cages for 7 days [31].

2.3. Behavioral Test. To evaluate the fear response (FCT), rats were acclimatized to a fear conditioned box (30 cm × 30 cm × 30 cm). On day 1, rats were initiated the conditioning trials for 1 min in the box. When a rat entered the box, a conditional stimulus (sound alarm: 30 sec, 85 dB) was applied. For fear stimulation, during the last 2 sec of the conditional stimulus, a single electric footshock (0.5 mA, 2 sec) was applied (as an unconditional stimulus, forming the learning trial). On day 2, when the rats entered the box from a railing, no conditional stimuli or shocks were applied, forming the extinction training. After 24 hours, for extinction recall, the rats were re-exposed to the conditional stimulus alone in the same chamber without any electric footshock, and we observed the rat's fear response for 5 min. The fear response was the duration of the freezing response with no movement other than breathing.

To evaluate the depression-like behavior (FST), rats were forced to swim in a water bath (20 cm diameter × 50 cm height) filled to a depth of 30 cm with 25°C water for 5 min. Rats that did not swim and were floating were considered to be in a depressed state (immobility), as described previously [31].

To evaluate anxiety-like behavior using the apparatus detailed in a previous study (EPM test) [32], rats were placed in the center of a maze and tested for 5 min. The apparatus consisted of two open arms (50 cm × 10 cm) and two closed arms with dark walls (50 cm × 10 cm × 50 cm). The maze was 50 cm above the ground and the arms were connected by a platform (10 × 10 cm). The time spent in and number of visits to the two open and two closed arms were measured. Behavior in the maze was measured by the S-MART program (PanLab, Barcelona, Spain).

Also, in the sucrose preference test, intake of water and sucrose solution (1%, w/v) was measured for 3 h, as described previously [31].

2.4. Enzyme-Linked Immunosorbent Assay to Assess Neurotransmitter Levels. Plasma levels of CORT (Novus Biologicals, LLC., Littleton, CO, USA), ACTH (Abcam, Cambridge, UK), and CRH (Biocompare, South San Francisco, CA, USA) and concentrations of 5-HT (Abcam) and NE (Novus Biologicals) in the PFC, HIP, and AMY were measured by competitive enzyme-linked immunosorbent assay (ELISA), as described previously [31]. Each group ($n = 4$/groups) was anesthetized through inhalation of isoflurane (2%; Hanlim Pharm., Seoul, Korea) for 5 to 10 seconds in random order and was killed by sacrifice one day

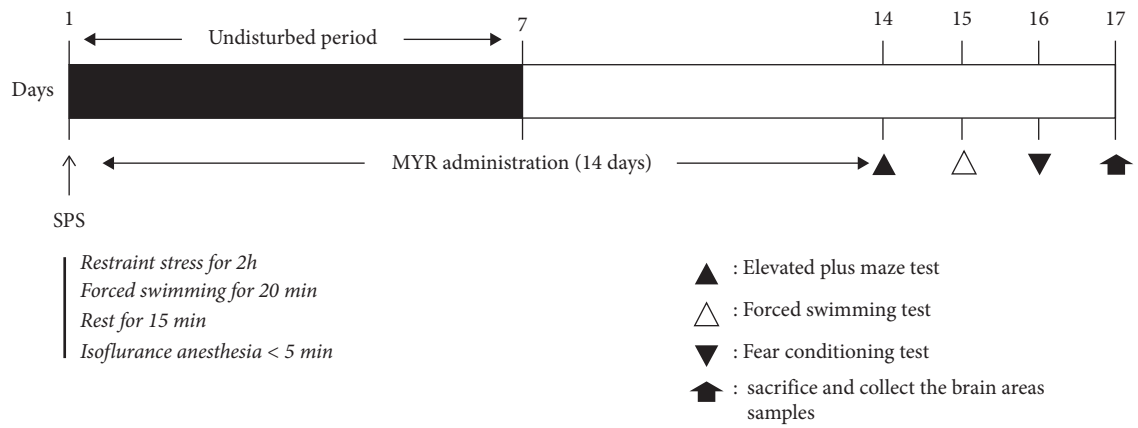

Figure 1: Experimental protocols for single prolonged stress (SPS)-induced anxiety-like behaviors and myricetin (MYR) treatment in rats. Different groups of rats ($n = 8$ per groups) were used for each experimental condition.

after the behavioral testing. Plasma was collected via the abdominal aorta ($n = 4$/groups), after which the PFC, HIP, and AMY were quickly dissected from the rat brains.

2.5. Immunohistochemistry. Immunohistochemistry was also performed to evaluate the number of BDNF-immunoreactive neurons in the HIP, as described previously [31]. The rats ($n = 4$/groups) were deeply anesthetized with sodium pentobarbital (80 mg/kg, by intraperitoneal injection) and perfused through the ascending aorta with normal saline (0.9%), followed by 300 ml (per rat) of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed, postfixed overnight, and cryoprotected with 20% sucrose in 0.1 M PBS at 4°C. Coronal sections of 30 μm thick were cut through the HIP using a cryostat (Leica CM1850; Leica Microsystems Ltd., Nussloch, Germany). The tissue sections were obtained according to the rat Atlas of Paxinos and Watson (HIP; between bregma −2.6 and −3.6) [33]. The tissue sections were incubated overnight with primary rabbit anti-BDNF antibody (1 : 200 dilution, Cell Signaling, Boston, MA, USA), and the sections were then incubated for 2 hours at room temperature with secondary antibodies (1 : 200 dilution; Vector Laboratories Co., Burlingame, CA, USA). Next, the sections were incubated with avidin-biotin-peroxidase complex (Vector Laboratories) for 1 hour at room temperature and then in a solution containing 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) and 0.03% hydrogen peroxide for 1 min. Images were captured using the AxioVision 3.0 imaging system (Carl Zeiss, Inc., Oberkochen, Germany) and processed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA). The sections were viewed at 200x magnification, and the numbers of cells within $100 \times 100\,\text{mm}^2$ grids were counted by observers blinded to the experimental groups. Hippocampal area cells were obtained according to the stereotactic Atlas of Paxinos and Watson [33]. The cells were counted in three sections per rat within the hippocampal area.

2.6. Total RNA Preparation and Reverse Transcription-Polymerase Chain Reaction. TRIzol reagent (Sigma-Aldrich) was added to the HIP isolated from the brain to recover the total RNA. After cDNA was synthesized from RNA by reverse transcription reaction, polymerase chain reaction (PCR) was performed as described previously [31]. The PCR products were separated on 1.2% agarose gel and stained with ethidium bromide after which the density of each band was evaluated using an image-analyzing system (i-Max™; CoreBio System Co., Seoul, Korea). Complementary DNA expression levels were determined by calculating the relative density of each BDNF and TrkB bands to GAPDH.

2.7. Western Blot Analysis. Total protein was extracted from the brain to detect ERK protein in the HIP. To extract total protein, brain tissue was homogenized with lysis buffer containing a phosphatase inhibitor and a protease inhibitor (CyQUANT; Invitrogen, Carlsbad, CA, USA). To separate proteins from the homogenized sample, the supernatant was collected by centrifugation (12,000 rpm, 15 min, 4°C). The protein concentration was measured using a colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (15–20 μg) were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 120 V and then electrotransferred to a nitrocellulose membrane (Schleicher and Schuell GmbH, Dassel, Germany). After incubation with a mouse p-ERK antibody (1 : 500; Cell Signaling, Danvers, MA, USA), the membrane was incubated with horseradish peroxidase conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA). A chemiluminescent kit (Super Signal West Pico; Pierce, Rockford, IL, USA) was used to detect the p-ERK protein. Protein content was analyzed using an enhanced chemiluminescence detection system (Santa Cruz), and the density was measured using the Tina 2.1 program.

2.8. Statistical Analysis. Data are presented as the mean ± standard error. To test for statistical differences between groups, all data were analyzed using SPSS software (version 23.0; SPSS, Inc., Chicago, IL, USA). Data sets were checked for normality and homogeneity of variances by Shapiro–Wilk and Brown–Forsythe tests, respectively. One-way

ANOVA followed by Tukey's *post hoc* test or Kruskal–Wallis test with Dunn's multiple comparison *post hoc* test was used to compare multiple comparisons such as the behavioral test, ELISA, PCR, western blot, and immunohistochemistry analysis. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Body Weight and Sucrose Intake according to SPS. Rats exposed to SPS showed a significant decrease in the change of body weight over time compared to the saline-treated normal (SAL) group ($t = 4.724$, $p < 0.01$). This change in body weight showed a significant difference from day 3 after exposure to SPS (Figure 2(a)).

Rats exposed to SPS showed a significant decrease in sucrose intake on days 7 and 14 compared to the SAL group ($t = 869$, $p < 0.01$; Figure 2(b)). The rats exhibited sufficient physiological changes and anhedonia due to traumatic stress following SPS exposure.

3.2. Effect of MYR in Behavioral Changes by SPS. In the FCT, the rats exposed to SPS showed a similar pattern of freezing responses to the fear stimulus (conditioning trials, C1–C3) compared to the SAL group ($t = 0.254$, $p = 0.808$; Figure 3(a)). During fear stimulus in both extinction (E1–E6) and extinction recall (R1; short-term recall), the rats exposed to SPS showed a significantly increased freezing response compared to the SAL group ($p < 0.01$ and $p < 0.001$, respectively). However, administration of 20 mg/kg MYR significantly decreased the freezing response during extinction recall compared to the SPS group ($p < 0.05$; Figure 3(b)), indicating that the freezing response of the MYR20 group was similar to that of the PAX group.

In the FST, the rats exposed to SPS had a significantly increased immobility time and decreased climbing time compared to the SAL group ($p < 0.01$; Figures 3(c) and 3(d)). Administration of 20 mg/kg MYR significantly decreased the immobility time ($p < 0.05$) and increased the climbing time ($p < 0.05$) compared to the SPS group. Similarly, rats in the PAX group decreased immobility time in the FST, but it was not statistically significant ($p = 0.078$, Figure 3(c)). Also, there was no difference in swimming time among all groups ($F_{4,34} = 0.245$, $p = 0.910$; Figure 3(e)).

In the EPM test, the percentage of time spent in and the number of entries into the open arms of rats exposed to SPS was significantly decreased compared to the SAL group ($p < 0.01$; Figures 3(f) and 3(h)). However, administration of 20 mg/kg MYR significantly increased the percentage of time spent in and the number of entries into the open arms compared to the SPS group ($p < 0.05$). Our results show that increase in the percentage of time spent in the open arms in the MYR20 group was comparable to the exploratory behavior. Similarly, the rats in the PAX group had significantly increased the percentage of time spent in the open arms of the maze ($p < 0.05$). Among all the groups, the time spent in and the number of entries into and in the closed arms did not differ ($F_{4,34} = 0.900$, $p = 0.476$ and $F_{4,34} = 2.477$, $p = 0.065$, respectively; Figures 3(g) and 3(i)). Administration of 20 mg/kg MYR significantly decreased the anxiety index compared to the SPS group ($p < 0.05$; Figure 3(j)), indicating that the anxiety-like behaviors of the MYR20 group were similar to those of the PAX group.

3.3. Effect of MYR in HPA Axis Activation and Neurotransmitters Changes by SPS. Rats exposed to SPS had significantly increased plasma CORT level compared to the SAL group ($p < 0.01$; Figure 4(a)). Compared to the SAL group, the rats exposed to SPS had significantly increased plasma ACTH level ($p < 0.01$; Figure 4(b)), but there was no significant difference in plasma CRH level ($p = 0.995$; Figure 4(c)). However, the administration of 20 mg/kg MYR significantly decreased the plasma CORT and ACTH levels compared to the SPS group ($p < 0.05$). Similarly, rats in the PAX group had significantly decreased the plasma CORT level compared to the SPS group ($p < 0.05$).

Rats exposed to SPS had significantly increased NE concentrations in the PFC, HIP, and AMY compared to the SAL group ($p < 0.05$ and $p < 0.01$, respectively; Figures 4(d)–4(f)). The administration of 20 mg/kg MYR significantly decreased the NE concentrations in the HIP and AMY compared to the SPS group ($p < 0.05$). Similarly, rats in the PAX group had significantly decreased the NE concentration in the AMY compared to the SPS group ($p < 0.05$).

Rats exposed to SPS had significantly decreased 5-HT concentrations in the PFC and HIP compared to the SAL group ($p < 0.05$ and $p < 0.01$, respectively; Figures 4(g) and 4(h)), but there was no significant difference in 5-HT concentration in the AMY ($p = 0.427$; Figure 4(i)). However, the administration of 20 mg/kg MYR significantly increased the 5-HT concentrations in the PFC and HIP compared to the SPS group ($p < 0.05$). Similarly, rats in the PAX group had significantly increased the 5-HT concentration in the HIP compared to the SPS group ($p < 0.05$).

3.4. Effect of MYR in Immunohistochemical Changes of BDNF by SPS. In the HIP, the number of BDNF-immunopositive neuronal cells in the SPS group was significantly decreased compared to those in the SAL group ($p < 0.05$; Figure 5). The BDNF-reactive neuronal activity in the HIP was significantly increased in the hippocampal region in the MYR20 group compared to those in the SPS group ($p < 0.05$).

3.5. Effect of MYR in BDNF and TrkB mRNA Expression by SPS. Compared to the SAL group, SPS exhibited significantly reduced BDNF mRNA expression in the HIP ($p < 0.01$; Figure 6(a)). However, the administration of 20 mg/kg MYR significantly increased the BDNF mRNA level in the HIP compared to the SPS group ($p < 0.05$; Figure 6(a)).

Compared to the SAL group, SPS exhibited significantly reduced TrkB mRNA expression in the HIP ($p < 0.05$). However, the administration of 20 mg/kg MYR increased the TrkB mRNA level in the HIP compared to the SPS group,

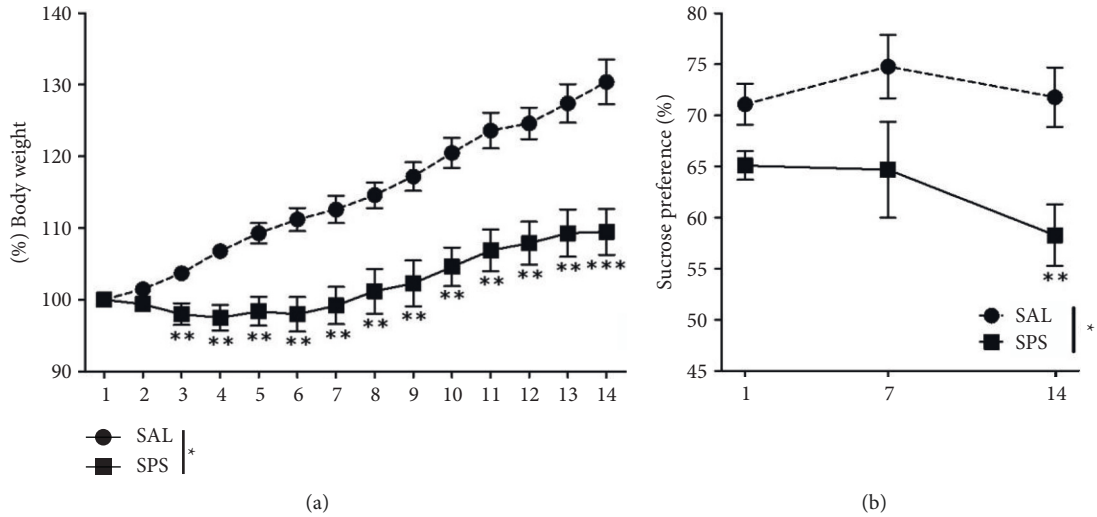

Figure 2: Results of body weight and sucrose intake analyses of rats subjected to 14 days of a SPS. Body weights and sucrose intake were significantly lower in SPS-exposed rats than in SAL-treated rats (significant main effect of SPS exposure *vs.* control handling). Data are shown as means ± standard errors of the mean. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* SAL group.

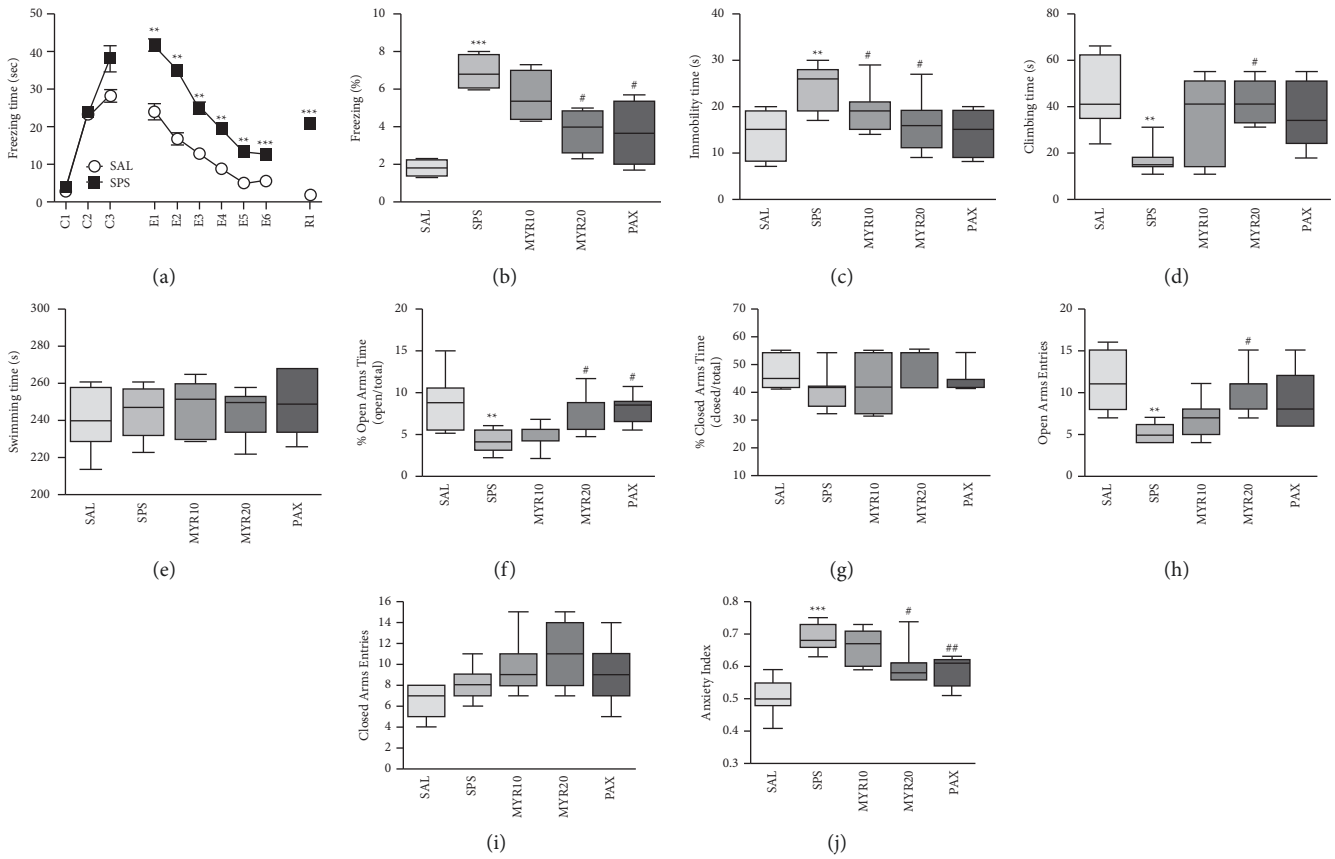

Figure 3: Effect of MYR on freezing behavior in response to conditioning (C1~C3), extinction (E1~E6), and short-term recall (R1) (a) and the percentage of time spent frozen during short-term recall (b) in the fear conditioning test, the immobility time (c), climbing behavior (d), and swimming time (e) in the forced swimming test (FST), the percentage of time spent in the open (f) and closed (g) arms, number of entries into the open (h) and closed (i) arms, and anxiety index (j) in the elevated plus maze (EPM) test after exposure to SPS in rats. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* SAL group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* SPS group.

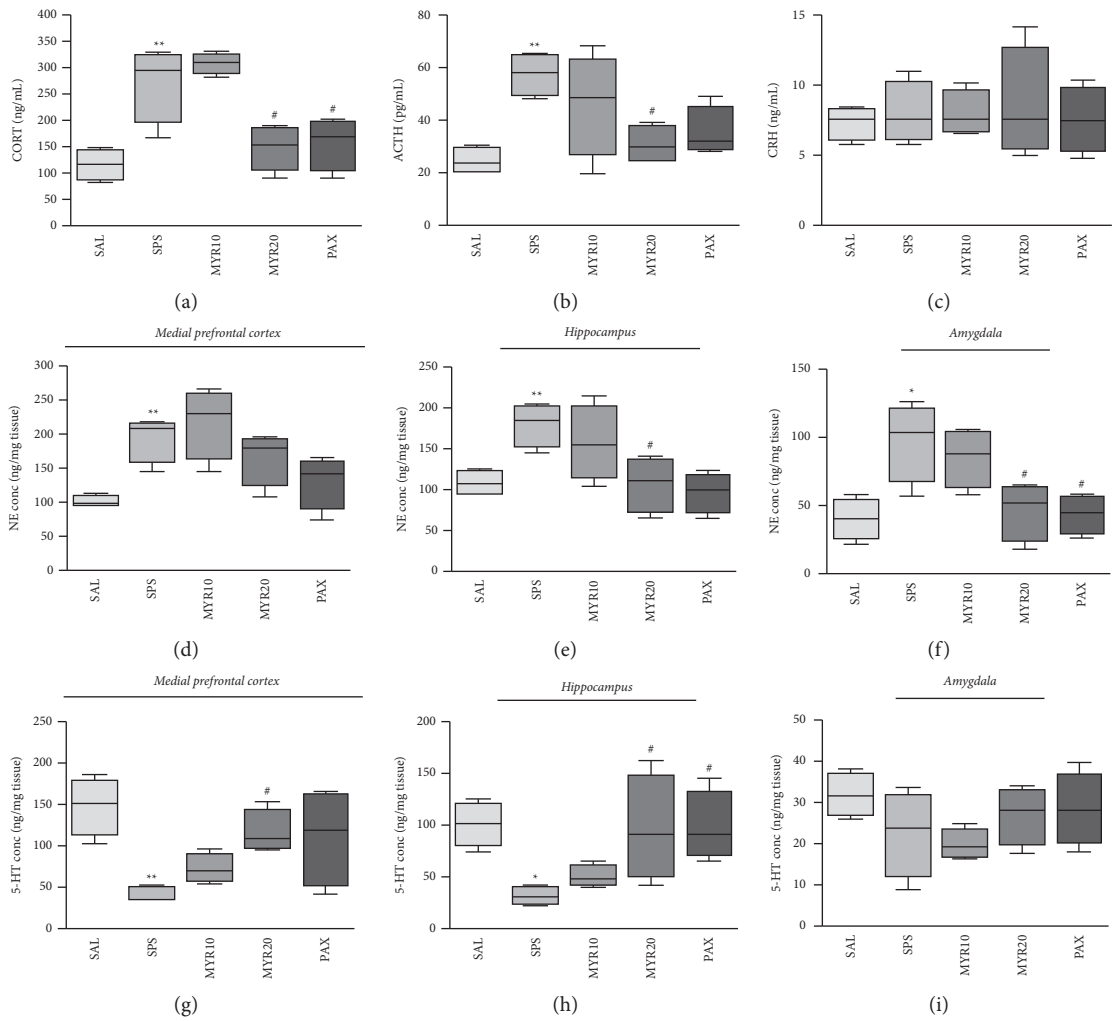

Figure 4: Effect of MYR on corticosterone (CORT (a)), adrenocorticotropic hormone (ACTH (b)), and corticotropin-releasing hormone corticosterone (CRH (c)) levels in the plasma and norepinephrine (NE) concentrations in the medial prefrontal cortex (d), hippocampus (e), and amygdala (f), and serotonin (5-HT) concentrations in the medial prefrontal cortex (g), hippocampus (h), and amygdala (i) of rats exposed to SPS for 14 days are shown. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* SAL group; $^{\#}p < 0.05$ *vs.* SPS group.

although this result was only marginally significant ($p = 0.196$).

3.6. Effect of MYR in p-ERK Activation by SPS. The effect of MYR on the protein expression level of p-ERK in hippocampal tissue were determined by Western blotting. The SPS group showed a significant decrease in p-ERK protein expression level in the HIP compared to the SAL group ($p < 0.01$; Figure 6(b)). However, the administration of 20 mg/kg MYR significantly increased the p-ERK protein expression level in the HIP compared to the SPS group ($p < 0.05$).

4. Discussion

In this study, the effects and possible mechanism of action of MYR on the fear response, depression, and anxiety-like behaviors induced by PTSD were evaluated using an SPS model. MYR administration after exposure to SPS significantly reduced the freezing response in the FCT, significantly increased the percentage of time spent in and the number of entries into the open arms of the EPM test, and significantly decreased immobility time in the FST. MYR administration also significantly reduced the increase in CORT and ACTH levels induced by HPA axis dysregulation and regulated the imbalance of NE and 5-HT concentrations in the fear circuit regions (PFC, HIP, and AMY). In addition, MYR treatment restored BDNF mRNA and p-ERK protein expression in the HIP. Thus, MYR relieved depression and anxiety through regulation of the HPA axis, neurotransmitters in the brain fear circuit regions, and the BDNF-ERK signaling pathway. These findings could lead to the development of a new therapeutic agent for PTSD.

SPS can induce cognitive dysfunction [34], an exaggerated startle response [35], heightened fear [36], and depressive and anxiety-like behaviors.

In this study, SPS exposure led to a decrease in the body weight and sucrose intake of rats compared to the controls. Increased anhedonia after SPS exposure in rats suggests that traumatic stress can induce depression [37]. Although body

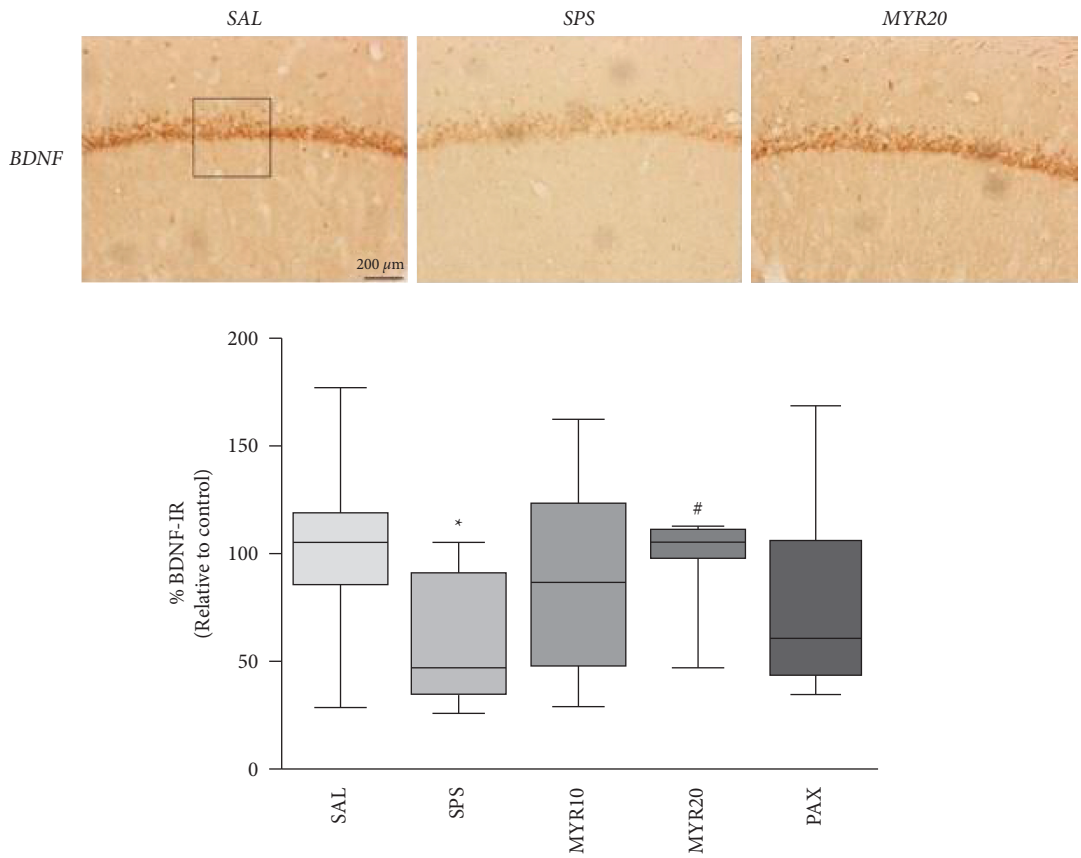

Figure 5: Effect of MYR on the mean number of brain-derived neurotrophic factor (BDNF)-stained hippocampal areas in rats with SPS-induced depression- and anxiety-like behaviors. Representative photographs and the relative percentage values are indicated. $^{*}p < 0.05$ *vs*. SAL group; $^{\#}p < 0.05$ *vs*. SPS group.

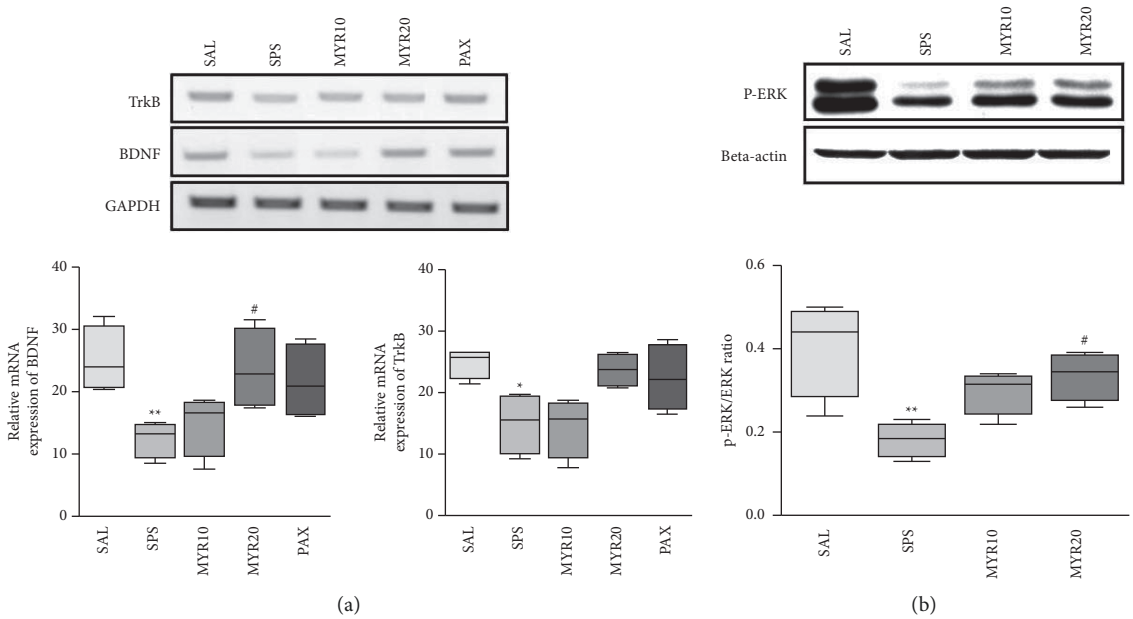

Figure 6: Effect of MYR on the expression levels of brain-derived neurotrophic factor (BDNF) and tropomyosin-related kinase B (TrkB) mRNAs in rats hippocampal with SPS-induced depression- and anxiety-like behaviors (a). The relative intensities of polymerase chain reaction amplification product bands on agarose gels are shown. The expression levels of BDNF and TrkB mRNAs were normalized to the expression level of glyceraldehyde 3-phosphate dehydrogenase mRNA as an internal control. Activation of phosphor-extracellular signal-regulated kinase (p-ERK) in the hippocampus after MYR treatment (b). Western blot analysis of protein expression levels of p-ERK. $^{*}p<0.05$ and $^{**}p<0.01$ vs. SAL group; $^{\#}p<0.05$ vs. SPS group.

weight loss is not a direct indicator of fear, depression, or anxiety, various physiological changes are associated with SPS exposure [37].

PTSD patients are known to develop a fear response due to impairment of the extinction of traumatic memory [38, 39]. This is consistent with the hypersensitivity and increased fear circuit activation seen in PTSD patients exposed to another potentially threatening stimulus [38, 39]. Several studies have demonstrated impaired fear extinction in the passive avoidance task in SPS-exposed rats, which also exhibited enhanced anxiety responses in the EPM test [40] and light/dark box task [34] due to the reinforcement of fear memory. In our study, in agreement with previous studies, rats exposed to single electric footshocks and conditional stimuli in the FCT had a significantly increased freezing response during fear extinction (E1~E6) and extinction recall (R1). The SPS-exposed rats did not learn to recognize a safe space or situation even when the threat was removed due to HPA-axis dysregulation and activation of the fear circuit regions (PFC and HIP) [41]. However, MYR administration alleviated the impaired fear extinction by reducing freezing behavior, followed by attenuation of fear during short-term extinction recall. Therefore, MYR administration may improve depression- and anxiety-like behaviors, as well as fear memory, by enhancing fear extinction.

Depression- and anxiety-like behaviors were comorbid symptoms in both human and animal studies of PTSD [38]. After SPS exposure, rats exhibited increased immobility time in the FST. A heightened state of fear and behavioral despair were observed following traumatic stress and forced swimming. In addition to the FST, exposure of rats to SPS in the EPM test significantly decreased the percentage of time spent in and the number of entries into the open arms. The decreased searching for the open arm reflected an increase in anxiety-like behavior due to an aversive experience, as is also seen when faced with another threatening situation [42]. These results suggested that increased depression- and anxiety-like behaviors were associated with changes in CORT and monoamine levels in various brain regions [43]. However, our results indicate that MYR administration can help restore the SPS-induced depression and anxiety-like behaviors.

Many studies have reported that the biochemical and endocrine abnormalities seen in PTSD patients are well reproduced in SPS animal models [36]. Long-term exposure to CORT has been also shown to produce neurotoxic effects in anxiety-related brain regions, such as the HIP and AMY [44]. In our study, SPS exposure increased plasma CORT and ACTH levels in rats, resulting in HPA axis dysfunction. PTSD-related HPA axis dysregulation implied impairment of important neuroendocrine systems regulating immune function, energy expenditure, emotion and mood, and stress [45]. Sustained CORT elevation may cause biochemical, metabolic, and structural changes in the HIP and AMY, leading to behavioral changes seen in PTSD, such as pathophysiological and anxiety-like behaviors [46]. However, MYR administration reduces plasma CORT and ACTH levels, spontaneous HPA axis excitability, and HPA axis dysregulation, thereby alleviating fear memory and depression- and anxiety-like behaviors.

The fear circuit of the brain is involved in fear extinction, which is an important feature of PTSD known to affect other systems related to fear extinction [47]. In PTSD patients, catecholamine imbalances occur due to disturbances in the adrenergic system in the fear circuit regions [48]. Increased NE is associated with avoidance and the re-experiencing of traumatic stress [49]. Our results also demonstrate that SPS-exposed rats had increased NE levels in the PFC, HIP, and AMY. However, MYR administration suppressed the SPS-related NE increase in HIP and AMY. This suggests that MYR administration could modulate the imbalance of catecholamines, which is a known pathophysiological cause of PTSD.

Many studies have shown that 5-HT regulates behavior and emotions and suppresses aggressive behavior caused by exposure to new environments [50]. The serotoninergic system plays an important role in anxiety-related behaviors [51]. Our results also demonstrate that 5-HT levels decreased in the fear circuit regions including PFC and HIP in SPS-exposed rats. However, MYR administration restored to normal 5-HT levels in the PFC and HIP. This suggests that MYR administration can modulate the 5-HT imbalance, which is a known pathophysiological mechanism of PTSD.

BDNF plays an important regulatory role in synaptic function and plasticity within specific circuitry of the HIP in the context of anxiety-like behaviors. Its expression was found to be decreased in the HIP of PTSD patients [34]. Therefore, we suggest that the functional disruption of the HIP induced by reduced BDNF expression may be closely related to anxiety-like symptoms [52]. MYR administration significantly reversed the SPS-induced reduction of BDNF mRNA expression, which suggests that its beneficial effects on depression- and anxiety-like behaviors were mediated by an increase in BDNF expression that may be associated with enhanced neuronal function.

BDNF reduction in animal models of PTSD augmented the response to traumatic stress and affected hippocampal function [53]. BDNF signaling is mediated by TrkB receptor activation, and phosphorylation of ERK protein could enhance contextual fear memory in PTSD [16, 54]. Therefore, modulation of the BDNF-ERK signaling pathway may increase fear-related freezing behavior and anxiety-like symptoms by enhancing hippocampal-dependent memory and promoting memory-related synaptic changes, as seen in long-term potentiation, for example [16, 54]. In our study, SPS-exposed rats exhibited decreased BDNF and TrkB mRNA expression and ERK phosphorylation in the HIP, but MYR administration reversed these reductions. The restoration of BDNF improved fear extinction and anxiety-like symptoms [54]. The antidepressant and anxiolytic effects of MYR may also be related to suppression of the ERK phosphorylation via modulation of BDNF levels. Our results show that MYR administration alleviated depression- and anxiety-like behaviors by regulating the BDNF-ERK signaling pathway.

MYR exerted antidepressant and anxiolytic effects in the FCT, FST, and EPM test, possibly by regulating the HPA axis and monoamines and activating the BDNF-ERK signaling pathway in our animal model of SPS. However, further studies are required to elucidate how MYR influences cell signaling pathways.

5. Conclusion

MYR is a potential therapeutic agent for fear memory, depression, and anxiety-like disorders caused by PTSD. MYR appeared to prevent monoamine imbalance through HPA axis regulation and activation of the BDNF-ERK signaling pathway.

Data Availability

All data supporting the conclusions of this article are included within the article. The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no potential conflicts of interests.

Acknowledgments

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Research Article

Rapid-Onset Antidepressant-Like Effect of *Nelumbinis semen* in Social Hierarchy Stress Model of Depression

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Depression is a disease with increasing prevalence worldwide, and it is necessary to develop a therapeutic agent with better efficacy than existing antidepressant drugs. Antidepressants that act on the glutamatergic nervous system, such as ketamine, have a rapid-onset antidepressant effect and are effective against treatment-resistant depression. However, because of the addictive potential of ketamine, alternative substances without psychological side effects are recommended. In particular, many natural compounds have been tested for their antidepressant effects. The antidepressant effects of *Nelumbinis semen* (NS) have been tested in many studies, along with the various actions of NS on the glutamatergic system. Thus, it was expected that NS might have a rapid-onset antidepressant effect. To test the antidepressant potential, despair and anhedonic behaviors were measured after administering NS to mice exposed to social hierarchy stress (SHS), and biochemical changes in the prefrontal cortex and hippocampus were analyzed. NS reduced despair-like responses in the forced swim test and tail suspension test. Mice exposed to SHS showed depression-like responses such as increased despair, reduced hedonia, and an anxiety-like response in the novelty suppressed feeding test. NS, but not fluoxetine, improved those depression-like behaviors after acute treatment, and NBQX, an AMPA receptor blocker, inhibited the antidepressant-like effects of NS. The antidepressant-like effect of NS was related to enhanced phosphorylation of mTOR in the prefrontal cortex and dephosphorylation of GluR1 S845 in the hippocampus. Since NS has shown antidepressant-like potential in a preclinical model, it may be considered as a candidate for the development of antidepressants in the future.

1. Introduction

Depression is one of the most prevalent health concerns, and unlike other diseases for which preventive treatment has been established, the number of patients is not expected to decrease soon [1]. This is because depression is caused by a combination of many physical, mental, and social factors [2]. Various methods were developed to treat depression; however, none of these methods could completely cure depression. Especially, conventional antidepressants are only effective in some patients, and clinical studies (STAR*D) have pointed out that they offer limited efficacy and a low rate of full remission [3, 4].

Interest in antidepressants that act on the glutamatergic system has been increasing since the discovery that a subanesthetic dose of ketamine has a rapid and lasting antidepressant effect [5, 6]. The antidepressant effect of ketamine and substances acting on the glutamatergic system was related in NMDA-to-AMPA throughput, mTOR activation, and inhibition of the reward stimulation circuit by the lateral habenula [7–9]. In addition, these drugs are effective in treatment-resistant depression, for which existing antidepressants do not work, so they are expected to replace existing antidepressants in the future [10].

Nelumbo nucifera Gaertner (NNG), a perennial aquatic herb, is common in Korea, Australia, China, India, Iran,

Japan, and regions of southeast Asia [11]. Almost all parts of NNG, i.e., leaves, flowers, seeds, roots, rhizomes, and embryo, are used as food garnishes and for various medicinal purposes. The seed of NNG, *Nelumbinis semen* (NS), was traditionally used as a sedative, antipyretic, and hemostatic to treat disorders such as insomnia, fevers, hypertension, arrhythmia, and postmenopausal depression [12–14]. It is also consumed as health-promoting foods that can treat ailments such as skin diseases and tissue inflammation and act as a poison antidote, astringent, emollient, and diuretic [15,16]. NS contains alkyl 4-hydroxybenzoates, bisbenzylisoquinoline alkaloids, benzylisoquinoline alkaloids, aporphine, and proaporphine alkaloids [17–21]. In previous studies, NS was shown to have antidepressant-like effects in rats; those effects were associated with local cholinergic and dopaminergic or noradrenergic neurotransmission in the hippocampus and prefrontal cortex [22, 23].

A recent publication has demonstrated that preparations containing NS have antidepressant effects via activation of the brain-derived neurotrophic factor through the Rac1-cofilin signaling pathway [24]. The stem part of NS exhibited antidepressant activity through NCAM and GAP-43 regulation [25]. Like the NMDA receptor antagonist ketamine, glutamatergic system changes are among the known effects of NS. It protected against glutamate-induced apoptosis in HT22 cells [26]. The learning and memory improvement effects of NS were associated with mTOR-induced neurogenesis enhancement in the hippocampal dentate gyrus [27]. Also, neferine, a component of NS, reduces hypoxic damage by activating the Akt/mTOR pathway [28]. On the other hand, in lung cancer cell lines, NS inhibited the PI3K/AkT/mTOR pathway [29]. As such, NS was expected to have a rapid antidepressant effect by being involved in the glutamatergic system and the mTOR pathway. Since NS exhibits different effects in each tissue, it is necessary to study whether NS would show a rapid-onset antidepressant effect.

Animal models to test the efficacy of antidepressant drugs are based on testing the construct validity of depressive symptoms such as behavioral despair and anhedonia and then determining whether the test drug improves those symptoms [30]. Among the depression models, rapid-onset antidepressant effects can be tested in models that do not respond to short-term but only to long-term administration of conventional antidepressants. In other words, if short-term administration of a drug improves depressive symptoms in those models, it can be said to have rapid antidepressant effects [8]. Appropriate depression models for those tests include learned helplessness, early life stress, social defeat stress, chronic unpredictable stress, and olfactory bulbectomy [31]. Also, in the novelty suppressed feeding test (NSFT), a decrease in feeding latency upon short-term administration of a test drug is considered to have an anti-anxiety effect, whereas a decrease in latency upon long-term administration is considered to have an antidepressant effect [32].

The model used in this study, the social hierarchy stress (SHS) model, forcibly puts animals that have a high rank in the social hierarchy into a low-rank state [33]. Subordinate males living in social hierarchies had significantly higher levels of plasma corticosterone than alpha males and pair-housed subordinate males [34]. Lowering an animal from a high rank to a low rank is a strong stressor that can induce depressive symptoms [35]. Rats maintaining a stable dominant status are more susceptible to the impact of status loss and showed more depressive behaviors, which were normalized by taking antidepressants for 3 weeks [36]. In addition, the socially dominant mouse is more vulnerable to chronic social defeat stress (CSDS) exposure than the subordinate mouse [37, 38]. The advantage of SHS as a depression model is that it requires less contact during the modeling process, and no direct physical harm is required, unlike the CSDS model [39].

Therefore, in this study, the SHS mice model was used to evaluate the antidepressant-like efficacy of NS on behavioral despair, anhedonia, and NSFT latency in comparison with the conventional antidepressant. We also measured whether the antidepressant effect of NS was inhibited by an AMPA receptor antagonist and measured the phosphorylation of mTOR and GluR1 in the prefrontal cortex and hippocampus.

2. Materials and Methods

2.1. Animals and Experimental Groups. Eight-week-old male C57Bl/6 mice were purchased from Daehan Bio-link (Chungbuk, Korea) ($n = 24$ for cohort 1, $n = 52$ for cohort 2, and $n = 24$ for cohort 3). The animals were housed 4 to 6 per cage in climate-controlled quarters ($24 \pm 1^{\circ}$C, $55 \pm 5\%$ relative humidity) maintained with a 12 h light : 12 h dark cycle. The animals were provided with standard rodent chow and water ad libitum. After a week of habituation, the mice were randomly divided into experimental groups. The mice in cohort 1 were assigned to the following groups: vehicle ($n = 6$): DW p.o.; 0.1 mg/kg ($n = 6$): NS 0.1 mg/kg p.o.; 1 mg/kg ($n = 6$): NS 1 mg/kg p.o.; and 10 mg/kg ($n = 6$): NS 10 mg/kg p.o. The mice in cohort 2 were assigned to the following groups: normal ($n = 4$): not exposed to SHS + DW p.o.; vehicle ($n = 4$): exposed to SHS + DW p.o.; NS ($n = 4$): exposed to SHS + NS 1 mg/kg p.o.; and fluoxetine ($n = 4$): exposed to SHS + fluoxetine 10 mg/kg i.p. The mice in cohort 3 were assigned to the following groups: vehicle ($n = 6$): DW p.o.; NS ($n = 6$): NS 1 mg/kg p.o.; NBQX ($n = 6$): NBQX 10 mg/kg i.p.; and NS + NBQX ($n = 6$): NS 1 mg/kg p.o. and NBQX 10 mg/kg i.p. The experimental procedures are schematically outlined in Figure 1. All animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The protocols were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUASP(GC)-19-040).

2.2. Preparation of NS Extract. Dried NS was purchased from Nong Lim Saengyak (Seoul, Korea). The NS (1.5 kg) were boiled at 121°C for 4 hours in eight times as much distilled water than NS by weight to produce an extract. After filtering the extract, the filtrate was decompressed to vapor in a rotary evaporator and freeze-dried. The yield of the extract was 36.7% (w/w).

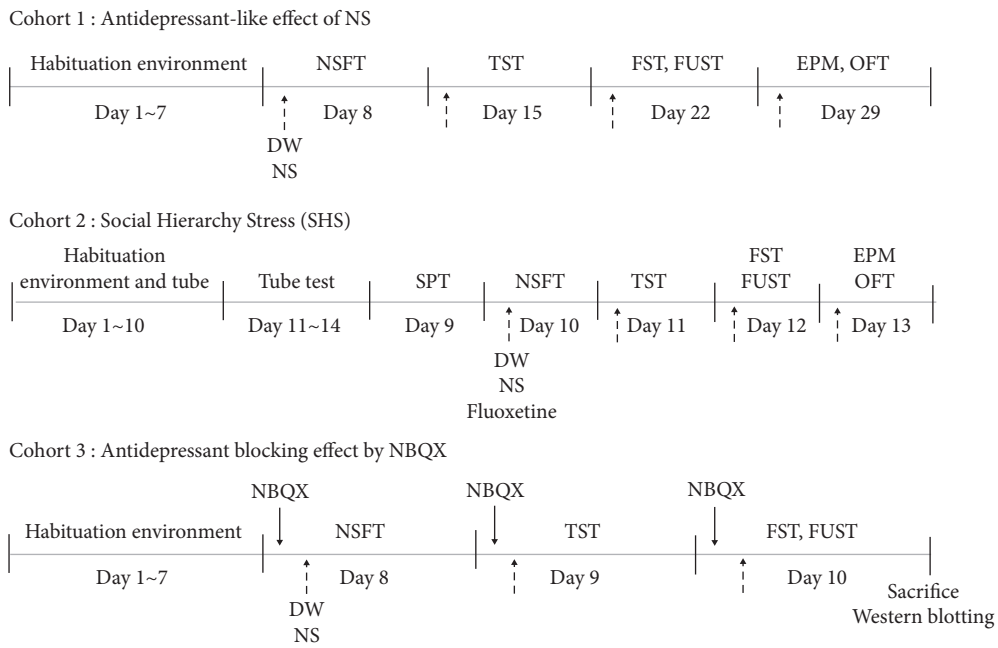

Figure 1: Schematic diagram of the experimental outline. NS (*Nelumbinis semen*), NSFT (novelty suppressed feeding test), TST (tail suspension test), FST (forced swim test), FUST (female urine sniffing test), EPM (elevated plus maze), OFT (open field test), SPT (sucrose preference test), and DW (distilled water).

2.3. Drug Administration and Treatment. The mice received a single oral administration of vehicle (DW) or NS extract (0.1, 1, and 10 mg/kg) dissolved in DW. Fluoxetine (10 mg/kg, Mallinckrdt Inc., St. Louis, MO, USA) was injected intraperitoneally 30 minutes before the test. NBQX (10 mg/kg, i.p., Tocris Bioscience, Bristol, UK) was given 30 minutes before administration of the NS extract.

2.4. Creating Model Animals (Social Hierarchy Stress Model). This model is based on the dominance hierarchy. It induces SHS by using social defeat; that is, the dominance of a higher status individual is destroyed by a lower status individual [33]. This experiment used transparent acrylic tubes (30 cm long, 3.0 cm outer diameter, and 2.4 cm inner diameter). On the first day, short transparent acrylic tubes (10 cm long, 3.0 cm outer diameter, and 2.4 cm inner diameter) were placed in the home cages of each group. On the second and third days, the experimental long transparent acrylic tubes were placed in the cages to allow the mice to become accustomed to passing through the tube at both ends. After that, the dominance hierarchy in each group of four mice was determined by allowing pairs of them to enter the tube from each end. In each pair, the one that backed out of the tube first was considered to be lower in the social hierarchy than the one that walked out forward. If dominance was not determined within 2 minutes, those mice were excluded from the test. The mouse that won the test four times in a row was considered to have the highest status. To induce SHS, the highest status mouse was put in the tube from one end while a lower status mouse was placed on the other end, and then both ends were closed. In this situation, the lower rank mouse, finding it impossible to escape, might instead attack the higher rank mouse. The defeated dominant mice were the SHS model animals used for the cohort 2 experiments. A sucrose preference test was conducted to see whether the social stress model was created, and then behavioral tests were implemented, as shown in Figure 1 (Cohort 2).

2.5. Behavioral Tests

2.5.1. Novelty Suppressed Feeding Test. The mice were fasted for 24 hours before the experiment started. For this test, a single food pellet was placed at the corner (60 cm × 60 cm) of a dimly lit (<20 lux) Plexiglas test chamber. The latency to feeding was measured for 5 minutes. If the mice had not eaten the food within 5 minutes, the feeding latency was considered to be 5 minutes. In this test, nonfeeding behaviors (e.g., touching and smelling) were not considered as eating.

2.5.2. Tail Suspension Test (TST). Each mouse was suspended 45 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. The movement of the mice was filmed for 6 minutes, and immobility was measured through a review of the video footage by a researcher blinded to the research condition. Immobility was defined as the absence of any limb or body movement with the exception being if mice climbed their own tails.

2.5.3. Forced Swimming Test (FST). A Plexiglas cylinder (35 cm high and 20 cm in diameter) was filled with water to a height of 20 cm (±22°C) and the movement of mice was filmed for 6 minutes. During the 6-minute test session,

immobility during the 4 minutes, from minutes two to six, was measured by a researcher blinded to the research condition. Immobility was defined as the minimal movement required for a mouse to keep its head above water.

2.5.4. Female Urine Sniffing Test (FUST). The mice were habituated to a sterile cotton-tipped applicator that was placed into their home cage for 1 hour. Then, each mouse was exposed to two cotton-tipped applicators, one dipped in water and the other dipped in the urine of female estrus, in a quiet, dimly lit room (<20 lux). This test lasted for 3 minutes, and the time the mice spent sniffing each cotton tip was measured.

2.5.5. Elevated Plus Maze (EPM). This apparatus consisted of a cross-shaped maze with two oppositely positioned arms, one open and one closed, with a 5 cm × 5 cm center. The device was a white opaque Plexiglas maze 40 cm above the ground; each arm was 30 cm × 5 cm × 30 cm. The mice were placed in the center of the maze with their heads facing the open arm. Their movement was filmed for 5 minutes, and the time they spent in the open arm was measured using video-tracking software (Smart 3.0, Panlab, Spain).

2.5.6. Open Field Test (OFT). The OFT was conducted in dimly illuminated conditions in a white opaque Plexiglas chamber (50 × 50 × 40 cm). The mice were gently placed inside the chamber, and their movements were filmed for an hour and analyzed using video-tracking software (Smart 3.0, Panlab, Spain). The chamber device inside was cleaned with 70% ethanol after each experiment.

2.5.7. Sucrose Preference Test (SPT). The SPT was based on the sucrose preference ratio of rodents, with reference to the study by Liu et al. [40]. Mice were exposed to two identical bottles overnight from 19:00 to 10:00 the next morning: one bottle contained water and the other contained a 1% sucrose solution. Both bottles were weighed before and after this period, and the sucrose preference was calculated as the ratio of the volume of sucrose to the total volume of sucrose and water consumed.

2.6. Western Blotting. Animals were deeply anesthetized and then rapidly sacrificed. Their dissected prefrontal cortex and hippocampus tissues were stored at −80°C until use. Tissues were homogenized with lysis buffer containing 1% each of phosphatase inhibitor cocktails 2 and 3 (P5726, P044, Sigma-Aldrich) in PRO-PREP™ (17081, iNtRON Biotechnology, Seongnam, South Korea) and centrifuged at 13,000 rpm for 10 minutes at 4°C. Protein concentrations were measured using DC™ Protein Assay Reagents A, B, and S (5000113, 5000114, and 5000115, BIO-RAD, Hercules, CA, USA). After electrophoresis using 10% SDS-polyacrylamide gel, the gel phase proteins were transferred to a nitrocellulose membrane (162-0115, BIO-RAD). Membranes were incubated for 1 hour at room temperature in 5% nonfat dry milk (170-6404, BIO-RAD) dissolved in TBS-T (0.1% tween 20 in TBS) for blocking. Primary antibodies were diluted in 5% nonfat dry milk in TBS-T and incubated overnight at 4°C. The next day, the membranes were washed thrice for 10 minutes with TBS-T. Secondary antibodies were diluted with 5% nonfat dry milk in TBS-T and incubated for 1 hour at room temperature. After washing thrice for 10 minutes with TBS-T, luminescence was induced using a Pierce™ ECL Western Blotting Substrate Kit (32106, Thermo Fisher Scientific, Waltham, MA, USA), and the samples were photographed with an EZ-Capture MG (ATTO, Tokyo, Japan). Band density was analyzed using ImageJ software (NIH, Bethesda, MD, USA). The antibodies used in this experiment were mTOR (#2972, 1:1000, Cell Signaling), p-mTOR (Ser2448, #2971, 1:1000, Cell Signaling), GluR1 (AP08676, 1:2500, Tocris), p-GluR1 (Ser845, #8084, 1:1000, Cell Signaling), beta-actin (sc-47778, 1:5000, Santa Cruz), anti-rabbit IgG (H + L) HRP conjugate (W4011, 1:1000, Promega, Madison, WI, USA), and anti-mouse IgG (H + L) HRP conjugate (W4021, 1:5000, Promega).

2.7. Statistical Analysis. The results are expressed as the mean ± SEM. Statistical comparisons were performed using unpaired T-testing (SPT) or one-way ANOVA (TST, FST, OFT, EPM, FUST, and NSFT) followed by LSD or Tukey's HSD post hoc multiple comparisons testing using SPSS 22 (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

3. Results

3.1. Antidepressant-Like Effect of NS in Normal Mice (Cohort 1). Behavioral measurements were performed in normal mice to test the antidepressant effect of NS (cohort 1). After acclimatizing to the environment for 7 days, each mouse was given one of three different doses of NS (0.1 mg/kg, 1 mg/kg, or 10 mg/kg), and the effects were measured using the NSFT, TST, FST, FUST, EPM, and OFT, as described in the methods and depicted in Figure 1.

In the NSFT, there were no differences among the experimental groups in the latency to feed (Figure 2(a)). In the TST, immobility was reduced by NS 0.1 mg/kg and 1 mg/kg compared with the control (Figure 2(b)), but the immobility reduction in the NS 10 mg/kg group was insignificant ($p = 0.228$). In the FST, immobility was reduced in all the NS-treated groups (Figure 2(c)). Among the NS doses, 1 mg/kg was the most effective. In the FUST, EPM, and OFT, no NS dose changed the behavioral responses (Figures 2(d)~2(f)) except that the 10 mg/kg group had higher locomotor activity than the 0.1 mg/kg group in the OFT ($p = 0.007$). These results indicate that NS might have antidepressant-like (but not anxiolytic) and activity-stimulating effects. Presenting better performance, 1 mg/kg of NS was used for further experiments.

3.2. Antidepressant Effect of NS in the Social Hierarchy Stress Model (Cohort 2). Behavioral measurements were made for SHS depression model mice to test the antidepressant effect of NS (cohort 2). SHS was performed as described in the methods, and the effects of NS 1 mg/kg were compared with

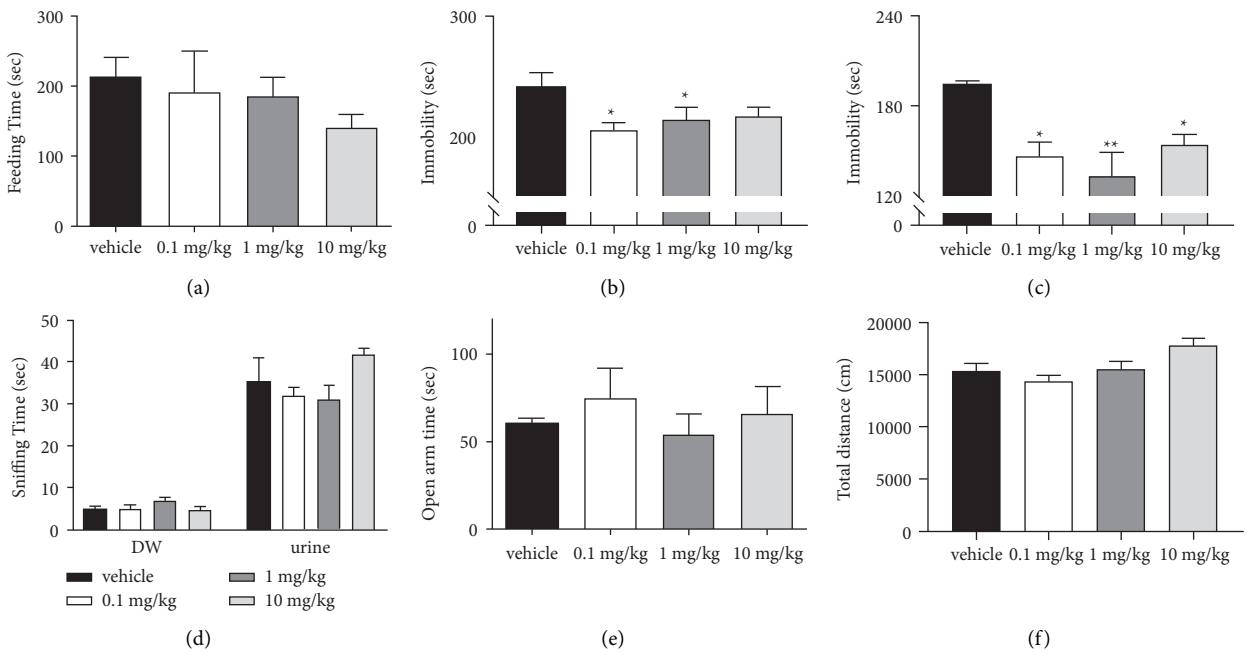

Figure 2: Antidepressant-like effects of *Nelumbinis semen* (NS). Tests were performed 30 minutes after NS administration (0.1, 1, and 10 mg/kg p.o.). (a) Novelty suppressed feeding test: the latency to feed did not differ among the groups ($F(3,20) = 1.718$, $p = 0.195$). (b) Tail suspension test: immobility time changed significantly ($F(3,20) = 3.685$, $p = 0.029$). Immobility was reduced in the 0.1 mg/kg ($p = 0.038$) and 1 mg/kg ($p = 0.047$) groups relative to the vehicle group. (c) Forced swim test: immobility time changed significantly ($F(3,20) = 7.229$, $p = 0.002$). Immobility was reduced in the 0.1 mg/kg ($p = 0.036$), 1 mg/kg ($p = 0.004$), and 10 mg/kg ($p = 0.039$) groups relative to the vehicle group. (d) Female urine sniffing test: the time spent sniffing DW ($F(3,20) = 1.988$, $p = 0.148$) and estrus female urine (F=$(3,20) = 2.042$, $p = 0.140$) did not differ among the experimental groups. (e) Elevated plus maze: time spent in the open arm was not significantly changed ($F(3,20) = 0.688$, $p = 0.57$). (f) Open field test: distance moved in the open field differed among the groups ($F(3,20) = 4.976$, $p = 0.01$), but there were no significant changes compared with the vehicle group. Data are shown as the mean ± SEM ($n = 6$/group). Vehicle: distilled water p.o.; 0.1 mg/kg: NS 0.1 mg/kg p.o.; 1 mg/kg: NS 1 mg/kg p.o.; and 10 mg/kg: NS 10 mg/kg p.o.; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. vehicle. ANOVA, Tukey's HSD post hoc test.

the effects of fluoxetine 10 mg/kg using the SPT, NSFT, TST, FST, FUST, EPM, and OFT, as depicted in Figure 1.

In the SPT, the sucrose preference in the SHS-exposed animals decreased, indicating that the SHS established a depression-like state in those mice (Figure 3(a)). In further experiments, DW (in the normal and vehicle groups) and NS 1 mg/kg were administered orally, and fluoxetine 10 mg/kg was injected intraperitoneally 30 minutes before each test. In the TST, immobility increased following SHS (normal vs. vehicle-treated SHS), and NS but not fluoxetine reduced the immobility of mice exposed to SHS (Figure 3(b)). In the FST, immobility also increased following SHS (normal vs. vehicle-treated SHS), and NS but not fluoxetine reduced that immobility (Figure 3(c)). In the FUST, the time spent smelling distilled water did not differ between the groups, but NS increased the time the SHS-exposed mice spent smelling estrus female urine (Figure 3(d)).

In the EPM, the anxiety-related behaviors did not change (Figure 4(a)). Likewise in the OFT, the locomotor activity did not differ among the experimental groups (Figure 4(b)). But in the NSFT, the latency to eat a pellet, which is an anxiety-related response, was prolonged in the SHS-exposed mice and reduced by NS (Figure 4(c)). No anxiety-related changes were detected in the EPM, which suggests an antidepressant effect.

3.3. AMPA Receptor Dependency of the Antidepressant-Like Effects of NS (Cohort 3). Behavioral measurements were performed in normal mice to evaluate the antidepressant effects of NS in relation to the AMPA receptor (cohort 3). After acclimatizing to the environment for 7 days, each mouse was given NS 1 mg/kg with or without NBQX (AMPA antagonist), and the effects were measured using the NSFT, TST, FST, and FUST, as depicted in Figure 1.

In the NSFT, feeding latency was shortened by NS. Although NBQX did not affect the feeding latency in untreated mice, it blocked the latency shortening effect of NS (Figure 5(a)). In the TST and FST, NBQX blocked the immobility-reducing effects of NS (Figures 5(b) and 5(c)). Also, NBQX blocked the NS-induced increase in the hedonic-like response to female urine (Figure 5(d)).

3.4. Involvement of mTOR and GluR1 Signaling in the Antidepressant-Like Effect of NS. Because NS demonstrated the possibility of acute-onset antidepressant-like properties, mTOR and GluR1, which are molecular signals related to acute-onset antidepressant effects, were measured in the prefrontal cortex and hippocampus tissues extracted from the cohort 3 mice.

In the prefrontal cortex, phospho-mTOR was increased by NS, and that increase was blocked by NBQX cotreatment

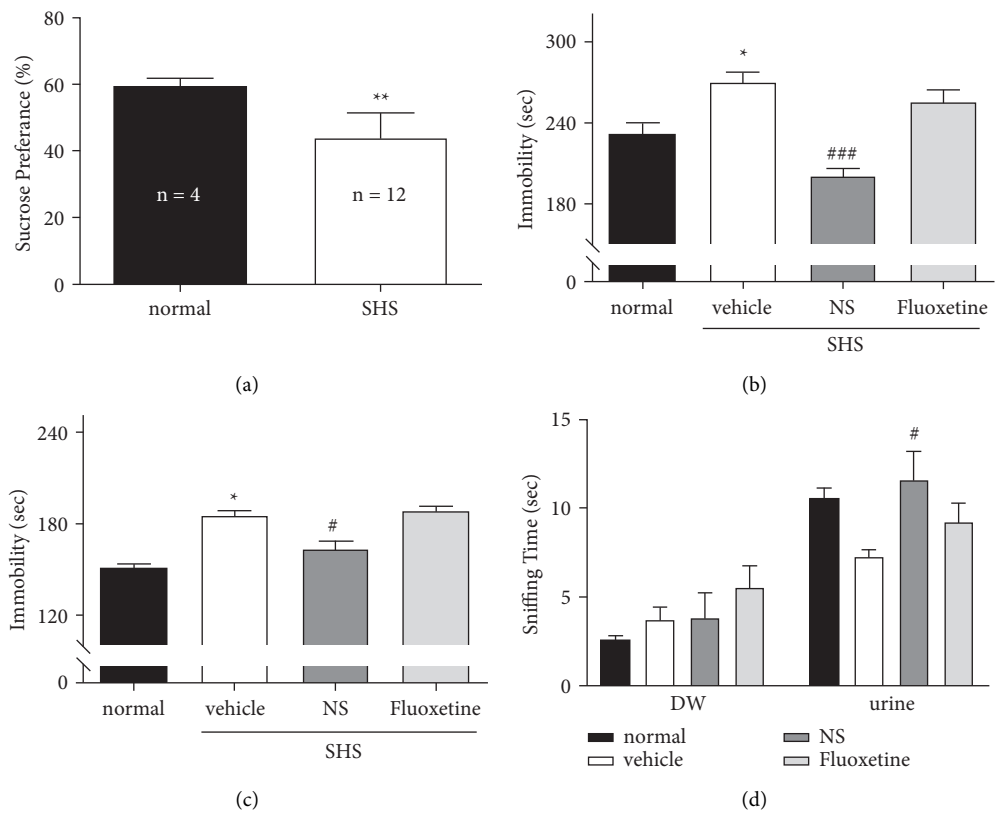

Figure 3: Antidepressant-like effect of *N. semen* (NS) measured in social hierarchy stress (SHS) depression model mice. Tests were performed 30 minutes after NS (1 mg/kg p.o.) or fluoxetine (10 mg/kg i.p.) administration. (a) Sucrose preference test: sucrose preference (%) was reduced in SHS-exposed mice compared with unexposed normal mice ($p = 0.001$). (b) Tail suspension test: immobility time differed significantly among groups ($F(3,12) = 17.264$, $p < 0.001$). The immobility time in the SHS animals increased (normal vs. vehicle, $p = 0.013$). NS reduced the immobility in the SHS model animals (vehicle vs. NS, $p < 0.001$), but fluoxetine did not (vehicle vs. fluoxetine, $p = 0.507$). (c) Forced swim test: immobility time differed significantly among groups ($F(3,12) = 26.584$, $p < 0.001$). Following SHS exposure, immobility increased compared with normal mice (normal vs. vehicle, $p < 0.001$). NS reduced the immobility in SHS model animals (vehicle vs. NS, $p = 0.004$), but fluoxetine did not (vehicle vs. fluoxetine, $p = 0.926$). (d) Female urine sniffing test: the time spent sniffing distilled water did not differ among the groups ($F(3,12) = 1.53$, $p = 0.257$), but the time spent sniffing the urine differed significantly ($F(3,12) = 3.534$, $p = 0.048$). The vehicle group spent less time sniffing the urine, but the difference was not significant (normal vs. vehicle, $p = 0.143$). Sniffing time was increased by NS in the SHS-exposed mice ($p = 0.041$). Data are shown as the mean ± SEM ($n = 4$). Normal: not exposed to SHS; SHS: exposed to social hierarchy stress; vehicle: SHS + distilled water p.o.; NS: SHS + NS 1 mg/kg p.o.; fluoxetine: SHS + fluoxetine 10 mg/kg i.p.; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. normal; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. vehicle. Student's t-test for (a), and ANOVA and Tukey's HSD post hoc test for (b), (c), and (d).

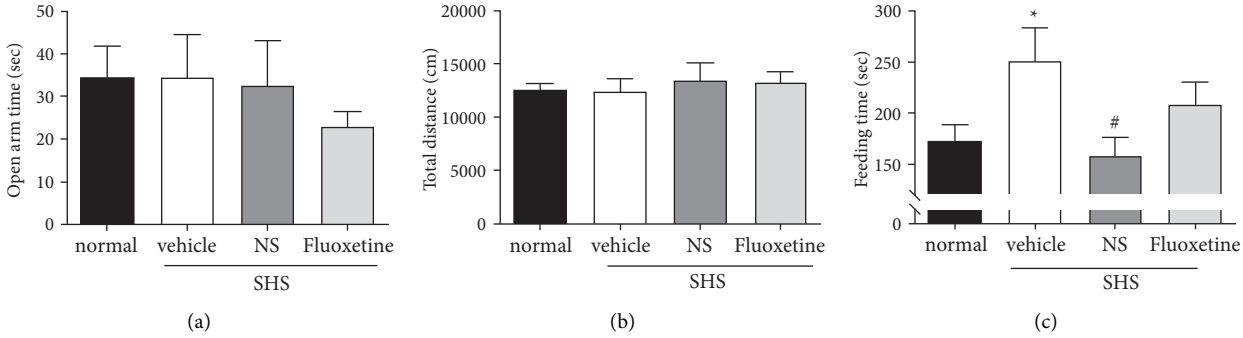

Figure 4: Acute-onset antidepressant-like effects of *Nelumbinis semen* (NS) measured using the social hierarchy stress (SHS) depression model. Tests were performed 30 minutes after NS (1 mg/kg p.o.) or fluoxetine (10 mg/kg i.p.) administration. (a) Elevated plus maze: the time spent in the open arm did not differ significantly among the experimental groups ($F(3,12) = 0.598$, $p = 0.625$). The reduced open-arm time in the fluoxetine group was insignificant (vehicle vs. fluoxetine, $p = 0.675$). (b) Open field test: the travel distance did not differ among the groups ($F(3,12) = 0.262$, $p = 0.852$). (c) Novelty suppressed feeding test (NSFT): the latency to take a food pellet differed significantly among the groups ($F(3,12) = 4.999$, $p = 0.018$). The latency increased in the SHS animals (normal vs. vehicle, $p = 0.046$). NS reduced the latency in the SHS animals (vehicle vs. NS, $p = 0.019$), but fluoxetine did not (vehicle vs. fluoxetine, $p = 0.081$). Data are shown as the mean ± SEM ($n = 4$). $^{*}p < 0.05$ vs. normal, $^{\#}p < 0.05$ vs. vehicle. ANOVA, Tukey's HSD post hoc test.

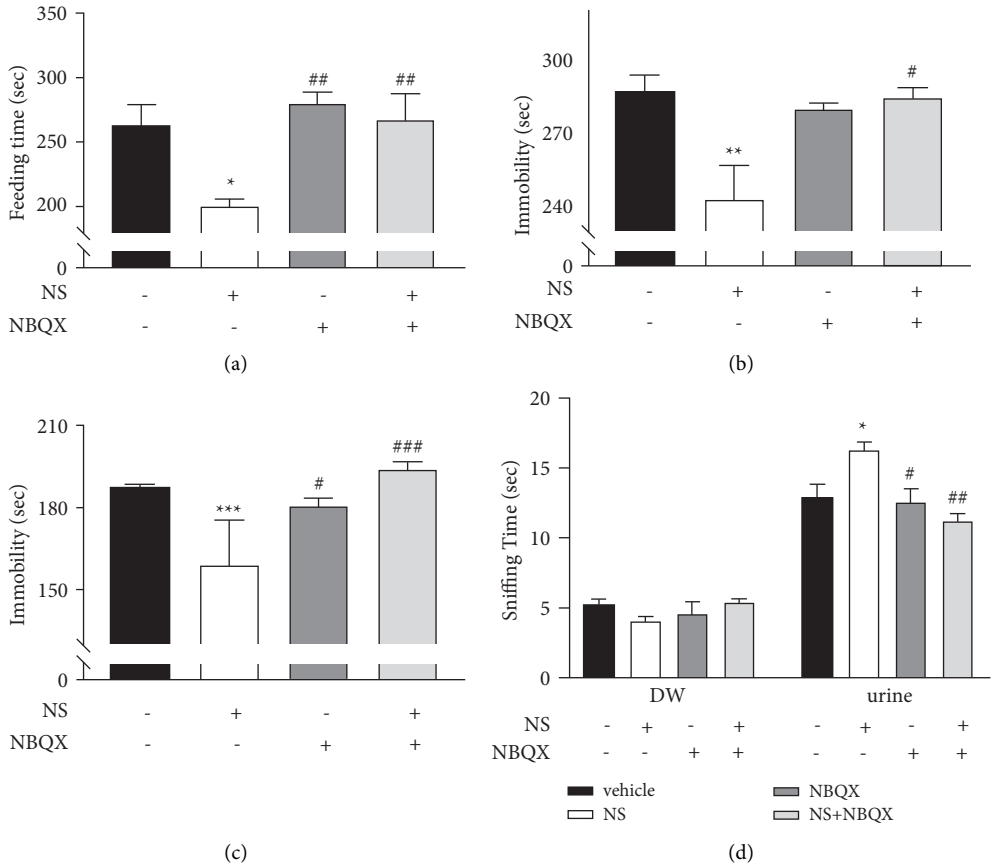

Figure 5: The role of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor in the antidepressant-like effect of *N. semen* (NS). Behavioral tests were performed 40 minutes after NBQX injection (10 mg/kg, i.p.) and 30 minutes after NS administration (1 mg/kg p.o.). (a) Novelty suppressed feeding test: there was no interaction between NS and NBQX ($F(1,20) = 3.882$, $p = 0.063$), but there was a main effect of NS ($F(1,20) = 8.613$, $p = 0.008$) and NBQX ($F(1,20) = 11.201$, $p = 0.003$). Latency to take a food pellet was reduced by NS ($p = 0.012$), and that reduction was blocked by NBQX ($p = 0.006$). (b) Tail suspension test: there was a group difference ($F(3,20) = 5.894$, $p < 0.005$) and an NS*NBQX interaction ($F(1,20) = 13.828$, $p = 0.001$). Immobility time was reduced in the NS group ($p = 0.007$ vs. vehicle), and the reduction in immobility caused by NS was blocked by NBQX ($p = 0.011$). (c) Forced swim test: NBQX had a significant main effect ($F(1,20) = 13.503$, $p = 0.002$) and NS*NBQX interaction ($F(1,20) = 30.266$, $p < 0.001$). The immobility was reduced by NS ($p < 0.001$), and that effect was blocked by NBQX ($p < 0.001$). (d) Female urine sniffing test: the time spent sniffing distilled water did not differ between the groups, but the time spent sniffing estrus female urine increased significantly in the NS group relative to the vehicle group ($p = 0.012$), and that antidepressant-like effect was reduced by NBQX ($p = 0.001$). There was also an NS*NBQX interaction in the time spent sniffing urine ($F(1,20) = 13.828$, $p = 0.001$). Data are shown as the mean ± SEM ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. NS. Two-way ANOVA, Tukey's HSD post hoc test.

(Figures 6(a) and 6(b)). However, the differences in total mTOR expression among groups were nonsignificant. In the hippocampus, no changes were detectable in the total or phospho forms of mTOR expression (Figures 6(d) and 6(e)).

In the prefrontal cortex, the total and phosphor-GluR1 expression was unaltered (Figures 7(a) and 7(b)). In the hippocampus, the total expression of GluR1 was not altered by NS or NBQX (Figure 7(d)), but the amount of phosphor-GluR1 in the hippocampus decreased after NS treatment (Figure 7(e)).

4. Discussion

To examine the antidepressant-like and rapid-onset antidepressant-like actions of NS, we conducted behavioral tests and biochemical analyses in normal and depression-model mice. We found that NS reduced the behavioral despair of normal mice in the range of 0.1 to 10 mg/kg, reduced behavioral despair and increased hedonic behavior in SHS-exposed mice, and showed rapid-onset antidepressant-like effects in the NSFT. Blockade of the AMPA receptor inhibited the antidepressant-like activity of NS. Also, NS increased the phosphorylation of mTOR in the prefrontal lobe and reduced the phosphorylation of GluR1-845 in the hippocampus.

NS has been widely used in oriental traditional medicine as a remedy for insomnia, anxiety, and depression [14]. Many preclinical studies have demonstrated its antidepressant-like effects since the discovery of serotonergic receptor ligands in this herbal medicine [41]. In nonstressed rats, three intraperitoneal injections of NS given at 400 mg/kg exerted antidepressant-like effects in the FST, but the effects of other doses (100, 200, and 1000 mg/kg) were

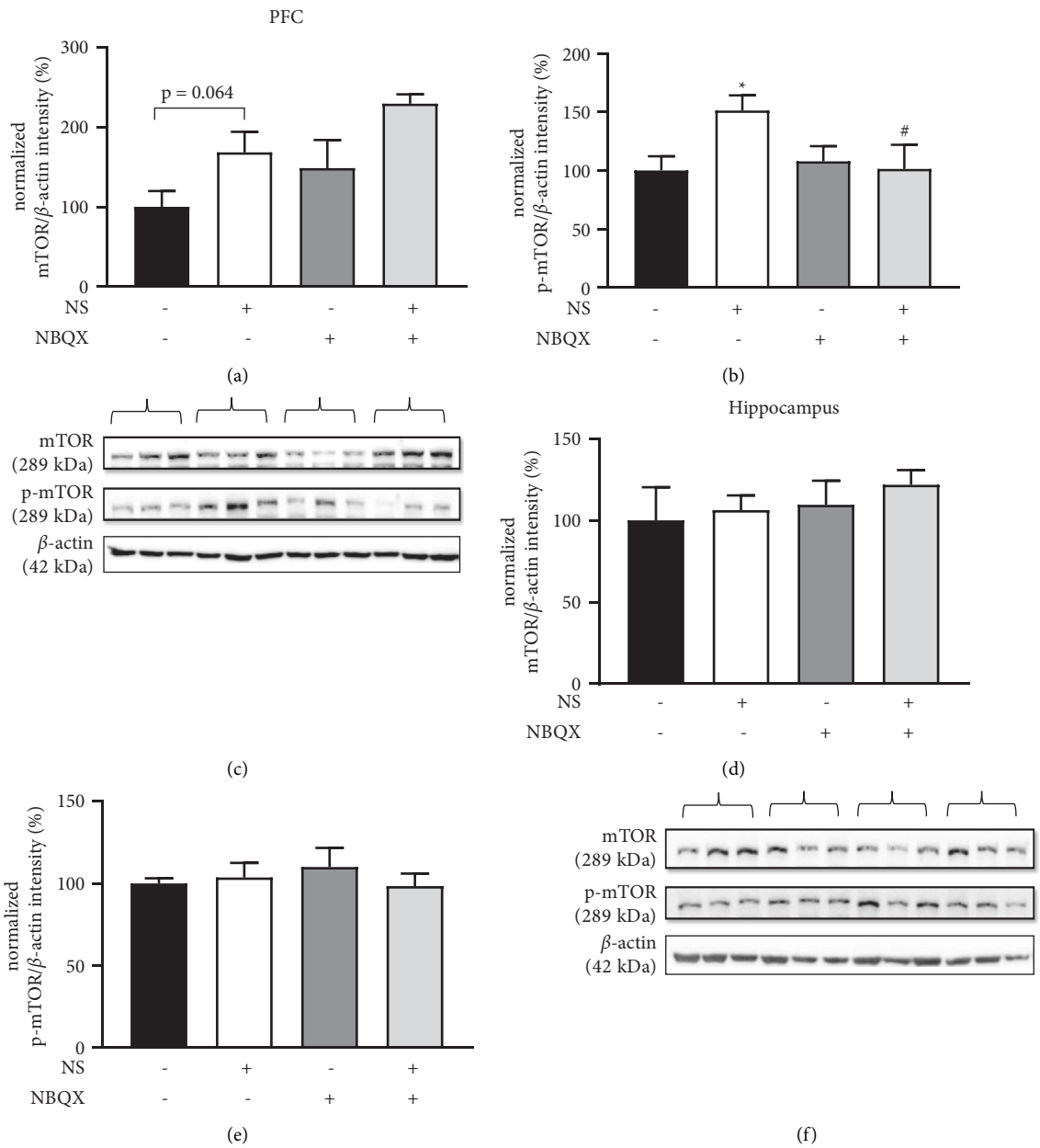

Figure 6: Effects of *Nelumbinis semen* (NS) and AMPA blockade on mTOR expression in the prefrontal cortex (PFC) and hippocampus. (a) Normalized mTOR/β-actin of PFC: there was a significant change among groups ($F(3,20) = 4.723$, $p = 0.012$), but mTOR levels were nonsignificantly increased by NS ($p = 0.064$), and NBQX did not affect the expression of mTOR in the NS-treated animals ($p = 0.095 p = 0.095$). (b) Normalized phospho-mTOR/β-actin of PFC: there was a significant change among groups ($F(3,20) = 2.613$, $p = 0.008$). p-mTOR expression increased with NS treatment compared with vehicle ($p = 0.026$), and NBQX decreased the p-mTOR expression in NS-treated animals ($p = 0.03$). (c) Representative blot image of total and phospho-mTOR expression in the PFC. (d) Normalized mTOR/β-actin of the hippocampus: there was no significant change among groups ($F(3,20) = 0.428$, $p = 0.735$). (e) Normalized phospho-mTOR/β-actin of the hippocampus. There was no significant change among groups ($F(3,20) = 0.383$, $p = 0.767$). (f) Representative blot image of total and phosphor-mTOR expression in the hippocampus. The parentheses in (c) and (f) correspond to the treatments indicated above them. Data are shown as the mean ± SEM ($n = 6$). $^{*}p < 0.05$ vs. vehicle, $^{\#}p < 0.05$ vs. NS. ANOVA, LSD post hoc test.

insignificant [14]. Another study demonstrated that among the different solvents used to prepare NS extracts, the n-butyl alcohol extract showed the strongest inhibitory effect on immobility in the FST in normal rats [42]. It was suggested that NS might exert an antidepressant effect by enhancing serotonin [43].

Unlike previous studies, we tested the antidepressant effect of NS in a dose range of 0.1 to 10 mg/kg because the dose of ketamine that produces rapid-onset antidepressant effects is 10 mg/kg, which is much lower than the dose that causes anesthetic effects. Similarly, *Radix polygalae*, which also produces rapid-onset antidepressant-like effects, is

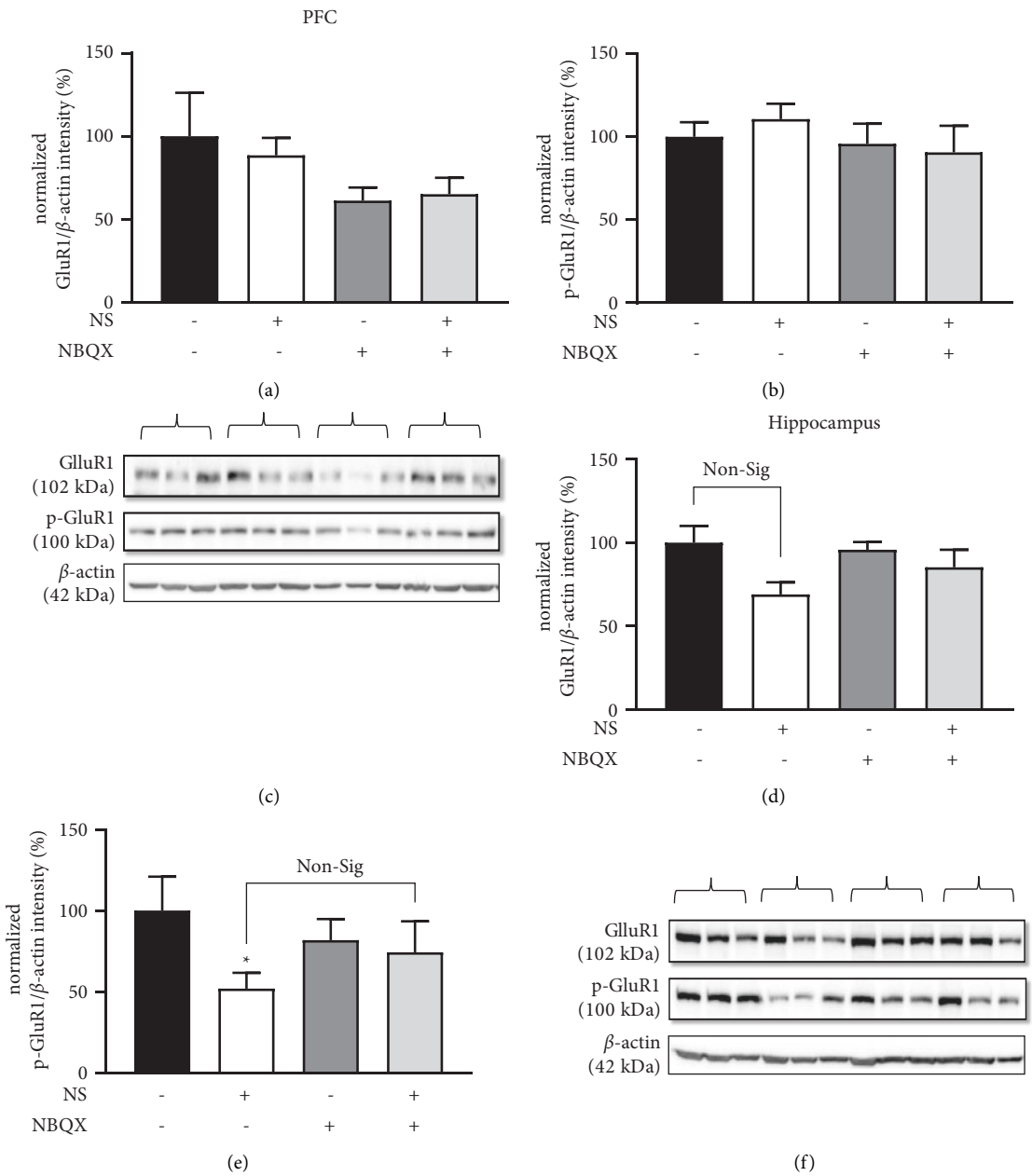

Figure 7: Effects of *Nelumbinis semen* (NS) and AMPA blockade on GluR1 expression in the prefrontal cortex (PFC) and hippocampus. (a) Normalized GluR1/ß-actin of PFC: there was no significant change ($F(3,20) = 1.145$, $p = 0.259$). (b) Normalized phospho-GluR1/ß-actin of PFC: there was no significant change ($F(3,20) = 0.517$, $p = 0.675$). (c) Representative blot image of the total and phospho-GluR1 expression in the PFC. The parentheses correspond to the treatments indicated above them. (d) Normalized GluR1/ß-actin of the hippocampus: ANOVA revealed no significant differences among groups ($F(3,20) = 2.692$, $p = 0.074$). (e) Normalized phospho-GluR1/ß-actin of the hippocampus: the phospho-GluR1 levels changed significantly ($F(3,20) = 4.48$, $p = 0.024$). Phospho-GluR1 decreased with NS treatment ($p = 0.04$), but the difference between the NS and NS + NBQX groups was nonsignificant. (f) Representative blot image of the total and phospho-GluR1 expression in the hippocampus. The parentheses in (c) and (f) correspond to the treatments indicated above them. Data are shown as the mean ± SEM ($n = 6$). $^{*}p < 0.05$ vs. vehicle, $^{\#}p < 0.05$ vs. NS. Non-sig: nonsignificant. ANOVA, LSD post hoc test.

effective at a dose of 1 mg/kg [7,44]. We found that NS reduced despair behaviors in the TST and FST after a single administration of 0.1 mg/kg and 1 mg/kg, and it reduced despair behavior in the FST at 10 mg/kg. In that dose range, NS had no anxiolytic- or psychomotor-stimulating effects. Also, it had no effect on the pleasure-seeking behavior evaluated in the FUST. Decreased pleasure-seeking behavior is one of the main symptoms of depression, and due to ceiling effects, NS might not have increased pleasure-seeking behavior in nondepressed animals. According to previous studies, NS had an antidepressant-like effect at a high dose (400 mg/kg) that is related to serotonergic signaling. Our

results suggest an antidepressant-like effect at a low dose (0.1 to 10 mg/kg) showing a possibility that the antidepressant-like effects of NS are biphasic.

Several methods are used to create models of depression in animals, but most commonly, stressors are applied to induce depression-like behaviors. Such methods have high construct validity in that long-term stress induces depression. However, animal models often use physical stressors, whereas the stress that people endure is often psychosocial. Therefore, depression models that use social stressors may be more valid and could reduce the gap between animal and clinical studies. As a model of social stress, the social defeat model is widely used, but we used the social hierarchy model because the social defeat model exposes animals to direct violence during the resident-intruder paradigm, which means that physical stress has a great influence on the formation of the model. In the SHS model, on the other hand, the depressed state is induced through frustration rather than exposure to direct physical harm. This method models experiences such as being pushed out of being an alpha male in chimpanzee society and celebrities moving away from public attention in human life, which is more appropriate than a social defeat for a depression model because depression occurs more often in those conditions rather than by becoming a victim of violence.

In mice exposed to SHS stress, anhedonia, measured as the preference for sweet water and time spent sniffing the urine of an estrus female, and despair behavior, measured using the FST and TST, were increased. Also, the latency to feeding was increased in the NSFT, but anxiety-like behavior and general activity did not change. Latency in the NSFT measures anxiety-like and depression-like behaviors, and locomotor activity can affect its outcome [32,45]. Because the anxiety and locomotor activity levels did not change, SHS induced a depressive phenotype in the mice.

When the effects of NS and fluoxetine were tested in this animal model of depression, a single dose of NS, but not a single dose of fluoxetine, reduced despair and anhedonic behaviors. Neither NS nor fluoxetine had any effect on anxiety or locomotion, but NS (but not fluoxetine) reduced the latency to feeding in the NSFT. This is also an antidepressant-like effect because NS did not affect anxiety or locomotion. In previous studies, the anti-anxiety effect of NS was detected in animals treated with normal and chronic unpredictable mild stress [25,46]. However, the doses of NS in those studies were 50 to 400 mg/kg, much higher than those we used in this study. Therefore, because low-dose NS did not affect anxiety or locomotion, the shortening of latency in the NSFT was an antidepressant-like effect. On the other hand, fluoxetine did not show any such antidepressant-like effect, and it is known that in the NSFT, fluoxetine exhibits antidepressant effects only when it is administered chronically [32,45,47]. Therefore, SHS seems to be a model for depression that responds to chronic administration of antidepressants, and NS seemed to exhibit rapid-onset antidepressant action in that model. So, our next step was to test its rapid-onset antidepressant effect.

To verify the rapid-onset antidepressant-like effect of NS, we pretreated mice with NBQX to block the AMPA receptor before behavior tests. An AMPA receptor blockade is known to abolish the rapid-onset antidepressant action of drugs that act on the glutamatergic system [8] such as ketamine, rapastinel, LY314597, (2R,6 R)-HNK, and scopolamine [48]. In our results, administration of NBQX alone did not affect depressive behaviors, but it suppressed the antidepressant effect of NS. In the NSFT, the reduced feeding latency seen with NS was blocked by NBQX pretreatment. As shown in Figure 4, the reduced latency in the NSFT caused by NS was not related to anxiety or spontaneous locomotion; therefore, NBQX blocked the rapid-onset antidepressant-like effect of NS. The despair response reduction caused by NS in the FST and TST was also blocked by NBQX. This result is parallel to that seen with ketamine [8,49]. In addition, the increase in urine sniffing time in the FUST was blocked by NBQX, indicating that the AMPA receptor blockade inhibited the hedonic-like behavior induced by NS.

A rapid-onset antidepressant effect is generally known to have a relationship with mTOR signaling, so mTOR phosphorylation was measured in the prefrontal cortex and hippocampus after NS administration. As expected, phosphorylated mTOR was increased by NS, and that effect was blocked by NBQX in the prefrontal cortex. The difference in the total amount of mTOR expressed in the prefrontal cortex was not statistically significant, and there was no difference in the amount of phosphorylated and total mTOR expressed in the hippocampus.

Li et al. found that the rapid antidepressant action of ketamine was due to an increase in phosphorylated mTOR in the prefrontal cortex [7], which indicates the synapse enhancement through mTORC1 signaling that has also been correlated with the rapid-onset antidepressant effect of Ro 25-6981 [7], mGluR2/3 antagonists [50], (2R,6R)-hydroxynorketamine [51], rapastinel [52], and scopolamine [53]. Such antidepressant effects also appear upon the activation of the p70S6 kinase, a downstream effector of mTORC1, or sestrin [48,54]. Studies have reported that mTOR phosphorylation is increased in the ventral hippocampus but not in the dorsal hippocampus [55]. In a schizophrenia model, the administration of 60 mg/kg of ketamine twice a day for 7 days decreased mTOR expression in the prefrontal cortex and hippocampus [56]. In our experimental results, the antidepressant action of NS appears to be related to mTOR phosphorylation in the prefrontal cortex but not in the hippocampus. However, it could be that no difference was found in the hippocampus because of measuring in the entire hippocampus instead of dividing it into ventral and dorsal parts.

It is known that rapid antidepressant action is associated with changes in AMPA receptors. Ketamine rapidly increases GluR1 expression in synaptosomes of the prefrontal cortex [7]. Antidepressant effect of ketamine was blocked by an AMPA receptor blockade, and phosphorylation of the GluR1 subunit was reduced [8]. In our results, the expression level and phosphorylated form of GluR1 were not affected by NS in the prefrontal cortex, but the phosphorylated form of GluR1 was reduced in the hippocampus. Ketamine was reported to reduce p845 on the GluR1 receptor subunit in the hippocampus [57], and similarly, an

mGluR7 agonist with rapid-onset antidepressant action also reduced the p845 of GluR1 [58].

Phosphorylation of S845 and S831 on the GluR1 subunit regulates the membrane expression of AMPA receptors [59]. In stress-induced depression models, the phosphorylation of S845 was decreased in some articles and increased in others [60,61]. When antidepressants such as imipramine and tianeptine were administered, the phosphorylation of S845 in the hippocampus increased [62,63]. Therefore, more research is needed to determine whether the results of this study fit the pharmacological mechanism of antidepressants in terms of the phosphorylation of GluR1 subunits. Also, the antidepressant effect of NS was found to have different actions in the prefrontal cortex and hippocampus.

One concern in this study was that the number of animals in the SHS model group was only 4, which was smaller than that of general animal behavioral experiments. This is because four times as many animals are needed to make SHS depression model mice. However, the mice induced with SHS showed a clear difference compared with the normal mice in sucrose preference, and the SHS mice treated with NS and fluoxetine also showed a clear difference from the untreated SHS mice. This effect was verified statistically as the p value below 0.05 indicates a good chance of difference in the distribution of data values.

5. Conclusions

NS reduced behavioral despair in the FST and TST. Under the condition of SHS, hedonic behavior was reduced, the despair response was increased, and the time to approach food in the NSFT was longer, indicating a condition of depression. Under the SHS condition, NS but not fluoxetine reduced those depression-like behaviors. Specifically, NS showed a rapid antidepressant-like effect by acutely reducing the access time to the food pellet in the NSFT. The administration of NBQX, an AMPA receptor blocker, inhibited the antidepressant-like effect of NS in the FST, TST, and NSFT, and the effects of NS were related to enhanced phosphorylation of mTOR in the prefrontal cortex and dephosphorylation of GluR1 S845 in the hippocampus. When the NS dose was increased, the tendency of the antidepressant effects to decrease was similar to that found with ketamine. Since NS has shown antidepressant-like potential in a preclinical model, it may be considered as a research candidate for the development of antidepressants in the future.

Data Availability

The data used to support the findings of this study are available from Seung-Yun Cha upon your request.

Ethical Approval

The experimental procedures were performed in accordance with the animal care guidelines of Kyung Hee University in the Institutional Animal Care and Use Committee (KHUASP(GC)-19-040).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

J.S. and S.M. conceptualized the study. J.L. and J.C. were responsible for methodology of the study. B.-T.A. and J.S.C. validated the study. S.-W.Y. performed formal analysis. D.-Y.K. performed the investigation. J.S. performed data curation. S.-Y.C. wrote the original draft preparation. S.M. reviewed and edited the manuscript. J.L. was responsible for visualization. S.M. and S.-Y.C supervised the study.

Acknowledgments

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Research Article

Analysis of the Underlying Mechanism of the Jiu Wei Zhen Xin Formula for Treating Generalized Anxiety Disorder Based on Network Pharmacology of Traditional Chinese Medicine

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Currently, there are many effective pharmacological treatments for generalized anxiety disorder (GAD), formulated herbal granule is also an alternative way. Our research intends to construct a pharmacological network on genetic targets and pathways between Jiu Wei Zhen Xin Formula (JWZXF) and GAD. Through the TCMSP database, we collected the active ingredients of JWZXF and potential targets of the active ingredients. The GAD-related proteins collected from GeneCards database and DisGeNET database were combined. Component-target protein networks were constructed and visualized using Cytoscape 3.8.2 software to comprehensively clarify the relationships between ingredients, components, and targets. The intersection targets were imported into the STRING database, and the protein-protein interaction (PPI) network was constructed. We constructed and analyzed the visualized "drug-target-disease" network. Gene Ontology (GO) enrichment together with Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis were conducted on the common target through R language. Forty-one effective components and 106 potential targets of JWZXF were found. There were top ten hub genes and multiple important signaling pathways involved in the treatment of GAD with the JWZXF. This study expounded the pharmacological actions and molecular mechanisms of the JWZXF in treating GAD from a holistic perspective. The potential pharmacological effects of the JWZXF are closely related to regulation because not only does it comprehensively analyze the possible mechanism of JWZXF treatment of GAD but it can also facilitate further in-depth research and provide a theoretical basis for the clinical expansion of its application.

1. Introduction

Generalized anxiety disorder (GAD) is a chronic mental disorder characterized by excessive tension, worry, and feelings of apprehension that last at least six months. The term GAD was first used in ICD-9; it got the attention of clinical researchers with the publication of DSM-III [1]. Later, DSM-V stated that individuals with GAD might experience restlessness, be easily fatigued, have difficulty concentrating, experience irritability, muscle tension, or sleep disturbance [2]. It is a chronic disease that is prevalent worldwide, with a combined lifetime prevalence of 3.7%, 12 months prevalence of 1.8%, 30 days prevalence of 0.8%, and comorbidity of 81.9%, respectively [3]. In urban China, the prevalence of GAD was 5.3%, with a low diagnosis rate [4]. Currently, many studies that focused on the effective pharmacological treatments of GAD mainly include selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), quetiapine, agomelatine, benzodiazepines, buspirone, and pregabalin [5, 6]. Among them, escitalopram and paroxetine of SSRIs, and venlafaxine and duloxetine of SNRIs are the most studied, respectively. However, those medicines may not be well tolerated in GAD patients [7], and both SSRIs and SNRIs are associated with decreased efficacy at higher doses [8]. Although there are many recommended pharmacological

treatments of GAD, they inevitably have some side effects and may even aggravate treatment-resistant patients. Complementary and alternative medicine (CAM) treatments are now recognized as an efficient alternative treatment [9]. Herbal medicine as a part of CAM is beneficial to the GAD, with an abundance of researchers highlighting the molecular mechanisms, signaling pathways, and neurotrophic factors in mental disorders [10–12]. Novel approaches like pharmacogenetics and pharmacoepigenetics are safer and may improve the treatment response while reducing the socioeconomic burden [13]. A meta-analysis revealed Jiu Wei Zhen Xin Formula (JWZXF) and concluded that a formulated herbal granule is less effective than SSRIs but is safer [14]. This research intends to construct a pharmacological network on JWZXF and GAD genetic targets and reveal their relationship.

2. Materials and Methods

2.1. Identification and Screening Strategy of Candidate Components in the JWZXF. The components of interest regarding the JWZXF were extracted based on their botanicals, which are *Panax ginseng C. A. Mey* (P.G.), Ziziphi Spinosae Semen (Z.S.S.), *Schisandrae chinensis Fructus* (S.C.F.), *Poria cocos Wolf* (P.C.W.), *Polygala tenuifolia Willd* (P.T.W.), Corydalis Rhizoma (C.R.), *Asparagi radix* (A.R.), *Rehmanniae radix Praeparata* (R.R.P.), and *Cinnanmomi cortex* (C.C.). The total component list of each ingredient was identified from the TCMSP [1] (Traditional Chinese Medicine Systems Pharmacology https://tcmsp-e.com/) database and TCM-ID (TCM-information database https://bidd.group/TCMID/index.html); due to the lack of component information of P.T.W in TCMSP database, we inquired and listed P.T.W component information from TCM-ID. Subsequently, we identified the ADME (absorption, distribution, metabolism, and excretion) properties from the TCMSP database based on the candidate components' information. Currently, drug-likeness (DL) evaluation (e.g., Lipinski's rule of five, Opera's rules of DL, and the ROES filter) is integrated into computational drug design/discovery pipelines. In this study, four main filtering criteria have been implemented in our research and used to screen components that could be involved in the central nervous regulation.

With a DL value higher than 0.18, oral bioavailability value higher than 30%, half-life time longer than four hours, and blood-brain barrier (BBB) penetration rate >0.3 can be retained as candidate components for subsequent analysis [15]. Finally, 41 active components were screened for target predictions within which three common components (CM1, CM2, and CM3), and 106 related targets were identified after removing repetitions, UniProt database (https://www.uniprot.org) was used to convert and calibrate protein names into gene official symbols that are potential genetic targets of the JWZXF.

2.2. Disease Target Prediction. With "generalized anxiety disorder" as the keyword, from the databases, GeneCards (https://www.genecards.org/) filtered from its highest "relevance score" to the third quartile that retrieved 1338 items and DisGeNET (https://www.disgenet.org/search, update by May 2020, v7.0) filtered by its "Score_gda" needed to be greater than and equal to 0.8 to help identify 1594 items. The GAD-related proteins collected from the GeneCards and the DisGeNET databases were combined to finally obtain 1977 related genetic targets (without duplication), after using the UniProt database to convert and calibrate gene names to official gene symbols, which are the potential genetic targets of GAD.

2.3. Network Construction and Analysis. Combining the result of potential target proteins of the JWZXF from above with the GAD-related proteins, we took the intersection of two datasets and found 50 drugs with shared targets. Component-target protein networks were constructed and visualized using Cytoscape 3.8.2 software to comprehensively clarify the relationships between ingredients, components, and targets. This showed common components of ingredients and off-target components.

The software allows data integration to analyze and visualize complex interactive networks. In these networks, nodes represent components, proteins, pathways, and GAD, while edges represent their interactions.

2.4. Construction of the Protein-Protein Interaction (PPI) Network and Analyses of Topological Properties. The intersection targets were imported into STRING (version 11.5 https://string-db.org/cgi/) database, with the "species" set as "Homo sapiens." Under the condition that the lowest interaction score was equal to 0.400, the TSV result file of protein to protein interactions was obtained, and the PPI network was constructed using the Cytoscape 3.8.2 software. The topological attributes of the PPI network were analyzed, and the value (degree) was calculated, representing the number of connected nodes.

Based on the PPI network, we can use the Cytoscape plug-in to extract subnetwork and hub genes. Cytoscape MCODE plug-in based on the K-core algorithm can be used to find clusters (highly interconnected regions) in a network (degree cutoff = 2, max. Depth = 100, K-core = 2 and node score cutoff = 0.2). After extracting the subclusters of PPI, we use the DAVID (version 6.7 October 31, 2020, https://david.ncifcrf.gov/) database to analyze those subcluster interactions and reveal the biological processes of each cluster.

Meanwhile, using CytoHubba plug-in to identify hub genes: we used the maximum cluster centrality (maximum clique centrality, MCC) algorithm to screen hub genes. MCC algorithm integrates 11 topology analysis methods and six centrality analysis methods, which produced high accuracy.

2.5. Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis. KEGG is a database that systematically analyzes the metabolic pathways and functions of gene products in cells. The KEGG database helps study genes and express information as a complete network by integrating data from genomes, chemical molecules, and biochemical systems, including metabolic pathways, drugs, diseases, gene

sequences, and genomes. We selected the DAVID online database to implement the KEGG enrichment analysis.

The intersection genes were introduced into the DAVID database, the "select identifier" was set to "official gene symbol," "list type" was set to "gene list," with the species defined as Homo sapiens, P-value ≤0.05 was set as the initial selection threshold. KEGG pathway enrichment analysis was realized, and the ascending order was sorted following the FDR to screen for the top 12 information pathways (ggplot2 package of R).

2.6. Gene Ontology (GO) Analysis. To study meaningful functional annotation and biological characteristics of potential targets, GO enrichment analysis was conducted to extract key GO terms (BP: biological process, MF: molecular function, CC: cellular components). In this case, we chose to use the DAVID database to conduct an online analysis that would allow researchers to use the KEGG enrichment together with the GO analysis. The targets, organized and condensed into several functional groups as denoted by their most significant leading term, were visualized in the network. The GO terms that had a P-value ≤0.05 were regarded significant and were studied further. Finally, we listed the top 15 GO terms sorted in the ascending order of FDR.

3. Results

3.1. Screening of the Effective Compounds and Potential Targets of JWZXF. The effective components were extracted based on the criteria we mentioned before, Table 1 lists out the potential effective components from the JWZXF. We also discovered that ingredient C.C did not contain any component that fulfilled our ADME selection criteria.

3.2. Component-Target Protein Network Construction and Analysis. We used the data extracted above and Cytoscape 3.8.2 software to build a component-target protein network, which contains 151 nodes (1 formula name, 9 ingredients, 41 effective components, and 106 potential targets of JWZXF), and 528 edges with the size of node based on its degree value is clearly represented in Figure 1. In the central layer of this network, the blue diamond represents target proteins from the center to the outside. Each layer represents common molecules with red hexagons, unique molecules of each ingredient with pink red, orange, yellow, green, lavender, rose red hexagons, ingredients with bluish-purple triangles, and off-target molecules of each ingredient, respectively. From the "common molecules" layer, stigmasterol (CM1) was present in P.G., A.R., and R.R.P.; sitosterol (CM2) was present in C.R., A.R., and R.R.P.; and beta-sitosterol (CM3) was present in P.G. and A.R. From the "molecules" layer, C.R. contains more active components than other ingredients with relatively higher degree values.

3.3. Protein-Protein Interaction Network of Targets. Proteins normally regulate their physiological functions through protein-protein interactions and other pathways. To better reveal the mechanism of the JWZXF in treating GAD, STRING, a database designed to collect and integrate all functional interactions between expressed proteins by integrating known and predicted protein-protein association data from a large number of organisms, was used. The intersection targets obtained above were entered into the STRING database, the PPI data obtained from the STRING database were imported into the software Cytoscape 3.8.2, to construct a PPI network related to GAD (with 49 nodes and 218 edges, one free node was removed) that is represented in

Table 1: Active components identified from nine herbs.

Ingredients	Number	Components (abbreviations)
Panax ginseng C. A. Mey. (P.G.)	7	Stigmasterol (CM1), Beta-sitosterol (CM3), Inermin (P.G.1), Arachidonate (P.G.2), Frutinone A (P.G.3), Girinimbin (P.G.4), Alexandrin_qt (P.G.5)
Ziziphi Spinosae semen (Z.S.S.)	2	Daucosterol (Z.S.S.1), Phytosterol (Z.S.S.2)
Schisandrae chinensis fructus (S.C.F.)	2	Angeloylgomisin O (S.C.F.1), Wuweizisu C (S.C.F.2)
Poria cocos (schw.) wolf. (P.C.W.)	2	Ergosta-7,22e-dien-3beta-ol (P.C.W.1), Hederagenin (P.C.W.2)
Polygala tenuifolia Willd (P.T.W.)	6	4-Methoxycinnamic acid (P.T.W.1), Onjixanthone I (P.T.W.2), Perlolyrine (P.T.W.3), Trans-asarone (P.T.W.4), 1,7-Dihydroxyxanthone (P.T.W.5), Trans-asarone (P.T.W.6)
Corydalis rhizoma (C.R.)	16	Stigmasterol (CM1), Sitosterol (CM2), Berberine (C.R.1), Coptisine (C.R.2), Cryptopin (C.R.3), Dihydrochelerythrine (C.R.4), Dihydrosanguinarine (C.R.5), Cavidine (C.R.6), (R)-canadine (C.R.7), (-)-alpha-N-methylcanadine (C.R.8), Dehydrocavidine (C.R.9), Leonticine (C.R.10), 24240-05-9 (C.R.11), Stylopine (C.R.12), Tetrahydrocorysamine (C.R.13), C09367 (C.R.14)
Asparagi radix (A.R.)	4	7-Methoxy-2-methylisoflavone (A.R.1), Stigmasterol (CM1), Sitosterol (CM2), Beta-sitosterol (CM3)
Rehmanniae radix praeparata (R.R.P.)	2	Stigmasterol (CM1), Sitosterol (CM2)
Cinnanmomi cortex (C.C.)	0	—

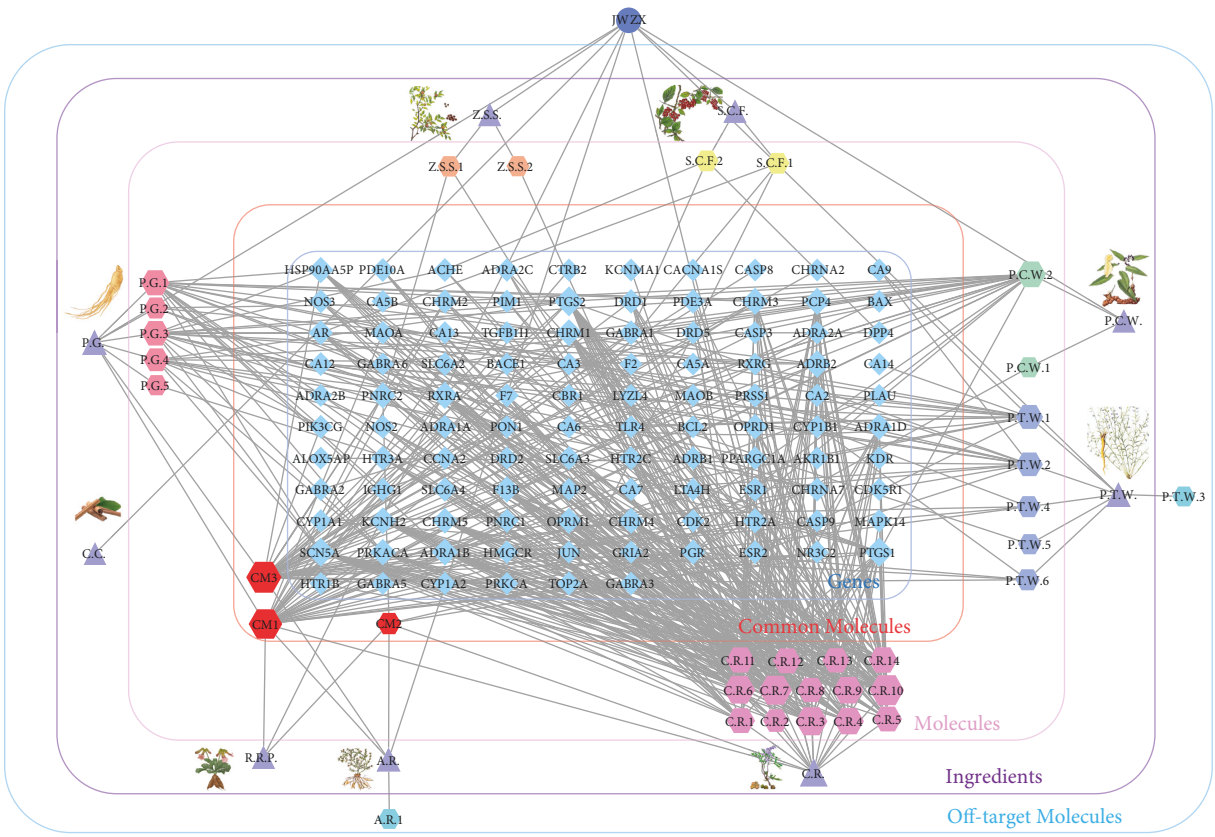

Figure 1: Component-target protein network. The hexagons represent the 41 candidate compounds in the Jiu Wei Zhen Xin formula (JWZXF). The blue diamond represents the genetic names of the target proteins of the nine herbs found by text mining.

Figure 2(a). Then, the Cytoscape MCODE plug-in based on the *K*-core algorithm was used to find clusters (highly interconnected regions) in a network. After calculation, five subcluster interactions were obtained which have network feature scores from 3.333 to 4.625, in the meantime, the DAVID database was used to analyze those subcluster interactions and to reveal the biological process of each cluster represented in Figure 2(b). With the help of Cytoscape CytoHubba plug-in under the algorithm of MCC, the top 10 hub genes found in the PPI network are represented in Table 2. The hub gene interaction with its neighbor genes are represented in Figure 3.

3.4. Kyoto Encyclopedia of Genes and Genomes Functional Enrichment Analysis and Gene Ontology Analysis. To macroscopically and comprehensively understand the biological function of the active ingredient target in the JWZXF, we implemented the GO functional enrichment analysis and KEGG pathway enrichment analysis on intersection targets. The results were mapped using the R software as bar plot and bubble plots for both KEGG and GO analysis. The top 12 pathways were screened based on the parameter of counts, as well as in combination with FDR-values in Figure 4, including neuroactive ligand-receptor interaction (hsa04080), serotonergic synapse (hsa04726), nicotine addiction (hsa05033), cocaine addiction (hsa05030), morphine addiction (hsa05032), retrograde endocannabinoid signaling (hsa04723), calcium signaling pathway (hsa04020), cAMP signaling pathway (hsa04024), amphetamine addiction (hsa05031), dopaminergic synapse (hsa04728), GABAergic synapse (hsa04727), and estrogen signaling pathway (hsa04915).

The GO analysis showed that the result of BP was significantly enriched in response to drug (GO:0042493), response to cocaine (GO:0042220), response to estradiol (GO:0032355), chemical synaptic transmission (GO:0007268), gamma-aminobutyric acid signaling pathway (GO:0007214), and so on. CC was significantly enriched in the plasma membrane (GO:0005886), integral component of the plasma membrane (GO:0005887), postsynaptic membrane (GO:0045211), GABA-A receptor complex (GO:1902711), cell junction (GO:0030054), and so forth. MF was significantly enriched in extracellular ligand-gated ion channel activity (GO:0005230), drug binding (GO:0008144), GABA-A receptor activity (GO:0004890), dopamine binding (GO:0035240), serotonin binding (GO:0051378), and the like. Based on the FDR value, the top 15th BPs, CCs, and MFs are, respectively, presented in Figures 5–7.

4. Summary and Discussion

Establishing the PPI network of the JWZXF and GAD revealed different pathways, as Figure 2(b) demonstrates that GABA signaling is a crucial pathway. The prolonged γ-aminobutyric acid (GABA) transmitter regulatory dysfunction in the animal

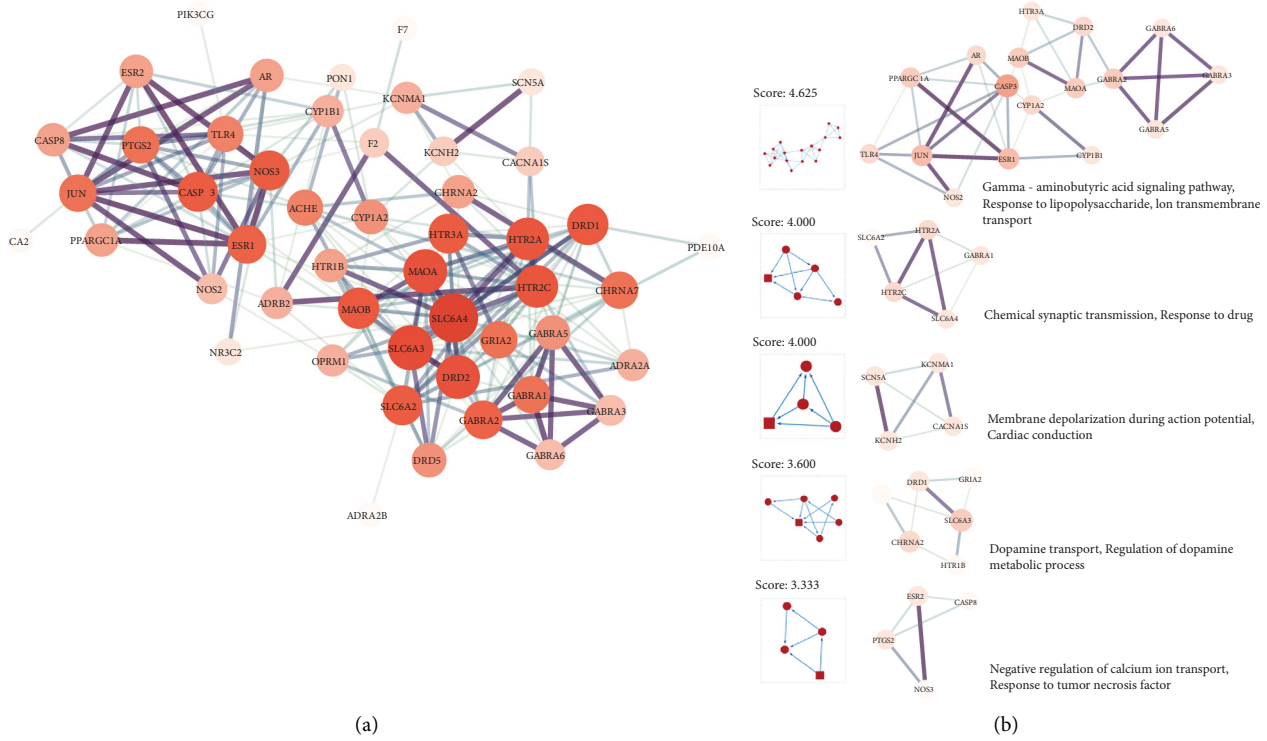

Figure 2: PPI network of JWZXF in the treatment of GAD-related proteins (a) and MCODE analysis of the PPI network and their corresponding GO biological processes (b).

Table 2: Top 10 hub genes ranked by MCC scores in the PPI network.

Ranks	Gene symbol	Score
1	*MAOA*	1536
2	*MAOB*	1394
3	*HTR3A*	1260
4	*DRD2*	1220
5	*HTR2A*	1038
6	*CASP3*	796
7	*SLC6A4*	717
8	*SLC6A3*	691
9	*JUN*	673
10	*ESR1*	629

and human brain contributes to anxiety disorders, especially in limbic systems. Brain circuits in the amygdala are thought to contain an inhibitory network of gamma-aminobutyric acid (GABAergic) interneurons, and thus this neurotransmitter plays a key role in regulating anxiety responses in both normal and pathological states [16]. Reducing GABA-mediated inhibition is one of the effective methods available for the modulation of neuronal excitability [17]. Many researchers have expressed great interest in developing novel medications acting on different subtypes of GABA receptors to manage GAD [18, 19] (Figure 8). Significantly, in second place are the pathways of chemical synaptic transmission and membrane depolarization during the action potential. The excitability and modulation of nerve synapses and the effective release of neurotransmitters also act on anti-anxiety [20, 21]. Psychiatric disorders are closely related to synaptic genes; additionally, mutations of the synaptic genes are also considered as risk factors for mental illness [22]. Moreover, GAD is mostly associated with peripheral inflammatory responses and changes in synaptic transmission profoundly affect these inflammatory responses [23]. Depolarization of the cell membrane potential can cause the alteration of neural connectivity, circuit, and neuron growth, which leads to neurological diseases [24]. Changes in the membrane potential that regulate brain metabolism can cause a series of pathophysiological processes, homeostatic alteration effectively acts on the whole body to reduce anxiety disorder [24, 25]. Moreover, evidence reveals that neuropsychiatric diseases bring about a dysregulation of the dopamine system and pathways [26].

Through the PPI network, the influence and effect of the JWZXF on several important pathways of GAD was observed. Many classical antipsychotic medications have a regulatory effect on the GABA pathway, and the JWZXF primarily acts on this pathway as well. Many studies now focus on finding new medications that selectively act on GABA subunit receptors of benzodiazepine (BZD) anxiolytics that have obvious side effects [18]. We must consider whether the active ingredients of JWZXF action GABA with a broad or selective spectrum are worthy of further discussion. As previously mentioned, selected active ingredients can all pass through the BBB which act on the central nervous system, but the peripheral regulation cannot be ignored in the treatment of GAD. In the future, more experiments and research should be conducted by applying JWZXF to influence both central nervous system and peripheral nerves.

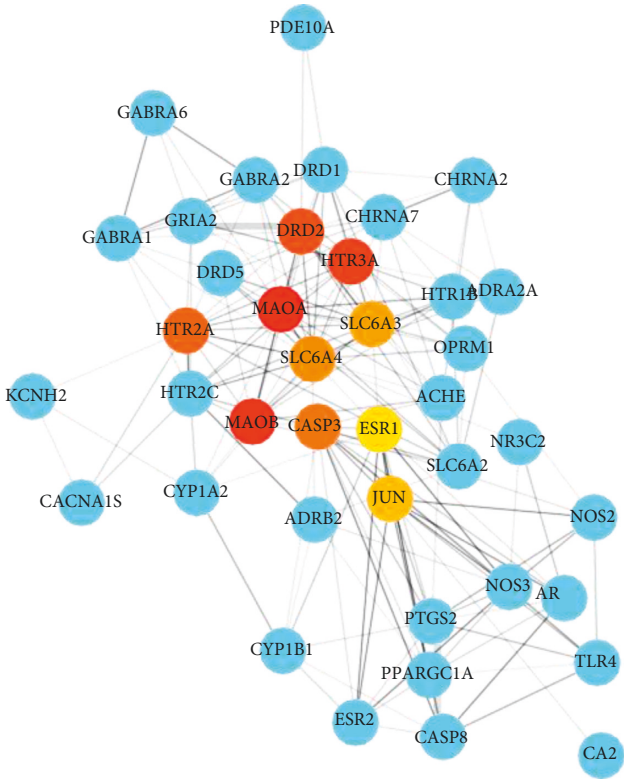

Figure 3: Top 10 hub genes with their neighboring gene interactions. The blue circles are the neighbor gene, red and yellow circles are the hub gene, and the color changes from dark to light according to the MCC score from high to low.

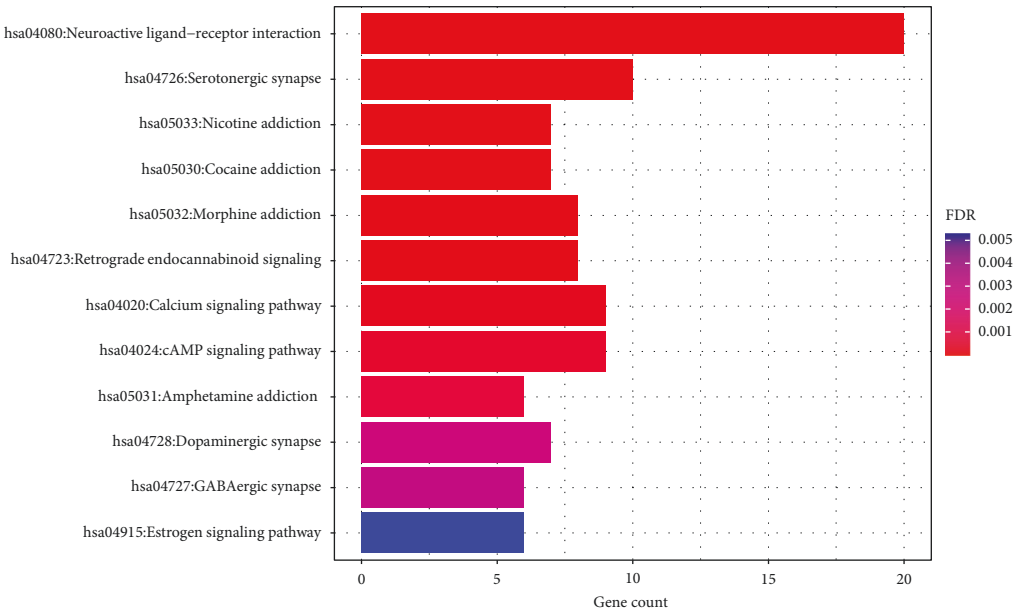

Figure 4: KEGG enrichment analysis of the identified JWZXF in GAD treatment by DAVID database, ranked by an FDR value from low to high.

Figure 3 depicts the relationship among hub genes and their neighbors, as Table 2 reveals that the top 10 hub genes ranked by MCC sores in the PPI network are *MAOA*, *MAOB*, *HTR3A*, *DRD2*, *HTR2A*, *CASP3*, *SLC6A4*, *SLC6A3*, *JUN*, and *ESR1*, respectively. GAD is a heritable disorder with a risk of series genes [27]. In the psychotherapy-epigenetic aspect, JWZXF is more involved in the monoamine oxidase genes, which are key enzymes to degrade neurotransmitters, and levels of *MAOA* gene methylation may be related to the categories and severity of the neuropsychiatric

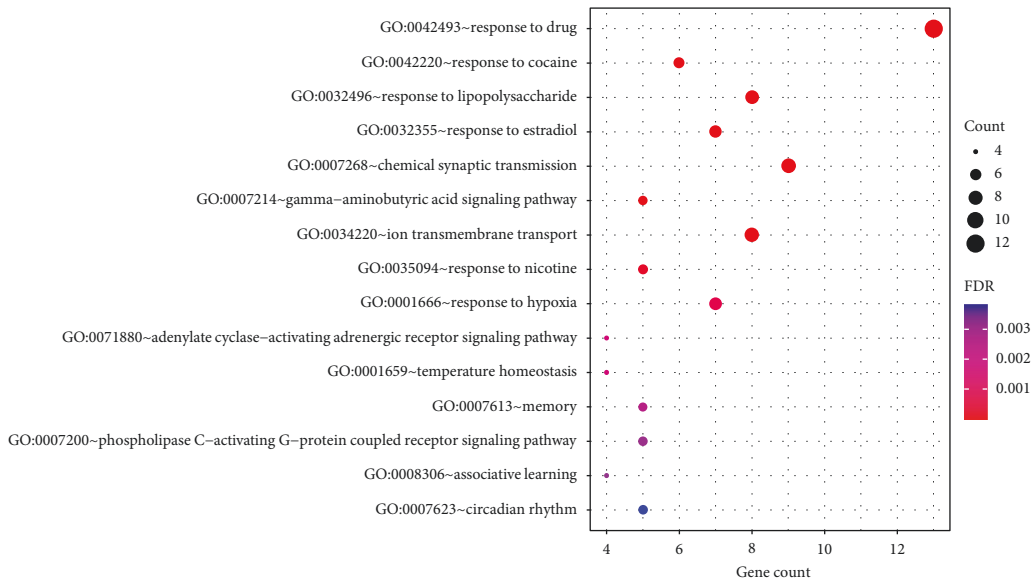

Figure 5: GO enrichment analysis of biological processes showing the top 15 items of 50 identified target proteins by DAVID database according to the FDR value sorted from small to large.

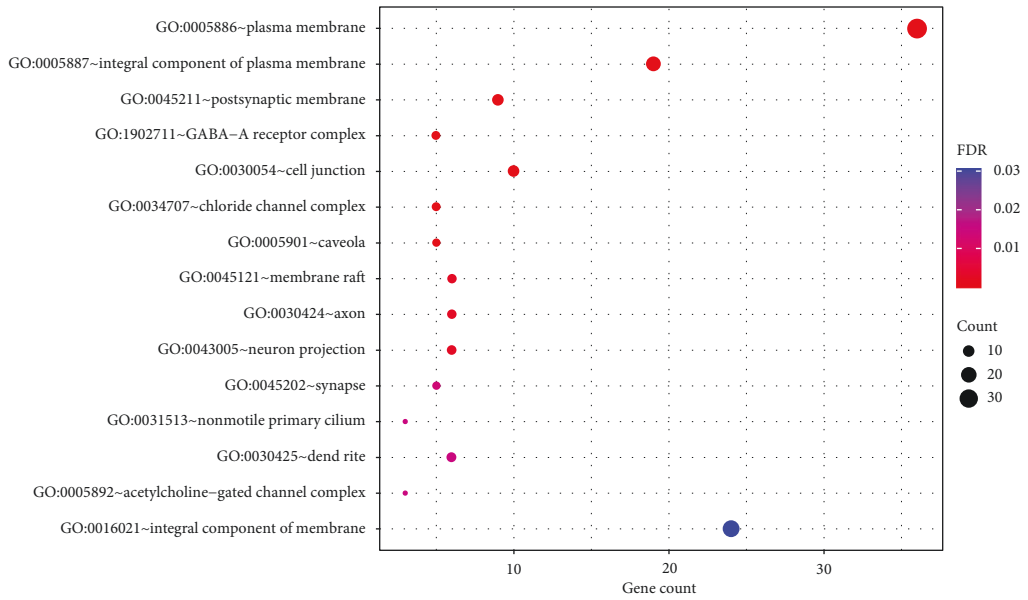

Figure 6: GO enrichment analysis of cellular components showing the top 15 items of 50 identified target proteins by DAVID database according to the FDR value sorted from small to large.

disorder [28, 29]. The activity genotype of the *MAOA* gene also correlates with emotional stability, impulse control, and emotion control [30]. *HTR3A* and HTR3B genes, which code subunits of serotonin receptors and their polymorphisms, may serve as predictors of 5-HT_3 antagonists and SSRIs as well [31–33]. Some researchers demonstrated that the loss of the *HTR3A* gene or *HTR3A* inactivation can induce anxiolytic-like features that have comparability of 5-HT_3 antagonists [34]. More interestingly, anxiety disorder and pain share the same pathway that gives us more chance to focus on the polymorphism of the *HTR3A* gene to manage chronic pain [31, 35, 36]. *DRD2* is also a risk gene related to GAD and may predict mental disorders at an early age [37, 38]. *SLC6A4* gene, which is a serotonin transporter gene, encodes a membrane protein through the transportation of serotonin to play a role in GAD [39, 40]. Additionally, caspase-3 action on neuronal metabolism is also significant to GAD [41].

The hub gene targets associated with the active components of the JWZXF mainly act on monoamine oxidase and serotonin receptors, especially selective 5-HT_3 receptors. It is difficult to determine how the JWZXF affects the pharmacokinetics of monoamine oxidase; so, JWZXF may have a monoamine oxidase inhibitor-like

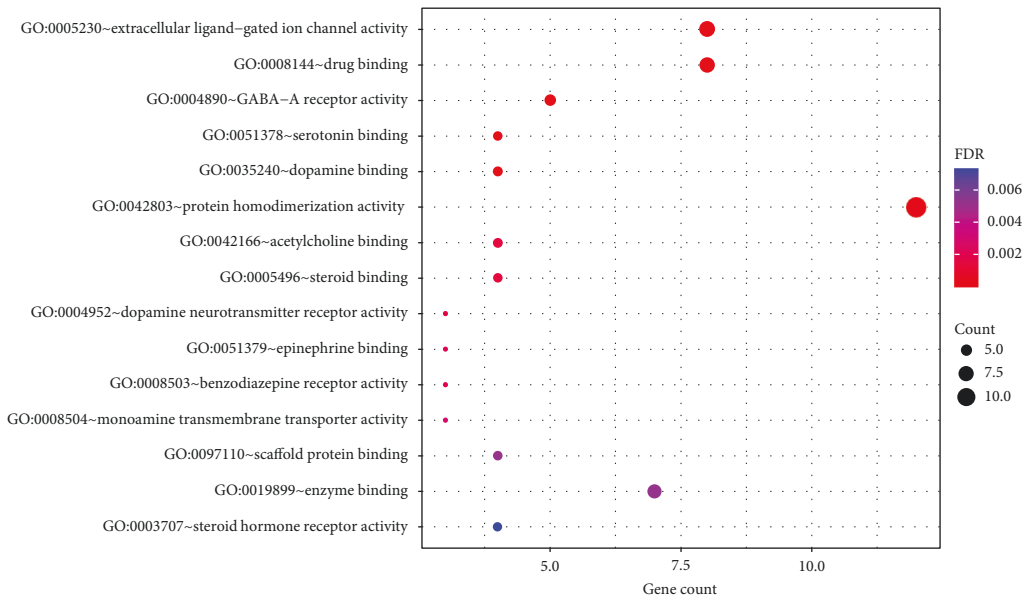

Figure 7: GO enrichment analysis of molecular functions showing the top 15 items of 50 identified target proteins by the DAVID database according to the FDR value sorted from small to large.

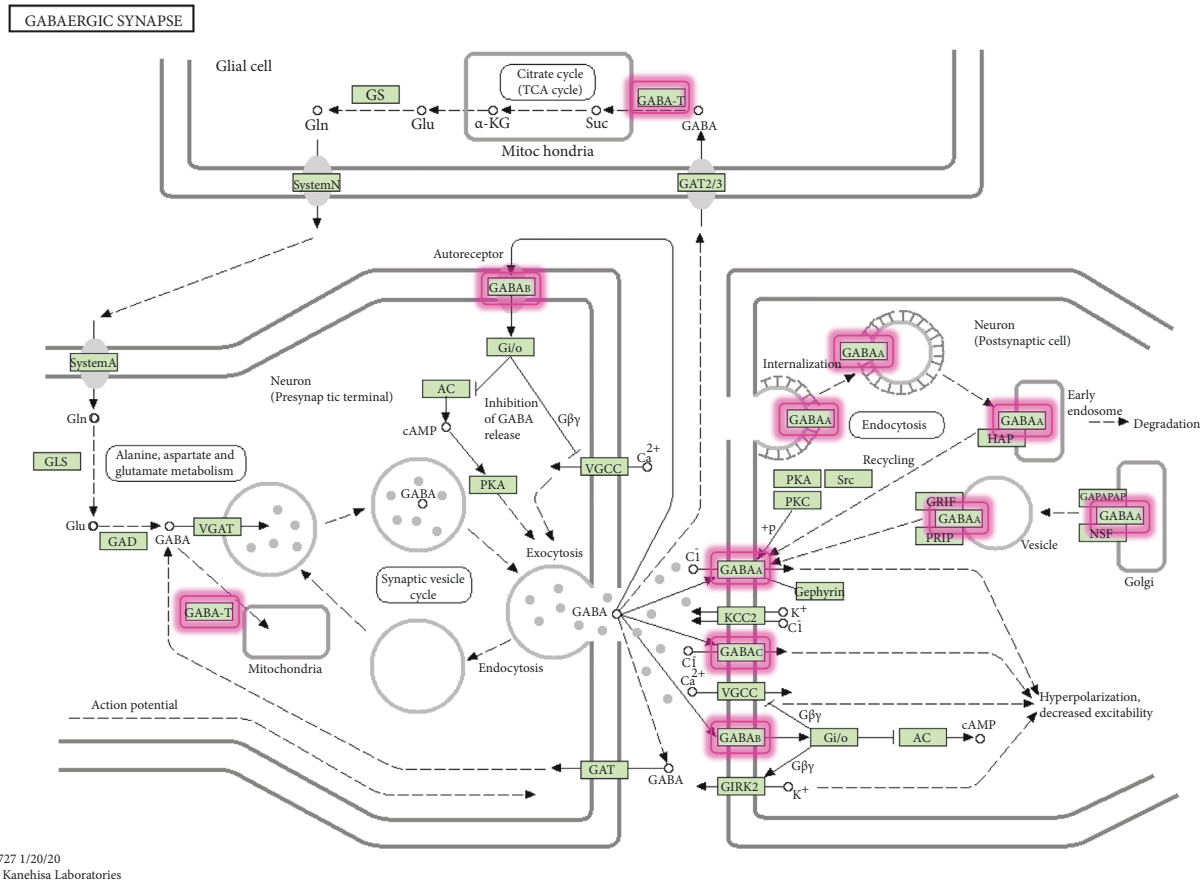

Figure 8: GABAergic synapse. The red rectangle represents the targets related to the component-target-pathway network, by Kanehisa Laboratories (https://www.kegg.jp/pathway/map04727).

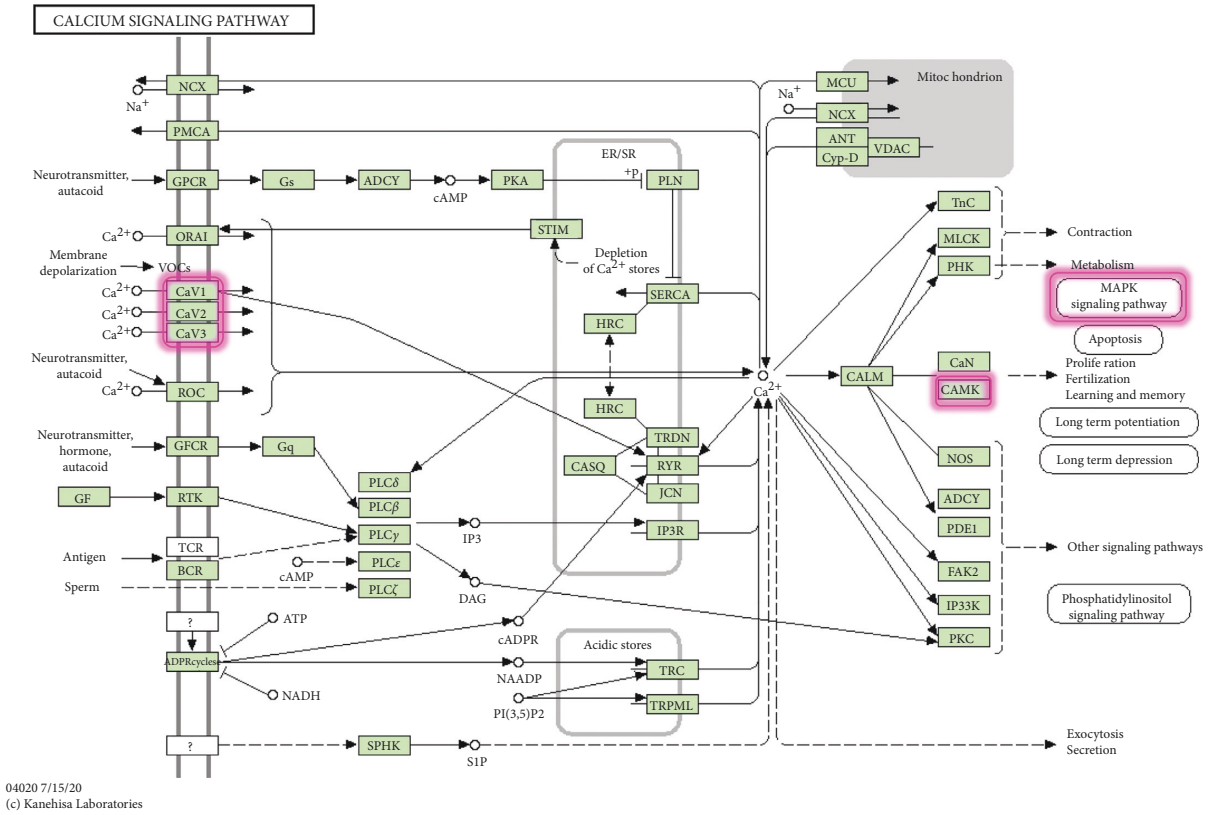

Figure 9: Calcium signaling pathway. The red rectangle represents the targets related to the component-target-pathway network, by Kanehisa Laboratories (https://www.kegg.jp/entry/map04020).

component. Drug interactions between the JWZXF and other drugs should be looked into [42, 43]. However, for patients with anxiety and chronic pain, such as fibromyalgia, JWZXF can regulate pain by acting on the HTR3 gene, making it a good choice when GAD is combined with chronic pain.

The KEGG pathway enrichment analysis showed multiple signaling pathways involved in the treatment of GAD with JWZXF. Particularly, the neuroactive ligand-receptor interaction, serotonergic synapse, calcium signaling pathway, and the cAMP signaling pathway, in which JWZXF-associated hub gene targets closely related to GAD were enriched. The neuroactive ligand-receptor interaction triggers intracellular signaling that modulates important gene expression dedicated to neural plasticity and stress processing that leads to emotional disorders [44, 45]. A higher level of the neuroactive ligand-receptor interaction gene expression can reduce anxiety-related behavior [46]. Through up- and downregulated signal pathways in the prefrontal cortex, neuroactive ligand-receptor interaction and serotonergic synapse may relate to the susceptibility and resilience of stress [47]. Notably, neurotransmitters also act on different calcium pathways which channels Cav 1.2 and Cav 1.3 are key signal pathways in neuro. Calcium activates calcium senor calmodulin, then, Ca^{+} combines with CaM-dependent protein kinases. The Ca^{+}/CaM-dependent protein kinases is activated in a successive way. Additionally, cascade pathways of Ras/mitogen-activated protein kinase and cAMP-responsive element-binding protein (CREB) to make gene expression are related to neuronal plasticity and GAD [48, 49] (Figure 9).

5. Conclusion

This study investigated the molecular mechanism of the JWZXF in the treatment of GAD by establishing multiple network models. Studies have shown that the most effective ingredient of the JWZXF in treating GAD is Corydalis, and the most involved compounds may be stigmasterol, sitosterol, and beta-sitosterol. The potential pharmacological effects of the abovementioned active compounds are closely related to regulated GABA receptors, dopamine receptors, chemical synaptic transmission, and membrane depolarization during action potential. Gene *MAOA*, *MAOB*, *HTR3A*, *DRD2*, *HTR2A*, *CASP3*, *SLC6A4*, and *SLC6A3* are possible targets of treatment by the JWZXF. Additionally, JWZXF may primarily modulate the neuroactive ligand-receptor interaction, serotonergic synapse, calcium signaling pathway, and cAMP signaling pathway. The network pharmacology analysis of JWZXF treatment of GAD is of great significance. Not only does it holistically analyze the possible mechanism of JWZXF treatment of GAD, it also facilitates further in-depth research and provide a theoretical basis for the clinical expansion of its application.

Abbreviations

GAD: Generalized anxiety disorder
SSRIs: Selective serotonin reuptake inhibitors
SNRIs: Erotonin-norepinephrine reuptake inhibitors
CAM: Complementary and alternative medicine
JWZXF: Jiu Wei Zhen Xin formula
P.G.: Panax ginseng C. A. Mey
Z.S.S.: Ziziphi spinosae semen
S.C.F.: Schisandrae chinensis fructus
P.C.W.: Poria cocos wolf
P.T.W.: Polygala tenuifolia willd
C.R.: Corydalis rhizoma
A.R.: Asparagi radix
R.R.P.: Rehmanniae radix praeparata
C.C.: Cinnanmomi cortex
TCMSP: Traditional Chinese Medicine Systems Pharmacology
TCM–ID: TCM-Information database
ADME: Absorption, distribution, metabolism, and excretion
DL: Drug-likeness
BBB: Blood-brain barrier
PPI: Protein-protein interaction
MCC: Maximum clique centrality
KEGG: Kyoto encyclopedia of genes and genomes
GO: Gene ontology
BP: Biological process
MF: Molecular function
CC: Cellular components
CM1: Stigmasterol
CM2: Sitosterol
CM3: Beta-sitosterol
P.G.1: Inermin
P.G.2: Arachidonate
P.G.3: Frutinone
P.G.4: Girinimbin
P.G.5: Alexandrin_qt
C.R.1: Berberine
C.R.2: Coptisine
C.R.3: Cryptopin
C.R.4: Dihydrochelerythrine
C.R.5: Dihydrosanguinarine
C.R.6: Cavidine
C.R.7: (R)-Canadine
C.R.8: (-)-Alpha-N-methylcanadine
C.R.9: Dehydrocavidine
C.R.10: Leonticine
C.R.11: 24240-05-9
C.R.12: Stylopine
C.R.13: Tetrahydrocorysamine
C.R.14: C09367 (C.R.14)
P.T.W.1: 4-Methoxycinnamic acid
P.T.W.2: Onjixanthone I
P.T.W.3: Perlolyrine
P.T.W.4: Trans-asarone
P.T.W.5: 1,7-Dihydroxyxanthone
P.T.W.6: Trans-asarone
A.R.1: 7-Methoxy-2-methylisoflavone
P.T.W.1: 4-Methoxycinnamic acid
P.T.W.2: Onjixanthone I
P.T.W.3: Perlolyrine
P.T.W.4: Trans-asarone
P.T.W.5: 1,7-Dihydroxyxanthone
P.T.W.6: Trans-asarone
P.C.W.1: Ergosta-7,22E-dien-3beta-ol
P.C.W.2: Hederagenin
Z.S.S.1: Daucosterol
Z.S.S.2: Phytosterol
S.C.F.1: Angeloylgomisin O
S.C.F.2: Wuweizisu C
GABA: γ-Aminobutyric acid
BZD: Benzodiazepine
CREB: CAMP-responsive element-binding protein.

Data Availability

The dataset generated for this study is available on request to the corresponding author.

Conflicts of Interest

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

Authors' Contributions

Mr. Heng Shao and Mr. QuanGan with Ms. Shasha Zhu did the analysis, developed the methodology and wrote the manuscript. Dr. Zhuangfei Chen and Ms. Yanqing Zhu were responsible for conceptualization and design of the work.

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Research Article

The 5-HT and PLC Signaling Pathways Regulate the Secretion of IL-1β, TNF-α and BDNF from NG2 Cells

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The present study was clarified the relationship between NG2 glial cells and 5-hydroxytryptamine (5-HT) to further revealed a role in the regulation of cortical excitability. The co-localization of NG2 cells and 5-HT in rat prefrontal cortex was determined using immunofluorescence. Different concentrations of 5-HT were applied to cultured NG2 cells. Real-time PCR measured the expression of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and brain-derived neurotrophic factor (BDNF). Changes in the expression of IL-1β, TNF-α, and BDNF in NG2 cells were detected after the addition of 5-HT receptor specific blockers and phospholipase C (PLC) specific activators and inhibitors. The results confirmed that the NG2 protein and 5-HT co-localized in the prefrontal cortex. 5-HT treatment of NG2 cells significantly reduced the expression of IL-1β and BDNF mRNA and increased the expression of TNF-α. The 5-HT receptor specific inhibitors alverine citrate, ketanserin, ondansetron and SB-399885 blocked the regulatory effects of 5-HT on NG2 cells. The PLC signal was linked to the secretion of IL-1β, TNF-α and BDNF in NG2 cells. These results indicated that 5-HT affected IL-1β, TNF-α, and BDNF secretion from NG2 cells via the 5-HT1A, 5-HT2A, 5-HT3, 5-HT6 receptors and the PLC signaling pathway.

1. Introduction

NG2 glia cells are widespread cell populations throughout gray and white matter in the central nervous system (CNS) and have distinct morphological and physiological traits [1–3]. These cells specifically express NG2 chondroitin sulfate proteoglycan (CSPG4) and platelet-derived growth factor receptor alpha (PDGFRα) [4–6]. They are considered to be an independent population of glial cells, otherwise known as oligodendrocyte progenitor cells (OPCs) given that they can differentiate into oligodendrocytes during development [7, 8]. The formation of direct synaptic associations of NG2 cells with glutamatergic and γ-aminobutylergic neurons is a vital process in the transmission of information in the CNS [9] .Moreover, NG2 cells also secret some factors, IL-1β, TNF-α and BDNF, which affect the activity of CNS neurons and glial cells [10, 11]. Recent studies revealed that IL-1β and TNF-α influenced neuronal excitability and participated in immunoregulatory functions [12]. BDNF was implicated in the regulation of neuroplasticity, synaptic function, neuroprotection and excitation/inhibition imbalance [13].

5-HT is an important monoamine neurotransmitter in the CNS, and it is synthesized primarily in the raphe nuclei [14]. This neurotransmitter is a broad player in numerous processes, inducing neuronal excitability and modulating excitatory synaptic transmission and sleep-wake behavior [15, 16]. Serotonergic neurons fire most actively during wakefulness, reduce their rate of activity during non-rapid eye movement sleep (non-REMS), and are silent during rapid eye movement sleep (REMS) [17]. 5-HT affects the development, proliferation and differentiation of NG2 cells. It was recently reported that fluoxetine increased 5-HT inhibits the basal proliferation and survival of OPCs in the fornix and corpus callosum of adult mice [18]. PLC is an enzyme that mediates cell signaling through various

metabolic receptors. Among several PLC subtypes, the $\beta 4$ subtype is uniquely localized in the geniculate nucleus of the thalamus, which is postulated to have a key role in the transition and maintenance of sleep stages. PLC-$\beta 4$−/− mice exhibited increased REM sleep and unusual wake-to-REM sleep transitions at night [19]. The actions of the 5-HT (2A), 5-HT (2B) and 5-HT (2C) receptor subtypes are mediated by the activation of PLC, resulting in depolarization of the host cell [16]. Our previous study revealed that the down-regulation of 5-HT synthesis via parachlorophenylalanine (PCPA) produced NG2 cell activation and proliferation. Other functional relationships between 5-HT and NG2 cells may exist, and further studies should investigate the relevant mechanisms of 5-HT.

The present study examined whether NG2 cell secretion of IL-1β, TNF-α and BDNF was associated with 5-HT. The results showed that the effects of 5-HT on NG2 cell secretion was related to 5-HT1A, 5-HT2A, 5-HT3 and 5-HT6 receptors and the PLC signaling pathway.

2. Materials and Methods

2.1. Experimental Animals. Adult male Sprague-Dawley (SD) rats (Tianqin Biotechnology Co. Ltd. China) were used for immunofluorescence study. All relevant experiments were performed in accordance with the protocol of the National Institutes of Health and institutional guidelines for the humane care of animals. All efforts were made to reduce the number of animals used and minimize any pain and discomfort.

2.2. Cell Culture and Treatment. NG2 cells, namely rat oligodendrocyte precursor cells (ROPCs), were provided by Qingqi (Shanghai, China) Biotechnology Development Co, Ltd. Cells were routinely cultivated in dulbecco's modified eagle medium (DMEM) with high glucose (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 humidified atmosphere.

For 5-HT-induced NG2 cell secretion assays, according to the results of the pre-experiment, the cells were treated with 5-HT (25, 50 and 100 μM) for 24 hours. NG2 cells were treated with various receptor (5-HT) inhibitors and specific activators/inhibitors of intracellular signals, such as 5-H1A, 5-HT2A, 5-HT3 and 5-HT6 receptors, including the 5-HT1AR, 5-HT2AR and 5-HT6R nonspecific antagonist asenapine maleate (Ase, 2.5 μM), the 5-HT1A specific receptor inhibitor alverine citrate (1.25 μM), the 5-HT2A specific receptor inhibitor ketanserin (37.5 μM), the 5-HT3 specific receptor inhibitor ondansetron (5 μM), the 5-HT6 specific receptor inhibitor SB-399885 (1 μM), the PLC specific activator m-3M3FBS (3 μM) and the specific inhibitor U-73122 (2.5 μM). These reagents were applied with 5-HT (100 μM) for 24 hours.

2.3. Immunofluorescence Double-Labeling. The brain was removed after perfusion and the prefrontal cortex was cut into 30-μM sections. Sections were blocked with 10% goat serum for 30 minutes at 37°C, and the liquid was aspirated without washing, and then incubated with primary antibodies for 2 hours at 37°C protected from light. The primary antibodies included anti-NG2 monoclonal antibodies (NG2-Ab, 1 : 80, Abcam, USA) and anti-TPH (TPH-Ab, 1 : 200, Abcam, USA). Subsequently, sections were incubated with secondary antibodies, including ALexa Fluor 488 (green, 1 : 100) and ALexa Fluor 594 (red, 1 : 100, both from Invitrogen, USA), for 1 hour at 37°C protected from light. These were followed by counterstained with a DAPI Mix (Beijing Solebro Technology Co, China). Finally, sections were observed under a fluorescence inverted microscope (Olympus, Japan). Fluorescence was detected using excitation wavelengths of 488 nm (green), 649 nm (red) and 358 nm (blue) , respectively. NG2 cells (red fluorescence) and 5-HT neuronal markers (green fluorescence) were observed. When two immunofluorescence images overlapped in yellow (red + green = yellow), the two markers exhibited co-expression [20].

2.4. ELISA Analysis. NG2 cells in the exponential growth phase (2×10^5 cells/well) were seeded in 6-well plates and treated with each group for 24 hours. The protein levels of TNF-α, IL-1β and BDNF in the cell supernatants were detected using ELISA kits, according to the manufacturer's instructions. ELISA kits for rat IL-1β (Shenzhen Xin Bosheng Biotechnology Co, China), TNF-α (Shenzhen Xin Bosheng Biotechnology Co, China) and BDNF (Wuhan Elixirite Corporation, China) [21].

2.5. Real-Time PCR Analysis. NG2 cells in the exponential growth phase (2×10^5 cells/well) were seeded in 6-well plates and treated with each group for 24 hours. Intracellular total RNA was extracted using a kit (Feiyang Biological Engineering Co, Guangzhou, China). cDNA was reverse transcribed with a kit (Feiyang Biological Engineering Co, Guangzhou, China), and 2× SYBR Green qPCR Mix (Yisun Biotechnology Co, Shanghai, China) was used for real-time PCR. Real-time PCR (StepOne Plus, ABI Corporation, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene. The amplified products were resolved by 1.5% agarose gel electrophoresis and visualized using ethidium bromide staining and a UV light source. Each experiment was repeated three times with three replicate wells, and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method to determine the relative expression. All primers and the related sequences used in the experiments are shown in Table 1.

2.6. Western Blot Analysis. NG2 cells in the exponential growth phase (2×10^5 cells/well) were seeded in 6-well plates, and all the experimental groups were incubated for 24 hours. The protein expression of PLC (Abclonal, China), IL-1β (Cell Signaling, China), TNF-α (Cell Signaling, China) and BDNF (Bioss, China) was determined using Western blot (WB) analysis. Protein extracts were separated using 5% gum concentrate and 12% separating

Table 1: Primer sequences used in this study.

Primer name		Sequences (5′-3′)	Product size (bp)
5-HT1AR	Forward	AAGGACCACGGCTACACCATCTAC	108
	Reverse	CTGACAGTCTTGCGGATTCGGAAG	
5-HT2AR	Forward	CTTCCAACGGTCCATCCACA	132
	Reverse	GGGCACCACATTACAACAAACAG	
5-HT3R	Forward	ACATTTCCCTGTGGCGAACA	189
	Reverse	CAGTGGTTTCCCATGGCTGAG	
5-HT5R	Forward	CGCTGTGCTCCTGGGATAT	78
	Reverse	CCTGTTGAACGCCGTGTAGAT	
5-HT6R	Forward	GCACGAACTGGGCAAAGCT	86
	Reverse	GGACGCCACGAGGACAAAA	
5-HT7R	Forward	TTCTGTCGGTCTGGCTGCTCTC	130
	Reverse	CCGCAGTGGAGTAGATCGTGTAG	
IL-1β	Forward	TGACAGGCAACCACTTACC	123
	Reverse	CCCATACACACGGACAACT	
TNF-α	Forward	GAAACAGTCTGCGAGGTGTG	158
	Reverse	TTCTTCTTGCAGCCACACAC	
BDNF	Forward	TCTACGAGACCAAGTGTAATCCCAT	166
	Reverse	GAAGTGTCTAT CCTTATGAACCGC	
PLCβ1	Forward	CTGAGGGCTCACGCAAGAA	102
	Reverse	GCAGCACGGTATAGGTGAA	
GAPDH	Forward	ACAGCAACAGGGTGGTGGAC	98
	Reverse	TTTGAGGGTGCAGCGAACTTT	

adhesive and transferred to PVDF membranes. The membranes were blocked with TBST buffer and kept at room temperature for 2 hours on a shaker. The corresponding primary antibody was diluted in the blocking solution and the PVDF membranes were immersed in the primary antibody incubation solution overnight at 4°C. The membranes were rinsed three times with TBST buffer and incubated with a secondary Ab for 2 hours at room temperature. After four rinses with TBST buffer, the PVDF membranes were scanned and imaged.

2.7. Statistical Analysis. SPSS software was used for statistical analysis (version 25.0). Outcomes are presented as the means ± SD from at least three separate experiments. Distinctions between groups were analyzed using one-way analysis of variance (one-way ANOVA) followed by post hoc tests using LSD or Dunnett's test. Statistical differences at $^{*}p < 0.05$ were deemed significant, and $^{**}p < 0.01$ was considered extremely significant.

3. Results

3.1. Colocalization of NG2 Cells with 5-HT Neuronal Markers (TPH) in the Prefrontal Cortex. To determine whether NG2 cells co-localized with 5-HT neuronal markers (TPHs) in the prefrontal cortex, double immunofluorescence staining was performed using NG2 and TPH antibodies. As shown in Figure 1, NG2 cell signals were identified as green fluorescence (Figure 1(a)), and 5-HT neuron marker (TPH) signals were recognized as red fluorescence (Figure 1(b)). The yellow color in double fluorescence detection showed that nearly all 5-HT neuron markers (TPH) co-labeled with NG2 cells in the prefrontal cortex (Figure 1(c)). These results suggest a functional relationship between NG2 cells and 5-HT.

3.2. Expression of 5-HT3, 5-HT5, 5-HT6, 5-HT7 Receptors and 5-HT1A, 5-HT2A Subtypes on NG2 Cells. To further investigate the functional relationship between NG2 cells and 5-HT, real-time PCR was used to detect 5-HT receptors expressed on NG2 cells. The expression of the 5-HT1A, 5-HT2A, 5-HT3, 5-HT5, 5-HT6 and 5-HT7 receptors was detected on NG2 cells (Figure 2).

3.3. 5-HT Induced IL-1β, TNF-α and BDNF Secretion from NG2 Cells. To investigate whether the secretion of IL-1β, TNF-α and BDNF in NG2 cells was associated with 5-HT, we used different concentrations of 5-HT (25, 50, and 100 μM) to stimulate NG2 cells.Figure 3 shows that 5-HT significantly inhibited the secretion of IL-1β and BDNF and significantly increased TNF-α secretion. These results showed that 5-HT induced the secretion of IL-1β, TNF-α and BDNF in NG2 cells.

3.4. 5-HT Modulates NG2 Cells Secretion of IL-1β, TNF-α and BDNF via 5-HT1AR, 5-HT2AR, 5-HT3R and 5-HT6R. There are multiple 5-HT receptor types on NG2 cells. Therefore, we investigated the receptors involved in IL-1β, TNF-α and BDNF secretion. We used the nonspecific antagonist asenapine maleate (Ase) to block 5-HT1AR, 5-HT2AR and 5-HT6R and found elevated secretion of IL-1β, TNF-α and BDNF in NG2 cells (Figure 4). We used specific antagonists alverine citrate (Alv), ketanserin (Ket), ondansetron (Ond) and SB-399885 (SB), to block 5-HT1AR, 5-HT2AR, 5-HT3R and 5-HT6R, respectively. Blockade of 5-HT1AR or 5-HT3R down-regulated the secretion of IL-1β

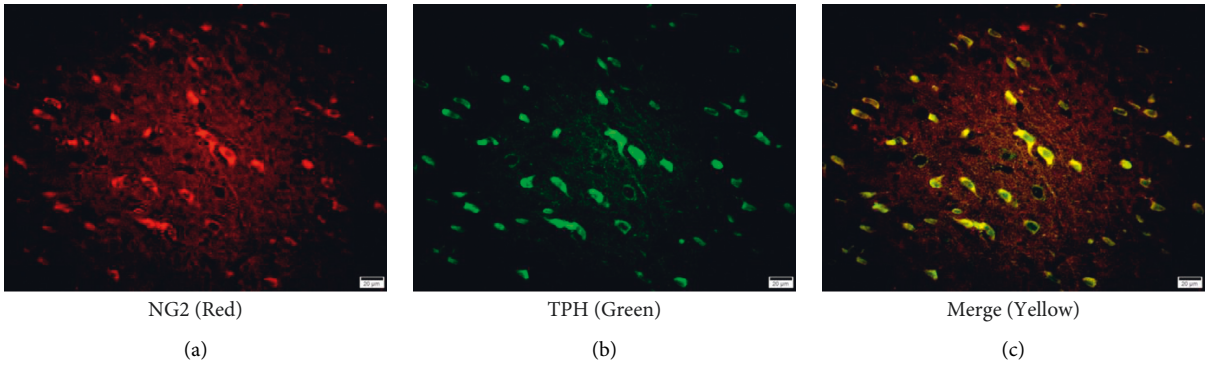

Figure 1: Colocalization of NG2 cells and 5-HT neuron markers in the rat prefrontal cortex. Double fluorescence detection of NG2 cells (red; (a)) and the 5-HT neuron marker TPH (green; (b)) in the prefrontal cortex. The merged images show colocalization of NG2 cells and 5-HT neurons in the prefrontal cortex (c). Scale bar = 20 μm.

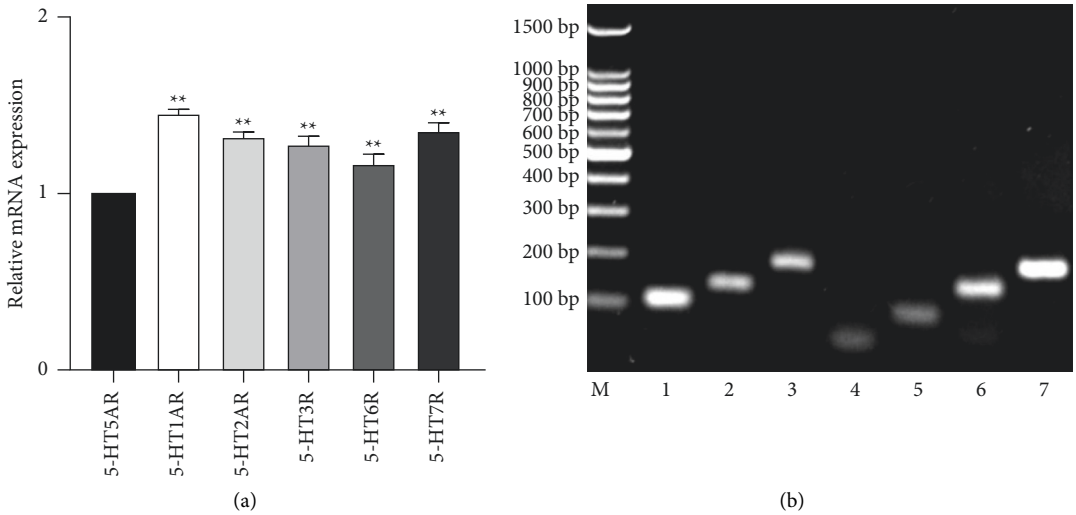

Figure 2: 5-HTRs expressed on NG2 cells. (a) Data presented as expression relative to 5-HT5. (Data are expressed as the means +/− s.d: $n = 3$ for each group. * $p < 0.05$, ** $p < 0.01$. s.d: standard deviation). (b) The PCR products were visualized in agarose gels. Comparison of the PCR products was performed using GAPDH as a positive control. Controls are labeled as follows: M, DNA marker; 1, 5-HT1AR; 2, 5-HT2AR; 3, 5-HT3R; 4, 5-HT5R; 5, 5-HT6R; 6, 5-HT7R; 7, GAPDH. The product size of each receptor matched the expected product size.

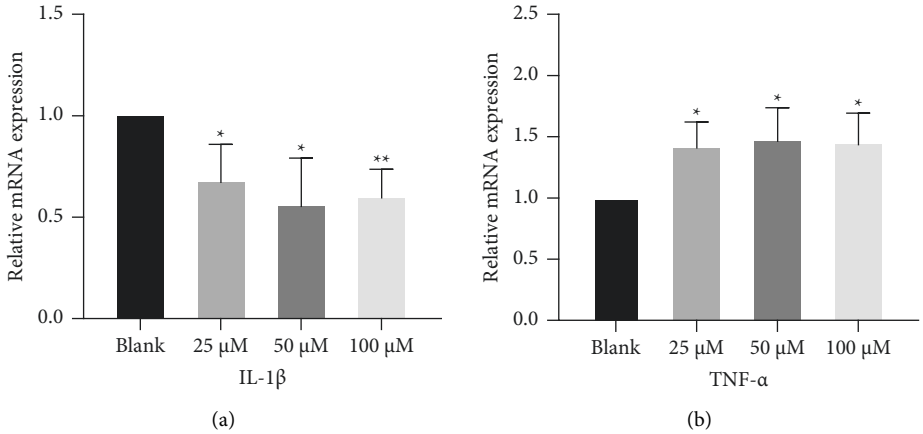

Figure 3: Continued.

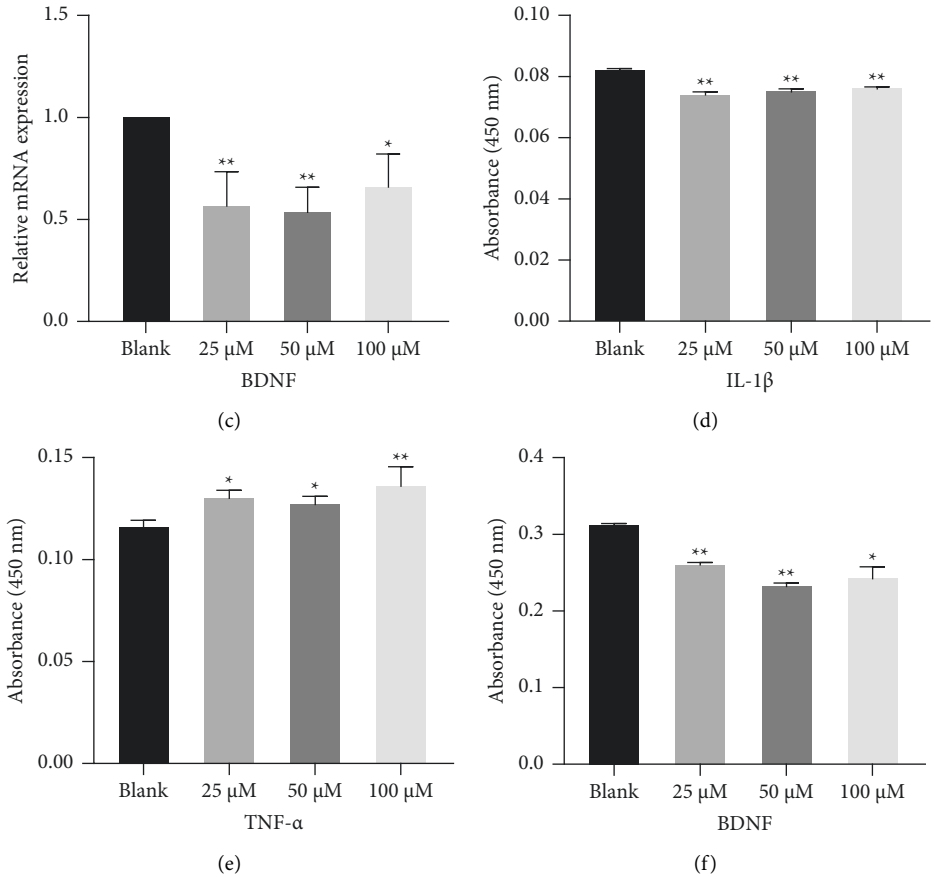

Figure 3: 5-HT treatment induces IL-1β, TNF-α and BDNF secretion in NG2 cells. (a-c) IL-1β, TNF-α and BDNF mRNA levels were determined using real-time PCR. (d-f) IL-1β, TNF-α and BDNF protein expression levels were measured using ELISA. (Data are expressed as the means +/− s.d. n = 3 for each group. $^{*}p < 0.05$, $^{**}p < 0.01$. s.d.: standard deviation).

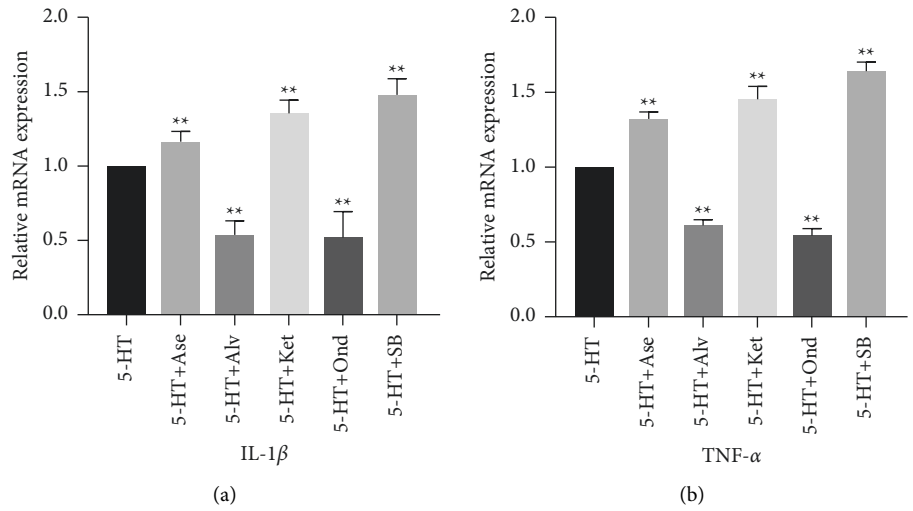

Figure 4: Continued.

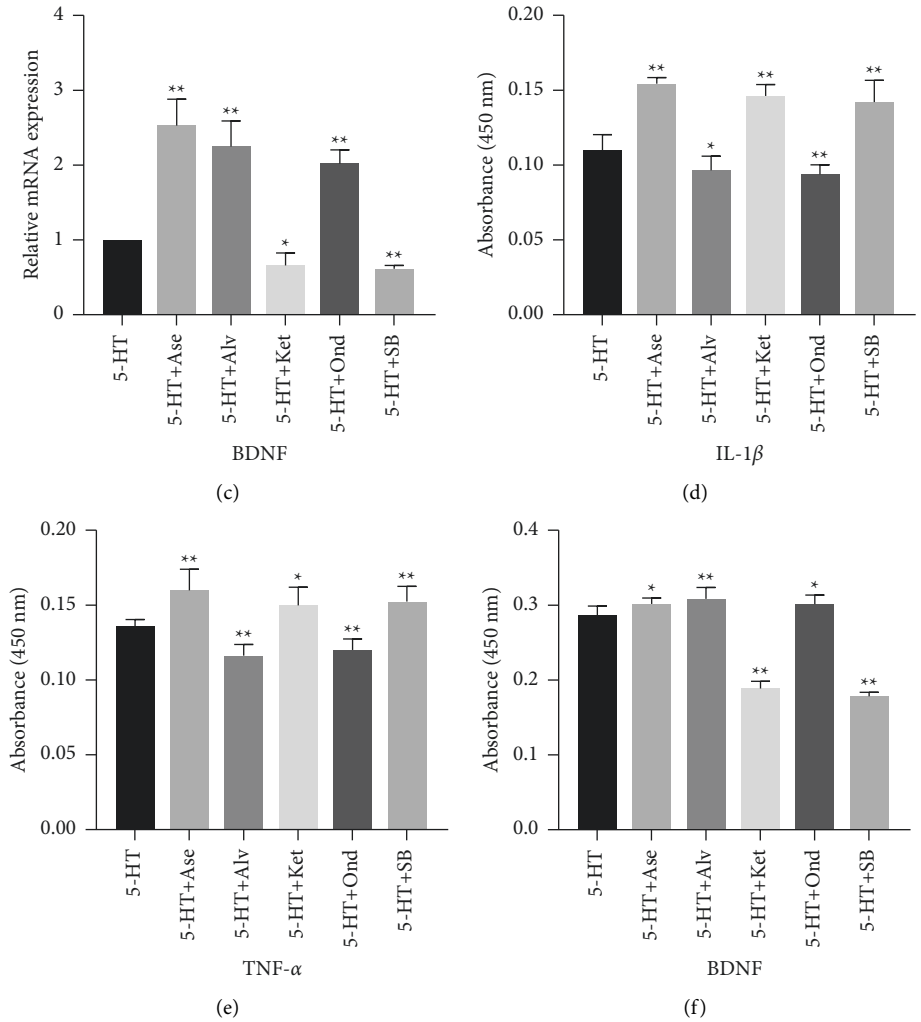

Figure 4: Effects of various antagonists of 5-HTRs on IL-1β, TNF-α and BDNF secretion from NG2 cells. (a-c) IL-1β, TNF-α and BDNF mRNA levels were estimated using real-time PCR. (d-f) IL-1β, TNF-α and BDNF protein expression was measured using ELISA. (Data are expressed as the means +/− s.d. n = 3 for each group. $^{*}p < 0.05$, $^{**}p < 0.01$. s.d.: standard deviation).

and TNF-α and elevated BDNF secretion (Figure 4). Blockade of 5-HT2AR and 5-HT6R reduced the secretion of BDNF and increased the secretion of IL-1β and TNF-α (Figure 4). These results indicated that 5-HT modulated IL-1β and TNF-α secretion via 5-HT1AR and 5-HT3R in NG2 cells. 5-HT modulated the secretion of BDNF via 5-HT2AR and 5-HT6R.

3.5. Role of PLC in the Secretion of Il-1β, TNF-α and BDNF in NG2 Cells. The effects of PLC on NG2 cell secretion of IL-1β, TNF-α and BDNF were determined using the PLC-specific activator m-3M3FBS and the specific inhibitor U-73122. As indicated in Figure 5, m-3M3FBS increased the expression of PLC in NG2 cells and increased the secretory level of BDNF but decreased IL-1β and TNF-α secretion from NG2 cells (Fig. 5(a)–5(d)). U-73122 decreased the expression of PLC and the secretion of BDNF but increased the levels of IL-1β and TNF-α in NG2 cells (Fig. 5(a)–5(d)). These results were confirmed using Western blotting (Figure 5(e)). These data demonstrated that secretion of IL-1β, TNF-α and BDNF in NG2 cells was associated with PLC signaling.

4. Discussion

With the widest distribution and the largest number of neural cells in the central nervous system, glial cells fulfil various physiological functions, including the orchestration of synaptic activity, maintenance of ion homeostasis, ensuring the integrity of the blood–brain barrier, and participating in neurotransmitter uptake [22, 23]. Previous studies confirmed the role of glial cells in the regulation of non-rapid eye movement sleep and cognitive functions [24]. Glial cells orchestrate the sleep-wake cycle by releasing different neurotransmitters, such as glutamate, serine, and adenosine triphosphate (ATP), and secrete pro-inflammatory cytokines, such as IL-1β and TNF-α, which aid in sleep. Glia also participate in the regulation of sleep homeostasis by regulating the level of extracellular adenosine and the activity of

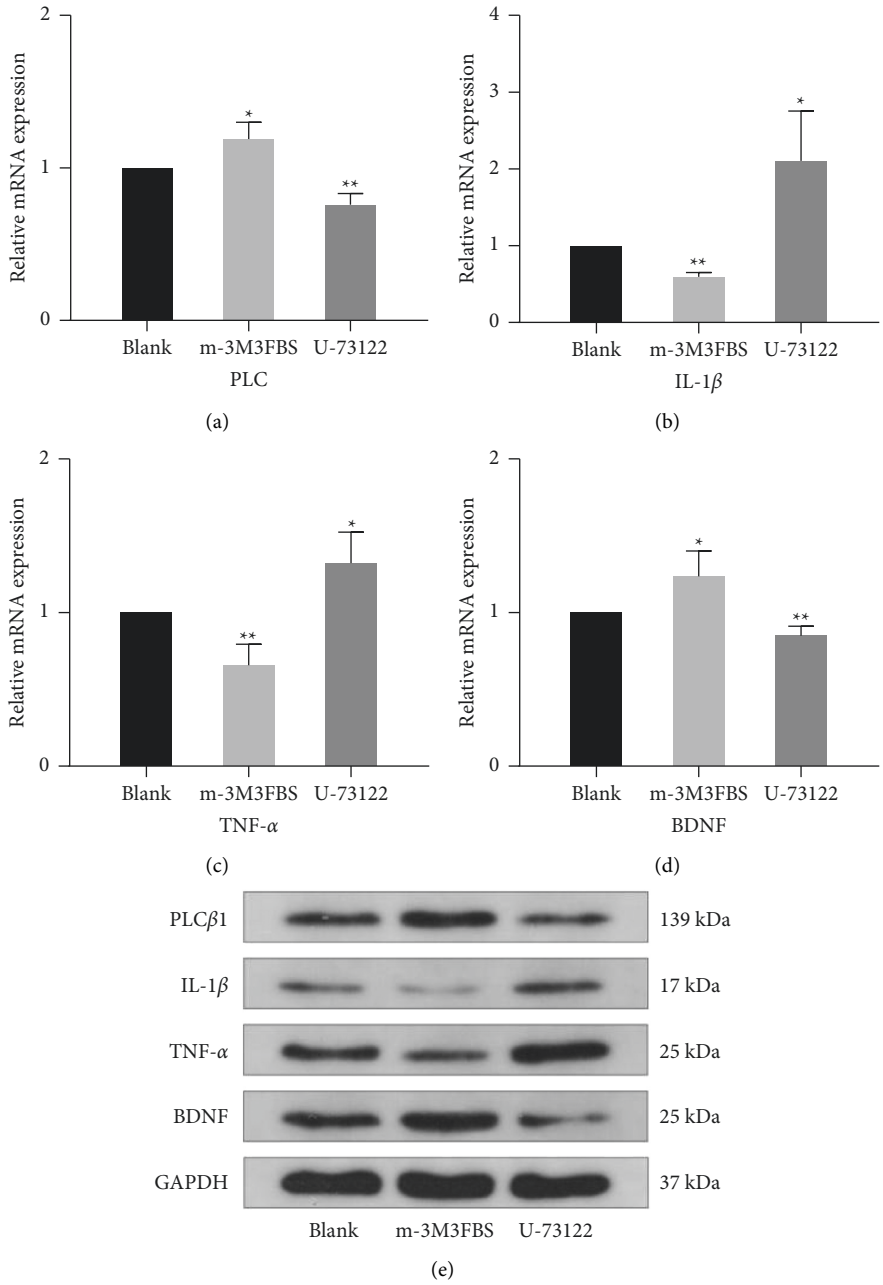

Figure 5: Effects of the specific activator m-3M3FBS and the specific inhibitor U-73122 of PLC on the secretion of IL-1β, TNF-α and BDNF in NG2 cells. (a-d) Real-time PCR was used to determine the expression of PLC, IL-1β, TNF-α and BDNF mRNA. (d-e) Western blotting was used to determine the expression of PLC, IL-1β, TNF-α and BDNF. GAPDH is shown as an internal control. (Data are expressed as the means +/− s.d. n = 3 for each group. $^{*}p < 0.05$, $^{**}p < 0.01$. s.d.: standard deviation).

adenosine receptor-1 (A1R) or adenosine receptor 2A (A2AR) on different neuronal membranes [25–27]. The number of NG2 cells increases during sleep increases compared to arousal and during sleep deprivation [28].The proliferation of NG2 cells is positively related to the frequency of rapid eye movement during sleep. The occurrence of this effect is orchestrated by Glu signal transduction initiated by neurons. This relationship suggests that the excitability of the central nervous system influences the proliferation and differentiation of NG2 cells [29, 30]. Activated NG2 cells secrete substances that affect the activity of neurons and glial cells [31, 32]. Ablation of NG2 cells in the prefrontal cortex adversely affects Glu receptors and Glu neurotransmission and disrupts Glu-related astrocytes and neuronal functions, which affect the excitability of the central nervous system.

In summary, NG2 cells may be related to central nervous system excitability and sleep-wake regulation. Notably, IL-1β, TNF-α and BDNF are associated with cortical excitability and sleep-wake. Increased expression of IL-1β and TNF-α in the brain was detected in rats completely deprived of sleep for 24 hours [33]. High levels of exogenous IL-1β and TNF-α expression induce slow-wave sleep [34]. Increased

expression of BDNF was detected in the hippocampus of mice after 1 day of sleep deprivation, and BDNF expression declined after 3 days of sleep deprivation [11]. These studies suggest that the roles of IL-1β, TNF-α and BDNF in sleep regulation are related to the degree of insomnia and the concentration and duration of IL-1β, TNF-α and BDNF effects. This study found that different concentrations of 5-HT (25, 50, 100 μM) stimulated NG2 cells to secrete IL-1β, TNF-α and BDNF. 5-HT (25, 50, 100 μM) significantly inhibited the secretion of IL-1β and BDNF, while 5-HT (25, 50, 100 μM) significantly increased the secretion of TNF-α. The results showed that 5-HT could significantly increase the secretion of TNF-α. NG2 cells were induced to secrete IL-1β, TNF-α and BDNF.

Our study revealed that 5-HT acted on NG2 cells to regulate the secretory levels of IL-1β, TNF-α and BDNF. 5-HT may regulate the release of IL-1β, TNF-α, BDNF and other sleep regulation-related active substances by affecting the glial system, which regulates the neuronal and glial cell activity involved in the regulation of cortical excitability. Their specific roles and mechanisms are discussed further below.

The raphe nucleus group, located in the reticular structure of the brainstem, contains the cell bodies of 5-HT neurons [35, 36]. Destruction of the raphe nucleus using electrocoagulation or serotonin receptor antagonists causes insomnia in animals. The use of p-chlorophenylalanine to deplete 5-HT in the brain also causes severe insomnia depending on the animal. This insomnia may be recovered by intracerebral injections of 5-HT. The injection of 5-HT into the fourth ventricle of a lightly anesthetized cat causes sleep-like brain waves [37, 38]. The RNA and protein of glial cells and neurons in the dorsal raphe nucleus of rats with sleep deprivation were found decreased 23% and 31%, respectively. After 48 hours, the RNA content reached normal levels, but the protein content was 21% lower than normal. Natural sleep increased the RNA concentration and absolute content of glial cells in locus coeruleus nucleus but reduced the absolute content of neuronal cytoplasmic protein. The protein concentration of neurons and glial cells increased, and the absolute content of glial cell protein increased. The concentration and absolute content of RNA were the same as the control group. Sleep regulation is closely related to 5-HT and glial cells, which affect cortical excitability [39, 40]. The present study explained, for the first time, the regulatory relationship between NG2 cells and 5-HT, which is consistent with previous studies on glial cells.

5-HT acts via 5-HT receptors. The 5-HTRs in mammals are organized into seven families (5-HT1-7R), and more than 16 subtypes have been described. 5-HT1AR and 5-HT1BR activation may hyperpolarize the cell membrane by inhibiting adenylate cyclase. 5-HT2AR and 5-HT2BR regulation by PLC leads to cell membrane depolarization. 5-HT3R activation depolarizes acetylcholine and amino acidergic neurons, and 5-HT6R and 5-HT7R receptor activation leads to depolarization of neurons that participate in the regulation of cortical excitability [41]. 5-HT functions as an activator in waking (W) but hinders REMS. The situation is similar in cats and rodents, and the process may be accomplished via 5-HT1R. 5-HT1AR and 5-HT1BR reduce REM sleep in mice, and 5-HT2AR, 5-HT2CR or 5-HT7R produce the opposite effect [42]. These studies suggest that 5-HT, acting on its receptors, is involved in cortical excitability that affects sleep regulation. PLC mediates cellular signaling via various metabotropic receptors. 5-HT2AR couples with the Gαq, Gαi/o and Gα12/13 proteins in vitro and in vivo. Stimulation of the Gαq pathway activates PLC, generates inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG), and releases calcium from intracellular stores to induce specific biological effects. The present study used 5-HT2AR receptor inhibitors and PLC signaling pathway inhibitors and showed consistent trends in the secretory levels of IL-1β, TNF-α and BDNF from NG2 cells. Our results suggest that 5-HT regulates the secretion of IL-1β, TNF-α and BDNF by affecting the 5-HT2AR-coupled PLC signaling pathway in NG2 cells. In summary, 5-HT may regulate cytokine activity via interaction with NG2 cells, which affects cortical excitability.

5. Conclusion

NG2 positive cells and 5-HT neuronal marker TPH were co-localized in the prefrontal cortex. 5-HT1A, 5-HT2A, 5-HT3, 5-HT6, and 5-HT7 receptors can be expressed on NG2 cells. 5-HT affected NG2 cells secretion of IL-1β, TNF-α, and BDNF via 5-HT1A, 5-HT2A, 5-HT3, and 5-HT6 receptors, through PLC signaling pathway.

Data Availability

The data used to support the findings of the study are available from the corresponding author upon reasonable request.

Ethical Approval

All animal experiments were carried out in accordance with the principles of experimental animal welfare ethics and were approved by the Experimental Animal Welfare Ethics Management Committee of Guizhou University of Traditional Chinese Medicine.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Tingting Yang and Yue Li contributed equally to this study.

Acknowledgments

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Research Article

Evaluation of the Safety and Efficacy of Xiao Yao San as a Treatment for Anxiety: A Systematic Review and Meta-Analysis

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Objective. Xiaoyao San (XYS) is a medicinal preparation that is commonly employed in China for the treatment of anxiety disorders (AD). Despite suggestions that it may offer certain advantages in this context, there are no reliable evidence-based studies regarding its efficacy at present. The present study was developed to gauge the efficacy and safety of XYS for the treatment of AD in a systematic manner. *Methods*. PubMed, the Cochrane Library, EMBASE, Web of Science, China National Knowledge Infrastructure (CNKI), Wanfang database, Weipu database, and China Biomedical Documentation Service System (CBM) databases were systematically searched for all randomized control trials (RCTs) evaluating the use of XYS for the treatment of AD published as of November 2021. Two investigators independently screened all studies, extracted data, and assessed the risk of bias for included studies using RevMan5.3. *Results*. In total, 9 RCTs incorporating 809 patients were included in the present meta-analysis, of which 3 compared oral XYS to anxiolytic treatment and 6 compared oral XYS + anxiolytics to anxiolytic treatment alone. The resultant meta-analysis revealed that XYS alone or in combination with anxiolytic treatment was associated with better improvements in anxiety-related symptoms and reduced adverse drug-related reactions as compared to anxiolytic treatment alone. *Conclusion*. The available evidence suggests that oral XYS alone or in combination with anxiolytic agents is more effective and safer than anxiolytic treatment alone when used for the treatment of AD. However, owing to the limited number and quality of the studies included in this analysis, further high-quality research will be essential to validate these results.

1. Introduction

Anxiety is an adverse emotional state in which individuals experience unease or nervousness that can be difficult to cope with. Anxiety disorders (ADs) are a group of psychological disorders characterized primarily by anxiety [1]. AD is associated with a lifetime prevalence of 13.6%–28.8% and an annual incidence rate of 5.6%–19.3%. An estimated 1% of all disability-adjusted life years are estimated to be lost due to anxiety-related factors such as panic attacks, obsessive-compulsive disorder, and posttraumatic stress disorder [2]. Rising levels of social pressure are resulting in rising annual AD incidence rates [3]. First-line pharmacological treatments for AD include a range of anxiolytics such as selective serotonin reuptake inhibitors (SSRIs), benzodiazepines, serotonin-norepinephrine reuptake inhibitors (SNRIs), noradrenergic and specific serotonergic antidepressants (NaSSAs), tricyclic antidepressants (TCAs), and azapirones [4]. While clinical trials have confirmed that these agents can very effectively treat AD [5–8], they are associated with adverse drug reactions and withdrawal symptoms that can make them undesirable for some patients [9], underscoring the need for the development of alternative safe and effective treatments. Traditional Chinese

medicine (TCM) approaches offer advantages including excellent safety profiles and multitarget multipathway mechanisms of action, providing effective complementary and alternative treatments for AD [10].

The etiological basis for psychological illnesses and associated treatment methods is highly varied [11]. In TCM theory, the pathogenesis of AD is primarily believed to be associated with the stagnation of the liver and qi together with the dysfunction of the five internal organs, with excess and deficiency also contributing to this condition [12]. Xiaoyao San (XYS) is a TCM preparation consisting of Chai Hu (*Bupleurum*), Dang Gui (*Angelica*), Bai Shao (white peony), Bai Zhu (*Atractylodes*), Fu Ling (Poria), Sheng Jiang (ginger), Bo He (peppermint), and Zhi Gan Cao (roasted licorice). XYS is widely used for the treatment of anxiety and has been reported to relieve depression, soothe the liver, and strengthen the blood and spleen [13]. A number of recent studies have evaluated the efficacy of XYS as a treatment for AD, and multiple randomized controlled trials (RCTs) have found that it is superior to control treatments in terms of improved efficacy, shorter duration of treatment, and lower rates of related adverse drug reactions. Several basic research studies have also suggested that XYS exhibits anxiolytic activity when used to treat AD. For example, Sun et al. employed XYS for the treatment of chronic stress injury model rats and assessed hippocampal *Gabra4* gene expression in these animals, revealing that such treatment was sufficient to downregulate *Gabra4* and to thereby alleviate chronic stress-related damage via soothing the liver and alleviating anxiety [14]. However, different studies have employed different XYS treatment strategies and study designs, making it challenging to draw reliable conclusions regarding the utility of this TCM preparation. The present systematic review was thus constructed to explore the safety and efficacy of XYS as a treatment for AD in an effort to provide an evidence-based foundation for future research and clinical treatment efforts.

2. Methods

This study was conducted as per the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines [14] and is registered in PROSPERO (registration number: CRD42021285024).

2.1. Search Strategy. The PubMed, the Cochrane Library, EMBASE, Web of Science, China National Knowledge Infrastructure (CNKI), Wanfang database, Weipu database, and China Biomedical Documentation Service System (CBM) databases were searched for all relevant studies published as of November 2021 using a combination of subject words and free words. Retrieval words included anxiety disorder, anxiety state, Xiaoyao San, and Xiaoyao Pill (Appendix 1). All literature searches were independently performed by two investigators (Jin Lin and Yue Ji), with disagreements being resolved through discussion with a third investigator (Jinhua Si).

2.2. Inclusion and Exclusion Criteria

2.2.1. Inclusion Criteria

(1) Study type: RCTs exploring the use of XYS for the treatment of AD.

(2) Diagnostic criteria: patients were diagnosed with AD as per the criteria included in the "Chinese Classification and Diagnostic Criteria for Mental Disorders" [15], with TCM diagnostic criteria being made in reference to the "Criteria for Diagnosis and Efficacy of TCM Diseases."

(3) Intervention measures: patients in the treatment group were treated with oral XYS either alone or in combination with other anxiolytic drugs, while patients in the control group were treated with the same anxiolytic agents used in the treatment group. XYS oral preparations eligible for inclusion in this analysis included XYS granules, XYS soup (XYS with the addition or removal of up to three herbs based on patient symptoms), and other dosage strategies.

(4) Outcome indicators: the primary outcome indicators for this study included: (1) the total efficacy rate as a means of gauging reductions in Hamilton anxiety scale (HAM-A) scores. For this endpoint, anxiety reduction rates were scored as follows: a reduction rate >75% was considered to be indicative of recovery, while a reduction rate >50% was considered a marked effect, a reduction rate ≥25% was considered effective, and a reduction rate <25% was considered ineffective. The total efficacy rate was calculated as follows: total efficacy = (recovery number + marked effect number + effective number)/total number * 100%. (2) HAM-A scores.

Secondary outcome indicators included: (1) Self-Rating Anxiety Scale (SAS) values; (2) Treatment Emergent Symptom Scale (TESS) values; (3) Traditional Chinese Medicine Symptom Observation Scale scores; and (4) adverse reaction rates.

2.2.2. Exclusion Criteria

(1) Duplicate studies or republished datasets were excluded, with only the most complete and highest quality study being included in the pooled analysis

(2) Studies with incomplete data or obvious errors that could not be corrected by contacting the corresponding author were excluded

(3) Studies that did not report observation outcome indicators were excluded

(4) The use of other TCM treatment techniques (such as acupuncture and massage) during the treatment process led to study exclusion regardless of whether it was in the treatment group or the control group

2.3. Study Selection and Data Extraction. Study screening was independently performed by two investigators with

reference to the above inclusion and exclusion criteria, with disagreements being resolved through discussion and consensus or consultation with a third investigator. The data extracted from included studies included: (i) basic information including title, first author, publication year, numbers of patients per group, and baseline patient characteristics; (ii) interventional measures and treatment courses for the treatment and control groups; (iii) outcome indicators; and (iv) elements necessary for risk of bias assessments.

2.4. Risk of Bias Analysis. The risk of bias for included studies was independently quantified by two investigators, with disagreements being resolved through discussion with a third investigator. The risk of bias was measured with the RCT bias risk assessment tool from the Cochrane Manual 5.1.0 [16].

2.5. Statistical Analysis. RevMan5.3 was used to conduct the present meta-analysis. When continuous data were measured using the same measurement tools and units, they were analyzed based on weighted mean difference (WMD) values, whereas they were otherwise analyzed using standard mean difference (SMD) values. Dichotomous variables were analyzed using relative risk (RR) values and 95% confidence intervals (95% CIs). The chi-squared test was used to detect heterogeneity among studies with the I^2 statistic. When no significant heterogeneity was detected ($P > 0.10$, $I^2 < 50\%$), results were analyzed with a fixed-effects model. When significant heterogeneity was detected ($P > 0.10$, $I^2 \geq 50\%$), subgroup or sensitivity analyses were used to explore potential sources of heterogeneity. When clear sources of clinical or methodological heterogeneity had been removed, a pooled meta-analysis was conducted using a random-effects model. The influence of individual studies on pooled results was assessed through sensitivity analyses, with individual studies being removed from the overall analysis to look for sources of heterogeneity. For primary outcome indicators, when 10 or more studies were available, publication bias was detected via visual inspection of funnel plots and through Egger's test and Begg's test.

3. Results

3.1. Study Characteristics. In total, the initial search strategy retrieved 1180 potentially relevant studies, of which 661 remained following the removal of duplicates. Of these, 612 were excluded following preliminary abstract and title reviews, while 40 were excluded following full-text review. The remaining 9 studies were included in the final analysis. The overall screening process is detailed in Figure 1.

The key characteristics of the included studies are listed in Table 1. The treatment group consists of patients treated with XYS alone or in combination with other antianxiety drugs, while the control group consists of patients treated with antianxiety drugs only. Interventional approaches for the included studies are listed in Table 2.

3.2. Risk of Bias Analysis. An RCT approach was employed to assess the quality of the 9 studies included in the present meta-analysis as per the Cochrane Manual 5.1.0. Just two studies employed appropriate random sequence generation methods [17, 18], while no studies mentioned the use of appropriate blinding techniques [17–25]. All studies exhibited an unclear risk of bias with respect to allocation concealment and outcome assessment blinding [17–25]. Moreover, all studies exhibited a low risk of bias with respect to selective reporting, incomplete outcome indicators, and other forms of bias [17–25]. The results of these analyses are listed in Figure 2.

3.3. Meta-Analysis Results. Of the 9 included studies, 3 compared oral XYS alone to anxiolytics, while 6 compared oral XYS + anxiolytics with anxiolytics.

3.3.1. Oral XYS Alone vs. Anxiolytics

(1) Total Efficacy Rates. Total efficacy rates were reported by 3 of the included RCTs, and significant heterogeneity was detected among the results of these analyses ($P = 0.07$, $I^2 = 63\%$). When studies were iteratively omitted from this analysis, the exclusion of the study conducted by Li et al. eliminated this heterogeneity ($P = 0.20$, $I^2 = 40\%$, Figure 3), indicating that this study was a source of substantial heterogeneity. The pooled data were then analyzed with a random-effects model, the efficacy of oral XYS was found to be significantly superior to that of oral anxiolytic treatment ([RR = 1.19, 95% CI: 1.01, 1.40, $P = 0.04$]).

(2) HAM-A Scores. HAM-A scores were only reported in a single trial, thus precluding the performance of a meta-analysis. Descriptive analysis of these results indicated that scores in the treatment group were significantly reduced relative to the control group ([MD = −6.52, 95%CI: −7.45, −5.59]), $P < 0.00001$]).

(3) TCM Syndrome Scale. The TCM Syndrome Scale was only reported in a single RCT, thus precluding the performance of a pooled meta-analysis. Descriptive analysis indicated that the scores for the treatment group were significantly lower than those for the control group ([MD = −3.30, 95%CI: −4.16, −2.44]), $P < 0.00001$]).

(4) Adverse Event Rates. Adverse event rates were reported by 2 RCTs, with no significant heterogeneity being observed for the pooled results ($P = 0.39$, $I^2 = 0\%$). Data were thus analyzed using a fixed-effects model, revealing that adverse event rates were significantly lower in the treatment group relative to the control group ([RR = 0.05, 95% CI: 0.01, 0.20, $P < 0.0001$]; Figure 4).

3.3.2. Oral XYS + Anxiolytics vs. Anxiolytics Alone

(1) Total Efficacy Rates. In total, 6 RCTs reported total efficacy rates, with no significant heterogeneity among these studies ($P = 0.85$, $I^2 = 0\%$). Data were analyzed with fixed-

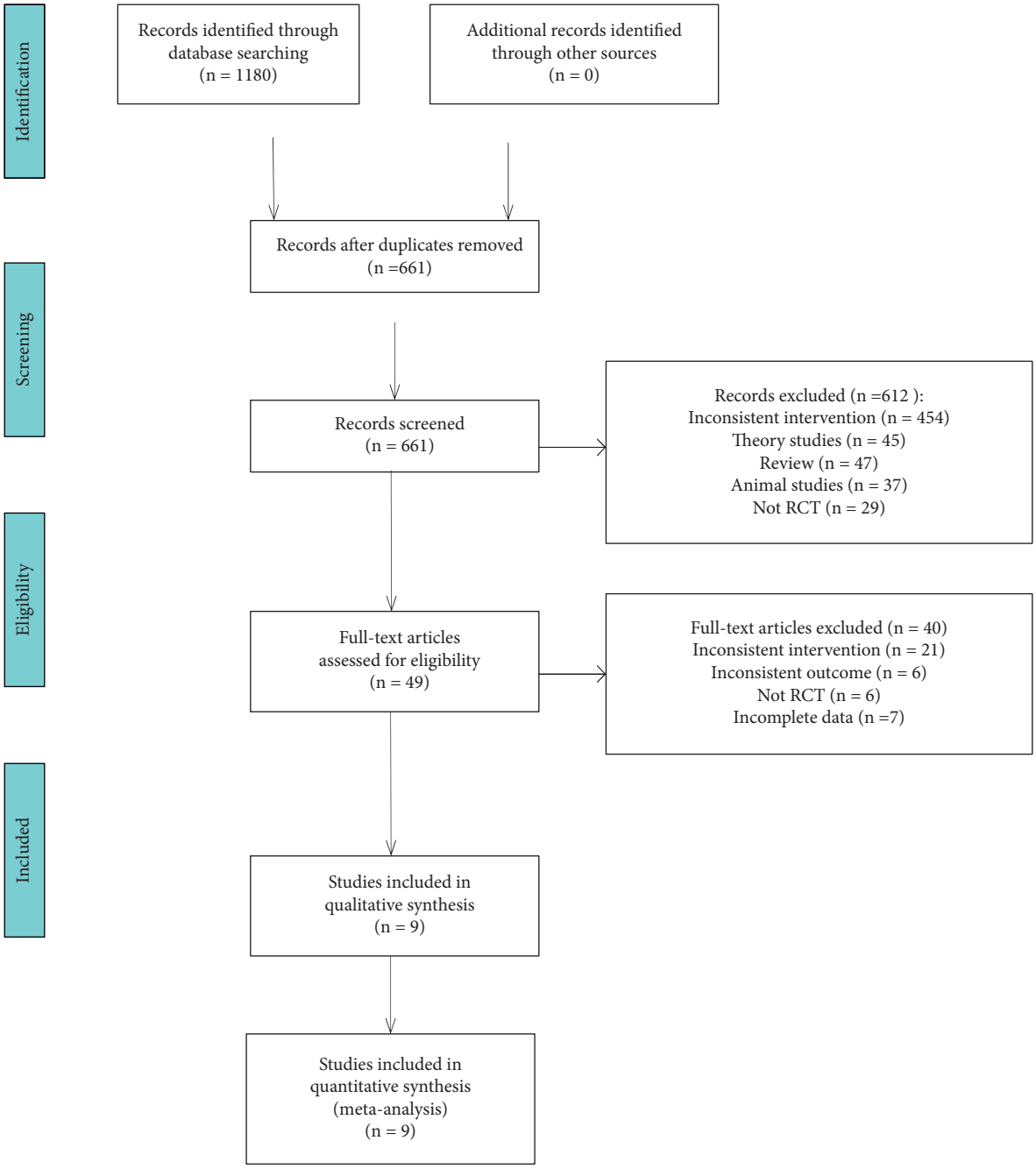

Figure 1: Flow diagram.

Table 1: Included study characteristics.

Study cohort	No (T/C)	Gender		Age		Course (day)	Outcome
		T	C	T	C		
Chen and Lj [17]	81/78	40/41	40/38	43.65 ± 8.01	43.93 ± 10.56	42	①②⑤
Deng [18]	59/57	26/33	30/27	43.81 ± 10.23	44.53 ± 11.65	28	①②⑥
Ding [19]	37/38	—	—	29.46 ± 6.82	27.62 ± 4.24	56	①②③
Li [20]	100/50	—	—	—	—	56	①⑥
Li [20]	32/32	15/17	14/18	41.6 ± 7.8	42.1 ± 7.2	42	①②⑥
Li [21]	30/30	22/8	19/11	43–79	41–80	28	①⑥
Wang [22]	32/31	18/14	20/11	19–57	20–56	56	①②
Wang [23]	30/30	16/14	15/15	18–48	20–51	42	①②⑥
Zhu [24]	28/28	—	—	—	—	42	①②④

Note: outcome: ① efficiency; ② HAMA; ③ SAS; ④ TESS; ⑤ symptom rating scale of TCM; ⑥ adverse reaction rate; —: unclear.

Table 2: Intervention characteristics.

Study	Interventions of treatment group		Interventions of control group	Days
	XYS	Anxiolytics	Anxiolytics	
Chen and Lj [17]	Xiaoyao san decoction, 150 ml Bid	None	Flupentixol and melitracen tablets, 10.5 mg Bid	42
Deng [18]	Xiaoyao san granule, 5 g Bid	Paroxetine, 20–40 mg Qd	Paroxetine, 20–40 mg Qd	28
Ding [19]	Xiaoyao san decoction Bid	Buspirone, 5–10 mg Tid	Buspirone, 5–10 mg Tid	56
Li [20]	Xiaoyao san granule, 8 granule Tid	None	Alprazolam, 0.4–1.2 mg/day	56
Li [20]	Xiaoyao san decoction Bid	Buspirone, 10 mg Tid	Buspirone, 10 mg Tid	42
Li [20]	Xiaoyao san decoction, 200 ml Bid	None	Paroxetine, 20 mg Qd	28
Wang [22]	Xiaoyao san decoction, 150 ml Bid	Doxepin 25 mg tid + alprazolam 0.4–1.2 mg Bid + oryzanol 20 mg Tid	Doxepin 25 mg tid + alprazolam 0.4–1.2 mg bid + oryzanol 20 mg Tid	56
Wang [23]	Xiaoyao san granule, 9 g Tid	Flupentixol and melitracen tablets, 10.5 mg Bid	Flupentixol and melitracen tablets, 10.5 mg Bid	42
Zhu [24]	Xiaoyao san decoction, 150 ml Bid	Buspirone, 10 mg Tid	Buspirone, 10 mg Tid	42

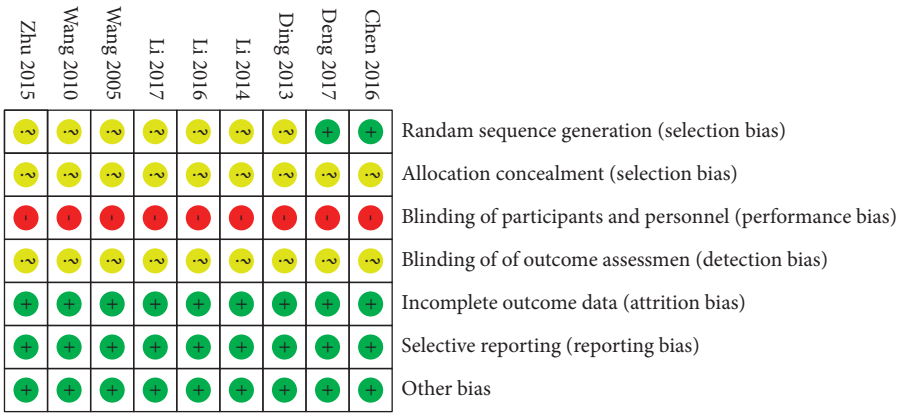

Figure 2: Risk of bias summary.

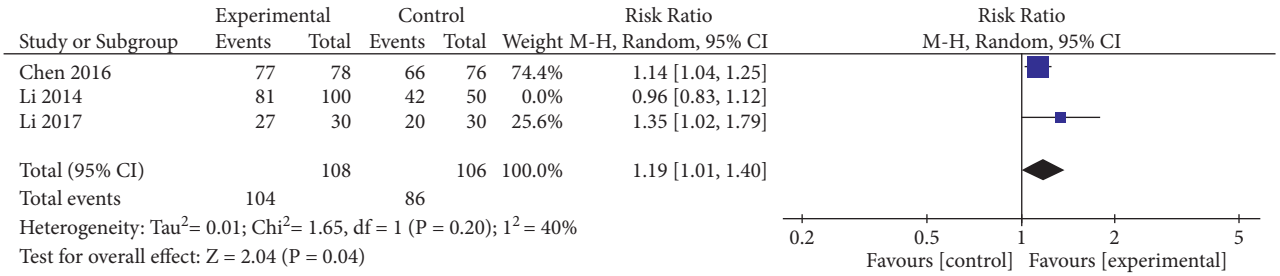

Figure 3: Meta-analysis of total efficacy rates for oral XYS alone vs. anxiolytics.

effects models, revealing significantly better total efficacy rates in the treatment group relative to the control group ([RR = 1.20, 95% CI: 1.11, 1.30, $P < 0.00001$], Figure 5). As there was substantial variability with respect to treatment duration among these different studies, we additionally conducted a subgroup analysis based on differences in treatment course by separating patients into those treated for ≤42 days and >42 days. Heterogeneity analyses revealed no significant heterogeneity for the ≤42 day ($P = 0.64$, $I^2 = 0\%$), or >42 day ($P = 0.72$, $I^2 = 0\%$) treatment groups (Figure 6), with fixed-effects models thus being used for pooled analysis. In both the ≤42 day and >42 day treatment groups, oral XYS + anxiolytic treatment was associated with better efficacy than that observed for oral anxiolytics alone (≤42 days

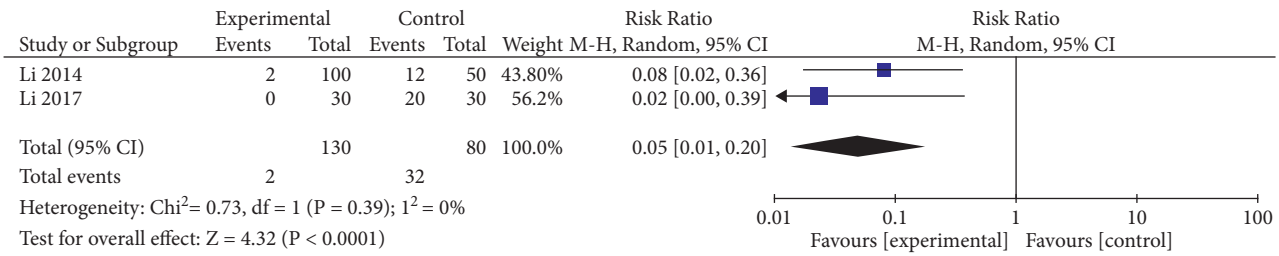

Figure 4: Meta-analysis of adverse event rates when comparing oral XYS alone vs. anxiolytics.

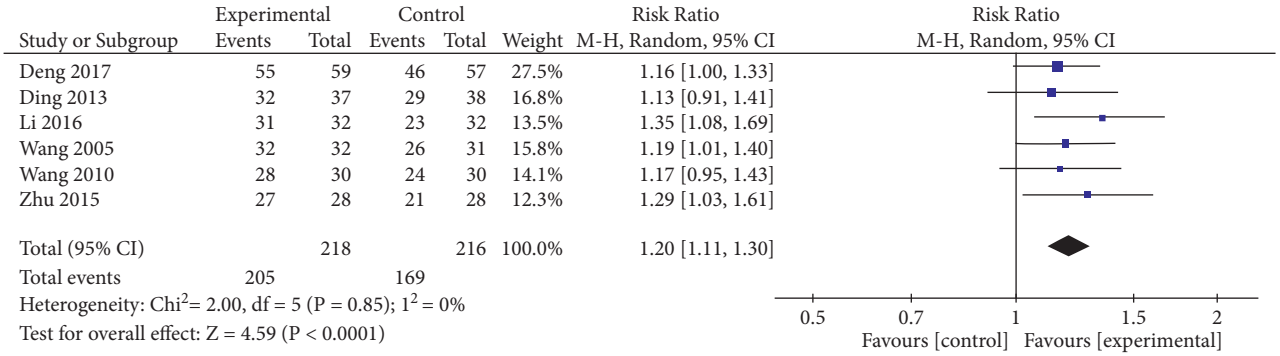

Figure 5: Meta-analysis of total efficacy rates when comparing oral XYS + anxiolytics vs. anxiolytics alone.

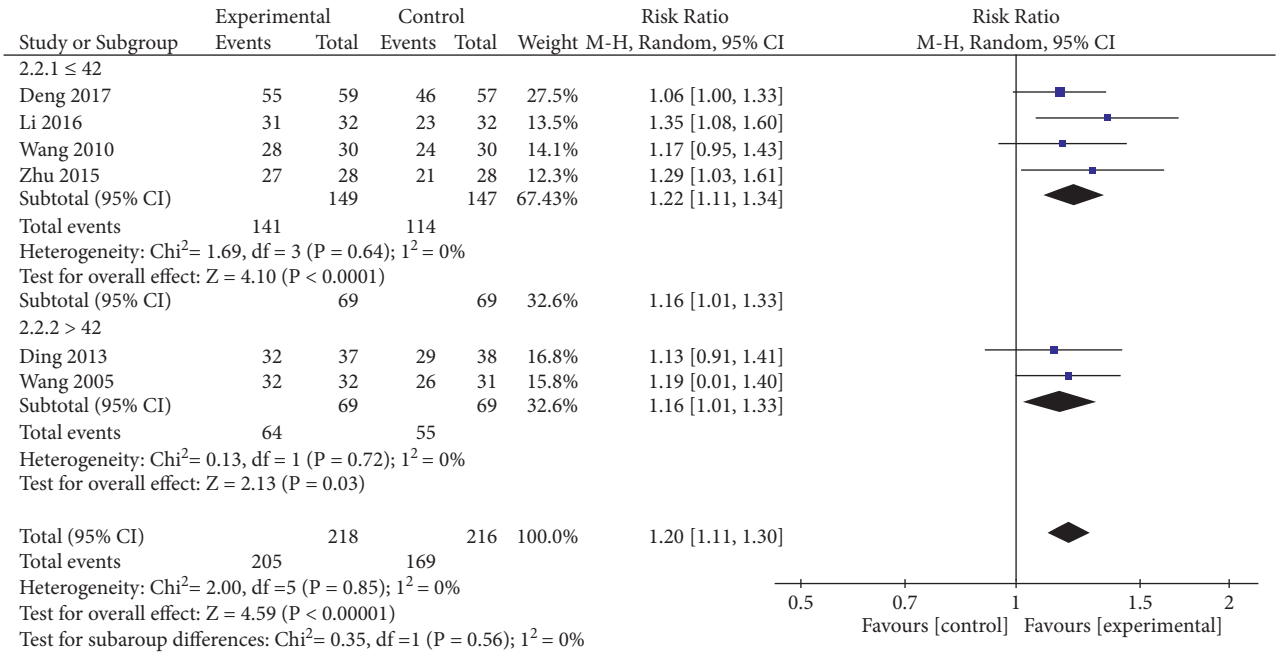

Figure 6: Subgroup analysis comparing total efficacy rates for oral XYS + anxiolytics to those for anxiolytics alone.

[RR = 1.22, 95% CI: 1.11, 1.34, $P < 0.0001$]; >42 days [RR = 1.16, 95% CI: 1.01,1.33, $P = 0.03$]).

(2) HAM-A Scores. HAM-A scores were reported in 6 studies, and significant heterogeneity was detected among these studies ($P < 0.00001$, $I^2 = 86\%$). However, the confidence interval for the forest plot was to the left of the invalid line, indicating that such heterogeneity had no impact on the overall results. Pooled data were thus analyzed with a random-effects model. Pooled analysis indicated that HAM-A scores were significantly lower in the treatment group relative to the control group ([MD = −4.22, 95% CI: −6.24, −2.19, $P < 0.0001$]; Figure 7). Next, subgroup analyses were conducted based on treatment course in order to identify sources of heterogeneity. Significant heterogeneity was detected in the >42 day treatment subgroup ($P = 0.007$, $I^2 = 86\%$), while no significant heterogeneity was detected in the ≤42 day treatment subgroup ($P = 0.13$, $I^2 = 48\%$) (Figure 8). Next,

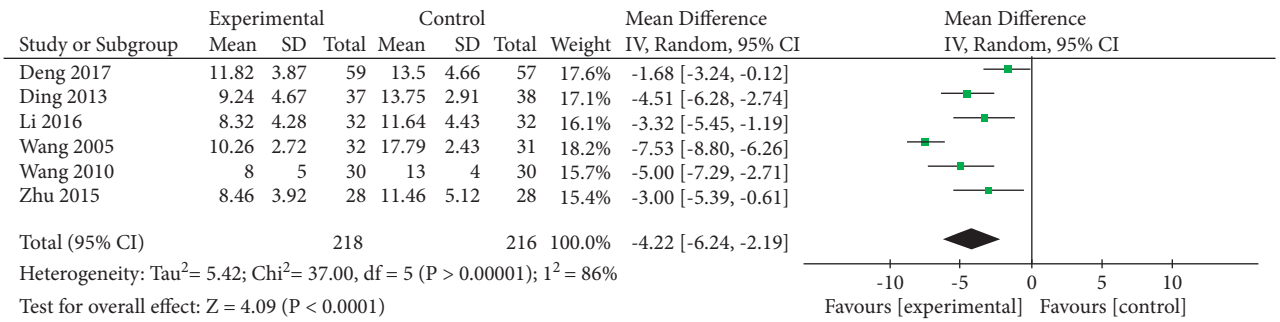

Figure 7: Meta-analysis comparing HAM-A scores for oral XYS + anxiolytics to those for Anxiolytic treatment alone.

heterogeneity was assessed for subgroups of patients with pretreatment HAM-A scores >29 points and pretreatment HAM-A scores >14 and ≤29 points. However, significant heterogeneity was still detected in this pooled analysis, indicating that HAM-A scores or numbers of treatment days were not the sources of heterogeneity in the pooled result analysis. Sensitivity analysis additionally failed to significantly decrease or increase heterogeneity or effect size. Given that the overall heterogeneity was relatively low and that no individual study biased these results, these results suggest that oral XYS + anxiolytic treatment can achieve superior efficacy to anxiolytic treatment alone as a means of lowering AD patient HAM-A scores.

(3) SAS. SAS scores were reported in just 1 RCT. As a meta-analysis could not be performed, descriptive analysis was instead conducted, revealing significantly lower SAS scores in the treatment group relative to the control group ([MD = −4.56, 95% CI: −7.19, −1.93]), $P = 0.0007$]).

(4) TESS. TESS scores were reported in 2 RCTs. No heterogeneity was detected when analyzing these data ($P = 0.89$, $I^2 = 0\%$), and results were thus analyzed with a fixed-effects model. Pooled analysis indicated that TESS scores in the treatment group were significantly lower than those in the control group ([MD = 3.64, 95% CI: −4.01, −3.26, $P < 0.00001$]; Figure 9).

(5) Adverse Event Rates. Adverse event rates were reported by two of the included RCTs. No heterogeneity was detected when evaluating these studies ($P = 0.69$, $I^2 = 0\%$), and results were thus analyzed with a fixed-effects model. The pooled meta-analysis indicated that adverse event rates in the treatment group were significantly lower than those in the control group ([RR = 0.18, 95% CI: 0.05, 0.67, $P = 0.01 < 0.05$]; Figure 10).

4. Discussion

Here, we conducted a pooled meta-analysis of 9 RCTs in which XYS was employed for the treatment of AD. The results of these analyses indicated that XYS treatment, either alone or in combination with anxiolytic agents, was superior to anxiolytic treatment alone with respect to total efficacy rates. Subgroup analysis further indicated that this effect remained evident irrespective of treatment duration. Moreover, we found that HAM-A scores, which are commonly used to assess anxiety symptoms, improved more significantly for patients treated with XYS than for patients treated with anxiolytics, irrespective of treatment duration or pretreatment HAM-A scores. SAS scores are used to assess anxiety severity, and while only one study assessed the scores in patients undergoing oral XYS + anxiolytic treatment, descriptive analysis indicated that such treatment was superior to anxiolytic treatment alone. The TCM Syndrome Scale is used to evaluate patient discomfort symptoms. As relatively few studies included this scale, a descriptive analysis was instead conducted, revealing that XYS treatment alone was superior to anxiolytic treatment. With respect to adverse event rates, fewer adverse reactions were reported in the XYS and XYS + anxiolytic groups compared to anxiolytic treatment alone. A meta-analysis of the results comparing the effects of oral XYS + anxiolytic treatment to anxiolytic treatment revealed scores were significantly lower in the treatment group relative to the control group. These data suggest that oral XYS is thus safe and effective as a treatment for AD, reducing drug treatment-related adverse reactions.

Anxiety is a psychological condition in which individuals experience episodes of distress and unease that can adversely affect their social function [26]. The prolonged alertness experienced by those with anxiety can increase the risk of cardiovascular disease and cerebrovascular disease [27]. Chinese medicinal approaches draw from thousands of years of experience, offering many advantages as treatments for psychological disorders [28].

XYS is among the most frequently utilized TCM preparations for the treatment of psychological illnesses, and it has been shown to exhibit psychotropic and anxiolytic activity in animal model studies [29]. Several reports have suggested that XYS may exert its anxiolytic activity in part through modulation of the intestinal microflora, increasing the relative abundance of anaerobic bacteria within the intestines and the associated production of intestinal-derived short-chain fatty acids (SCFAs). These changes can prevent bacterial migration and reduce peripheral inflammation, with neuroinflammation ultimately being alleviated through changes in both central and peripheral immunity, thereby mediating an antianxiety effect [30]. *Bupleurum* is the key drug in many spiritual prescriptions, and several pharmacological studies have suggested that it can relieve

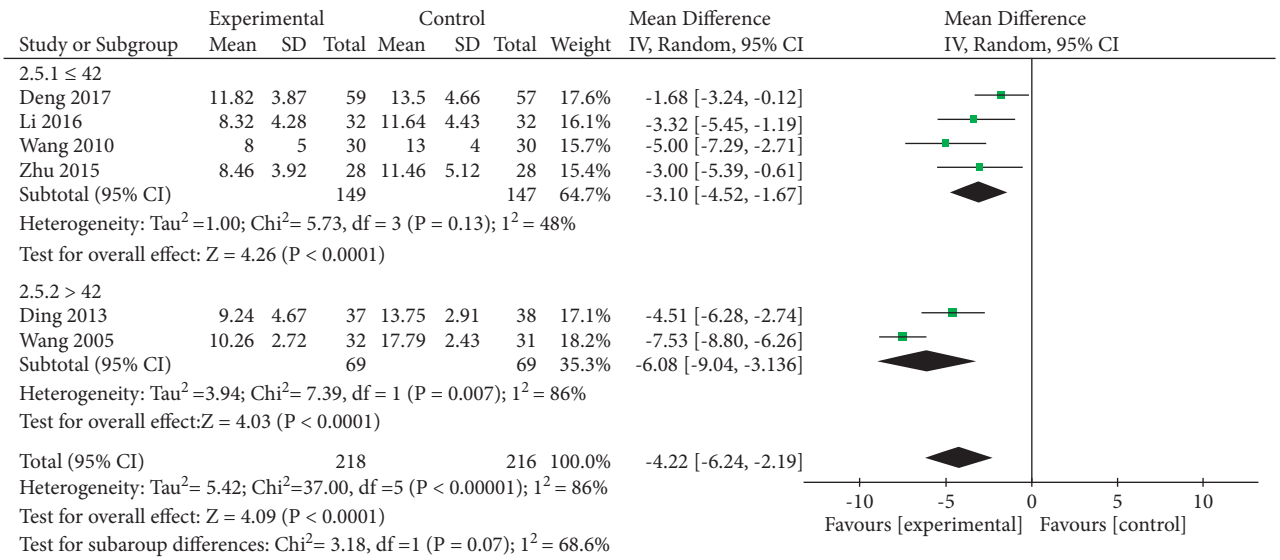

Figure 8: Subgroup analysis comparing HAM-A scores for oral XYS + anxiolytic treatment to those for Anxiolytics alone.

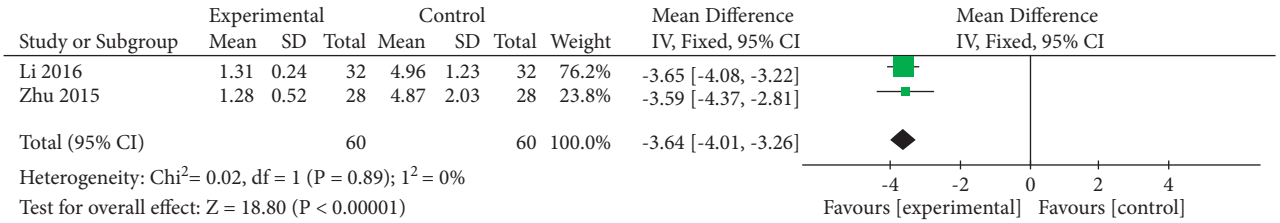

Figure 9: Meta-analysis comparing TESS scores for Oral XYS + Anxiolytic treatment to those for Anxiolytics alone.

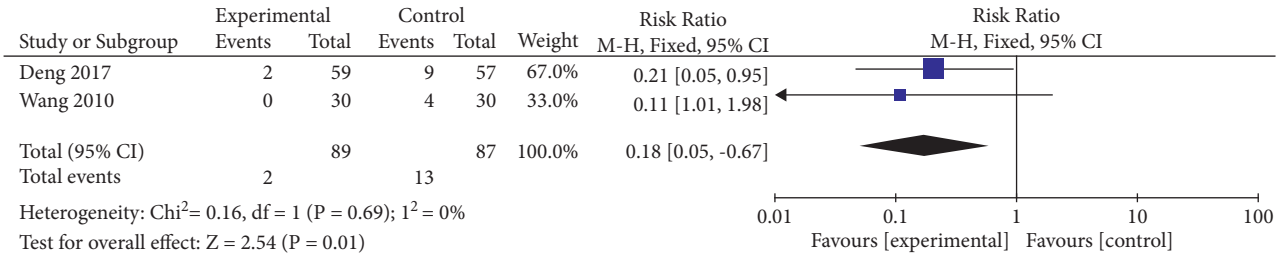

Figure 10: Meta-analysis comparing adverse event rates for oral XYS + anxiolytic treatment to those for anxiolytics alone.

neuronal apoptosis and associated neuroinflammation [31], increasing concentrations of nerve growth factors and brain-derived neurotrophic factor [32].

This study is subject to several limitations. For one, many of the included studies did not specify the allocation concealment or blinding approaches employed, and the results may thus be susceptible to measurement bias and selection basis. In addition, this study did not assess the degree of anxiety, education level, previous life experience, or concomitant diseases in the included patients. These factors will affect the treatment of anxiety, and their omission may lead to heterogeneity among study results. In addition, all the studies were from China and may thus not be generalizable. There were also differences in the dosage and composition of XYS used in these different studies, potentially influencing pooled analysis results.

5. Conclusions

Current research suggests that XYS treatment can effectively alleviate AD patients' anxiety symptoms while reducing rates of adverse drug reactions as compared to anxiolytic treatment. The overall efficacy of XYS alone or in combination with anxiolytic agents was no less than that of anxiolytic agents alone in our pooled analysis. However, to validate these results, additional large-scale multicenter high-quality clinical trials will be essential, thereby providing a foundation for future patient treatment.

Abbreviations

AD: Anxiety disorders
SSRI: Selective serotonin reuptake inhibitor

SNRI: Serotonin norepinephrine reuptake inhibitor
NaSSA: Noradrenergic and specific serotonergic antidepressant
TCAs: Tricyclic antidepressants
TCM: Traditional Chinese medicine
XYS: Xiao yao san
RCTs: Randomized controlled trials
HAMA: Hamilton Anxiety Scale
SAS: Self-Rating Anxiety Scale
TESS: Treatment Emergent Symptom Scale
WMD: Weighted mean difference
SMD: Standard mean difference
RR: Relative risk.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Jin Lin and Yue Ji are the co-first authors.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Jin Lin and Yue Ji contributed equally to this work.

Acknowledgments

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Supplementary Materials

Appendix 1. Search strategy in PubMed database. (Supplementary Materials). Appendix 2. The PRISMA checklist. (*Supplementary Materials*)

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Review Article

Ethnobotany, Phytochemistry, Biological Activities, and Health-Promoting Effects of the Genus *Bulbophyllum*

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The genus *Bulbophyllum* is of scientific interest due to the phytochemical components and diverse biological activities found across species of the genus. Most *Bulbophyllum* species are epiphytic and located in habitats that range from subtropical dry forests to wet montane cloud forests. In many cultures, the genus *Bulbophyllum* has a religious, protective, ornamenting, cosmetic, and medicinal role. Detailed investigations into the molecular pharmacological mechanisms and numerous biological effects of *Bulbophyllum* spp. remain ambiguous. The review focuses on an in-depth discussion of studies containing data on phytochemistry and preclinical pharmacology. Thus, the purpose of this review was to summarize the therapeutic potential of *Bulbophyllum* spp. biocompounds. Data were collected from several scientific databases such as PubMed and ScienceDirect,

other professional websites, and traditional medicine books to obtain the necessary information. Evidence from pharmacological studies has shown that various phytoconstituents in some *Bulbophyllum* species have different biological health-promoting activities such as antimicrobial, antifungal, antioxidant, anti-inflammatory, anticancer, and neuroprotective. No toxicological effects have been reported to date. Future clinical trials are needed for the clinical confirmation of biological activities proven in preclinical studies. Although orchid species are cultivated for ornamental purposes and have a wide traditional use, the novelty of this review is a summary of biological actions from preclinical studies, thus supporting ethnopharmacological data.

1. Introduction

Orchids are the largest group of angiosperms consisting of nearly 28,000 species with over 736 genera [1]. Although orchids are found in natural habitats in several parts of the world, their presence is decreasing due to great demand by the population [2]. Due to habitat destruction and indiscriminate collection, Orchid species are at a steady loss [3]. One of the most represented genera is *Bulbophyllum* Thouars (*Orchidaceae*: subfamily *Epidendroideae*, subtribe *Bulbophyllinae*) with ca. 2,200 species distributed in Africa and Asia (China, Nepal, India, Thailand, Laos, and Vietnam) [4]. The taxonomic history of *Bulbophyllum* has been complex since its establishment [5]. The great dimension and indefinite infrageneric systematics of this genus bring significant difficulties, reducing evolution, ecology, and morphology research [6]. Taxonomists have reported at least 24 closely related genera, classified based on floral morphology [7]. Only recently, molecular biology techniques have allowed recognizing this genus as monophyletic, i.e., belonging to a common ancestor [8].

Most *Bulbophyllum* species are epiphytic and located in habitats that range from subtropical dry forests to wet montane cloud forests [9, 10]. They exclusively obtain water and nutrients from air, rain, and debris and thus must be able to overcome difficult environmental conditions by storing water in the pseudobulbs [11]. In addition to the economic importance attributable to ornamental uses, herbal medicinal properties of phytochemical substances of biological interest (such as flavonoids, sterols-terpenoids, and phenolic acids) in *Bulbophyllum* species have also been reported [12, 13]. Several studies report both the phytochemical compounds and the molecules' biological effects extracted from *Bulbophyllum* leaf, pseudobulb, and root, for traditional medicine treatments [14].

In a continuous effort by researchers to discover the biological activities of Bulbophyllum species, we describe and summarize recent research in ethnobotanical knowledge, phytochemistry, and pharmacological properties, along with the limitations of the research in our article.

2. Methodology

To conduct this review, data were collected from research in several scientific databases, such as PubMed and ScienceDirect, using the following MeSH terms: "Orchidaceae/chemistry∗," "Plant Extracts/chemistry," "Drugs, Herbal/chemistry," "Molecular Structure," "Anti-Inflammatory Agents/chemistry," "Antineoplastic Agents/chemistry." The study included documents written in English language that discussed ethnopharmacology, phytochemistry, and biological activities of these species. The taxonomy of the species has been validated according to the Plant List [15, 16].

3. Botany, Species, and Distribution

3.1. Botanical Aspects. Plants of the genus *Bulbophyllum* are epilithic herbs, sympodial with roots creeping over the surface of the substrate or aerial, and filamentous to fibrous [17]. Orchid plants, such as Australian *Bulbophyllum minutissimum*, differ in size and weight from a gram to a few millimetres (1–1.5 mm across) [18]. The stems differentiate into rhizome and pseudobulb, and the leaves may be ovate, lanceolate, or orbiculate and variable in size on an individual plant [19]. Leaves are terminal on the pseudobulb (one or several per shoots) and conduplicate to the substrate. Recently, Piazza et al. [20] studied the vegetative anatomy of 13 species of *Bulbophyllum* belonging to sections *Didactyle* and *Xiphizusa* to elucidate the anatomical characters between and among the sections. The results revealed the anatomical differences among the species of both lipophilic secretion sections in young leaf trichomes and the presence of xeromorphic characters. Adaptations of both species to different environmental conditions were among the main differences described by the authors [20].

The inflorescence is racemose and presents many lateral flowers emerging from the rhizome, often at the base of the pseudobulb [19]. Some orchid species in the genus *Bulbophyllum* show an elaborated floral architecture, in addition to their characteristic floral odours that attract and bring specific pollinators [21]. The dorsal sepal is free, similar, or smaller than the lateral sepals. The latter are united basally to each other and a *column* foot forms a *mentum*, free or fused further [19]. The lateral petals are free and smaller than the dorsal sepals, and the lip of the flowers is trilobed with a large callus. In some species, the lip flaps with the wind, to simulate a fly shaking its wings to attract insects [9]. The *labellum* is hinged to the tip of the column foot; the lamina is either not lobed, obscurely 3-lobed, unornamented, or with 2 longitudinal keels. The column lacks the free filament and style; the column wings are fused to the column and reduced to teeth that project beside the anther [9]. The ovary, composed of three fused carpels and the mature seed pod, opens down the middle between the lines of juncture. The ovules are arranged along the ridges inside the ovary and do not develop until sometime after the flower [9]. Orchid flowers attract specific insect species by deceiving males with

an imitation signal of female odour and mimetic appearance [22]. For example, the ginger orchid (*Bulbophyllum patens* King) flower releases a ginger essence called zingerone that attracts fruit flies sensitive to methyl eugenol and raspberry ketone [22, 23]. Also, the flowers of *Bulbophyllum apertum* Schltr. release the volatile compound raspberry ketone with the function of attracting raspberry ketone-sensitive *Bactrocera* species [23].

The seeds of *Bulbophyllum* species consist of a dry, outer coat enclosing a small mass of undifferentiated cells that form a proembryo [17]. They are fusiform, spindle, and narrowly ellipsoidal shaped and less than 1 mm in length without endosperm [24]. This extremely small and light unit can easily be carried in through air currents and may travel long distances before coming to rest [17]. Seed volume is related to seed size and *Bulbophyllum* species, and the higher seed volume is a result of greater width rather than *testa* length [24]. In natural conditions, the seeds have specific germination requirements provided by mycorrhizal fungi. For instance, seeds infected by a specific fungus can either germinate or be destroyed [25]. There are different seed morphologies among the plants of the genus *Bulbophyllum*. For example, *Bulbophyllum mysorense* seeds are transparent, short, and spindle to oblong with blunt ends, and the *testa* cells have marginal clavate ridges, smooth on the outer face with longitudinally oriented cells resembling a twisted rope [26].

3.2. Bulbophyllum Species and Cultivation. Every year, some new *Bulbophyllum* species are described, making this genus grow steadily [27]. Due to the extraordinary diversity, a general description would be too extensive [1, 27]. Over 50 generic names have been proposed, in addition to *Bulbophyllum* [19].

In 2005, the Federal Ministry of Agriculture, Forestry, Environment, and Water Management as the CITES Management Authority of Austria elaborated three lists (names, accepted names, and the checklist) for the *Bulbophyllum* genus, produced by the Botanical Garden of Vienna [28]. The list is an approach to handle the vast number of species of this genus [28]. Recently, *Bulbophyllum* is into a single genus [7, 19]. In 2019, the World Checklist of Selected Plant Families [29] published an updated and exhaustive list that comprises over 2000 epiphytic *Bulbophyllum* species. There have been many efforts to split off segregate genera based on morphological characteristics. As reported by the American Orchid Society [30], some of the common *Bulbophyllum* species are as follows: *Bulbophyllum dearei, Bulbophyllum echinolabium, Bulbophyllum falcatum, Bulbophyllum fascinator, Bulbophyllum flabellum-veneris, Bulbophyllum guttulatum, Bulbophyllum lasiochilum, Bulbophyllum lobbii, Bulbophyllum longissimum, Bulbophyllum makoyanum, Bulbophyllum medusae,* and *Bulbophyllum putidum*. Two hybrids, Bulbophyllum Daisy Chain and Bulbophyllum Elizabeth discovered recently, are particularly attractive [30]. Among this ecological group, new taxa were also discovered: *Bulbophyllum cariniflorum* Reichenbach var. *orlovii* Aver., *Bulbophyllum sonii* Aver. and N. V. Duy, *Bulbophyllum ustulata* Aver., *Bulbophyllum cariniflorum*, *Bulbophyllum flavescens* Lindley, *Bulbophyllum ovatum* Seidenfaden, *Bulbophyllum physocoryphum* Seidenfaden, and *Bulbophyllum wendlandianum* Dammer.

Regarding the environmental conditions of cultivation, the warm temperature is suitable for *Bulbophyllum* plants that grow with a minimum of 22°C in winter, though species from temperate regions are grown 5–10 degrees cooler [30]. Light can be moderate to bright (2000 to 3500 candle feet): a higher light, which does not damage the leaves, seems to produce a better and more frequent flowering [30]. *Bulbophyllum* species tend to prefer a minimum of repotting using fern or cork slabs, baskets, and well-draining pots and the recommended impregnation media are sphagnum moss, coconut (flakes or coconut fibres), and tree fern both horizontally and vertically, whereas the relative humidity should be around 90% inside a greenhouse [31]. High relative humidity with a high air movement rate and constant fresh air are decisive parameters for the health and successful cultivation of the plants [31]. During the growth cycle, plants need adequate nutrition using standard fertilizers at 1/4 of the concentration given for houseplants [30]. The plants are usually rapid growers, reporting no major problems with pests. Propagation is through the division of the bulbs or via seeds of excellent germination rates [31]. Manual pollination of *Bulbophyllum phalaenopsis*, *Bulbophyllum spies,* and *Bulbophyllum strontium* has so far not been successful [31].

3.3. Geographical Distribution. The taxa of the genus *Bulbophyllum* are pantropical, spreading across Africa, Australia, India, Madagascar, Southeast Asia, and tropical South and Central America [32] (Figure 1).

The orchids, as epiphytes, are believed to be less adaptable to anthropogenic environmental variations than other plants [33]. The availability of the specificity host tree plays a central role in influencing the distribution and abundance of epiphytic orchids, often causing their unequal geographical distribution, even within a single tree [11].

Most of the *Bulbophyllum* species is epiphytes and is present in the virgin pantropical forest in the lower Montane forest at 1000 m above sea level [32]. However, a major number of species arise in the Indo-Malayan region [34]. Of 1000 species from India, 62 species are in the Northeastern region, 14 species in South India, and the remaining from other parts of India [35]. Madagascar is the hub of *Bulbophyllum* diversity (over 210 spp.), found mainly as epiphytes in a wide range of rainforest environments [36].

Madagascan *Bulbophyllum* molecular studies evidenced the existence of a monophyletic group of Late Miocene age with two major lineages: a species-rich core clade, mainly distributed in eastern rainforest of mid-to-high elevation (c. 800–1300 m), and a species-poor clade that is ecogeographically wide-ranging [37].

According to research, about 105 species of *Bulbophyllum* are present in China [38, 39]. *Bulbophyllum* Thouars is one of the largest genera among the orchid family, with around 2000 species in the tropical and subtropical zone of the world [40].

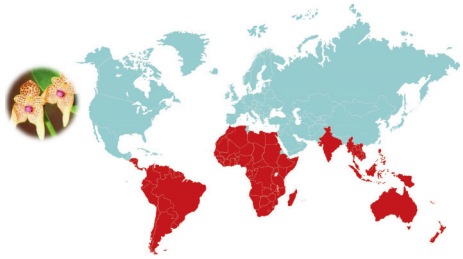

Figure 1: Geographical distribution of *Bulbophyllum* species.

Up to date, Vietnam has documented 122 species of the genus *Bulbophyllum* [41]. Recent studies have allowed identifying four new species of *Bulbophyllum* in Vietnam, namely *Bulbophyllum flavescens* (sect. *Aphanobulbon*), *Bulbophyllum ovatum* (sect. *Desmosanthes*), *Bulbophyllum physocoryphum* (sect. *Macrocaulia*), and *Bulbophyllum wendlandianum* (sect. *Cirrhopetalum*) [40]. These species are endemic of the Indochinese Peninsula, except for *Bulbophyllum flavescens,* which is widely distributed in western Malesia [40]. The "vinaceous orchid" (*Bulbophyllum vinaceum* Ames and C. Schweinf) is a rare epiphytic plant endemic to the highlands of Borneo Island, such as the Crocker Range and Mt. Kinabalu of Sabah [21].

4. Ethnopharmacology

The usage of orchids in Ayurvedic or folklore treatment is common in many parts of the world, which raised immense attention to explore their pharmacological properties and bioactive constituents in-depth. The genus *Bulbophyllum* has an important role in many cultures acting as a religious, protective, ornamenting, cosmetic, and medicinal means. Leaves, pseudobulbs, and flowers of *Bulbophyllum* sp. have ethnobotanical importance and are used for various ailments, for both external and internal applications and administrations by tribal peoples. *B. neilgherrense* is an epiphytic plant used by the South Indian tribes to treat heart disease, leukoderma, skin allergy, and rheumatism; its pseudobulb is the most used part for traditional remedies.

The uses of *B. scaberulum* in South African traditional medicine were observed for pain-related ailments, recommending further studies to explore the chemical profile and interactions between different classes of compounds and biological/pharmacological activities.

In Cameroon, *Bulbophyllum falcatum* and *Bulbophyllum lupulinum* are used against sorcery [2]. At the same time, leaves of *Bulbophyllum falcatum* are used for predictions, and the whole plant of *Bulbophyllum simonii* is used as a luck potion [2]. *Bulbophyllum shanicum* is offered in many religious ceremonies and used by Kayaladies for ornamenting their hair [42]. *Bulbophyllum simonii* and *Bulbophyllum lilacinum* are mixed in body lotion to keep the body fresh and cool [2, 12].

Fluids from cleaned pseudobulbs of *Bulbophyllum lilacinum* are extracted by the press, kept in a sealed jar overnight, and then mixed with water and taken as a cool drink [12]. Powder of *Bulbophyllum melinostachyum* is recommended as anti-poisons [2].

In Zimbabwe, bark from species of the *Bulbophyllum* genus is tied around a fracture as a supporting pad [43]. Also, in different countries, many species of the *Bulbophyllum* genus are used as traditional herbal medicines (Table 1).

5. Phytochemistry

The spectroscopic analyses carried out by several investigations highlighted the phytochemicals of plants belonging to the genus *Bulbophyllum*. Although these studies are rare, their identification results have revealed a remarkable richness in the chemical composition of the genus *Bulbophyllum*. The identified compounds are shown in Table 2, and the molecular chemical structures (majority compounds) are schematized by ChemDraw and are shown in Figure 2. Note in Table 2, the 17 species belong to the genus *Bulbophyllum (B. odoratissimum, B. kaitense, B. weddellii, B. involutum, B. ipanemense, B. retusiusculum, B. neilgherrens, B. variegatum, B. vaginatum, B. protractum, B. reptans, B. cheiri, B. retusiusculum, B. kwangtungense, B. taeniophyllum, B. ambrosia, and B. echinolabium).*

Numerous chemical compounds *in B. odoratissimum* belong to different chemical families, in particular phenanthrene and phenanthraquinone. Chen et al. [56] identified 3,7-dihydroxy-2,4,6-trimethoxyphenanthrene, while the same author identified, 7-hydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene, coelonin, densiflorol B, gigantol, batatasin III, tristin, vanillic acid, and syringaldehyde as major compounds in 2008 Moscatin [56]. Additionally, Xu et al. [59] identified bulbophythrin A and bulbophythrin B. *B. odoratissimum* was also reported to contain 5-(2-benzo[1,3]dioxole-5-ylethyl)-6-methoxy benzo[1,3]dioxole-4-ol (1) and 5-(2-benzo[1,3]dioxole-5-ylethyl)benzo[1,3]dioxole-4,7-diol [60].

The chemical compounds of *Bulbophyllum weddellii, Bulbophyllum involutum,* and *Bulbophyllum ipanemense* were characterized by Da Silva et al. [62]. In this study, several chemical compounds were identified including furfural, 2-furanomethanol, 5-methyl-2(3H)-furanone, 2,3-dihydro-4-hydroxy-2,5-dimethyl-3-furanone, 2-methoxyphenol, maltol, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyrene-4-one, 1,2-benzenediol, 2,3,5,6-tetramethylphenol, 2,6-dimethoxyphenol, 4-hydroxy methyl benzoate, 4-hydroxy-3-methoxy methyl benzoate, 2,6-dimethyl-3-methoxymethyl-p-benzoquinone, tetradecanoic acid, pentadecanoic acid, and hexadecanoic acid [62].

The chemical compounds of the plant *B. kaitense* were reported by Kalaiarasan et al. [61]. The authors observed that this plant contained numerous compounds belonging to different classes such as ether compound (propane, 1,1-diethoxy), hydrocarbon (cyclopentane, 2-methylbutyl), plasticizer compound (1,2-benzenedicarboxylic acid, butyl 2-methylpropyl ester), ketone compound (2-nonanone, 9-hydroxy), aromatic alcoholic compound (2,4-dimethylcyclopentanol), alcoholic compound (3-buten-2-ol), iodo compound (nonane, 1-iodo), plasticizer compound (didodecyl phthalate), alcoholic compound (3,4-hexanediol, 2,5-dimethyl), ketone compound (fluorenone, 2,3,4,7-tetramethoxy), triterpene (squalene),

Table 1: Traditional and folk medical usage of the *Bulbophyllum* species.

Bulbophyllum species	Country	Usage	References
Bulbophyllum albidum	India	Strengthening of a weak uterus for conception	[44]
Bulbophyllum barbigerum	Cameroon	Side pain (whole plant), ear pain (leaves)	[2]
Bulbophyllum calyptratum	Cameroon	Skin diseases (measles, poxes abscesses, rashes) (leaves), wounds, burns (whole plant)	[2]
Bulbophyllum careyanum	Nepal	Burns (pseudobulb), abortion, and recovery during childbirth (leaves)	[3]
	India	Burns (pseudobulb), abortion, and recovery during childbirth (leaves)	[45]
Bulbophyllum cariniflorum	Not specified	Induce abortion (root)	[10]
Bulbophyllum intertextum	Cameroon	Side pain (whole plant)	[2]
Bulbophyllum kaitesse	India	Cancer, inflammatory, bacterial infection (pseudobulb)	[46]
Bulbophyllum kwangtungense	China	Pulmonary tuberculosis, bleeding, fever (tuber)	[47]
	Japan	Pulmonary tuberculosis, bleeding, fever (tuber)	[47]
Bulbophyllum leopardinum	Nepal	Burns	[48]
	India	Burns	[45]
Bulbophyllum lilacinum	Bangladesh	Tiredness, anxiety, aphrodisiac, inflammation, rheumatism, hypertension, diabetes, anemia, tuberculosis, cough, asthma, jaundice, heavy menstruation, leucorrhoea, eye disease, wound (pseudobulb, whole plant)	[10, 49, 50]
Bulbophyllum modestum	Thailand	Ear infection (stem)	[51]
Bulbophyllum mutabile	Malaysia	Fever (leaves)	[52]
Bulbophyllum neilgherrense	India	Heart diseases, rheumatism, leukoderma (pseudobulb), weakness (juice), tuberculosis, chronic inflammation, fractures, scabies (whole plant)	[53–55]
	Bangladesh	Tonic	[50]
Bulbophyllum odoratissimum	Bhutan	Tuberculosis, chronic inflammation, fracture (whole plant)	[47]
	Burma	Tuberculosis, chronic inflammation, fracture (whole plant)	[47]
	China	Cough, toothache, tuberculosis, chronic inflammation, fracture (whole plant)	[47], 15]
	India	Tuberculosis, chronic inflammation, fracture (whole plant)	[45, 47]
	Laos	Tuberculosis, chronic inflammation, fracture (whole plant)	[47]
	Nepal	Tuberculosis, chronic inflammation, fracture (whole plant)	[47, 48]
	Thailand	Tuberculosis, chronic inflammation, fracture (whole plant)	[47]
	Vietnam	Tuberculosis, chronic inflammation, fracture (whole plant)	[47]
Bulbophyllum pumilum	Cameroon	Epilepsy (whole plant)	[2]
Bulbophyllum sterile	India	Rheumatism, swellings (pseudobulb)	[45]

bromo compound (methyl 3-bromo-1-adamantaneacetate), and aromatic compound (1,3-bis(trimethylsilyl)benzene) [77].

The identification of chemical compounds of *B. vaginatum* begun in 1997 by the study of Yuan-Wah Leong et al. [68]. This work revealed the presence of different chemical compounds belonging to several chemical family such as phenanthrenes (4,9-dimethoxyphenanthrene-2,5-diol and 4,6 dimethoxyphenanthrene-2,3,7-triol,3,4,6-trimethenanthrene-2,7-diol, 3,4-dimethoxyphenanthrene-2,7-diol (nudol), 2,4 dimethoxyphenanthrene-3,7-diol, 3,5-dimeth-oxyphenanthrene-2,7-diol, 4 methoxyphenanthrene-2,3,5-triol (fimbriol B),4-methoxyphenan-threne-2,7-diol (flavanthrinin)), less bibenzyls (3,4′-dihydroxy-5,5′-dimethoxybibenzyl and 3,3′-dihydroxy-5-methoxybibenzyl) (batatasin III), and triterpenoid friedelin [68].

In the same year, *B. protractum was found to contain numerous bioactive compounds including* bulbophyllin, bulbophyllidin, batatasin III (3,3′-dihydroxy-5-methoxy bibenzyl), 3,3′,5-trimethoxybibenzyl, aloifol-I (3′,4-dihydroxy-3,5-dimethoxybibenzyl), 3,3′-dimethoxy-4,5-methylenedioxybibenzyl, flavidin (2,7-dihydroxy-9,10-dihydro-5H-phenanthro[4,5-bcd]pyran), dihydroconiferyl alcohol, stigmasterol, and sitosterol [69]. In addition, *B. reptans* was found to be rich *in* dimeric phenanthrenes (reptanthrin and isoreptanthrin) and stilbenoids (gymnopusin, confusarin, 2,7 dihydroxy-3,4,6-trimethoxyphenanthrene, flavanthrinin, cirrhopetalanthrin) [70].

Furthermore, *B. cheiri* contains several phenylpropanoids (eugenol, methyl eugenol, cis-methyl isoeugenol, trans-methyl isoeugenol, 2-allyl-4,5-dimethoxyphenol, 5-allyl-1,2,4-trimethoxybenzene (euasarone), and trans-3,4-dimethoxycinnamyl acetate) [71], while only two phenylpropanoid (bobulretulate A, bobulretulate B) esters were identified in *B. retusiusculum* (extracts) [72]. In contrast, six dihydrodibenzoxepins (7,8-dihydro-5-hydroxy-12,13-methylenedioxy-11-methoxyldibenz[B,F]oxepin, 7,8-dihydro-4-hydroxy-12,13-methylenedioxy-11-methoxyldibenz[B,F]oxepin, 7,8-dihydro-3-hydroxy-12,13-methylenedioxy-11-

Table 2: Phytochemistry of *Bulbophyllum* species.

Bulbophyllum species	Chemical classes	Compounds	References
Bulbophyllum Odoratissimum	Phenanthrene	3,7-Dihydroxy-2,4,6-trimethoxyphenanthrene	[56]
		Moscatin, 7-hydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene, coelonin, densiflorol B, gigantol, batatasin III, tristin, vanillic acid, syringaldehyde	[57]
	Phenanthraquinone	Bulbophyllanthrone	[58]
	Biphenanthrenes	Bulbophythrin A and bulbophythrin B	[59]
	Dihydrostilbenes	5-(2-Benzo[1,3]dioxole-5-ylethyl)-6-methoxy benzo[1,3]dioxole-4-ol (1) and 5-(2-benzo[1,3]dioxole-5-ylethyl)benzo[1,3]dioxole-4,7-diol	[60]
Bulbophyllum kaitense	Ether compound	Propane, 1,1-diethoxy	[61]
	Hydrocarbon	Cyclopentane, (2-methylbutyl)	
	Plasticizer compound	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	
	Ketone compound	2-Nonanone, 9-hydroxy	
	Aromatic alcoholic compound	2,4-Dimethylcyclopentanol	
	Alcoholic compound	3-Buten-2-ol	
	Iodo compound	Nonane, 1-iodo	
	Plasticizer compound	Didodecyl phthalate	
	Alcoholic compound	3,4-Hexanediol, 2,5-dimethyl	
	Ketone compound	Fluorenone, 2,3,4,7-tetramethoxy	
	Triterpene	Squalene	
	Bromo compound	Methyl 3-bromo-1-adamantaneacetate	
	Aromatic compound	1,3-bis(Trimethylsilyl)benzene	
Bulbophyllum weddellii		Furfural, 2-furanomethanol, 5-methyl-2(3H)-furanone, 2,3-dihydro-4-hydroxy-2,5-dimethyl-3-furanone, 2-methoxy-phenol, maltol, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyrene-4-one, 1,2-benzenediol, 2,3,5,6-tetramethylphenol, 2,6-dimethoxyphenol, 4-hydroxy methyl benzoate, 4-hydroxy-3-methoxy methyl benzoate, 2,6-dimethyl-3-methoxymethyl-p-benzoquinone, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid	[62]
Bulbophyllum involutum		Furfural, 2-furanomethanol, 5-methyl-furfural, 2,3-dihydro-4-hydroxy-2,5-dimethyl-3-furanone, 2-methoxy-phenol, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyrene-4-one, 1,2-benzenediol, 5-hydroxymethyl-2-furan-carboxyaldehyde, tetradecanoic acid, pentadecanoic acid, 14-methyl methyl pentadecanoate, 9-hexadecenoic acid, hexadecanoic acid	[62]
Bulbophyllum ipanemense		Hexanal, 2-furanomethanol, 2-pentyl-furan, 1,2-benzenediol, 2,4-decadienal-(E,Z), 2,3,5,6-tetramethylphenol, 4-decadienal-(E,E), 2,6-dimethoxyphenol, tridecanone, tridecanol, methyl tetradecanoate, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, ethyl hexadecanoate	[62]
Bulbophyllum retusiusculum	Phenylpropanoids	Retusiusine A, retusiusine B, (±)-retusiusine C, dihydroconiferyl dihydro-p-coumarate, methyl 3-(4-hydroxyphenyl) propionate, 3-(4-hydroxyphenyl)-propanoic acid, dihydroferulic acid, methyl 3-(4-methoxyphenyl) propionate, 3-(3,4-dimethoxyphenyl)-2-propenal, trans-p-coumaric acid, dihydroconiferyl alcohol	[63]
	Bibenzyl	Bulbotetusine	[64]
	Flavone C-glycoside	Apigenin 6-C-α-arabinofuranosyl 8-C-α-arabinopyranoside	
Bulbophyllum neilgherrens	Alkaloids	+	[65]
	Saponin glycosides	+	
	Tannins	+	
	Phenols	+	
	Flavonoids	+	
	Steroids	+	
	Reducing sugar	+	

Table 2: Continued.

Bulbophyllum species	Chemical classes	Compounds	References
Bulbophyllum variegatum	Alcohols	2-Ethylhexanol, 2-nonanol	[66]
	Aldehydes	Nonanal, decanal	
	Ketones	2-Heptanone, 2-nonanone	
	Acids	Acetic acid, propanoic acid	
	Sesquiterpene hydrocarbons	Beta-elemene, (E)-caryophyllene, alpha-humulene	
	Nitrogenous compounds	Trimethylamine, methoxyphenyloxime, indole	
	Sulphur compounds	Methyl thioacetate, benzothiazole	
	Aromatic compounds	Toluene, p-cresol, p-cresyl acetate	
Bulbophyllum vaginatum	Phenanthrenes	Biphenanthrene, phenanthro[4,3-b]furan derivative	[67]
		4,9-Dimethoxyphenanthrene-2,5-diol and 4,6 dimethoxyphenanthrene-2,3,7-triol, 3,4,6-trimethenanthrene-2,7-diol, 3,4-dimethoxyphenanthrene-2,7-diol (nudol), 2,4-dimethoxyphenanthrene-3,7-diol, 3,5-dimethoxyphenanthrene-2,7-diol, 4-methoxyphenanthrene-2,3,5-triol (fimbriol B), 4-methoxyphenan-threne-2,7-diol (flavanthrinin)	[68]
	Dihydrophenanthrenes	4-Methoxy-9,10-dihydrophenanthrene-2,3,7-triol and 4,6-dimethoxy-9,10-dihydrophenanthrene-2,3,7-trio, 9,10-dihydrophenanthrenes 3,4,6-trimethoxy-9,10-dihydrophenanthrene-2,7-diol, 4-methoxy-9,10-dihydrophenanthrene-2,7-diol (coelonin), 3,5-di-methoxy-9,10-dihydrophenanthrene-2,7-diol (6-methoxycoelonin), 3,4-dimethoxy-9,10-dihydrophenanthrene-2,7-diol (erianthridin)	
	Triterpenoid	Friedelin	
	Bibenzyls	3,4′-Dihydroxy-5,5′-dimethoxybibenzyl and 3,3′-dihydroxy-5-methoxybibenzyl (batatasin III)	
Bulbophyllum protractum		Bulbophyllin, bulbophyllidin, batatasin III (3,3′-dihydroxy-5-methoxy bibenzyl), 3,3′,5-trimethoxybibenzyl, aloifol-I (3′,4-dihydroxy-3,5-dimethoxybibenzyl), 3,3′-dimethoxy-4,5-methylenedioxybibenzyl, flavidin (2,7-dihydroxy-9,10-dihydro-5H-phenanthro[4,5-bcd]pyran), dihydroconiferyl alcohol, stigmasterol, and sitosterol	[69]
Bulbophyllum reptans	Dimeric phenanthrenes	Reptanthrin and isoreptanthrin	[70]
	Stilbenoids	Gymnopusin, confusarin, 2,7 dihydroxy-3,4,6-trimethoxyphenanthrene, flavanthrinin, cirrhopetalanthrin	
Bulbophyllum cheiri	Phenylpropanoids	Eugenol, methyl eugenol, cis-methyl isoeugenol, trans-methyl isoeugenol, 2-allyl-4,5-dimethoxyphenol, 5-allyl-1,2,4-trimethoxybenzene (euasarone), trans-3,4-dimethoxycinnamyl acetate	[71]
Bulbophyllum retusiusculum	Phenylpropanoid esters	Bobulretulate A, bobulretulate B	[72]
Bulbophyllum kwangtungense	Dihydrodibenzoxepins	7,8-Dihydro-5-hydroxy-12,13-methylenedioxy-11-methoxyldibenz[B,F] oxepin, 7,8-dihydro-4-hydroxy-12,13-methylenedioxy-11-methoxyldibenz [B,F]oxepin, 7,8-dihydro-3-hydroxy-12,13-methylenedioxy-11-methoxyldibenz[B,F]oxepin, cumulatin, densiflorol A, and plicatol B	[73]
Bulbophyllum taeniophyllum	Bibenzyls	Batatasin III and cirrhopetalidin	[74]
Bulbophyllum ambrosia		Moscatin,3,7-dihydroxy-2,4-dimethoxyphenanthrene, 2,7-dihydroxy-3,4-dimethoxy-phenanthrene, 2,5-dihydroxy-4-methoxy-9,10-dihydrophenanthrene,ephemeranthol-A, 3,7-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene, 2,4-dihydroxy-7-methoxy-9,10-dihydrophenanthrene, coelonin, rotundatin,4′,5-dihydroxyl-3,3′-dimethoxybibenzyl, moscatilin, batatasin III, and tristin	[75]
Bulbophyllum echinolabium	Diptera attractants	Cholest-5-en-3-ol, glycerol 1-palmitate, hexadecane, tridecane, decanal, nonanal, undecane, beta-linalool, limonene, 2-hexenal	[76]
	Amino acids	L-Phenylalanine, serine, norleucine, L-threonine	
	Saccharides	Alpha-D-glucopyranoside, D-turanose, sucrose, D-glucose, hydroquinone-beta-d-glucopyranoside, D-galactose, glucopyranose, 2,3,4-trihydroxybutyric acid, glycoside, alpha-methyl, ribonic acid, D-xylopyranose, D-(−)-tagatofuranose, inositol, D-ribofuranose, erythritol, D-(+)-xylose, myo-inositol, fructose	
	Lipids	Stigmasterol, campesterol, cholesterol, 1-monopalmitin	
	Others	Cyanuric acid, malic acid, 1-cyclohexene-1-carboxylic acid, 3,4,5-hydroxy, citric acid, alpha-hydroxyglutaric acid, mannonic acid, 1,4-lactone, benzoic acid, 3-methoxy, beta-hydroxy-beta-methylglutaric acid, p-hydroxybenzoic acid, pantothenic acid, alpha-aminoadipic acid	

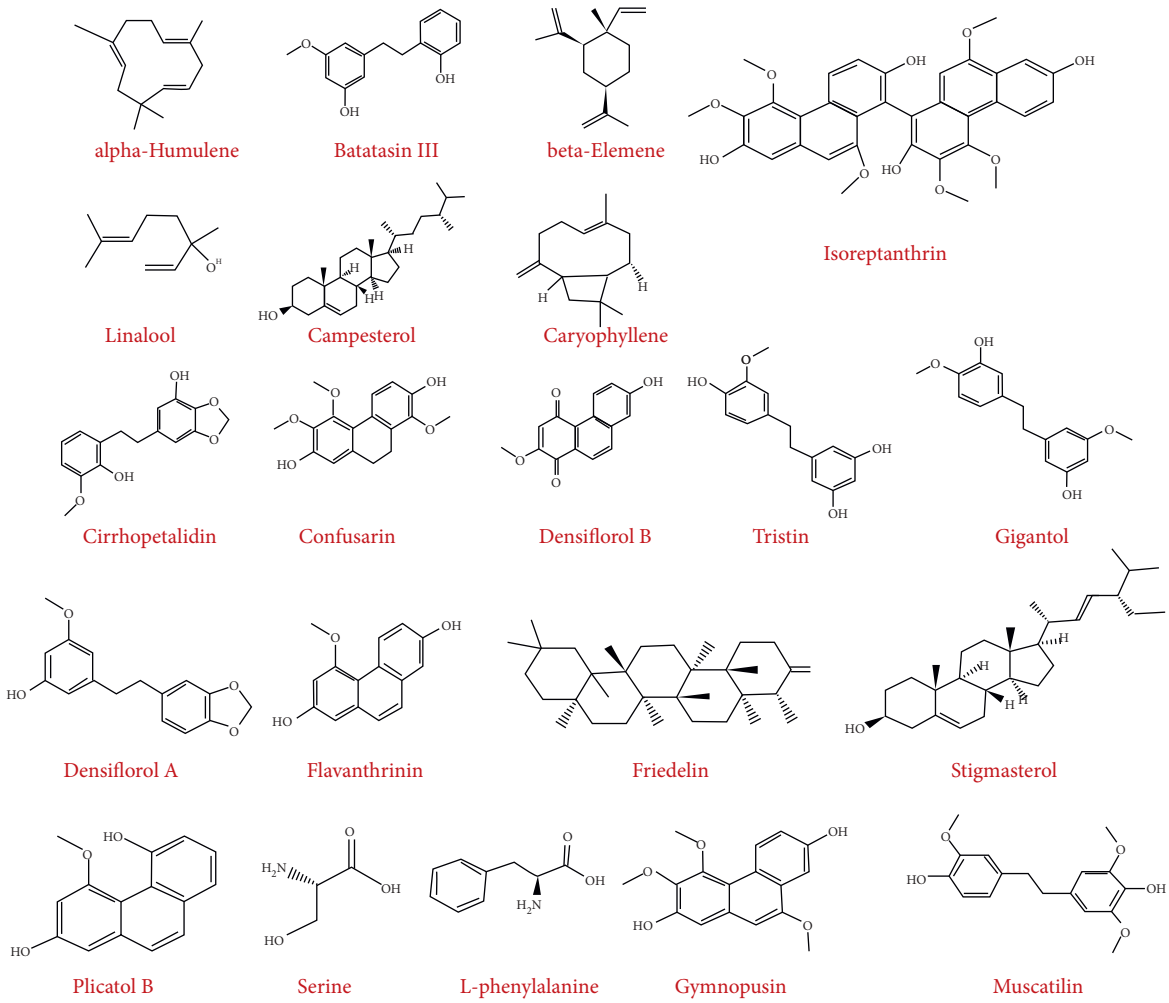

Figure 2: Phytochemical compounds identified in different *Bulbophyllum* species schematized by ChemDraw.

methoxyldibenz[B,F]oxepin, cumulatin, densiflorol A, and plicatol B) were identified from *B. kwangtungense* [73].

The extracts of *B. retusiusculum* contain several phytochemical compounds belonging to phenylpropanoids such as retusiusine A, retusiusine B, (±)-retusiusine C, dihydroconiferyl dihydro-p-coumarate, methyl 3-(4-hydroxyphenyl) propionate, 3-(4-hydroxyphenyl)-propionic acid, dihydroferulic acid, methyl 3-(4-methoxyphenyl) propionate, 3-(3,4-dimethoxyphenyl)-2-propenal, trans-p-coumaric acid, and dihydroconiferyl alcohol [63]. In addition, bibenzyl (bulbotetusine) and flavone C-glycoside (apigenin 6-C-α-arabinofuranosyl 8-C-α-arabinopyranoside) have also been identified in this plant [64].

The phytochemical compounds of *Bulbophyllum variegatum* were also identified [66]. The finding revealed the presence of several chemical families in this plant such as alcohols (2-ethylhexanol, 2-nonanol), aldehydes (nonanal and decanal), ketones (2-heptanone, 2-nonanone), acids (acetic acid, propanoic acid), sesquiterpene hydrocarbons (beta-elemene, *(E)*-caryophyllene, alpha-humulene), nitrogenous compounds (trimethylamine, methoxyphenyloxime, indole), sulphur compounds (methyl thioacetate, benzothiazole), and aromatic compounds (toluene, *p*-cresol, *p*-cresyl acetate) [66]. *B. neilgherrens* is another medicinal species of the *genus Bulbophyllum*. Only qualitative analysis of the chemical contents of this species was carried out by Kumari et al. [65]. The results showed the presence of different chemical classes in this plant such as alkaloids, saponin glycosides, tannins, phenols, flavonoids, and steroids [65].

Both the quantitative and qualitative differences in chemical composition across species of the genus *Bulbophyllum* are due to the physiology and genetics of the species. However, other factors such as climate, soil, parts used, and phenological stages of the plants can also affect the secondary metabolite synthesis in these species.

Recent studies show that chemical composition can vary within the same species, in different environments, and in extraction systems.

6. Biological Activities

6.1. Antimicrobial and Antifungal Activities. Phytochemical screening of different solvent extracts (petroleum ether, chloroform, ethanol, and water) collected from stems of terrestrial orchid *B. kaitense* in Kolli Hills, India, confirms the presence of terpenoids, flavonoids, reducing sugars, phenols, catechins, saponins, tannins, anthraquinone, quinine, coumarin, glycosides, and

carbohydrates. Ethanol and chloroform extracts show greater antifungal activity than petroleum ether and aqueous extracts, whereas the antibacterial activity in petroleum ether extract showed less effect than that reported in chloroform, ethanol, and aqueous extracts [61].

There are many orchids with different medicinal properties and antibacterial activity. *B. neilgherrense was* tested for antibacterial activity against five bacterial species (*Escherichia coli, Staphylococcus aureus, Bacillus pumilus, Pseudomonas aeruginosa*, and *Pseudomonas putida*). Ethanolic, chloroform, and aqueous extracts (concentration of 5.50 w/v) from leaves and pseudobulbs were prepared for the disk diffusion method of antimicrobial sensitivity testing. The ethanolic extract of both leaves and pseudobulbs was more effective. The bacterial species *P. aeruginosa* and *P. putida* showed greater sensitivity to the pseudobulb ethanolic extract, while the ethanolic extract from leaves was effective against *E. coli, S. aureus*, and *P. aeruginosa*. However, all extracts were less effective than standard antibiotic streptomycin, when tested with the disk diffusion method *in vitro* [78].

In another study, Fang et al. [63] identified new phenylpropanoids in tubers of *B. retusiusculum*, which were then tested for their antimicrobial activities. The antimicrobial tests were performed against *E. coli, B. subtilis*, and *Candida albicans*, where kanamycin (4 μg/mL) and nystatin (4 μg/mL) were positive controls for measuring antibacterial and antifungal activity potencies, respectively. Retusiusine B exhibited potent antifungal activity against *C. albicans* (16 μg/mL), and (±)-retusiusine C enantiomers showed moderate antibacterial activity against *B. subtilis* (64 μg/mL) [63].

B. affine has moderate bactericidal activity against *Staphylococcus aureus* (a common cause of skin infections) but none against *Bacillus subtilis, Klebsiella pneumonia, Escherichia coli, or Vibrio cholera* [79].

The orchid species *B. careyanum* and *B. leopardinum* are commonly used in burn treatments [80] although antimicrobial testing has not been reported for these species. In a similar study, *B. neilgherrense* exhibited moderate antibacterial activity against five infectious bacterial species [78], while promising antifungal activity against ten pathogenic fungal strains was reported from the same orchid species [81].

Phenylpropanoids isolated from *Bulbophyllum retusiusculum* exhibited moderate antibacterial activity against *Bacillus subtilis* (64 μg/mL) and potent antifungal activity against *Candida albicans* (16 μg/mL) [63]. *Bulbophyllum kaitense* stem extract was also effective against 10 different bacterial strains with greater sensitivity to fungal strains [61].

In the light of these results, the antibacterial activity of orchids is a good alternative for preventing/treating infections instead of using antibiotics, which have many side effects.

6.2. Antioxidant Activity. Natural plant products have been used as poultices and/or anti-inflammatory drugs and antioxidants for many years [82, 83].

Chinsamy et al. [84] reported antioxidant activity of *Bulbophyllum scaberulum* higher than other South African orchid species. The overall average antioxidant activity (% ANT) of *B. scaberulum* pseudobulb and root extracts was higher than 90%, which might validate the use of species to treat certain inflammatory disorders.

The antioxidant effects and inhibition of acetylcholinesterase (AchE) enzyme from several indigenous orchid species (including *B. Scaberulum)* were evaluated. In their studies, the methanolic extracts of leaves, pseudobulbs, and roots of *B. Scaberulum* (similar to other orchids the extracts) showed high antioxidant potential with 100% average antioxidant activity (ANT) as compared to the standard BHT drug (95.88%), when tested with β-carotene bleaching assay. They also found that the ethanol root extract at 5 and 0.5 mg/m exhibited significant mutagenic effects comparable to the 4NQO drug. Similarly, the dichloromethane extract of roots significantly inhibit AchE with the lowest IC_{50} value of 0.02 mg/ml, while the ethanol extract showed less activity against AchE.

Recently, Sun et al. [85] purified several compounds from *B. retusiusculum* whole plants. New phenanthrene, bobulretin, and two bibenzyls were evaluated against α-glucosidase activity *in vitro*. All three compounds showed less than 20% inhibition when tested at the final concentration of 4.37×10^{-4} mol/L.

Similarly, *Bulbophyllum* sp. exhibited higher antioxidant activity when compared to four other epiphytic orchids [86]. Plants of *B. kaitense* also presented good antioxidant activity and are considered a source of plant-derived antioxidants [87].

Polyphenols such as chrysin and pinobanksin found in orchid species of the genus *Bulbophyllum* have antioxidant effects [65, 88, 89], thus supporting their use in various heart diseases by the folklore traditions.

6.3. Anti-Inflammatory Activity. Nair et al. [54] used the pseudobulb powder of *B. neilgherrense* to examine analgesic and anti-inflammatory activities using different rat models. The pseudobulb powder mixed with honey and water revealed central analgesic activity against radiant heat-induced pain, moderate anti-inflammatory activity against carrageenan-induced acute inflammation, and mild or negligible activity against formalin-induced subacute inflammation and pain in rats. Pseudobulb contains flavonoids (chrysin and quercetin), glycosides, tannin, phenolic compounds, and calcium and may play a fundamental role in observed analgesic and anti-inflammatory activities [54].

A study run by Kumari et al. [65] reported that the pseudobulb of *B. neilgherrense* contains alkaloids, tannis, phenols, flavonoids, steroids, saponin glycosides, and reducing sugar (almost the same compounds as found earlier by Nair and his coworkers). The amounts of tannins, sugars, and alkaloids were 0.828%, 8.96%, and 0.3%, respectively (w/w). Of these, chrysin (a flavonoid) inhibited COX-2 expression and IL-6 signalling, indicating that *B. neilgherrense* pseudobulbs have anti-inflammatory potential [65].

B. kaitense was examined *in vitro* for its anti-inflammatory activity using the human red blood cell (HRBC) method [77]. While petroleum ether, chloroform, and aqueous extracts exhibited various anti-inflammatory activities, the ethanolic extract of *B. kaitense* pseudobulbs showed potent anti-inflammatory activity. As south Indian orchids, some South African medicinal orchids also demonstrated notable anti-inflammatory effects, suggesting their potential in treating inflammation and related disorders.

A study conducted by Chinsamy et al. [84] reported that the dichloromethane, ethanol, and aqueous root extract of *B. scaberulum* had selective and significant COX-2 inhibition effects. Inhibition was 100.00, 93.31, and 58.09%, respectively, and dichloromethane and ethanol extract IC_{50} values against COX-2 were 1.43 and 0.44 mg/ml, respectively. Surprisingly, the dichloromethane root extracts showed a greater inhibition performance than the commercial drug galantamine for COX-2. In contrast, water extracts from leaves and pseudobulbs exhibited moderate effects on COX-2, but no effect on COX-1. The COX-2 inhibition effects of *B. scaberulum* suggested that it was due to condensed tannins present in the stems and/or roots [84].

Gowlis of Karnataka use a paste of pseudobulbs of *Bulbophyllum neilgherrense Wight.* for arthritis [90]. A study validated this trait and concluded that the pseudobulb powder had central analgesic activity against radiant heat-induced pain and moderate anti-inflammatory activity against carrageenan-induced acute inflammation [54]. The authors suggest that the potential mode of action of B. neilgherrense Wight. pseudobulb was due to the presence of flavonoids (chrysin and quercetin) in the plants [65] as several flavonoids such as hesperidin, luteolin, and quercetin have anti-inflammatory and analgesic effects [91]. Similarly, chrysin and quercetin have significant analgesic and anti-inflammatory activities [92]. However, a 50% ethanolic extract of *B. gymnopus Hook f.* failed to show antimicrobial or anti-inflammatory activity and did not affect either respiration, cardiovascular system, or central nervous system, in experimental animals [93].

Aqueous pseudobulb extracts of *B. scaberulum* showed poor or no COX-1 and COX-2 inhibition [84]. Interestingly, the organic extracts (petroleum ether, dichloromethane, and ethanol) of *B. scaberulum* showed higher activity in the same study.

Bulbophyllum kaitense has been used in indigenous medicine by local healers of the Kolli hills. Its anti-inflammatory validation has been reported using human red blood cell (HRBC) membrane stabilization method [46]. The HRBC membrane stabilization assay uses the HRBC method as analogous to lysosomal membrane components, and thus, the inhibition of hypotonicity or heat-induced red blood cell membrane lysis may be taken as a measure of the anti-inflammatory activity mechanism of extracts.

6.4. Anticancer Activity. Numerous studies report that *Bulbophyllum* species have *in vitro* cytotoxicity activity. The phenanthrenes isolated from *Bulbophyllum odoratissimum* and *Bulbophyllum inconspicuum* showed significant cytotoxicity against various cancer lines such as the human leukaemia cell lines K562, HL-60, and SMMC-7721 [59, 94]. Similarly, dihydrodibenzoxepins isolated from *Bulbophyllum kwangtungense* also exhibited antitumour activities against HeLa and K562 human tumour cell lines [73]. Chen et al. [56] isolated and studied various phenolic compounds from *Bulbophyllum odoratissimum* as their inhibitory ability against the growth of human leukaemia cell lines K562 and HL-60, human lung adenocarcinoma A549, human hepatoma BEL-7402, and human stomach cancer SGC-7901. The results indicated a high activity against selective cell lines, i.e., in three compounds where densiflorol was the most active compound, followed by syringaldehyde and tristin [57]. The other compounds evaluated were weak or either inactive.

Biswas et al. [95] reported that *Bulbophyllum* sterile petroleum ether fraction induces apoptosis *in vitro* and ameliorates tumour progression *in vivo*, suggesting that the active fractions of bulbs and roots have anticancer activity likely by inducing apoptosis through the phospho-p53-dependent pathway [95].

The extract of *B. kwantungense* also exhibited antitumour activity *in vitro* against cultivated human cervical carcinoma cells (HeLa) [96]. Chen et al. [56] isolated phenanthrene derivative 3,7-dihydroxy-2,4,6-trimethoxyphenanthrene from *B. odoratissimum*, and its structure was elucidated by extensive chemical transformations and nuclear magnetic resonance (NMR) spectroscopy spectrum studies. The isolated compound showed significant cytotoxicity against the growth of human leukaemia cell lines HL-60 and K562, human stomach cancer cell line SGC-7901, human hepatoma BEL-7402, and human lung adenocarcinoma A549 with IC_{50} values of 10.02, 14.23, 1.13, 15.36, and 3.42 mg/ml, respectively.

In an investigation to evaluate the cytotoxicity of *B. kwangtungense*, Wu et al. [73] purified three new compounds, dihydrodibenzoxepins 7,8-dihydro-5-hydroxy-12,13-methylenedioxy-11-methoxydibenz[*bf*]oxepin (D5MO), 7,8-dihydro-4-hydroxy-12,13-methylenedioxy-11-methoxydibenz[*bf*]oxepin (D4MO), and 7,8-dihydro-3-hydroxy-12,13-methylenedioxy-11-methoxyldibenz[*bf*]oxepin (D3MO), along with compounds cumulatin, densiflorol A, and plicatol B. Within the compounds tested for antitumour properties, D4MO and D3MO exhibited the highest activity against HeLa cells with the IC_{50} values of 78.3 and 61.2 μM, respectively, while activities against K562 tumour cells, D3MO, and densiflorol A showed the highest activity values with the IC_{50} of 64.7 and 67.6 μM, respectively. In another study, two dihydrostillbenes, 3-(2-(7-methoxybenzo[d][1,3]dioxol-5-yl)ethyl)phenol (3MDP) and 6-(3-hydroxyphenethyl)benzo[d][1,3]dioxol-4-ol (6HBD), previously purified from *B. odoratissimum*, were synthesized via the Wittig–Horner reaction and used for developing nine synthetic analogues by Zhang et al. [97]. 3MDP and 6HBD together with their two analogues bearing an amino acid moiety in place of the phenolic OH of 3MDP and 6HBD were found to have significant anti-proliferative activity selective to two cancer cell lines, SGC-7901 and KB, with IC_{50} value of $<10.0\,\mu$M, while the other analogues showed a

markedly reduced cytotoxicity towards all tumour cell lines, SGC-7901, KB, and HT-1080.

Chen et al. [57] purified nine phenolic compounds, including moscatin, 7-hydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene, coelonin, densiflorol B, gigantol, batatasin III, tristin, vanillic acid, and syringaldehyde, from the ethyl acetate extract of *B. odoratissimum* whole plant and investigated the cell growth inhibition effects on different tumour cell lines such as human leukaemia cell lines K562 and HL-60, human lung adenocarcinoma A549, human hepatoma BEL-7402, and human stomach cancer SGC-7901. Except for three compounds such as densiflorol B, tristin, and syringaldehyde, all other compounds showed weak or no activity on tumour cells. Densiflorol B was the most active compound against all tumour cells showing the IC_{50} values ranging from 0.08 to 3.52 μg/ml. Tristin displayed selective cytotoxicity against SGC-7901, with an IC_{50} value of 2.08 μg/ml, whereas syringaldehyde exerted a strong activity against BEL-7402 cells with an IC_{50} value of 1.54 μg/ml.

Two new dimeric phenanthrenes, bulbophythrins A and B, were then purified from *B. odoratissimum*, and their inhibitory ability against the growth of the same tumour cell lines was evaluated, as previously tested by Chen and his coworkers [57], with both compounds exerting significant cytotoxicity against all tumour cell lines [59]. However, bulbophythrin A exhibited some selective cytotoxicity against HL-60 and BEL-7402 with IC_{50} values of 1.27×10^{-3} and 1.22×10^{-3} μM, respectively, whereas bulbophythrin A appeared to be most active against A549 with an IC_{50} value of 1.18×10^{-3} μM, suggesting their potential use as a novel class of antitumour candidate in tumour disease.

Petroleum fraction, compared with the alcoholic extracts, from bulbs (PFB) and roots (PFR) of *B. sterile*, was found to be the most active in three different cancer cell lines, HCT-116, MDA-MB-231, and A549, by [95]. However, there was significant cytotoxicity in HCT-116 cells with IC_{50} values of 94.2 and 75.7 μg/ml for PFB and PFR, respectively, likely attributed to the effects on the cell cycle G2/M phase with 32.6% and 49.4% arrest. In addition, PFB and PFR treatments showed 48% and 38% apoptosis, respectively, when tested with acridine orange/ethidium bromide (AO/EB) staining assay. Their apoptosis induction was carried out through phospho-p53-dependent pathway. Both fractions lowered tumour volume and increased life span and hepatic antioxidant level in Ehrlich ascites carcinoma (EAC) bearing mice, resulting in lower EAC-induced mortality.

In a different report by Yang et al. [64], two compounds, a flavone C-glycoside and bibenzyl purified from B. retusiusculum tubers, did not show evident cytotoxicity on six cancer cell lines, including HL-60, SMMC-7721, A549, MCF-7, and SW-480. Their IC_{50} value was greater than 40 μM, and their noncytotoxic effects were previously thought to be attributed to their structural differences to other bibenzyl compounds previously reported to have cytotoxic effects.

6.5. Neuroprotective Effect. Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with memory impairment and cognitive deficit [98]. Various mechanisms, such as AChE inhibition, modification of monoamine levels, anti-amyloid aggregation, and antioxidant activities, are the strategies that have been employed for the amelioration of AD symptoms [99, 100]. Of these, one of the major approaches has involved addressing the levels of acetylcholine in the AD-depressed brain using AChE inhibitors [101]. Most of the currently available drugs are AchEi, and some are related to natural products with an important therapeutic strategy for the treatment of AD. Many research groups have focused their studies on naturally occurring compounds from plants as potential sources of either new or more effective AChEi. Due to the presence of flavonoids and tannins, orchid extracts that display significant effects in anti-inflammatory, antioxidant, and AChE inhibitory assays could be potential natural plant products in the inflammatory treatment of neurodegenerative disorders.

Chinsamy et al. [84] reported anticholinesterase activity (EC_{50}) for different extraction solvents as 0.02 ± 0.00 and 0.26 ± 0.007 mg/ml, respectively, in dichloromethane and ethanol root extracts of *B. scaberulum*. The authors also found that *B. scaberulum* root extract effectively inhibited AChE as compared to the commercial product galantamine.

6.6. Other Activities. Myanmar women prepare a hair tonic and shampoo by mixing ground pseudobulbs of various species of *Bulbophyllum* with pulverized bark, seeds, and fruit (species not identified) when washing their hair. This mixture is claimed to cure dandruff, promote hair growth, and improve hair colour [102].

Imbricatin, a stilbenoid isolated previously from *Bulbophyllum* and other orchid genera, was one of three isolated stilbenoids recommended for use as skin photoprotectants based on their antioxidant, anti-inflammatory, and immunomodulatory effects [103].

A summarized scheme of the most representative biological activities is summarized in Figure 3.

7. Safety Data

No reports were found in the literature concerning health safety issues after the consumption of *Bulbophyllum*. This plant has been used in ethnomedicine and folk medicine by villagers in different conditions for ages; however, the toxicity profile of the *Bulbophyllum* species is still undiscovered. With the increasing cases of poisoning associated with the use of herbal medicines in many parts of the world in recent times [104], it is essential to ensure safety through toxicity assessment alongside active pharmacovigilance to promote their safe use and protect public health. Therefore, it is necessary to identify the risks associated with the use of such herbal plants, and in this regard, the safety of these products has become an issue of great public health importance and thus a key moment for considering it in pharmacovigilance systems.

8. Limitations and Future Perspective

The data from this review validate the use of certain orchid species in ethnopharmacology for various conditions. More comprehensive studies of the bioactive compounds of

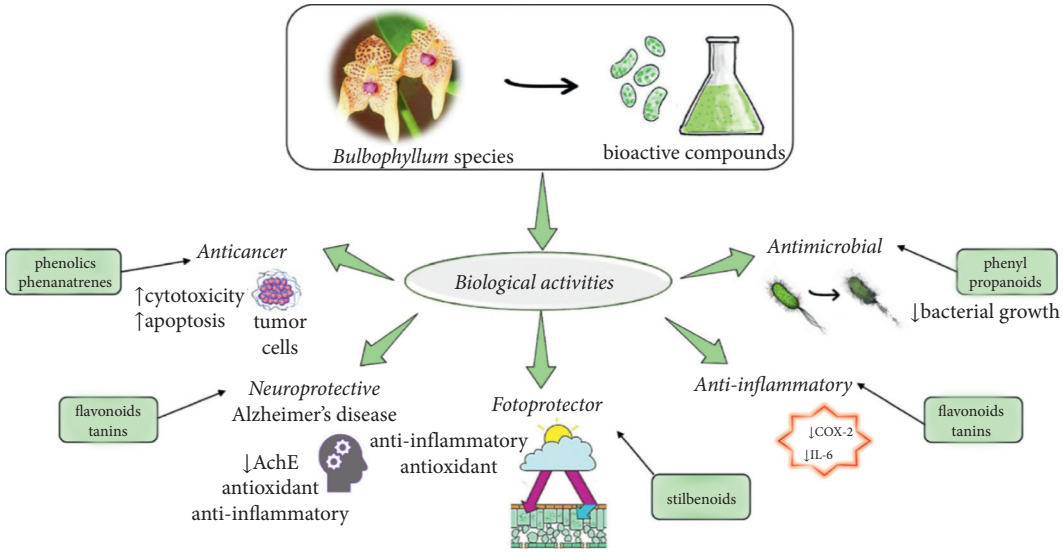

Figure 3: Illustrative scheme with the most important biological activities of *Bulbophyllum* species and correlations with their bioactive compounds. Abbreviations and symbols: ↑, increase, ↓, decrease, COX-2, cyclooxygenase 2, IL-6, interleukin-6, and AchE, acetylcholinesterase.

several *Bulbophyllum* species would open a new perspective on the relationships between chemical profiles, interactions between different classes of chemical compounds, biological properties, and correlation with geographical location. Therapeutic limitations of *Bulbophyllum* species are due to ignorance of long-term adverse effects, toxicity, and mutagenic potential. Future experimental pharmacological research is needed to further investigate the molecular mechanisms and action targets of the bioactive compounds contained in *Bulbophyllum* species. Another limitation is the lack of clinical trials.

9. Conclusion

In recent years, the genus *Bulbophyllum* is gaining the attention of plant researchers because of its rich phytochemical profile and variety of biological activities reported across species of the genus. Most *Bulbophyllum* species are epiphytic and found in habitats that range from subtropical dry forests to wet montane cloud forests. Plants belonging to the genus *Bulbophyllum* are mostly epilithic herbs and sympodial with roots creeping over the surface of the substrate or aerial, filamentous to fibrous. The genus *Bulbophyllum* has been utilized for protective, religious, cosmetic, ornamenting, and medicinal applications. Numerous investigations report the phytochemical compounds extracted from *Bulbophyllum* root, pseudobulb, and leaf, and their biological effect in traditional medicine treatments. Several phytochemical compounds of biological interest such as flavonoids, sterols-terpenoids, and phenolic acids have been reported in *Bulbophyllum* species. As summarized in the present review, the phytochemical composition of species of the genus *Bulbophyllum* offers insights into providing information on the antioxidant, anti-inflammatory, cytotoxic, antimicrobial, anticancer, and anticholinesterase activity. Furthermore, investigations are warranted to extract the various health-promoting phytochemicals and to identify their bioactivities, which will help to boost the utilization of *Bulbophyllum* species.

Data Availability

The data supporting this review are from previously reported studies and datasets, which have been cited. The processed data are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

All authors made a significant contribution to the work.

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Research Article

Baccharis dracunculifolia DC Hydroalcoholic Extract Improves Intestinal and Hippocampal Inflammation and Decreases Behavioral Changes of Colitis Mice

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The hydroalcoholic extract of B. dracunculifolia (HEBD) and its major compound p-coumaric acid were evaluated against the severity of intestinal inflammation and behavioral changes like depressive and anxious behavior in colitis mice. Colitis was induced in Swiss mice by oral dextran sulfate sodium (DSS) administration for five days. The mice received vehicle (10 ml/kg), HEBD (3, 30, or 300 mg/kg), or p-coumaric acid (15 mg/kg) orally, once a day for twelve days. Behavioral tests were performed on the 11th and 12th days after the beginning of the treatments. Moreover, the colon, cortex, and hippocampus were collected to analyze oxidative and inflammatory parameters. The treatment with HEBD (300 mg/Kg), but not p-coumaric acid, showed decreased disease activity index (DAI) values compared to the vehicle group and partially preserved the villi architecture and mucin levels. Furthermore, the HEBD increased the antioxidant defenses in the colon and hippocampus and reduced the myeloperoxidase activity and IL-6 levels in the colon from colitis mice. Colitis mice treated with HEBD did not show depressive-like behavior in the tail suspension test. HEBD reduced colon inflammation, while it maintains antioxidant defenses and mucin levels in this tissue. It may reduce neuropsychiatric comorbidities associated with colitis through its antioxidant effects.

1. Introduction

Ulcerative colitis (UC) is an idiopathic disease defined as a chronic colon and rectum inflammatory process. Despite the variety of existing drugs available for its treatment, it is estimated that 15–20% of the patients will require hospitalization [1]. The incidence and prevalence of UC differ by region, with an accelerated incidence rate in Western and newly industrialized countries [2]. The pathogenesis of this disease can be related to some environmental factors, oxidative stress, disruption of the epithelial barrier, dysregulation in the immune response, and impairment of the intestinal microflora [3].

The symptoms of UC may include weight loss, abdominal pain, diarrhea, rectal bleeding, and fatigue, and when the disease is limited to the rectum, the symptoms are more related to fecal incontinence and urgency of defecation. In severe cases, episodes of vomiting, fever, anorexia, and abdominal distension can occur [3, 4]. In addition to these symptoms, patients with UC are at higher risk of

developing colorectal cancer [5] and are more likely to develop psychological conditions such as anxiety and depression and have impaired social interactions or careers [6].

Herbal medicine has become a frequent therapeutic resource in patients with UC [7]; therefore, the development of studies related to the pharmacological potential of medicinal plants becomes essential and a fertile field for increasing the therapeutic arsenal to the treatment of inflammatory bowel disease (IBD). In this context, Baccharis dracunculifolia DC (Asteraceae), known as "alecrim-do-campo," a medicinal shrub native to Brazil, can be a natural resource in obtaining herbal medicines or phytopharmaceuticals for IBD treatment, especially UC.

Baccharis dracunculifolia has been described as the primary botanical source of Brazilian green propolis, responsible for many bioactive constituents of this propolis [8]. In addition, this Brazilian native plant has been widely used for medicinal purposes, including to treat gastrointestinal diseases, inflammation, and liver disorders [9, 10]. Regarding its anti-inflammatory potential, a few studies have described that B. dracunculifolia mitigates the inflammatory process in macrophages [11] and in rodents [12]. Furthermore, Cestari et al. [13] evaluated the intestinal anti-inflammatory of a B. dracunculifolia [14, 15] ethyl acetate extract in rats exposed to trinitrobenzene sulfonic acid (TNBS), a model of intestinal inflammation that triggers an immune response like Crohn disease in humans [16].

The pharmacological research about natural products could benefit current therapies for IBD or be integrated into conventional therapy, reducing the dosages of current therapies and their undesirable effects. Therefore, given the traditional use of B. dracunculifolia and the results from Cestari et al. [13], this study was performed to evaluate the efficacy of the hydroalcoholic extract of B. dracunculifolia (HEBD) against intestinal inflammation induced by oral intake of dextran sulfate sodium (DSS) in mice. It is noteworthy that the choice of this model reflects the fact that it triggers predominantly a TH2 immune response, which is prevalent in UC in humans [16–18]. In addition, the effect of p-coumaric acid, a major compound in HEBD, was evaluated on mice submitted to DSS-induced colitis, along with the effects of HEBD on behavioral changes in colitis mice. In this way, this study expands the knowledge about the pharmacological potential of HEBD in the treatment of UC in an adequate murine model.

2. Material and Methods

2.1. Plant Material and Extract Obtaining. Baccharis dracunculifolia aerial parts were collected in Ribeirão Preto at the University of São Paulo (USP) campus. The voucher specimen of B. dracunculifolia was identified by Milton Groppo Junior and deposited in the herbarium of the Department of Biology of Faculty of Philosophy, Science and Letters of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil, under the number SPFR 06143. As previously described by Lemos et al. [9], the dried ground leaves (350 g) were extracted by maceration using (ethanol/water 7:3, v/v) exhaustively. The hydroalcoholic extract of B. dracunculifolia was concentrated under vacuum and lyophilized, furnishing 50.2 g (14.3%) of the crude hydroalcoholic extract (HEBD).

2.2. p-Coumaric Acid Isolation. The p-coumaric acid was isolated from HEBD with purity greater than 98%. For a detailed protocol of these procedures, see Lemos et al. [9] and Costa et al. [14]. In summary, the isolated compound was identified by spectroscopic analysis of ^{1}H and 1^{3}C NMR using deuterated solvents (chloroform $CDCl_3$, dimethyl sulfoxide-DMSO-d6, and acetone-d6) in Bruker spectrometers (DRX-400 or DRX500), working at 400 or 500 MHz for ^{1}H and at 100 or 125 MHz for 1^{3}C. Tetramethylsilane was used as an internal reference. High-resolution mass spectral (MS) data were acquired using Thermo Scientific Exactive PlusTM H-ESI II Instrument with OrbitrapTM System at spray voltage 3.6 kV in negative ionization mode and 3.2 kV in positive ionization mode. Automatic gain control was set at 3E6 loads and 250 ms maximum injection time, 70,000 resolutions, and a scan time of 2.5 scans/s. The content of coumaric acid in HEBD was previously verified and was 5% in the dry extract, as Costa et al. [14] described.

2.3. In Vivo Trials

2.3.1. Animals. All experiments were carried out following the International Standards and Ethical Guidelines on Animal Welfare and the ARRIVE guidelines and were previously approved by the Ethics Committee on the Use of Animals of the University of Vale do Itajaí (038/18p and 024/20p). Swiss male mice (60 days old, 30–40 g) from the central vivarium of UNIVALI were used. The animals were acclimated to controlled conditions for at least seven days before experimental handling, with free access to feed and water, at 23°C and under a 12-hour light/dark cycle. The study was undertaken by using 37 animals distributed in different groups.

2.3.2. Colitis Induction. The experimental colitis was induced by adding 3% dextran sulfate sodium (DSS) to the animals' drinking water for seven consecutive days, followed by five days in which they received drinking water without DSS, according to the used by Meurer et al. [19]. The vehicle group (water plus 1% Tween-80, 10 mL/kg, $n = 6$), the HEBD groups (3, 30, or 300 mg/kg, $n = 6$), and the p-coumaric acid group (15 mg/kg, $n = 6$) received orally the treatments once a day for twelve days. The treatments started simultaneously with DSS intake.

The animals were weighed daily, and the presence of rectal blood and fecal consistency was individually analyzed, and each parameter was assigned a score according to Utrilla et al. [20], and these data were used to calculate the disease activity index (DAI). A noncolitis group (naïve group, $n = 7$) was monitored throughout the experiment and did not receive DSS in the drinking water but was treated with a vehicle. Finally, to verify whether the extract was able to change the

behavior of the mice, an experiment was also carried out in noncolitis mice receiving HEBD (300 mg/kg, p.o).

2.3.3. Histological and Histochemical Evaluation. On the 13th day after the treatments, all animals were euthanized, and the colon was removed, measured, and weighted. A segment of approximately 0.5 cm was fixed in a solution composed of 85% alcohol, 10% formaldehyde, and 5% acetic acid. Subsequently, these samples were dehydrated, cleared, and embedded in paraffin. Sections of 5 μm were obtained, deparaffinized, rehydrated, and submitted to staining in eosin-hematoxylin for morphological analysis. The histological damage was performed according to Utrilla et al. [20] and Camuesco et al. [21], considering epithelial loss, edema, cellular infiltration, goblet cells depletion, and the condition of the crypts.

The mucin levels were quantified through histochemical analysis on the sections obtained as described above using the periodic acid Schiff's (PAS) technique, which stained glycoproteins like mucins in pink. After the PAS procedure, the slides were observed under an optical microscope at 400 × magnification and photographed to quantify the mucin levels using the ImageJ® software, according to Pereira et al. [22].

2.3.4. Biochemical Analyses. Samples from each mouse's colon, cortex, and hippocampus were homogenized with 200 mM potassium phosphate buffer (pH 6.5). Then, this homogenate was used to determine glutathione reduced (GSH) in accordance with Sedlak and Lindsay [23] and lipoperoxides (LOOH) as described by. The remaining material was centrifuged again at 9000 g for 20 minutes. The activity of glutathione S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT), and the levels of reactive oxygen species (ROS), interleukin (IL)-4, and tumor necrosis factor (TNF) levels were determined in the supernatant, and the precipitate was used to determine myeloperoxidase (MPO) activity according to Bradley and collaborators [24] and modified by De Young and collaborators [25]. The SOD activity was determined based on the inhibitory capability of autoxidation of pyrogallol [26], whereas the determination of CAT and GST activities was according to the methods described by AEBI [27] and Habig et al. [28], respectively. A mouse cytokine kit acquired of BD Biosciences (Franklin Lakes, New Jersey, USA) was used to estimate TNF and IL-6 levels by enzyme-linked immunosorbent assay following the manufacturer's instructions. The Bradford method was adopted using bovine serum albumin (0.125 to 1.0 mg/mL) as standard to determine the protein concentration in each sample.

2.3.5. Behavioral Tests. Behavioral tests were undertaken on the 11th and 12th days of the treatments to measure anxious and depressive behavior, respectively. Three different experimental models were used to assess depressive and anxious-like behavior in mice: elevated plus maze (EPM), tail suspension test (TST), and open field test (OFT).

The effects of HEBD in depressive-like behavior of health and colitis mice were evaluated using the TST following Steru et al. [29]. On 11th day of treatments, the animals were suspended by the tail 30 cm above the floor using adhesive tape (1 cm from the tip of the end). The time of mouse immobility was recorded (in seconds) during the last 4 min of a 6-min session.

The spontaneous locomotor activity of mice was evaluated in the OFT to determine whether HEBD promotes changes in the locomotory activity of health or colitis mice. This test was performed on the 11th day. Animals were individually placed in an acrylic box (50 × 50 × 50 cm), with the floor being divided into 24 equal squares. The number of squares crossed with the four paws (crossing) and the rearing (number of times the rat raised on its hind legs) was recorded in a 6-min session, similarly to.

On the 12th day from the beginning of the treatments, the anxiety-like behavior of health and colitis mice was measured using the EPM (40 cm length, 10 cm width, and 50 cm height). Each mouse was placed in the central area of the maze facing one of the open arms, and all movements were recorded by the observer for 6 min [30]. The closed arms were enclosed by a black wall 20 cm in height.

2.4. Statistical Analysis. Analyses were performed using the GraphPad Prism 7.0 program (San Diego, USA). When applicable, statistical analysis was performed using one-way or two-way analysis of variance (ANOVA), followed by Bonferroni post-test. The results were presented as means ± standard error of means (S.E.M). Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effect of HEBD and p-Coumaric Acid on Weight Loss and DAI Score in DSS-Induced Colitis Mice. The DAI score in the colitis mice treated with a vehicle reached media equal to 10.5 ± 1.05, whereas the colitis mice treated with HEBD (300 mg/kg, p.o) experienced a decrease in this value from 10th day of the treatment, compared with the vehicle group (Figure 1(a)). Moreover, it was observed 39.2% of reduction in DAI score in the mice treated with HEBD (300 mg/kg, p.o) after the end of the treatment period, compared with the vehicle group (Figure 1(b)). However, the colitis group treated with p-coumaric acid showed no difference in the DAI score compared with the vehicle-treated colitis group (Figure 1(a)).

Regarding the weight loss of the animals, the difference between the naive group and the vehicle-treated colitis group was evidenced ($p < 0.05$), and the treatment with HEBD (300 mg/kg, p.o) prevented this weight loss compared with the vehicle-treated colitis group (Figure 1(c)).

3.2. Effects of HEBD and p-Coumaric Acid on Colon Length and in the Spleen, Liver, and Colon Weight of DSS-Induced Colitis Mice. The DSS intake reduced the colon length of the vehicle-treated colitis mice by 68% compared with the naive group (9.27 ± 0.70 cm) (Table 1). However, HEBD at the

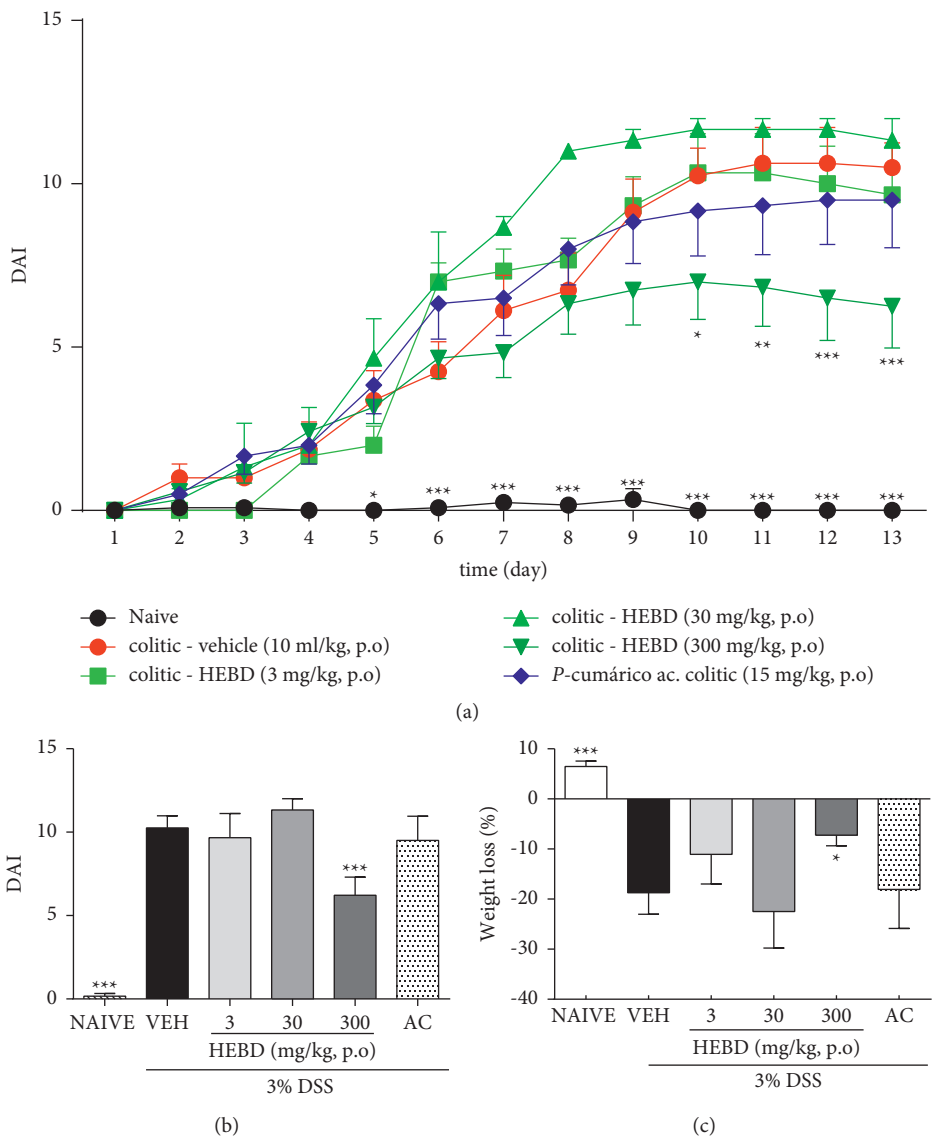


Figure 1: HEBD, but not p-coumaric acid, reduces colitis severity (panels A and B and prevents the weight loss (panel C in DSS-induced colitis mice). (a): DAI score over the entire treatment period. (b): DAI values at the 13th after the beginning of treatments. (c): Weight loss at the 13th after the beginning of treatments. Colitis mice were treated with the vehicle (VEH: water plus 1% Tween-80, 10 ml/kg, p.o) or hydroalcoholic extract of *B. dracunculifolia* (HEBD: 3–300 mg/kg, p.o). The naïve group was composed of noncolitis mice treated with the vehicle. Results are presented as the means ± S.E.M. ($n = 6$). One-way (panels B and C) or two-way (panel C) ANOVA followed by Bonferroni's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.05$, and $^{***}p < 0.05$ when compared to VEH.

Table 1: Effects of HEBD and *p*-coumaric acid on colon length and in the spleen, liver, and colon weight of DSS-induced colitis mice.

Groups	Dose	Colon length (cm)	Colon weight (g/100 g)	Spleen weight (g/100 g)	Liver weight (g/100 g)
Naive	10 mL/Kg	9.3 ± 0.7	0.06 ± 0.01	0.05 ± 0.01	0.50 ± 0.10
Colitis—vehicle	10 mL/Kg	6.3 ± 0.9[d]	0.07 ± 0.01	0.08 ± 0.01[c]	0.41 ± 0.01
Colitis—HEBD	3 mg/Kg	7.4 ± 0.8[a]	0.08 ± 0.01	0.06 ± 0.01	0.43 ± 0.01
Colitis—HEBD	30 mg/Kg	6.0 ± 0.8[d]	0.11 ± 0.01[b,c]	0.08 ± 0.01	0.53 ± 0.10
Colitis—HEBD	300 mg/kg	6.9 ± 0.8[d]	0.09 ± 0.01[b,c]	0.08 ± 0.0[c]	0.44 ± 0.01
p-Coumaric acid	15 mg/kg	6.9 ± 1.5[d]	0.07 ± 0.01	0.07 ± 0.01	0.43 ± 0.01

dose of 300 mg/kg did not prevent this decrease compared with the vehicle-treated colitis group. Interestingly, the HEBD at 3 mg/kg prevented by 16% the decrease in colon length compared with the vehicle-treated colitis group. The treatment with p-coumaric acid does not change the reduction of colon length in colitis animals (Table 1). Furthermore, the results also showed that there was a difference concerning the weight of the colon from the colitis mice

treated with HEBD at doses of 30 and 300 mg/kg compared with the colitis mice treated with the vehicle or the naïve group ($p < 0.05$) (Table 1).

3.3. Effect of HEBD on Histopathological Changes in the Colon of DSS-Induced Colitis Mice. The colon histological architecture was intensely altered by the DSS intake in the vehicle group, presenting villus height and crypt depth reduced (points highlighted by black arrows) (Figure 2). In addition, intense edema in the lamina propria with significant inflammatory infiltrate was highlighted in the colitis mice treated with the vehicle (points indicated by asterisks). On the other hand, the treatment with HEBD (300 mg/kg) partially preserved the villi architecture and decreased the deleterious effect of DSS on intestinal tunics.

3.4. Effect of HEBD on DSS-Induced Changes in Colonic Mucin Levels. Representative images of each experimental group are shown in Figure 3(b). As quantified in Figure 4(a), the PAS staining in the colon of vehicle-treated colitis mice decreased by 46% compared with the noncolitis group (NV: $26 \pm 4 \times 103$ pixels/field) (Figure 3(a)). In contrast, the treatment with HEBD (300 mg/kg) increased the mucin levels by 318% compared with the colitis mice treated with the vehicle.

3.5. Effect of HEBD on Behavioral of Colitis Mice Evaluated in the EPM Test. It was not observed a significant difference between the groups in the percentages of entry into the open or closed arms, respectively, Figures 4(a) and 4(b). However, the vehicle-treated colitis mice had an average of 14% in the length of stay in the open arms, which was significantly increased compared with the naive (noncolitis) group ($p < 0.001$). In contrast, the colitis group treated with HEBD presented a decrease in the length of stay in the open arms with the colitis group treated with the vehicle (Figure 4(c), $p < 0.05$), but with similar values to found in noncolitis mice ($p > 0.05$). The analysis of the length of stay in the closed arms was similar between the naive group and the colitis group treated with HEBD ($p > 0.05$); in contrast, the vehicle group had an increased average stay in the closed arms compared with the noncolitis mice (Figure 4(d), $p < 0.05$).

3.6. Effects of HEBD on Behavioral Parameters of Colitis Mice Evaluated in the TST. There was no statistical difference between groups in the frequency of immobility in the tail suspension test (Figure 5(a)), but it was possible to observe an increase in the immobility time in the vehicle-treated colitis mice compared with the naive group or the colitis group treated with HEBD ($p < 0.001$, Figure 5(b)).

3.7. Effects of HEBD on Behavioral Parameters of Colitis Mice Evaluated in the OFT. The vehicle-treated colitis group showed a reduced number of crossings with the naive group ($p < 0.01$) (Figure 6(a)). Likewise, there was a significant difference between the naive and colitis groups treated with HEBD ($p < 0.05$) regarding the crossing activity. Further, the rearing activities of the colitis group treated with the vehicle ($p < 0.05$) or HEBD ($p < 0.05$) also were reduced compared with the naive (noncolitis) group (Figure 6(b)).

3.8. Effects of HEBD on DSS-Induced Biochemical Changes in the Colon of Mice. The GSH levels were decreased by 38% in the colitis group treated with the vehicle compared with the naïve (noncolitis) group, whereas the colitis group treated with HEBD (300 mg/kg, p.o) showed an increase of 51% in the GSH levels related to the vehicle-treated colitis group (Table 2).

The SOD activity was increased by 39% in the vehicle-treated colitis group compared with the naïve (noncolitis) group, and an increase of 107% in this parameter was evidenced in the colitis group that received HEBD (300 mg/kg) compared with the vehicle-treated colitis group ($p < 0.01$). Moreover, the CAT activity was decreased by 33% in the vehicle-treated colitis group compared with the naive (noncolitis) group, and the treatment with HEBD (300 mg/kg) increased by 41% the activity of this enzyme compared with the vehicle group (Table 2).

Regarding GST activity, no differences were observed between the vehicle-treated colitis group and the naïve (noncolitis) group, but there was a reduction of 32,4% in the GST activity in the colitis animals treated with HEBD (300 mg/kg). The LOOH and ROS levels did not differ between experimental groups (Table 2).

The MPO analysis showed a 53% increase in the vehicle-treated colitis group compared with the naive ($p < 0.05$). When comparing the colitis group treated with vehicle and the colitis group treated with HEBD, there is a 50% decrease ($p < 0.01$) in MPO levels in the colitis group treated with HEBD.

3.9. Effect of HEBD on Oxidative Stress Parameters in the Cortex and Hippocampus of Colitis Mice. The effect of HEBD on oxidative stress in the cortex and hippocampus is shown in Table 3. In the cortex, a decrease of 44% and 38% was observed in the colitis groups treated with the vehicle and HEBD in the GSH levels, with the naive group, respectively. There was a 24% decrease in the GSH content of the vehicle-treated colitis group in hippocampal samples compared with the naive group. On the other hand, when compared to the vehicle-treated colitis group, the treatment with HEBD increased by 44% of the GSH levels in the hippocampus of colitis mice.

In the cortex samples, a significant decrease in the SOD activity in colitis mice treated with the vehicle or HEBD was not observed compared with the naive (noncolitis) group. Similarly, the vehicle-treated colitis group had a 36% decrease in SOD activity in the hippocampus, compared with the naive group; however, the HEBD-treated colitis group showed an increase of 43% in the activity of this enzyme, compared with the vehicle-treated colitis mice.

It was not verified a significant difference in the CAT activity in both the cortex and the hippocampus of experimental groups (Table 3). Compared with the naive group,

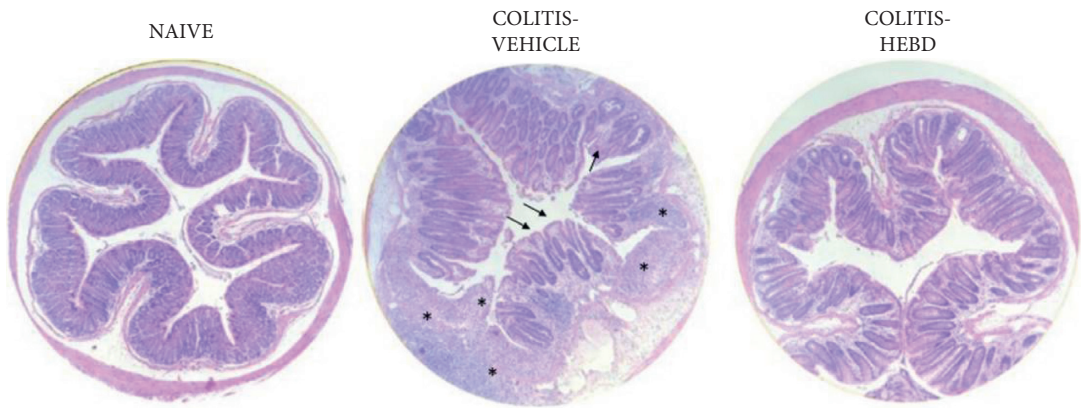
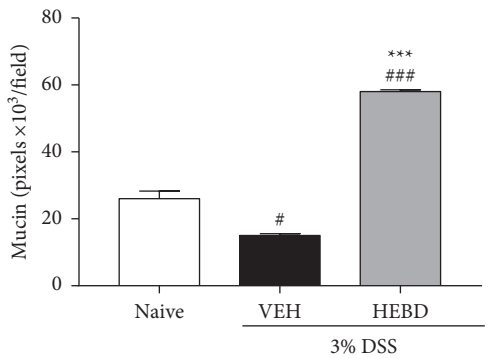
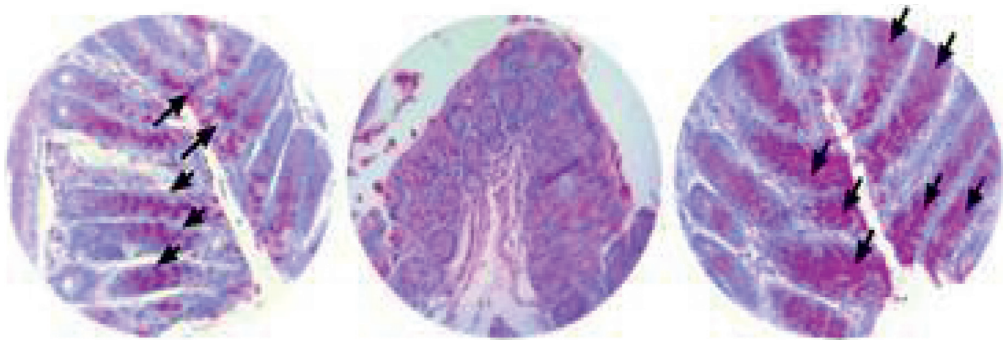


Figure 2: HEBD reduces on histological changes in colon tissue from DSS-induced colitis mice. NV: naive (noncolitis mice treated with the vehicle, water plus 1% Tween-80, 10 ml/kg, p.o); VEH: colitis mice treated with the vehicle; HEBD: hydroalcoholic extract of *B. dracunculifolia* (300 mg/kg). The asterisks indicate edema and inflammatory infiltrate, and black arrows indicate reduced villus height and crypt depth.



(a)



(b)

Figure 3: HEBD increased the mucin staining by PAS method in colon tissue from DSS-induced colitis mice. Colitis mice were treated with the vehicle (VEH: water plus 1% Tween-80, 10 ml/kg, p.o) or hydroalcoholic extract of *B. dracunculifolia* (HEBD: 300 mg/kg, p.o). (a) Results are presented as the means ± S.E.M. ($n = 6$), one-way ANOVA followed by Bonferroni post-test. $^{###}p < 0.001$ *and* $^{\#}p < 0.05$ compared with the naive (noncolitis) group (NV). $^{***}p < 0.0001$ compared with the colitis group treated with the vehicle (VEH). (b) Microscopic representative image from each experimental. Arrows indicate positive points for mucin labeling (stained in pink).

there was a 35% decrease in GST activity in the cortex and 22% in the hippocampus. In addition, the treatment with HEBD did not prevent the diminished GST activity in the cortex or hippocampus of colitis mice.

3.10. Effect of HEBD on TNF and IL-6 Levels in the Colon, Cortex, and Hippocampus of Colitis Mice. There was an increase in TNF levels in the hippocampus of vehicle-treated colitis mice compared with the naive group ($p < 0.05$)

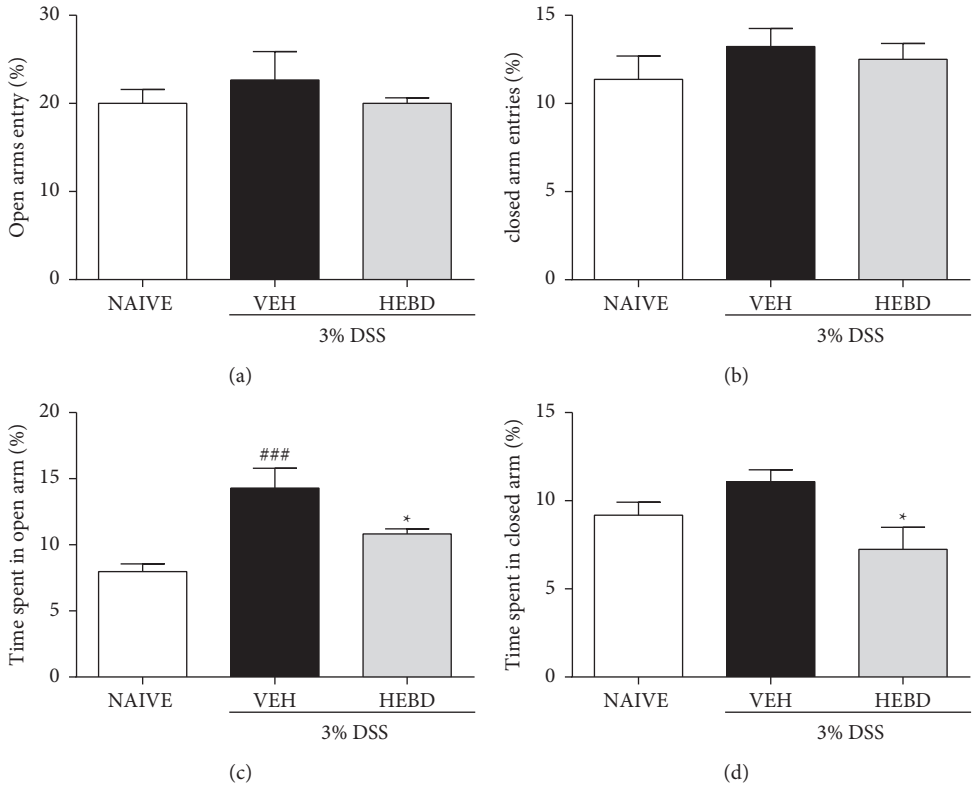


Figure 4: HEBD changes behavioral parameters of colitis mice in the EPM test. Colitis mice were treated with the vehicle (VEH: water plus 1% Tween-80, 10 ml/kg, p.o) or hydroalcoholic extract of *B. dracunculifolia* (HEBD: 300 mg/kg, p.o). Results are presented as the means ± S.E.M. ($n = 6$). One-way ANOVA followed by Bonferroni's post-test. $^{###}p < 0.001$ and $^{\#}p < 0.05$ compared with the naive (noncolitis) group (NV). $^{*}p < 0.05$ compared with the colitis group treated with the vehicle (VEH).

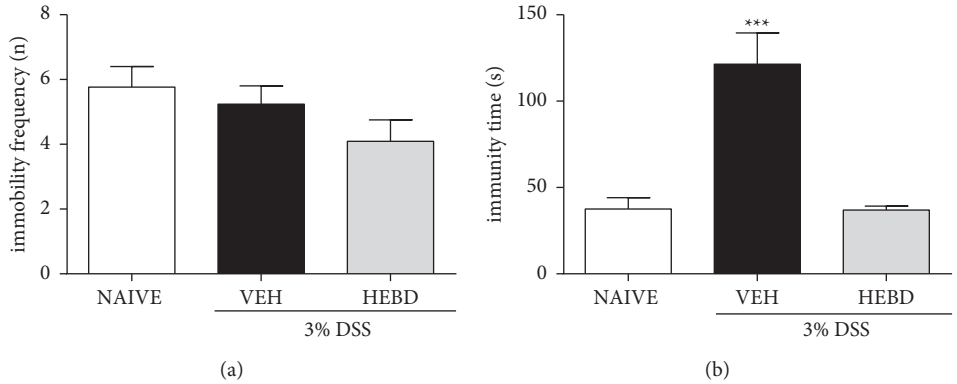


Figure 5: HEBD changes behavioral parameters of colitis mice in the TST. Colitis mice were treated with the vehicle (VEH: water plus 1% Tween-80, 10 ml/kg, p.o) or hydroalcoholic extract of *B. dracunculifolia* (HEBD: 300 mg/kg, p.o). Results are presented as the means ± S.E.M. ($n = 6$). One-way ANOVA followed by Bonferroni's post-test. $^{***}p < 0.001$ compared with the noncolitis mice (NAIVE) or the colitis group treated with HEBD.

(Figure 7(b)). A difference ($p < 0.05$) was also observed between the colitis group treated with HEBD and the colitis group treated with the vehicle, reducing TNF levels to values like the naive group. However, there was no difference between TNF levels in colon and cortex samples from different experimental groups.

Regarding IL-6 levels, the results observed in Figure 8(a) demonstrate a significant decrease ($p < 0.05$) in IL-6 levels in the colon of the colitis mice treated with the HEBD-treated compared with the vehicle-treated colitis group. Furthermore, there was a difference ($p < 0.05$) with increased levels of IL-6 in the hippocampus of colitis mice treated with HEBD and the naive group compared with the vehicle-treated colitis group (Figure 8(b)). Finally, there was no significant difference in IL-6 levels in the cortex of mice in any of the experimental groups (Figure 8(c)).

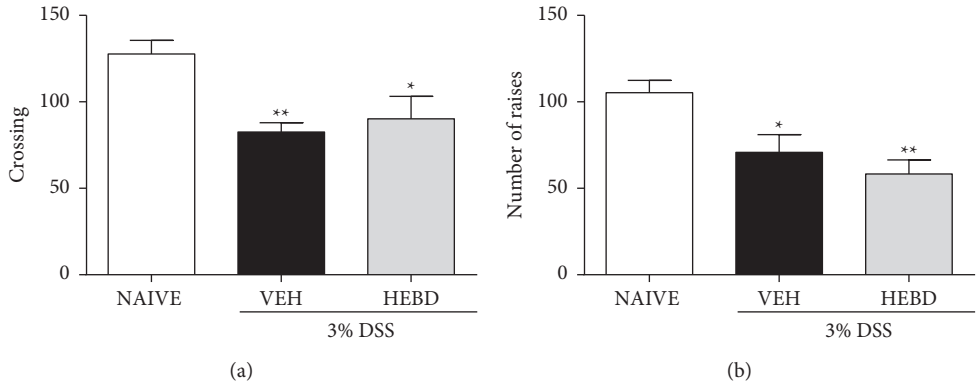


Figure 6: HEBD has not changed the behavioral parameters of colitis mice in the OFT. Colitis mice were treated with the vehicle (VEH: water plus 1% Tween-80, 10 ml/kg, p.o) or hydroalcoholic extract of *B. dracunculifolia* (HEBD: 300 mg/kg, p.o). Results are presented as the means ± S.E.M. ($n = 6$). One-way ANOVA followed by Bonferroni's posttest. * $p < 0.05$ and * * $p < 0.01$ compared with the noncolitis mice (NAIVE).

Table 2: Effect of HEBD on biochemical parameters in colon of colitis mice.

	Naive	Colitis vehicle	Colitis HEBD
GSH	831.7 ± 67.5	509.5 ± 65.7[b]	772.3 ± 84.7[b]
ROS	2315 ± 347	2079 ± 624	1813 ± 155.3
LOOH	3.8 ± 0.3	3.6 ± 0.8	1.9 ± 0.1
SOD	18.7 ± 3.6	11.4 ± 1.5[c]	23.6 ± 6.4[b]
CAT	6.1 ± 1.4	4.0 ± 1.2[c]	5.7 ± 1.2[a]
GST	3.3 ± 0.1	3.2 ± 0.3	2.1 ± 0.6[b,d]
MPO	0.03 ± 0.01[a]	0.04 ± 0.01	0.03 ± 0.01[b]

Colitis mice were treated with the vehicle (VEH: water plus 1% Tween-80, 10 ml/kg, p.o) or *B. dracunculifolia* hydroalcoholic extract (HEBD: 300 mg/kg, p.o). GSH: reduced glutathione (μg/g tissue). ROS: reactive oxygen species (fluorescence units). LOOH: lipid hydroperoxide (mmol/g of tissue). SOD: superoxide dismutase (U/mg protein). CAT: catalase (mmol/mg protein). GST: glutathione S-transferase (mmol/mg protein). MPO: myeloperoxidase: m O.D/mg protein. Results are presented as the means ± S.E.M. ($n = 6$). One-way ANOVA followed by Bonferroni's posttest. [a]$p < 0.05$ and [b]$p < 0.01$ vs. vehicle-treated colitis and [c]$p < 0.05$ and [d]$p < 0.01$ vs. naive (noncolitis) groups.

Table 3: Effect of HEBD on oxidative stress parameters in the cortex and hippocampus of colitis mice.

	Naive	Colitis vehicle	Colitis HEBD	Naïve	Colitis vehicle	Colitis HEBD
		Cortex			*Hippocampus*	
GSH	419.7 ± 49.0	234.2 ± 143[a]	261.3 ± 8.5[a]	352.9 ± 36.6	268.6 ± 31.7[a]	386.9 ± 53.2[b]
SOD	4.1 ± 0.4	2.2 ± 0.1[a]	2.4 ± 0.5[a]	4.3 ± 0.4	2.7 ± 0.1[a]	3.9 ± 0.3[b]
CAT	0.7 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.7 ± 0.2	0.8 ± 0.2	1.0 ± 0.2
GST	9.0 ± 0.1	5.8 ± 0.5[a]	6.5 ± 0.4[a]	6.3 ± 1.1	4.9 ± 0.5[a]	4.6 ± 0.6[a]

Colitis mice were treated with the vehicle (VEH: water plus 1% Tween-80, 10 ml/kg, p.o) or *B. dracunculifolia* hydroalcoholic extract (HEBD: 300 mg/kg, p.o). GSH: reduced glutathione (μg/g tissue); SOD: superoxide dismutase (U/mg protein); CAT: catalase (mmol/mg protein); and GST: glutathione S-transferase (mmol/mg protein). Results are presented as the means ± S.E.M. ($n = 6$). One-way ANOVA followed by Bonferroni's posttest. [a]$p < 0.05$ vs. vehicle-treated colitis and [b]$p < 0.05$ vs. naïve (noncolitis) group.

4. Discussion

The B. dracunculifolia has been used in the south and southeast of Brazil as a medicinal plant to treat inflammatory conditions; indeed, some authors have validated such use and described the pharmacological potential of preparations of this plant in models of inflammatory diseases [31, 32]. Nevertheless, a single experimental study reported the effects of the ethyl acetate extract from B. dracunculifolia against intestinal inflammation induced by TNBS in rats [13].

Strengthening the hypothesis that B. dracunculifolia is helpful as an ethnopharmacological resource in treating intestinal inflammation, this research described the hydroalcoholic extract of B. dracunculifolia (HEBD) attenuated the DSS-induced ulcerative colitis in mice. The DSS-induced ulcerative colitis model has the advantage of easy reproducibility and promotes colonic damage mediated by a predominantly TH2 immune response, like what occurs in humans affected by ulcerative colitis.

It is interesting to highlight that the results obtained by Cestari et al. [13] were achieved using an extract soluble in ethyl acetate and that the described effects of this study were obtained by the administration of a hydroalcoholic extract, obtained with water and alcohol, which is closest to the folkloric use of this plant. Moreover, corroborating with Cestari et al. [13], a reduction in the DAI values in colitis

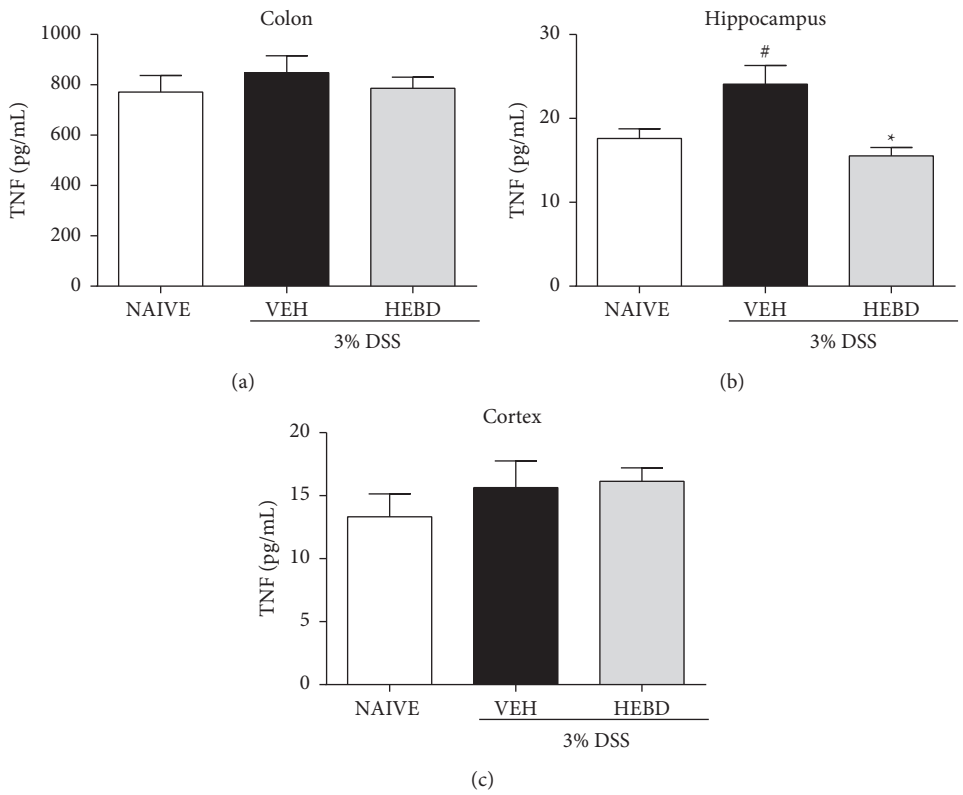


Figure 7: Effects of HEBD on TNF levels in colon (a), hippocampus (b), and cortex (c) of colitis mice. Colitis mice were treated with the vehicle (VEH: water plus 1% Tween-80, 10 ml/kg, p.o) or hydroalcoholic extract of *B. dracunculifolia* (HEBD: 300 mg/kg, p.o). Results are presented as the means ± S.E.M. ($n = 6$). One-way ANOVA followed by Bonferroni's posttest. $^{\#}p < 0.05$ compared with naïve (noncolitis) group, and $^{*}p < 0.05$ compared to colitis group treated with the vehicle.

mice treated with HEBD at a dose of 300 mg/Kg was also observed, evidencing the pharmacological potential of HEBD to treat IBD.

The DAI is a parameter that assesses the remission or exacerbation of the disease during the experimental period and includes changes in stool consistency, body weight, and rectal bleeding [33]. In addition to the reduction in DAI, HEBD attenuates the weight loss of colitis mice, which also positively impacted DAI reduction in the HEBD-treated group. The main physical signs presented by individuals affected by UC are excessive weight loss leading to malnutrition [34], which compromises their quality of life [5]. Therefore, alternative treatments to mitigate weight loss are necessary.

The UC also compromises the colon's integrity, and the treatment with HEBD minimized the colon shortening in colitis mice, but only observed at the lowest dose tested, although this dose did not reduce the DAI. In addition, p-coumaric acid (15 mg/Kg), the major compound of B. dracunculifolia [35, 36], was not able to attenuate the DAI or prevent the decrease in colon length in colitis animals, which can be due to the dose administered chosen according to its content in the HEBD and the minimum effective dose of this extract in reducing DAI. p-Coumaric acid doses were established based on the concentration of this compound in HEBD. Luceri et al. [37] described that the p-coumaric acid at 50 mg/kg attenuated the intestinal inflammation induced by DSS due to its ability to suppress COX-2 expression and activity. Moreover, our research group showed the gastric healing effects of p-coumaric acid at a 15 mg/kg dose in rats [14]. Therefore, it is possible to infer that the p-coumaric acid may participate in the intestinal anti-inflammatory effects of the extract along with other constituents and not as an isolated mediator.

The main histological changes observed in the colon of mice after exposure to DSS were a decrease in mucin levels, changes in epithelial integrity with some necrosis areas, and neutrophil infiltration in the lamina propria and submucosa [38]. In fact, our slices were found edema and inflammatory infiltrate in the lamina propria, loss of crypts, and goblet cells, in addition to multifocal areas of erosions and ulcerations demonstrating significant tissue damage in the colon after exposure of the mice to DSS. Despite the ability of DSS to alter the colon histology integrity, it was possible to observe that the treatment with HEBD (300 mg/kg) partially preserved the villi architecture and reduced the deleterious effect of DSS on the intestinal tunics, corroborating with the diminished in the DAI values reached in these mice.

Epithelial mucins are mucus glycoproteins that make a layer that lines the gastrointestinal tract as a protective barrier that guarantees intestinal homeostasis acting as the first line of defense against xenobiotics, bacteria, viruses, fungi, or protozoa. The Goblet cells produce different types of mucins basally or in response to some stimulus (toxins,

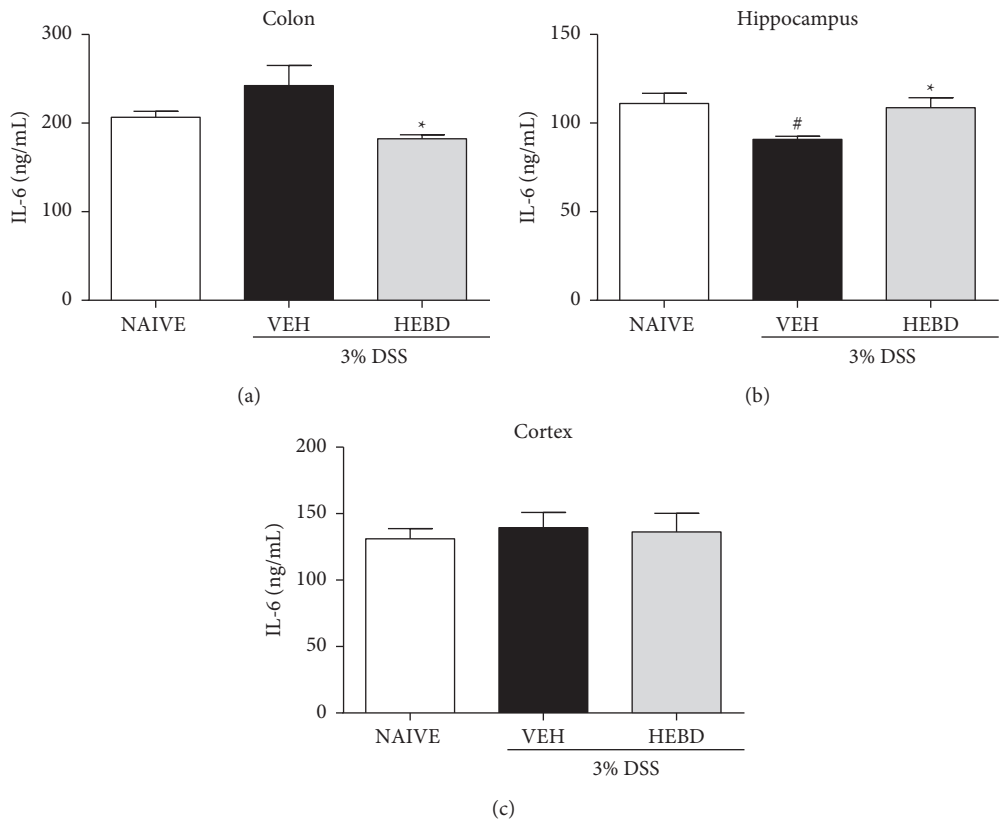


Figure 8: Effects of HEBD on IL-6 levels in colon (a), hippocampus (b), and cortex (c) of colitis mice. Colitis mice were treated with the vehicle (VEH: water, 10 ml/kg, p.o) or hydroalcoholic extract of *B. dracunculifolia* (HEBD: 300 mg/kg, p.o). Results are presented as the means ± S.E.M. ($n = 6$). One-way ANOVA followed by Bonferroni's posttest. $^{\#}p < 0.05$ compared with naïve (noncolitis) group, and $^{*}p < 0.05$ compared with colitis group treated with the vehicle.

cytokines, neuropeptides, and growth factors). Our results showed that mucin levels in the colon of mice from the colitis group treated with the vehicle decreased. This reduction in mucin levels due to an inflammatory injury affects the mucus barrier. It increases intestinal permeability, which facilitates the passage of bacteria, microbial products, and toxins that cause damage to the epithelial cells, initiating a systemic inflammatory stimulus [39]. In contrast, the HEBD (300 mg/kg) prevented the decrease in mucin levels even in the intake of DSS. This effect occurred in the stomach of rats submitted to acetic acid–induced ulcer and treated with HEBD (300 mg/kg) [14], attesting that it favors the mucin secretion in the gastrointestinal tract mucosa and can play a protective factor during injurious processes in these tissues.

Dorofeyev et al. [39] described that in patients with UC and Crohn's disease, there was a depletion in mucin expression, compromising its protective function in the colon, and Liu et al. [40] showed the relationship with intestinal diseases and reduction in MUC2 expression. These data corroborate our results because colitis mice that received vehicle had greater DAI and more significant depletion in colonic mucin levels, while colitis animals treated with extract showed a reduction in DAI in parallel to mucin preservation.

In addition to the action of HEBD against intestinal damage due to DSS-induced colitis, we also investigated the neuroprotective effect of the extract in these colitis mice because of a relationship between IBD and neuropsychiatric comorbidities [41]. Byrne et al. [42] assessed the prevalence of anxiety and depression in patients with IBD; they found a high prevalence of these diseases in those populations and demonstrated that the active phase of the disease was significantly associated with an increased risk of depression and anxiety. A depressive-like behavior was observed in vehicle-treated colitis mice, evidenced by the increase in the immobility time in the TST. It was not observed in the group treated with HEBD.

Rivet-Noor [43] reviewed the evidence about the relationship between disruption of intestinal mucins and depression and addressed that stress can modify the delicate mucus-microbiome balance, initiating dysbiosis and ultimately leading to depression. Moreover, conducted a clinical with patients suffering from major depressive disorder (MDD) investigating pro-inflammatory pathways related to the "likable bowel" hypothesis associated with MDD, defined as an intestinal translocation of bacterial and an abnormal toll-like receptor-mediated immune response initiating a systemic inflammatory state, microglial activation, and neuroinflammation. Therefore, the similar behavior to the healthy group found in colitis mice treated with HEBD may be due to the preservation of the integrity of the intestinal barrier promoted by the extract or other effects that mitigate inflammatory events in the central nervous system.

The debility state of colitis mice treated with the vehicle might interfere with the data obtained. Therefore, the animals were evaluated in the OFT to verify the impact of locomotor activity on the obtained data. Furthermore, in the EPM test, the colitis group treated with the vehicle had a higher average stay in both open and closed arms when compared to the naive group or HEBD colitis group. Indeed, there was a reduction in exploratory activity in the colitis mice treated with the vehicle; however, the colitis group treated with HEBD also showed reduced in the crossing and rearing number, which could mean that although the extract reduced the severity of colitis, a reduction in mobility in this group remains but not to the degree that it impacts the data obtained in the TST and EPM test. Besides, the extract administration in healthy animals did not promote any behavior change.

The excessive production of reactive oxygen species (ROS) is related to the development of colitis, and endogenous antioxidant defenses, especially GSH, play an essential role in the attenuation of oxidative stress that is related to cytotoxicity, cell death, and inflammatory process due to intestinal inflammation [44]. In parallel to the increase in intestinal mucin, the HEBD also increased GSH levels and SOD activity in the colon. In agreement with these data, Mariano et al. [45] found that hydroalcoholic extract of Brazilian green propolis increased colonic GSH and SOD activity in the colitis mice, keeping these antioxidants at levels like those found in the noncolitis group.

Guimarães et al. [35] investigated the effects of the glycolic extract of B. dracunculifolia against oxidative stress in the liver of rodents. They attributed its beneficial effects to its antioxidant capacity, which is related to the compounds present in the extract, mainly phenols, and flavonoids. The SOD acts to convert the superoxide anion into hydrogen peroxide, and CAT, in turn, neutralizes it in water [46]. In addition to the increase in SOD activity, the colitis mice treated with HEBD showed an increase in CAT activity, demonstrating the favor of antioxidant defenses mediated the HEBD actions in the colon.

The central nervous system (CNS) is highly susceptible to oxidative stress, and the interactions that occur in the intestinal microbiota can also influence the oxidative state of the CNS [47]. In parallel to attenuating the colonic oxidative stress, the HEBD treatment also increased the levels of GSH and the SOD activity in the hippocampus from colitis mice. In contrast, the extract did not prevent GSH depletion and SOD activity reduction in the cortex of colitis animals, evidencing specificity in the site of action.

An increase in MPO activity, a biomarker of neutrophil infiltration, was found in the colitis group treated with the vehicle; however, the HEBD treatment reduced this inflammatory marker. Previously, Costa et al. [14] demonstrated that the hydroalcoholic extract of Brazilian green propolis also reduced the MPO activity in gastric tissues and attributed the intestinal anti-inflammatory effects of this propolis to its ability to inhibit TH1 differentiation in a model of colitis induced by TNBS.

Our results also agree with Cestari et al. [13] using TNBS-induced UC in rats treated with an extract of B. dracunculifolia. Another study also associated the anti-inflammatory effect of the HEBD with its ability to inhibit cyclooxygenase (COX)-2, which is often increased in inflammatory processes [48]. Moreover, the inflammatory process associated with colitis increases the production of inflammatory cytokines, mainly TNF and IL-6 [18]. TNF plays an essential role in the progression of UC as it stimulates immune system cells to produce different chemokines, cytokines, and prostaglandin E2, in addition to ROS and RNS, which culminates in an increase in intestinal permeability and consequent recruitment of neutrophils to the inflamed colonic tissue [49]. However, unexpectedly, in our study, we did not observe a significant difference in TNF levels in the colon of colitis mice.

As TNF, IL-6 is also involved in the inflammatory process related to colitis and is associated with the regulation of the adaptive immune response [49]. In the study by Wang et al. [50], in which the authors also used a murine model of ulcerative colitis induced by DSS and azoxymethane, they found significantly higher levels of TNF-α and IL-6 in colitis mice compared with the control group. These data partially corroborate our results, although no difference was observed in TNF-α levels, IL-6 levels were significantly lower in the colon of colitis mice treated with HEBD, confirming once more the intestinal anti-inflammatory capacity of this extract.

A recent study discussed that high serum levels of IL-6 and TNF may be related to depressive symptoms [51]. A significant increase in TNF levels in the hippocampus, but not cortex, of vehicle-treated colitis mice was observed, along with a depressive-like behavior assessed by the TST in this experimental group. Interestingly, Do and Woo [52] described that the hippocampus is more susceptible to neuroinflammation in animal models of DSS-induced colitis, and later, this inflammation can progress to other brain regions. Furthermore, it has been shown that rats with colitis have increased excitability in the hippocampal lamina.

Opposite to TNF, there was a reduction in IL-6 levels in the hippocampus of vehicle-treated colitis mice. However, although IL-6 is a pro-inflammatory cytokine, it can also act in regenerative or anti-inflammatory activities, depending on the type of signaling [53]. However, it is essential to note that animals treated with extract had similar levels of IL-6 to healthy animals in both the colon and hippocampus.

5. Conclusions

In conclusion, HEBD by oral route reduced the severity of colitis due to its ability to minimize weight loss and colon shortening and preserve the colonic histology. The adequate level of colon mucin in colitis mice treated with HEBD, along with the reduction in inflammation and oxidative stress in the colon of treated animals, placed this extract as a natural resource to treat intestinal inflammatory diseases, corroborating the medicinal use of B. dracunculifolia as anti-inflammatory.

Furthermore, HEBD has also been shown to be effective in preventing behavioral changes in colitis mice, mainly attenuating depressive behaviors that may have been

mediated by the inhibitory effects in inflammatory and oxidative markers in the hippocampus in parallel to the degree of preservation in the intestinal barrier in colitis mice that received the extract.

Data Availability

The data that support the findings of this study are available from the corresponding author, LMS, upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

LMS and TCSF conceived and designed the research. TCSF, LNBM, ACS, LV, RKSN, and CC contributed to experiment data collection. LMS, MMS, and JKB analyzed and interpreted the data. LMS and TCSF wrote the paper. LNBM, MMS, and JKB obtained the plant extract and revised the manuscript. All authors have read and approved the final manuscript.

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Review Article

An Insight into Phytochemical, Pharmacological, and Nutritional Properties of *Arbutus unedo* L. from Morocco

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Arbutus unedo L. (Ericaceae) is an evergreen shrub widely distributed in the Mediterranean region, particularly through the Moroccan forests. It is an important medicinal plant of great scientific interest due to its nutritional, pharmacological, and chemical properties. The objective of this review is to provide insights into traditional medicinal uses and phytochemical and pharmacological properties of *A. unedo* from Morocco. In Morocco, the plant has been used as a traditional medicine to treat several pathological conditions. Many phytochemical compounds have been reported in the plant, of which vitamins, carotenoids, flavonoids, polyphenols, tannins, and their derivatives are the most prevalent. Leaves and fruits of *A. unedo* contain the most significant number of phytochemicals among the species. Furthermore, researchers have demonstrated that *A. unedo* exhibited antioxidant, anticancer, antibacterial, antidiabetic, antiaggregant, and antihypertensive activities due to the presence of many biochemical compounds with health-promoting properties. According to different toxicity tests, the use of *A. unedo* is devoid of any significant side effects and/or toxicity. Despite its nutraceutical and health-promoting properties, Moroccan *A. unedo* remains underexploited mainly, and most of its traditional uses have not yet undergone scientific evidence-based research; therefore, improved knowledge about the potential value of the plant would allow understanding of its biological activity based on its phytochemical compounds that may contribute to the species preservation and valorization.

1. Introduction

During recent years, there has been an increasing reliance on phytonutrients and medicinal plants, due to their wide range significance in human health, food industry, and human nutrition [1–4]. According to the World Health Organization (WHO), 80% of the world's population depends on traditional medicine for primary health care, especially in Asian and African countries [5]. In fact, various reports from epidemiological studies have consistently shown an inverse association between regular consumption of fruits and vegetables and the risk of cardiovascular diseases (CVD) and certain types of cancer [6–10]. Furthermore, it has been reported that herbal remedies have fewer side effects and are better tolerated by patients than synthetic medications [11]. In addition, the WHO has recognized that traditional medicine is an important component in health care and has been encouraging research on development involving medicinal plants [12]. The health beneficial properties of medicinal plants are in part attributed to the antioxidant activity of their phytochemical compounds, such as phenols, flavonoids, tannins, alkaloids, carotenoids, vitamins, and endogenous metabolites [13–15].

Arbutus unedo (*A. unedo*), the strawberry tree (Ericaceae), is widely distributed in the Mediterranean region. It can be also found in Canary Islands and western Asia, where climate is adequate to its development [16, 17]. This fruit tree species is known by different vernacular names, such as sasnou and bakhanou in Morocco, madroño in Spain, arbousier in France, Koumaria in Greek, and corbezzolo in Italy [18].

In Morocco, strawberry trees grow wild in different bioclimatic regions extending from the subhumid to the semiarid regions (Figure 1). *A. unedo* has been extensively found in the region of the Northwest, Central Plateau, Pre-Rif and Western Rif, High and Middle Atlas, mainly associated with *Quercus ilex* and sometimes with *Pinus halepensis* and *Tetraclinis articulate*. A fragmented-like distribution is common in the Central, Souss plateau, and Northeastern regions. *A. unedo* shows strong resistance to hard environmental conditions and has the ability to regenerate after forest fires. This plant populations are widespread on all types of substrates but most often on siliceous and calcareous soils at altitudes varying from 150 to 1613 m. The average of annual temperature and rainfall varied from 12.4°C to 18.4°C and from 337 to 1115 mm, respectively [19].

Traditionally, the fruits, leaves, and roots of *A. unedo* are well-known and used by the Moroccan population as diuretic, astringent, antidiarrheal, antiasthmatic, antiinflammatory, antidiabetic, antihypertensive, against rheumatism and gastrointestinal and renal diseases [20–27]. However, all these uses remain more as traditional habits than having economical purposes.

A diversity of pharmacological properties is ascribed to *A. unedo*, such as astringent, depurative, anti-inflammatory, haemostatic, antitumor, antioxidant, antimicrobial, spasmolytic, and neuroprotective [28–40].

Furthermore, experimental investigations of our group have shown that *A. unedo* extracts exhibited different biological properties including antioxidant, platelet antiaggregant, vasorelaxant, antihypertensive, and antidiabetic activities [37, 41–49]. In addition, the plant has been shown to contain different phytoconstituents such as flavonoids, tannins, phenolic acids, organic acids, α-tocopherol, carotenoids, anthocyanins, triterpenoids, fatty acids, sterols, vitamin c, fibers, calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P), and other bioactive compounds [37, 47, 50–55], which contribute to its various pharmacological and nutritional properties.

In Morocco, no economic importance was attributed to *A. unedo*, and the plant remains largely underexploited, in comparison to other Mediterranean countries, where the fruit of the *Arbutus* tree is highly sought after for its nutritional qualities [56–58]. Furthermore, *A. unedo* tree populations are severely destroyed due to deforestation and overcollecting. In addition, review publications related to *A. unedo* from other countries can be found in the literature; however, to the best of our knowledge, there are no comprehensive scientific reviews to cover all aspects of information about *A. unedo* grown in Morocco. Thus, an improved knowledge about the potential value of the plant could contribute to enhance the production, marketing, and consumption of *A. unedo* derived products, which may contribute to the species preservation and valorization. In this context, the aim of this work is to provide a comprehensive review concerning the ethnobotanical, pharmacological, nutritional, phytochemical, and toxicological properties of *A. unedo* grown spontaneously in Morocco, which may encourage interested researchers to conduct further investigations to evaluate the health-promising benefits and nutritional properties of Moroccan *A. unedo*, its active constituents, and their derivatives.

2. Search Strategy

Available information was collected from different scientific databases such as PubMed, Science direct, Springer, Web of Science, and Wiley using keywords: *Arbutus unedo* L., *A. unedo* L., strawberry tree, ethnobotanical, phytochemical and Pharmacological properties. Additional references were hand-searched. This review is limited to scientific research concerning the ethnobotanical, pharmacological, and phytochemical properties of *A. unedo* L. grown in Morocco.

3. Botanical Description

A. unedo belongs to family Ericaceae that is in the major group Angiosperms (flowering plants). It is an evergreen shrub with erect and branched stems (Figure 2). The plant can reach a height of 12 meters, but it is normally a shrub between 1.5 and 3 meters tall [16]. It is an important ornamental bush due to its nice look and fragrance of the white flowers and the red fruit, which appear simultaneously in autumn and winter. The leaves are evergreen, entire, oblong-lanceolate, short-stalked, and coriaceous with a dark green color and finely serrated margin [59]. The flowers are

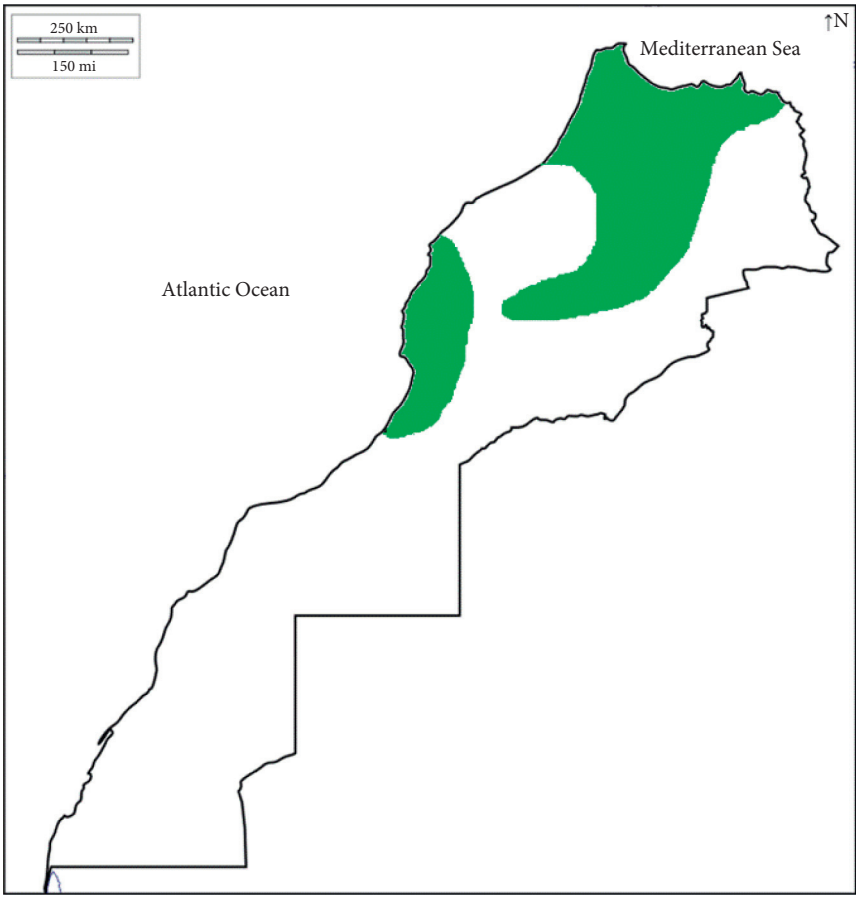


Figure 1: Approximate distribution of *Arbutus unedo* L. throughout Morocco.

Figure 2: The whole plant (a) and flowers (b) of *Arbutus unedo* L.

hermaphrodites, fragrant, white, and like little bells, with 5 green sepals fused at the base, 5 united petals, and 10 stamens [18]. *A. unedo* fruit is a spherical berry about 1–2 cm in diameter, with a rough surface, green to yellow to bright red when fully ripe. The strawberry tree bears both flowers and fruits at varying degrees of maturity. This is due to the long ripening period of the fruits, which is more than one year [59].

4. Traditional Uses

In Morocco, different parts of *A. unedo* are used in folk medicine to treat various ailments (Table 1). A survey of medicinal plants in Oriental Morocco showed that *A. unedo* roots and leaves are widely used in the treatment of hypertension and diabetes [22]. Authors reported that the phytotherapy is widely adopted in northeastern Morocco. About 67.5% of total identified patients regularly utilized phytotherapy to treat their diseases. In another ethnobotanical survey realized in Northern Morocco, the leaves and the roots of the plant were used traditionally to treat diabetes, hypertension, and cardiac diseases [23]. Furthermore, the roots and cortex of the plant have been also used as a remedy for cholesterol-lowering, against digestive problems and CVD [60]. In another ethnobotanical study, El-Hilaly et al. reported that the leaves and fruits of *A. unedo* are orally administered in the form of decoction as well as raw by the population in the region of Taounate for the treatment of renal diseases [24]. In Ouezzane (North-West of Morocco), the leaves of the plant have been used as decoction in the treatment of diabetes [61]. Other ethnobotanical surveys realized through Morocco revealed that the plant is also used by the population as stomachic, diuretic, astringent, antidiarrheal, antiasthmatic, and anti-inflammatory medicine, and against rheumatism [20, 25, 27]. There are numerous reports, which showed widespread utilization of *A. unedo* to heal various disease conditions in Morocco. Therefore, there is an urgent need to utilize *A. unedo* as an effective alternative to modern allopathy medicines to heal several diseases.

5. Phytoconstituents

The plant is also an important source of phytochemical compounds; therefore, many bioactive compounds are isolated. It is revealed that genetic variability and environmental factors may affect the phytochemistry of *A. unedo*. Phytochemicals found in various parts of Moroccan *A. unedo* are given in Table 2, and the structures are shown in Figure 3.

5.1. Leaves. A number of phytochemical studies revealed that *A. unedo* leaves contain various classes of chemical compounds such as phenolic compounds, terpenoids, anthocyanins, flavonoids, and tannins. In a previous study, we have demonstrated the presence of genins and heterosidic flavonoids in the extracts of *A. unedo* leaves collected from Tazzeka Mountain (region of Taza) [37]. In another study, we have isolated tannins from *A. unedo* leaf extract [48]. Other phytochemical analysis reported that *A. unedo* leaves contain various polyphenolic compounds, such as epicatechin, catechin, and catechin gallate [47].

In a screening study, Mrabti et al. reported that *A. unedo* leaves from the Beni Mellal region contain tannins, flavonoids, and anthraquinones at high concentrations, and free quinones and terpenoids in lower concentrations. A study of Kachkoul et al. found that *A. unedo* leaf extracts from Taounate region contain polyphenols, flavonoids, flavonols, and anthocyanins [62]. The level of anthocyanins was low with regard to other compounds. The authors used ultra-performance liquid chromatography (UPLC-PDA-ESI-MS) to characterize the chemical compounds in the leaves. The UPLC profile indicated the presence of 22 compounds of gallic acid derivatives and flavonoids [62]. In another study, it has been reported that hydroalcoholic and aqueous extracts from *A. unedo* leaves contain tannins, polyphenols, and flavonoids [63]. Thus, the consumption of *A. unedo* leaves as raw or decoction would provide a sufficient amount of health-promoting phytochemicals.

5.2. Fruits. Little information is known about the chemical composition of Moroccan *A. unedo* fruits. Recently, Zitouni and coworkers [64] have analyzed the phenolic compounds in five strawberry tree fruits from different locations in Morocco and found that total phenols varied from 25.37 to 39.06 mg GAE/g D.W, while total flavonoid ranged from 3.30 to 7.07 mg GAE/g D.W. In total, 17 phenolic compounds have been identified in sampled cultivars, of which gallocatechol and catechin were the most abundant.

El Cadi et al. investigated the phytochemical profile of strawberry tree fruits grown in different locations of Northern Morocco (Achakar, Qsar Kbir and Chaoun-Qalaa). Results indicate that the chemical composition of the fruits varied between the three regions. Indeed, fruits from Achakar showed a high amount of polyphenols and flavonoids (127.5 ± 7.14 mg GAE/g (w/w) (D.W) and 105.9 ± 3.2 mg QE/g (w/w) (D.W), respectively), while berries from Qsar Kbir presented a high content of anthocyanins and tannins (1.48 ± 0.09 mg Pg-3-glu/g (w/w) (D.W) and 1.22 ± 0.1 mg EC/g (w/w) (D.W), respectively). In addition, chemical analysis of the three fruits using HPLC-DAD-ESI/MS revealed the presence of 75 chemical compounds, including hydroxybenzoic acids, hydroxycinnamic acids, flavone, flavan-3-ols, flavonols, and dihydroflavonols [65]. It was suggested that the variability observed in phytochemical composition among the different fruit extracts could be due to the geographical location of the samples.

In another study, Zitouni et al. determined the content of the total phenolic compounds and flavonoids, condensed and hydrolyzable tannins, and anthocyanins in twelve genotypes of *A. unedo* fruits collected from different locations of Morocco. Results showed that total phenolic compounds and flavonoids varied from 22.63 ± 1.74 to 39.06 ± 2.44 mg GAE/g D.W and from 3.30 ± 0.60 to 8.62 ± 1.10 mg RE/g D.W, respectively, while anthocyanins varied from 0.12 ± 0.06 to 0.66 ± 0.15 mg cya-3-glu/100 g D.W; however, condensed tannins and hydrolyzable tannins ranged between 10.41 ± 1.07–16.08 ± 1.50 mg TAE/g D.W and 4.08 ± 2.43–6.34 ± 3.47 TAE/g D.W, respectively. Using the HPLC assay, the authors also identified 17 phenolic compounds in the studied fruits, of which gallocatechol and catechin were the most abundant [66]. It was concluded that genetic factors seem to be responsible for the chemical profile of the analyzed fruits. The significant amounts of phytochemicals in *A. unedo* fruits confirm the nutritional and medicinal value of the plant.

Table 1: Traditional uses of *A. unedo* L. in Morocco.

Part used	Traditional medicinal uses	Mode of preparation/administration	References
Leaves	Kidney diseases	Decoction	[24]
	Antidiabetic, antihypertensive	Decoction/infusion	[22, 108]
	Cardiac disease, diabetes, hypertension	N.D.	[23]
	Antidiabetic, astringent, antidiarrheal	Decoction	[25, 26, 61]
	Stomachic, antiasthmatic	Decoction	[27]
Roots	Antidiabetic, antihypertensive, anti-inflammatory, against rheumatism	Decoction/infusion	[22, 25, 108]
	Cardiac disease, diabetes	N.D.	[23]
	Cholesterol-lowering, digestive problems, cardiovascular diseases	Decoction	[60]
	Diabetes	Decoction	[26]
	Stomachic, antiasthmatic	Decoction	[20, 27]
Fruits	Kidney diseases	Raw	[24]
	Digestive diseases and diarrhea	Infusion, fresh fruit	[19, 21]
Bark	Cholesterol-lowering, digestive problems, cardiovascular diseases	Decoction	[60]
	Diuretic, astringent, antidiarrheal	Decoction	[25]

N.D: not determined.

Table 2: Major phytochemical compounds reported from A. unedo *L.*

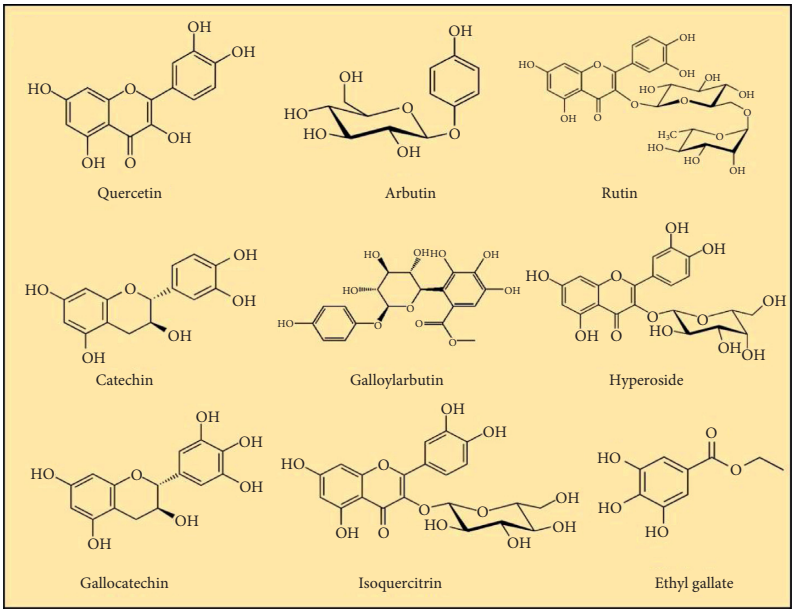
Plant part	Major components	Extraction method	References
Leaves	Genins and heterosidic flavonoids	Solid-liquid extraction Colorimetric method	[37]
	Tannins	Soxhlet extraction method Soxhlet extraction method	[47, 48]
	Arbutoside, quercetin, epicatechin, catechin, catechin gallate, hyperoside and gallic acid	HPLC coupled with mass spectrometry	[47]
	Tannins, flavonoids, anthraquinones, terpenoids, free quinones	Colorimetric method	[67]
	Polyphenols, flavonoids, tannins.	Infusion Soxhlet extraction method	[63]
	Phenolic compounds, flavonoids, flavonols, and anthocyanins	Colorimetric method Colorimetric method and UPLC-PDA-ESI-MS	[62]
	Gallic acid derivatives and flavonoids (22 compounds)		
Roots	Tannins, flavonoids, anthraquinones, terpenoids, free quinones	Colorimetric method	[67]
	Catechin	XAD-16 resin HPLC	[50]
Fruit	Flavonoids, tannins, anthocyanins, anthraquinones, sterols, steroids, deoxysugars, and glycosides	Colorimetric method	[65]
	75 compounds identified as hydroxybenzoic acids, hydroxycinnamic acids, flavone, flavan-3-ols, flavonols, and dihydroflavonols.	HPLC UPLC-PDA-ESI-MS	
	Phenols, flavonoids, condensed tannins, hydrolyzable tannins, and anthocyanins.	Spectrophotometric methods	[66]
	17 phenolic compounds, of which gallocatechol and catechin were the most abundant	HPLC	

UPLC-PDA-ESI-MS, Ultra-performance liquid chromatography with photodiode array and electrospray ionization tandem mass spectrometry; HPLC, high-performance liquid chromatography.

5.3. Roots. There are few studies that have focused on the chemical composition of the Moroccan *A. unedo* roots. Using Zippertex technology, Mrabti et al. isolated catechin (the therapeutic active compound) from *A. unedo* roots (Figure 4). The chemical structure of this compound was characterized by MS and NMR (^{1}H and ^{13}C) analyses [50]. In addition, similar to leaves, the roots of *A. unedo* were shown also to contain tannins, anthraquinones, and flavonoids [67]. Furthermore, scientific investigations are needed to unearth the phytochemical profile of *A. unedo* roots and its applications to treat several diseases.

6. Nutritional Characterization

The berries of *A. unedo* are popular in Morocco and consumed by the population as food since a long time, while the leaves and roots are commonly used for medicinal applications to treat ailments. The fruits have a sweet taste and contain a wide variety of molecules with an excellent nutritional quality including phenolic compounds, sugars, dietary fiber, α-tocopherol, proteins, unsaturated fatty acids, organic and phenolic acids, arbutine, vitamins, and carotenoids [56, 57, 68–71].



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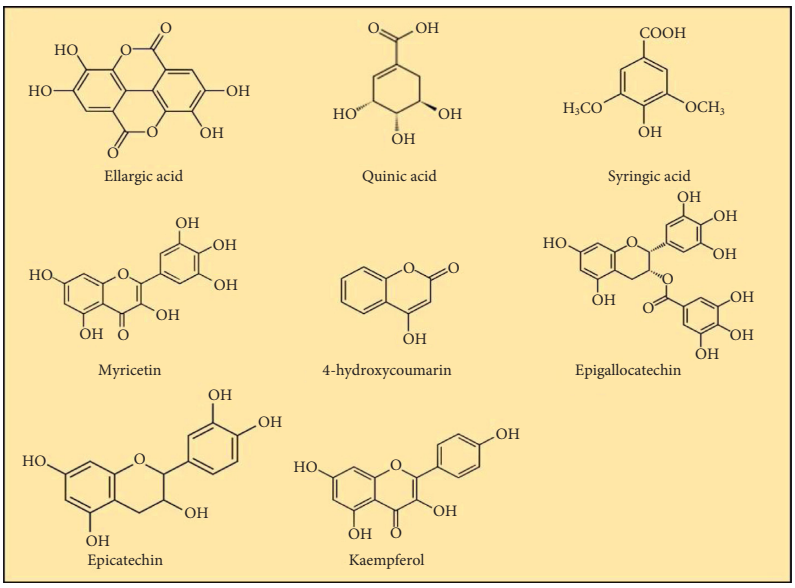

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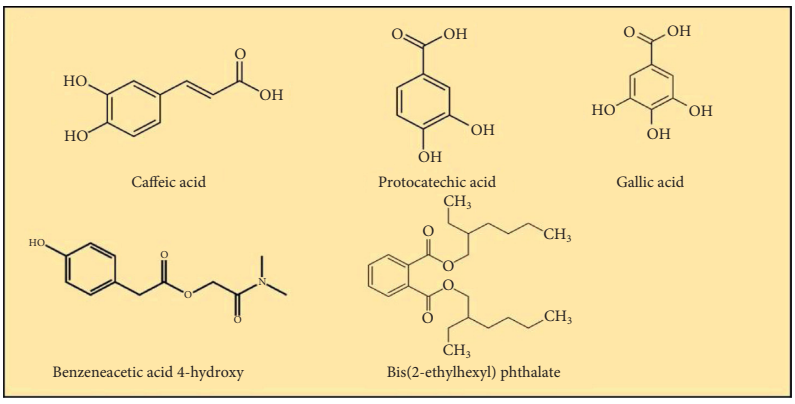

(c)

Figure 3: Structure of various bioactive compounds present in leaves, roots, and fruits of strawberry trees.

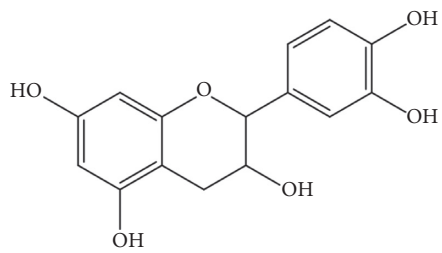

Figure 4: Chemical structure of catechin isolated from *A. unedo* roots [50].

In Morocco, ripened fruits of *A. unedo* are eaten as a remedy for some health problems such as gastrointestinal, infective, and urological diseases [21, 24]. Their consumption provides the body with a significant amount of nutrients, vitamins, minerals, sugars, and other bioactive compounds with health beneficial effects. The fruits are not consumed very often, but they can be enjoyed when eaten in moderate quantities. However, if taken in excess, on fasting, they would be purgative. They are usually eaten in the field as fresh fruits and sometimes taken home for dessert or sold by rural people at the weekly markets or by the roadside in small drum or reed baskets. Often this product constitutes a complement to rural income.

A study by El Cadi et al. demonstrated that *A. unedo* fruits collected from different parts of Morocco are with high antioxidant capacity. It has been concluded that this fruit is a source of various potentially therapeutic compounds for the treatment of many diseases [65]. In addition, several studies reported that *A. unedo* leaves constitute valuable flavonoids and minerals, especially K, Ca, P, and Mg [47, 67]. It was suggested that the rich mineral and polyphenols content of *A. unedo* leaves can constitute an interesting addition to human diet and therapy.

Despite its high nutritional value, strawberry fruit valuation is still timid in Morocco and is limited to the production of honey, jams, and pastries by some cooperatives located in the Western Rif of Morocco [19].

7. Pharmacological Activities

The documented pharmacological activities of Moroccan *A. unedo*, its extracts, and compounds isolated from this species are detailed below and are summarized in Table 3, and some of the pharmacological effects are illustrated in Figure 5.

7.1. Antidiabetic Activity. Type 2 diabetes mellitus (DM) is a complex syndrome characterized by chronic hyperglycemia as a consequence of a disorder of insulin secretion, insulin resistance/action, or combination of both of these factors. It is estimated that 25% of the world population is affected by this disease [72]. Furthermore, DM is considered as the major risk factor for CVD including heart ischemic disease, stroke, atherosclerosis, and heart failure [73]. Therefore, the identification of natural antidiabetic compounds with insignificant toxicity and no side effects is of great interest [74]. In this regard, several medicinal plants have been used traditionally for the treatment and/or prevention of DM [75].

The antidiabetic activity of *A. unedo* roots extract has been tested in rats using the oral glucose tolerance test (OGTT) and the intravenous glucose tolerance test (IVGTT). Oral administration (500 mg/Kg b.w) of the extract to rats submitted to OGTT produced a significant decrease in glycaemia after glucose overload. This effect was not confirmed by the IVGTT. Furthermore, *A. unedo* extract exhibited significant reduction of intestinal glucose absorption, which may justify in part the reduction of glycaemia observed in the OGTT model [41].

In a pre-clinical study, it has been demonstrated that the oral administration of water extracts from *A. unedo* roots (400 mg/L, drink water) showed significant reduction of glycaemia in neonatal streptozotocin (n-stz)-induced diabetic rats under chronic treatment [46]. Furthermore, the authors reported that the combination of the plant extract with insulin enhanced the peripheral utilization of glucose and potentiated the activity of insulin, which may explain in part the hypoglycemic effect observed with the *in vivo* test [46]. It is concluded that *A. unedo* roots contain bioactive compounds, which contribute to their antidiabetic activity and validate their utilization in traditional medicine.

In another study, Mrabti et al. assessed the antidiabetic properties of *A. unedo* root extract *in vitro* and *in vivo*. In the *in vitro* test, results showed that *A. unedo* root extract exerted more potent inhibitory effect against α-glucosidase than acarbose (positive control) with IC_{50} (median inhibitory concentration) values of 94.81 ± 5.99 µg/mL and 199.53 ± 1.12 µg/mL respectively. In the *in vivo* antidiabetic study, it has been found that *A. unedo* extract (500 mg/Kg b.w) produced a significant decrease in glycaemia similar to that of metformin (positive control) in STZ–NA induced-diabetic mice after chronic oral administration for 4 weeks. In addition, treatment with the extract (500 mg/day/kg b.w) resulted in the restoration of the histological architecture of the islets of Langerhans in diabetic mice [76]. The possible action mechanism by which *A. unedo* exerts its hypoglycemic effect may be related to the pancreatic secretion of insulin from the existing beta cells or by its release from the bound form [77].

The effects of the root bark aqueous extract of *A. unedo* on intestinal glucose absorption was studied by Mrabti and coworkers *in vitro* and *in vivo*. Results showed that the tested extract (10 μg/mL to 1 mg/mL) inhibited dose-dependent sodium-dependent glucose transport across isolated mouse jejunum with an IC_{50} value close to 216 μg/mL. In addition, the *A. unedo* extract (2 g/kg/day) improved oral glucose tolerance and reduced body weight after chronic oral administration during 4 weeks in rats [78]. These results support the traditional use of *A. unedo* in the treatment of diabetes.

In another study, Mrabti and colleagues isolated catechin from *A. unedo* roots and evaluated its antidiabetic activity using the α-glucosidase test. Results showed that the isolated compound exerted antidiabetic activity through inhibition of α-glucosidase enzyme activity with an IC_{50} value of

Table 3: Pharmacological activities of A. unedo *L*.

Pharmacological effect	Plant part	Extract/fraction	Model applied	Effect/mechanism(s) of action	References
Antidiabetic activity	Roots	Water	OGTT IVGTT	Antihyperglycemic effect Inhibition of jejunal glucose absorption	[41]
	Roots	Water	OGTT n-stz-induced diabetic rats (*in vivo*)	Hypoglycemic effect in n-stz induced-diabetic rats (*in vivo*) Potentiation of the insulin activity Improved glucose peripheral consumption	[46]
	Roots	Catechin	α-glucosidase assay	Antidiabetic effect through inhibition of α-glucosidase enzyme	[50]
	Roots	Water	α-glucosidase and α-amylase assays STZ-NA-induced-diabetic mice (*in vivo*)	Inhibition of α-glucosidase and α-amylase Regeneration of pancreatic β-cells	[76]
	Roots bark	Water	OGTT Short-circuit current technique (*in vitro*)	Inhibition of SGLT Improved oral glucose tolerance	[78]
Antihypertensive and vasorelaxant activity	Roots and leaves	Water	*In vivo* determination of both blood pressure and baroreflex sensitivity. *Ex vivo* analysis of vascular reactivity.	Reduces the development of increased SBP Ameliorates vascular reactivity and baroreflex sensitivity Normalizes renal function Prevents the myocardial hypertrophy (roots extract)	[42]
	Leaves	Aqueous extract soxhlet extraction: Hexane, dichloromethane, ethyl acetate, methanol and water	*In vitro* study of vasorelaxant effect	Endothelium-dependent vasorelaxant activity mediated by NO. This effect is due to the presence of condensed tannins and catechin gallate.	[47]
	Roots	Water extract	*In vitro* study of vasodilator effect and mechanisms of action	Endothelium-dependent relaxation of aorta mainly mediated by a stimulation of endothelial NO synthase	[43]
	Roots	Water	*In vivo* study of hypertension Measurement of diuresis	Delayed the development of hypertension Attenuated the pressor responses to phenylephrine and angiotensin I diuretic effect	[49]

Table 3: Continued.

Pharmacological effect	Plant part	Extract/fraction	Model applied	Effect/mechanism(s) of action	References
Antiaggregant activity	Leaves	Genins (free flavonoids) heterosidic flavonoids	*In vitro* measurement of platelet aggregation	Inhibition of rat platelet aggregation induced by thrombin (effect mainly due to flavonoids)	[44]
	Leaves	Water Genins (free flavonoids) Heterosidic flavonoids	*In vitro* measurement of platelet aggregation	Inhibition of human platelet aggregation Attenuation of Ca^{2+} mobilization Reduction of ROS production Inhibition of protein tyrosine phosphorylation	[37]
	Leaves	Water Petroleum ether Dichloromethane Ethyl acetate Methanol Tannins	*In vitro* measurement of platelet aggregation	Inhibition of rat platelet aggregation induced by thrombin (effect probably due to tannins)	[48]
	Roots	Water	*In vitro* measurement of platelet aggregation	Inhibition of thrombin- and ADP-induced rat platelet aggregation	[45]
Antioxidant activity	Fruits	Phenolic Flavonoids Condensed tannins Hydrolyzable tannins Anthocyanins	ABTS assay	Radical scavenging activity (ABTS) Antioxidant capacity varies with the genotypes and variations in plants chemical composition	[66]
	Leaves	Water Hydroalcoholic extractHydroalcoholic extract	DPPH and FRAP assays	Radical scavenging activity (DPPH and FRAP) Potent antioxidant activity with hydroalcoholic extract	[62]
	Roots and leaves	Aqueous extract	DPPH assay *In vivo* antioxidant assay (dosage of MDA and SOD)	In vitro antioxidant activity in DPPH assay *In vivo* antioxidant activity Roots exhibited better activity than leaves	[67]
	Fruits from three different regions (AA, AC and AQ)	Ethyl acetate MeOH/water 80 : 20 (v/v)	DPPH assay	Antiradical ability of all *A. unedo* samples Higher antioxidant activity with samples extracted by MeOH:water Higher antioxidant activity with AC fruits Antioxidant activity depends on the geographic origin of fruit and its phenolic content	[65]
	Leaves	Ethyl acetate, ethanol, methanol, and n-hexane	DPPH assay	Ability to act as DPPH radicals scavenger for all the extracts n-Hexane and methanol extracts showed the highest radical scavenging activity The antioxidant activity is correlated with phytochemical content	[87]

Table 3: Continued.

Pharmacological effect	Plant part	Extract/fraction	Model applied	Effect/mechanism(s) of action	References
Antibacterial and antiparasitic activity	Leaves	Ethyl acetate, ethanol, methanol, and *n*-hexane.	Determination of MIC by the dilution agar method. Agar well diffusion assay	Antibacterial activity against gram-negative (*Escherichia coli, Pseudomonas aeruginosa)* and gram-positive (*Staphylococcus aureus, Listeria monocytogenes*) bacteria	[87]
	Leaves	Water Ethanol	Determination of growth inhibition values by paper disc	Antimycobacterial activity against *Mycobacterium bovis, Mycobacterium smegmatis and Mycobacterium aurum*	[90]
	Leaves	*n*-hexane, methanol, and ethanol	MTT assay	Antileishmanial activity using *Leishmania major, Leishmania tropica, and Leishmania infantum*	[93]
Anticancer and cytotoxic activity	Leaves	*n*-hexane, methanol, and ethanol	MTT assay Cancerous cell lines: L20B, RD and vero Normal cells: PBMC	Cytotoxic effects against L20 B, RD and vero cell lines with *A. unedo n*-hexane extract	[94]
	Leaves	Water	Cell viability evaluated using calcein and trypan blue.	No toxic effect was observed in human platelets cell viability with a concentration of 1.5 mg/mL	[37]
	Leaves	Ethanol	Neutral red uptake assay	No toxic effect was observed in peritoneal macrophages with a concentration of 6.02 ± 0.76 mg/mL	[90]
Antilithiasic activity	Leaves	Water Hydroalcoholic extract	Measurement of litholytic activity using a model structure resembling the urinary circuit	Litholytic activity against calcium oxalate stones Higher litholytic activity with aqueous extract	[62]
	Leaves	Water Hydroalcoholic extract	*In vitro* measurement of the crystal formation by using a UV-Visible spectrophotometer Microscopic observation of the crystals	Inhibitory activity against calcium oxalate crystallization Higher litholytic activity with aqueous extract This effect is due to the presence of polar compounds in the plant extracts such as polyphenols	[63]
	Fruits (seeds)	Water	Polarizing optical microscope (PLM)	Inhibition of the crystallization of calcium oxalate	[100]

OGTT, oral glucose tolerance test; n-stz, neonatal streptozotocin; IVGTT, intravenous glucose tolerance test; STZ-NA, streptozotocin-nicotinamide; SGLT, sodium-dependent glucose transporter; SBP, systolic blood pressure; NO, nitric oxide; Ca2+, calcium; ROS, reactive oxygen species; ABTS, 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing/antioxidant power; ADP, adenosine diphosphate; SOD, superoxide dismutase; MDA, malondialdehyde; AA, achakar; AQ, qsar Kbir; AC, chaoun-Qalaa; MIC, minimal inhibitory concentration; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide).

$87.55 \pm 2.23\,\mu$g/mL [50]. The inhibitory potential of catechin was greater than that of the positive control acarbose ($IC_{50} = 199.53 \pm 1.12\,\mu$g/mL). The antidiabetic activity for root extract of *A. unedo* is very well established by various researchers. Therefore, there is need to explore the potential of *A. unedo* leaves to treat diabetes. However, there is no adequate studies on the effect of *A. unedo extracts* with respect to molecular aspects.

7.2. Antihypertensive and Vasorelaxant Activity. Hypertension is a major risk factor for CVD, such as coronary heart disease and stroke, the two leading causes of death among adults worldwide [79]. In addition, current antihypertensive agents have limited effectiveness and various side effects. Recently, various studies have been focused to find new drugs from medicinal plants with antihypertensive properties.

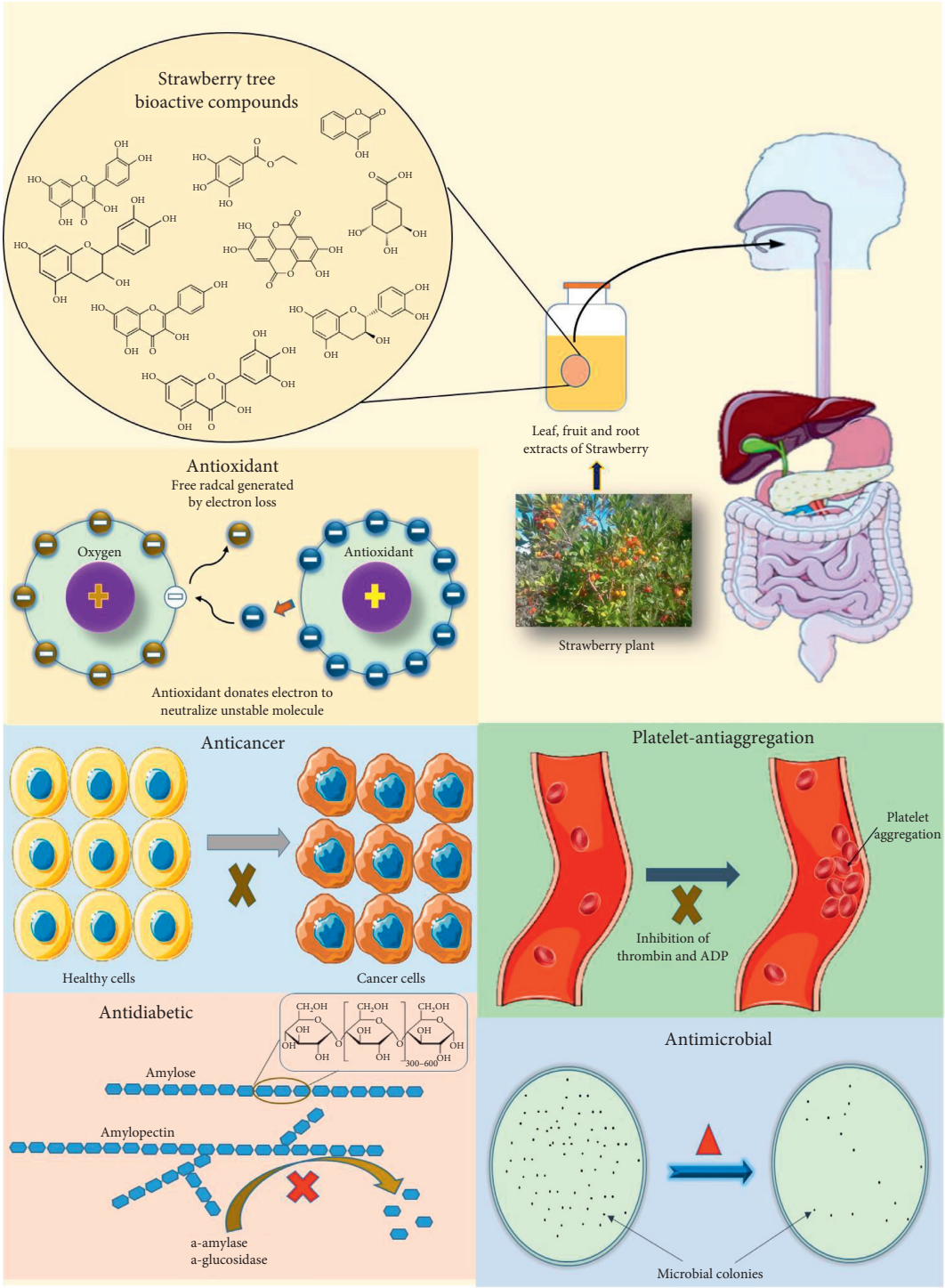

Figure 5: Representation of some pharmacological activities of *Arbutus unedo* L.

A. unedo has been tested for antihypertensive activity. Afkir et al. reported that the simultaneous oral treatment of rats with L-NG-nitroarginine methyl ester (L-NAME) and *A. unedo* leaf or root aqueous extract (250 mg/Kg/day) for 4 weeks inhibited hypertension development and reduced ventricular hypertrophy (root extract). Furthermore, the extracts normalized renal function, improved vascular reactivity, and baroreflex sensitivity in rats [42].

In another pre-clinical study, it has been reported that the chronic treatment of spontaneously hypertensive rats (SHR) with *A. unedo* root aqueous extracts (5, 50 and 250 mg/kg/24 h) delayed the development of hypertension but did not change the final level of blood pressure and heart rate. A diuretic effect was observed in the group receiving the highest dose (250 mg/kg/24 h) of *A. unedo* [49].

The vascular activity of *A. unedo* roots was also examined *in vitro* using isolated rat aorta. It has been found that the root aqueous extract (0.25 mg/mL) induced an endothelium-dependent vasodilation of the isolated rat aorta, and this effect was mainly attributed to the activation of the endothelial nitric oxide synthase (NOS) [43].

In another *in vitro* study, Legssyer and colleagues examined the vascular effect of aqueous extract from *A. unedo* leaves on the isolated rat aorta. Results showed that the *Arbutus* leaf extract (0.01 g/L) produced a strong endothelium-dependent vasorelaxant activity. A chromatographic fractionation of the methanolic extract of *A. unedo* leaves showed that this vasorelaxant activity was probably assigned to polyphenolic compounds, such as tannins and catechin gallate [47]. It was suggested that these findings may account for the antihypertensive property of *A. unedo* reported in folk medicine. Furthermore, studies are required to unravel the molecular mechanism of action of phytochemicals present in *A. unedo* extracts.

7.3. Antiaggregant Effect. Blood platelets play a crucial role in the primary hemostasis through the formation of a platelet clot. However, platelet hyperactivity is mainly involved in thrombosis and atherosclerosis [37, 80, 81]. Furthermore, the use of anti-platelet agents is often associated with various adverse effects including bleeding problem and gastric problems [82]. In this context, the development of anti-platelet drugs of natural origin is of great interest [2].

In a study, the platelet antiaggregant effect of *A. unedo* was investigated *in vitro* by a group of researchers using different extract preparations. In a screening study, it was demonstrated that *A. unedo* root extract from Tazekka Mountain inhibited *in vitro* platelet aggregation induced by thrombin or ADP in a dose-dependent manner [45]. In another investigation, it was reported that the leaf aqueous extract of the plant inhibited thrombin-induced rat platelet aggregation *in vitro* with an IC_{50} value of 1.8 g/l. Successive extraction of *A. unedo* leaves with solvents of increasing polarity (petroleum ether, dichloromethane, ethyl acetate, and methanol) revealed that ethyl acetate and the methanolic extracts were most active against platelet aggregation with IC_{50} values of 0.6 ± 0.05 and 0.7 ± 0.08 g/L, respectively [48]. In order to verify if the tannins were implicated in the obtained antiaggregant effects, these compounds were isolated from the methanolic extract, and their anti-platelet effect was evaluated *in vitro*. The obtained results indicated that tannins precipitated by caffeine induced an important antiaggregant effect, whereas the adsorption of these compounds by skin powder in the methanol extract reduced significantly its activity. Thus, the antiplatelet aggregation effect of the *A. unedo* leaf extract was mainly attributed to condensed tannins [48].

In another study, researchers examined the anti-platelet effect of flavonoids from *A. unedo* leaves *in vitro*. It has been found that genins (free flavonoids) hetrosidic flavonoids reduced significantly thrombin-induced platelet aggregation with IC50 values of 0.22 ± 0.03 and 0.36 ± 0.05 mg/ml, respectively. In addition, at 1 mg/ml, these compounds reduced significantly the initial rate of platelet aggregation by $97.8 \pm 0.74\%$ and $90.8 \pm 1.55\%$ for genins and heterosidic flavonoids, respectively [44]. Thus, these findings clearly demonstrate that the anti-platelet effect of *A. unedo* is mainly attributed to flavonoids.

In addition to our work on rat platelets, we have investigated the effects of flavonoids from *A. unedo* leaves on human platelets. It has been found that the aqueous extract and flavonoids from *A. unedo* leaves exerted anti-platelet effects in human platelets [37]. Furthermore, it was demonstrated that at 0.05 mg/mL, the tested compounds reduced thrombin-induced endogenous ROS production, as well as Ca^{2+} mobilization and protein tyrosine phosphorylation, two processes that have been revealed to be regulated by the redox potential in platelets [83, 84]. These findings are in agreement with the antiaggregant effect of *A. unedo* observed in rat platelets [45, 48], although, it should be noted that human platelets seems to be more sensitive to the extracts than rat platelets. It was concluded that *A. unedo* extracts exhibited anti-platelet activity by a mechanism implying the reduction of Ca^{2+} mobilization, ROS production, and protein tyrosine phosphorylation, which might support the traditional use of this plant in the treatment and/or prevention of CVD. However, further studies to understand its molecular mechanism and in-depth in vivo investigations may help to include *A. unedo* as an alternative to modern medicine.

7.4. Antioxidant Activity. Oxidative stress has been mainly implied in the development of chronic and degenerative diseases such as cancer, diabetes, and cardiovascular and neurological diseases [85]. Increased consumption of plant-based foods (fruits, vegetables, and nuts) has been reported to reduce and prevent damage caused by free radicals [86].

Numerous studies have evaluated the antioxidant activity of *A. unedo in vitro* and *in vivo*. Different compounds were isolated from the plant and were evaluated for their antioxidant potential. Recently, Zitouni and coworkers [64] evaluated *in vitro* the antioxidant properties of *A. unedo* fruits from different areas of Morocco using three antioxidant essays (1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and β-Carotene bleaching). Results revealed significant differences among sampled fruits. The average radical scavenging capacities were 3.33–21.08, 2.25–19.58, and 1.08–13 mg Ascorbic Acid Equivalent (AAE)/g d.w) for the DPPH, ABTS, and β-carotene bleaching assays, respectively. The authors suggested that the difference in results might be due to difference in varieties and/or growing region of the *A. unedo* samples.

In another recent study, Zitouni and colleagues assessed the antioxidant activity of phenolic, flavonoids, condensed tannins, hydrolyzable tannins and anthocyanins from twelve *A. unedo* fruits genotypes using ABTS. Significant variations were observed in the antioxidant activity among the studied extracts. The IC_{50} value (ABTS) of the tested compounds

varied from 1.75 to 19.58 mg AAE/g d.w [66]. It is noticeable that the antioxidant activities of the tested compounds depend on the region and diversity of geographical environments.

Kachkoul and coworkers analyzed the antioxidant activity of aqueous and hydroalcoholic extracts from *A. unedo* leaves using DPPH and the ferric reducing/antioxidant power (FRAP) assays. It has been found that the hydroalcoholic extract exerted higher antioxidant capacity than the aqueous extract with IC_{50} values of $76.14 \pm 0.91\,\mu$g/mL for hydroalcoholic extract versus $202.64 \pm 5.77\,\mu$g/mL for aqueous extract using the DPPH assay, and $53.77 \pm 0.81\,\mu$g/mL for hydroalcoholic extract versus $236.86 \pm 31.90\,\mu$g/mL for aqueous extract using the FRAP test [62].

The antioxidant activity of *A. unedo* roots and leaves aqueous extracts was determined by Mrabti et al. *in vitro* and *in vivo*. The *in vitro* antioxidant activity was determined using the DPPH assay, while the *in vivo* activity was analyzed by dosage of malondialdehyde (MDA) and superoxide dismutase (SOD) in diabetic mice. The IC_{50} values for the *in vitro* antioxidant activity of the aqueous extract was 4.52 and 7.24 μg/mL in roots and leaves, respectively [67]. In addition, there was no significant difference between *A. unedo* and metformin group (positive control) in all of the *in vivo* examined parameters (MDA and SOD) in liver and kidney of diabetic mice [67]. The high antioxidant potential of the roots is compatible with their higher content in tannins, anthraquinones, terpenoids, and flavonoids. These results suggest that *A. unedo* may strengthen the enzymatic antioxidant system and reduce tissue damage due to oxidative stress.

In another study, Bouyahya and coauthors assessed the antioxidant activity of methanolic, ethanolic, ethyl acetate and n-hexanic extract from *A. unedo* leaves using DPPH assay. It has been found that the plant extracts showed an antioxidant effect against DPPH radicals. In addition, n-hexane and methanol extracts were found to exert the highest radical scavenging activity with IC_{50} values of 73.73 μg/mL and 95.25 μg/mL, respectively [87]. The differences of the obtained results could be related to the type of solvents and the nature of phytochemical content of each extract.

In a recent study, El Cadi et al. determined the antioxidant capacity of *A. unedo* fruits from three locations of northern Morocco (Achakar, Qsar Kbir and Chaoun-Qalaa) using the DPPH assay. Results showed that all the samples have antioxidant activity (EC50 values between 1.37 ± 0.2 and 17.82 ± 0.12 mg/mL (w/v)). Higher antioxidant capacity was observed with samples extracted by MeOH:water. In addition, Chaoun-Qalaa fruits were found to exhibit the highest antioxidant activity, which is compatible with their higher total polyphenol and flavonoid content [65]. The authors concluded that *A. unedo* fruits contain various phytochemicals with high antioxidant capacity, and could be, therefore, used in pharmacological and nutritional research. The antioxidant potential of *A. unedo* extracts has been justified by several in vitro and in vivo investigations, still there is enough scope to study the effect of phytochemicals present in *A. unedo* on gene expression levels. Additionally, these investigations mainly used the whole crude extracts of *A. unedo* root and leaves instead of the bioactive compounds.

7.5. Antibacterial and Antiparasitic Activity. Antimicrobial agents play a major role against infectious diseases. However, the consumption of antibiotics can lead over time to the development of a more resistant bacteria [88]. Antimicrobial resistance makes antibiotics less effective, and infections become difficult to be treated. Thus, the need to find new and efficient antimicrobial agents is of great interest. In this sense, various medicinal herbs have been used due to their antimicrobial properties [89].

Bouyahya and coworkers determined the antimicrobial activity of methanolic, *n*-hexanic, ethyl acetate, and ethanolic extract from *A. unedo* leaves against *Escherichia coli* K12 MBLA, *Staphylococus aureus* CECT 976, *Listeria monocytogenes* Serovar 4b CECT 4032, and *Pseudomonas aeruginosa* IH using the agar well diffusion assay. A significant difference in activities was observed against the studied bacterial strains. The highest activity was obtained with the *n*-hexanic extract, particularly against *S. aureus* and *L. monocytogenes* (zones of inhibition varied from 34.42 ± 0.26 mm to 40 ± 0.19 mm) [87]. The inhibition was related to the presence of phenolic and flavonoid compounds.

In another study, the extra- and intracellular antimycobacterial activities of aqueous and ethanol extracts from *A. unedo* leaves were studied against the growth of three mycobacteria (*Mycobacterium bovis*, *Mycobacterium smegmatis*, and *Mycobacterium aurum*). Results showed that the tested extracts exhibited extracellular antimycobacterial activity against the growth of three *mycobacteria*. The minimum inhibitory concentration (MIC) of the ethanolic extract was 5.59 ± 0.69 mg/ml for *Mycobacterium aurum* A+ and 6.02 ± 0.76 mg/ml for *Mycobacterium smegmatis* MC2 and *Mycobacterium bovis* PPI. Moreover, ethanol extract used at 6.02 ± 0.76 mg/ml showed potent intracellular antimycobacterial activity against *M. smegmatis* MC2 located within the rat peritoneal macrophages [90]. The antimycobacterial activity of the plant could be assigned to a number of phytochemicals, such as phenolic compounds.

Leishmaniasis is a parasitic disease caused by *Leishmania* parasites, which are transmitted by phlebotomy insects. It constitutes a serious health problem worldwide, especially in Africa [91, 92]. Therefore, the search for antileishmanial drugs with no side effects is of great interest. In this sense, the antileishmanial activity of methanolic, *n*-hexanic, and ethanolic extract of four medicinal plants including *A. unedo* was examined by Bouyahya et al. against *Leishmania major*, *Leishmania tropica*, and *Leishmania infantum* using the MTT (3-(4.5-dimethylthiazol- 2yl)-2.5-diphenyltetrazolium bromide) assay. Results indicated that the *n*-hexanic extract from *A. unedo* leaves exhibited profound and highly significant inhibitory effect against *L. infantum* ($IC_{50} = 64.05 \pm 1.44$ μ/mL) and *L. tropica* ($IC_{50} = 79.57 \pm 2.66$ µg/mL) [93]. The authors suggested that *A. unedo* could constitute a potential source for

antileishmanial agents. However, further research is needed to identify the bioactive compounds that are responsible for the antileishmanial activity and to determine their modes of action.

7.6. Anticancer and Cytotoxic Activity. The anticancer activity of methanolic, *n*-hexanic, and ethanolic extract from five medicinal plants including *A. unedo* was investigated *in vitro* against different cancer cell lines (L20B, RD and Vero) using the MTT assay. The pharmacological selectivity index (PSI) was calculated using PBMC as the normal cell line. It has been found that the n-hexanic extract of *A. unedo* leaves significantly inhibited the proliferation of RD ($IC_{50} = 27.83 \pm 1.20\,\mu g/mL$; PSI = 0.69), L20 B ($IC_{50} = 25.32 \pm 1.26\,\mu g/mL$; PSI = 0.76), and Vero cell lines ($IC_{50} = 18.37 \pm 1.23\,\mu g/ml$; PSI = 1.05) [94]. As there are limited studies on the anticancer activity of the *A. unedo* extract, furthermore, in vitro as well as in vivo investigations are required to consolidate the role of *A. unedo* extract as a potential anticancerous drug. In addition, most of researchers focused on the whole crude extracts of *A. unedo* instead of the bioactive components with molecular mechanisms mostly unclear.

7.7. Antilithiasis Activity. Urolithiasis is a pathological problem that involves the formation of crystalline aggregates called "urinary stones" in the kidneys or in the urinary tract. The etiology of this disorder is related to a variety of metabolic and environmental disturbances [95]. Until now, there has been no satisfactory treatment for urolithiasis. Furthermore, medical management of urolithiasis can lead to side effects, which are sometimes very serious [96]. Thus, several medicinal plants have been used for the treatment and/or prevention of urolithiasis [97, 98].

Little information was recorded with the antilithiasic activity of *A. unedo*. A study by Kachkoul and coworkers assessed the effects of *A. unedo* leaf extracts (aqueous and hydroalcoholic extracts) on calcium oxalate crystallization *in vitro*. It has been found that the aqueous extract was more effective in the dissolution of calcium oxalate stones than the hydroalcoholic extract with dissolution values of 31.05% and 14.55%, respectively [62]. Another *in vitro* study on calcium oxalate crystallization has been performed with aqueous and hydroalcoholic extracts from *A. unedo* leaves using UV-visible spectrophotometric method. Results showed a higher potency of the plant aqueous extract compared to the hydroalcoholic extract against crystallization or nucleation at percentages of 69.41 ± 0.24 or $19.76 \pm 0.27\%$ and at 93.92 ± 2.61 and $45.16 \pm 3.06\%$ against the aggregation for both the aqueous and the hydroalcoholic extract, respectively [63]. This antilithiasic activity could be attributed to the high level of phenolic components contained in the leaves [63], which are known for their anticrystallizing property according to studies of both human urine as well as on animal models [99]. The authors concluded that *A. unedo* is a promising and effective remedy against "urinary stones".

In a recent study, Baddade and colleagues investigated the antilithiasic activity of *A. unedo* fruits *in vitro*. Fruit samples were collected from six zones from the Beni Mellal-Khenifra region, and the antilithiasic activity was evaluated against the aggregation of calcium oxalate. Results showed that the fruit seed aqueous extract (3 mg/ml) inhibited the crystallization of calcium oxalate *in vitro*. At 10 mg/ml, the extract showed significant dissociation of the aggregates [100]. The authors suggested that *A. unedo* extracts seem to be promising against the crystallization of calcium oxalate, especially in the aggregation and nucleation stages.

8. Toxicological Properties

According to different toxicity tests, the use of *A. unedo* is devoid of any significant side effects and/or toxicity. Indeed, in an acute toxicity test performed in mice, we have demonstrated that the oral administration of *A. unedo* leaf aqueous extract at a dose up to 1200 mg/kg caused no mortality and no signs of adverse effects, demonstrating that the LD_{50} value of the *A. unedo* aqueous extract was higher than 1200 mg/kg b.w in oral administration in mice [44]. In another acute toxicity test realized in mice, it has been reported that the *A. unedo* root aqueous extract exhibited LD_{50} higher than 2000 mg/kg b.w with no adverse effect of this dose shown after single oral administration in mice [76]. Furthermore, in a study to analyze the acute toxicity of *A. unedo* and to evaluate its safety, an aqueous extract from *A. unedo* roots was intraperitoneally administrated in mice. The results did not show visible signs of toxicity or adverse effects even at a dose of 6 g/kg b.w [41].

The cytotoxicity of rat peritoneal macrophages treated with the ethanolic extract of *A. unedo* leaves was measured by the neutral red uptake assay. Results showed that rat peritoneal macrophages treated with the extract (6.02 ± 0.76 mg/ml) were able to uptake the red neutral dye after 3 days of incubation. It is concluded that the extract used at 6.02 ± 0.76 mg/ml had no cytotoxic effect [90]. In a previous study, we have examined the cytotoxic effect of *A. unedo* on platelet cell viability using calcein and trypan blue. Results showed that the incubation of human platelets with *A. unedo* leaf aqueous extracts (1.5 mg/ml) did not affect their viability [37].

9. Discussion

In the current review, we report the traditional medicinal uses and phytochemical and pharmacological properties of *A. unedo* from Morocco. Various *A. unedo* extract preparations have been broadly used in folk medicine for the management of several ailments. Different parts of the plant are used in the prevention and treatment of several complications, especially microbial infection, diabetes, hypertension, and gastrointestinal and renal diseases. In contrast, limited studies have been devoted to the plant fruits, although they represent an abundant source of phytochemicals with potent antioxidant capacity. Different traditional uses have been confirmed by pharmacological activities of the plant extracts and its identified compounds using different animal models *in vitro* and *in vivo*. Among the bioactive compounds, flavonoids and tannins are the most important, being responsible for most of the

pharmacological activities reported, namely, antioxidant, antiaggregant, antidiabetic, antihypertensive, antimicrobial, anticancer, or antidiarrheal activities. These activities generally agree with traditional knowledge and folk medicine.

The studied *A. unedo* extracts have been shown to be a rich source of epicatechin, catechin, catechin gallate, flavonoids, anthocyanins and tannins, and phytochemicals with well-recognized antioxidant capacity. The antioxidant properties of *A. unedo* extracts have been evidenced in different *in vitro* systems and *in vivo* studies in rats, mice, and cell culture systems. The antihypertensive, anti-platelet, anticancer, and antidiabetic properties of the plant are all believed to be related to its antioxidant activity, although no direct associations have been made.

Various studies involving catechin, epicatechin, tannins, anthocyanins, and flavonoid-rich extracts from other plants have demonstrated antiaggregant [101–104], antidiabetic [105], anticancer [106], and vasodilation properties [107]. Therefore, it is not surprising that various *A. unedo* extracts exhibit these properties due to the presence of catechins, anthocyanins, and flavonoids. Significant variations were observed in phytochemical composition among investigated *A. unedo* extracts, which are mainly due to genetic variability and environmental factors, such as bioclimatic or geographical origin [39, 54, 55, 64]. Furthermore, it was revealed that the biological properties of *A. unedo* depend on the extraction method, the nature of phytochemicals, and their levels in the plant extracts.

The retrieved studies were insufficient to substantiate all the traditional medicinal claims of the plant. Moreover, most of the studies that highlighted the beneficial properties of *A. unedo* on health were *in vitro* and *in vivo* investigations on animal models as well as several cell lines, which represent one of the limitations in this review. Thus, more clinical studies are necessary to validate the traditional uses and pharmacological activities of the selected plant.

In addition to details regarding the traditional use of *A. unedo*, this review also focuses on the nutritional characteristics of the plant and its potential uses for other industries, such as food, cosmetics, or pharmaceutical ones, especially the fruit, which constitutes an important source of compounds with beneficial health properties such as minerals, vitamins, fiber, flavonoids, and tannins.

Different parts of the plant have been used for a long time without any toxicity or side effects. In addition, *A. unedo* fruits have been consumed for many years in folk medicine due to its medicinal properties and nutritional value with no known toxicity. Furthermore, toxicity tests carried out on animals or *in vitro* did not demonstrate any toxic effects on the plant. However, we note the absence of long-term studies either on animals or on humans that could address its long-term safety. Thus, unlike pharmaceutical drugs, *A. unedo* not only has no side effects, but has various medicinal properties against several diseases.

10. Conclusion

Numerous studies have reported that *A. unedo* is an important source of catechins, tannins, anthocyanins, as well as flavonoids with potent antioxidant capacity. The antioxidant properties of the plant have been well demonstrated through both *in vitro* and *in vivo* investigations. The antioxidant activity of the plant seems to be responsible for the antidiabetic, anticancer, antiaggregant, and antihypertensive activities that have been demonstrated. However, the majority of the phytochemical investigations was limited to screening studies rather than the isolation of bioactive constituents. In addition, several researchers focused on the whole crude extracts of *A. unedo* instead of the bioactive components, with molecular mechanisms mostly unclear. Additionally, no human clinical studies have been reported. In light of this, relevant goals for future research are the identification of bioactive molecules from *A. unedo* using bioguided isolation assays and finding evidence for their beneficial health effects through preclinical and clinical studies. The physical properties of the equipment used in the transport, storage, harvesting, and processing of *A. unedo* fruits must also be investigated.

Abbreviations

WHO: World health organization
DM: Diabetes mellitus
A. unedo: Arbutus unedo
B.W: Body weight
Ca^{2+}: Calcium
IC_{50}: Median inhibitory concentration
D.W: Dry weight
ROS: Reactive oxygen species
CVD: Cardiovascular diseases.

Data Availability

The data supporting this review were taken from previously reported studies and datasets, which have been cited. The processed data are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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