Study on antidepressant-like effect of protoilludane sesquiterpenoid aromatic esters from *Armillaria mellea*

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Abstract

*Armillaria mellea*, also known as Hazel mushroom, is a delicious food material and traditional herbal medicine in East Asia. Protoilludane sesquiterpenoid aromatic esters from *A. mellea* (PSAM) are the main active components with antibacterial and anticancer activities. This study explored the antidepressant-like activities of PSAM and its possible mechanisms of action using the open field test (OFT), tail suspension test (TST) and forced swimming test (FST) in mice for the first time. The results revealed that PSAM (1 mg/kg, i.p.) exhibited markedly antidepressant-like activity, which could be reversed by pretreatment with haloperidol (a non-selective D2 receptor antagonist), bicuculline (a competitive GABA antagonist), NMDA (an agonist at the glutamate site). Meanwhile, PSAM also effectively increased the hippocampus dopamine (DA) and γ-aminobutyric acid (GABA) and decreased the hippocampus glutamate (Glu) levels of mice, indicating that the antidepressant-like effect of PSAM might be mediated by the DAergic, GABAergic and Gluergic systems.

Keywords: *Armillaria mellea*, antidepressant-like effect, tail suspension test, open field test, GABAergic, DAergic, Gluergic
Experimental Methods

Animal

Adult male ICR mice (18 – 22 g), were provided by Changchun Yisi Laboratory Animal Technology Co., Ltd. Mice were put on standardized laboratory conditions (temperature 23 ± 2 °C, relative humidity 55 % ± 5 %, 12 h light/dark cycle) with food and water available ad libitum. Mice were divided into different groups after 1 week of adaptation, with 10 mice in each group. All experiments were performed in accordance with the guidelines for animal experiments at Jilin Agricultural University. The agreement was approved by the Animal Management and Use Committee of Jilin Agricultural University.

Protoilludane sesquiterpenoid aromatic esters of Armillaria mellea (PSAM)

The fermentation broth of A. mellea was extracted by ethyl acetate. The extract was filtered and concentrated under reduced pressure, and the residue was dissolved in methanol and passed through a polyamide chromatographic column, then washed with methanol-chloroform (99:1-3:1), and the contents of sesquiterpene esters in these fractions were determined by ultraviolet spectrophotometry. It can be concluded that the fraction of methanol-chloroform (19:1) had the highest sesquiterpene esters’ content (54.5 %), therefore, this fraction was collected as PSAM for subsequent experiments. According to the previous studies, PSAM was mainly composed of nearly 50 protoilludane sesquiterpenoid aromatic esters such as armillarin, armillaridin and melleolide shown as Figure S1 (Rui et al. 2016). Structurally, the typical protoilludane sesquiterpenoid aromatic esters in PSAM consist of two characteristic parts, the protoilludane unit and o-hydroxybenzyl unit. The ultra-violet (UV) absorption spectrum of PSAM showed four signals λmax (logε): 211 (4.15), 221 (3.92), 260 (3.87) and 298 (3.57) which indicated π→π* transition of carbon-carbon double bond of benzene ring and n→π* transition of carbonyl group, respectively, indicating the existence of the characteristic structure o-hydroxybenzyl unit in the protoilludane sesquiterpenoid aromatic esters of PSAM. Infrared absorption spectrum (IR, KBr, νmax) of PSAM showed an absorption at 3398 cm⁻¹ indicated the existence of hydroxyl (-OH), the signals at 2945 ~ 2741 cm⁻¹ revealed the saturated
carbon-hydrogen and aldehyde carbon-hydrogen stretching vibrations, 1728 and 1701 cm\(^{-1}\) indicated carbonyl stretching vibrations, 1635 cm\(^{-1}\) demonstrated carbon-carbon double bond stretching vibration, 1608 cm\(^{-1}\) and 1505 cm\(^{-1}\) indicated the stretching vibration of carbon-carbon double bond of benzene ring, 1371 cm\(^{-1}\) demonstrated symmetric deformational vibration of methyl, and 1079 cm\(^{-1}\) revealed carbon-oxygen stretching vibration, indicating the existence of the typical structures in the protoilludane sesquiterpenoid aromatic esters of PSAM. According to the UV spectrum characteristics of o-hydroxybenzoyl unit, which is the characteristic unit in the structure of sesquiterpenoid in PSAM, an ultraviolet spectrophotometric method suitable for the determination of protoilludane sesquiterpenoid aromatic esters was established (Zhao Y. 2018). The sesquiterpenes’ content in PSAM was measured by an UV-VIS spectrophotometer at 260 nm. The sesquiterpenes’ content in PSAM calculated based on armillarin was 54.5 %.

**Drugs**

Fluoxetine (5 mg/kg, 20 mg/kg, an antidepressant drug belonging to SSRIs) and reboxetine (2.5 mg/kg, an antidepressant drug belonging to NA reuptake inhibitors) were gained from Sigma (St. Louis, MO, USA); receptor antagonists and agonist: para-chlorophenylalanine (100 mg/kg, an inhibitor of 5-HT synthesis), haloperidol (0.2 mg/kg, a non-selective D\(_2\) receptor antagonist), Im-hydrochloride (20 mg/kg, NA and 5-HT reuptake inhibitors), agomelatine (40 mg/kg, 5-HT receptor antagonists, 5-HT\(_2\)C receptor antagonists), ondansetron (8 mg/kg, 5-HT reuptake inhibitor; 5-HT\(_3\) receptor antagonist), prazosin (1 mg/kg, \(\alpha_1\)-adrenoceptor antagonist), bicuculline (4 mg/kg, a competitive GABA antagonist), N-methyl-D-aspartic acid (NMDA, 75 mg/kg, an agonist at the glutamate site) and ramelteon (16 mg/kg, melatonin receptor agonists) were also from Sigma. ELISA kits for the detection of GABA, 5-HT, DA, Glu and NA were obtained from US R&D Systems, Ltd. (Minneapolis, USA). All reagents used in this study were of analytical grade. Ltd.

**Spontaneous Locomotor Activity Test (SLT)**

In order to rule out the changes in immobility time in the forced swimming test (FST) and tail suspension test (TST) are the possibility of interference due to athletic
activity. Mice were placed in a ZZ-6 mouse autonomic activity tester 30 minutes after administration to observe the autonomic activity of mice. The device was placed in a sound attenuation laboratory. The activities of mice were recorded within 20 min using a video recorder (Pesarico et al. 2014). Each animal was used only once.

**Open Field Test (OFT)**

Reduced locomotor activity is another core symptom of depression (Ding et al. 2015). Open field test is used to evaluate the autonomous activities, inquiry behavior and tension of animals in new environments. The device consists of two parts: the field reaction box and the analysis system of camera recording. The mouse field reaction box’s length, width and height are 80 cm, which is no cover and the inner wall is black. Camera above it covers the entire interior of the box. The bottom plate was set to 25 squares, and the active area was set. Putting mice in the center of the bottom of the box and adapted for 6 min, the time of movement, the distance of movement and the time of center residence time 4 min after the recording in the OFT. The experiment was carried out in a quiet environment, removing feces and removing the odor with alcohol to prevent it from affecting the experimental results.

**Tail Suspension Test (TST)**

The mice were suspended at a distance of 50 cm above the floor. The adhesive tape was pasted at a distance of 2 cm from the tip of the tail. All tests were recorded directly by the camera. We tested for 30 minutes in the control group, fluoxetine group (20 mg/kg) and PSAM group (1 mg/kg), and recorded the immobility time per minute for 30 minutes. Based on the results, it was found that each mouse was suspended for 6 min, and the sum of the immobility time was observed within the last 4 min.

**Forced Swimming Test (FST)**

According to the improved method of Lin et al determined the immobility time of mice (Lin et al. 2017). Each mouse was forced into a cylindrical glass vessel (20 cm in diameter, 50 cm in height) of 30 cm water (22 ± 1 °C). First, we tested in control group, fluoxetine group (20 mg/kg) and PSAM group (1 mg/kg) for 30 min, recording the immobility time per minute within 30 min. According to the results, it
was found that the total duration of immobility was recorded during the next 4 min of a total 6 min test.

**Drug treatments and experimental design**

PSAM and other drugs were dissolved in physiological saline (0.9 % NaCl aq) before administration. The control group of mice was given the same volume of physiological saline. All mice were administered PSAM and other drugs in each group by intraperitoneal (i.p.) injection. The specific experimental arrangements are as follows:

Control group: administered the same volume of physiological saline (i.p.) 60 min prior to the experiment.

P-chlorophenylalanine group: administered p-chlorophenylalanine (100 mg/kg, i.p.) 60 min prior to the experiment; PSAM (1 mg/kg, i.p.) + p-chlorophenylalanine group: administered p-chlorophenylalanine (100 mg/kg, i.p.) 30 min prior to PSAM (1 mg/kg, i.p.), and performed behavioral experiment after 60 min.

Haloperidol group: administered haloperidol (0.2 mg/kg, i.p.) 60 min prior to the experiment; PSAM (1 mg/kg, i.p.) + haloperidol group: administered haloperidol (0.2 mg/kg, i.p.) 30 min prior to PSAM (1 mg/kg, i.p.), and performed behavioral experiment after 60 min.

Im-hydrochlorid group: administered Im-hydrochlorid (20 mg/kg, i.p.) 60 min prior to the experiment; PSAM (1 mg/kg, i.p.) + Im-hydrochlorid group: administered Im-hydrochlorid (20 mg/kg, i.p.) 30 min prior to PSAM (1 mg/kg, i.p.), and performed behavioral experiment after 60 min.

Agomelatine group: administered agomelatine (40 mg/kg, i.p.) 60 min prior to the experiment; PSAM (1 mg/kg, i.p.) + agomelatine group: administered agomelatine (40 mg/kg, i.p.) 30 min prior to PSAM (1 mg/kg, i.p.), and performed behavioral experiment after 60 min.

Ondansetron group: administered ondansetron (8 mg/kg, i.p.) 60 min prior to the experiment; PSAM (1 mg/kg, i.p.) + ondansetron group: administered ondansetron (8 mg/kg, i.p.) 30 min prior to PSAM (1 mg/kg, i.p.), and performed behavioral experiment after 60 min.
Prazosin group: administered prazosin (1 mg/kg, i.p.) 60 min prior to the experiment; PSAM (1 mg/kg, i.p.) + prazosin group: administered prazosin (1 mg/kg, i.p.) 30 min prior to PSAM (1 mg/kg, i.p.), and performed behavioral experiment after 60 min.

Bicuculline group: administered bicuculline (4 mg/kg, i.p.) 60 min prior to the experiment; PSAM (1 mg/kg, i.p.) + bicuculline group: administered bicuculline (4 mg/kg, i.p.) 30 min prior to PSAM (1 mg/kg, i.p.), and performed behavioral experiment after 60 min.

NMDA group: administered NMDA (75 mg/kg, i.p.) 60 min prior to the experiment; PSAM (1 mg/kg, i.p.) + NMDA: administered NMDA (75 mg/kg, i.p.) 30 min prior to PSAM (1 mg/kg, i.p.), and performed behavioral experiment after 60 min.

Ramelteon group: administered ramelteon (16 mg/kg, i.p.) 60 min prior to the experiment; PSAM (1 mg/kg, i.p.) + ramelteon group: administered ramelteon (16 mg/kg, i.p.) 30 min prior to PSAM (1 mg/kg, i.p.), and performed behavioral experiment after 60 min.

**Biochemical analysis**

After behavioral experiments, immediately dissect the hippocampus part of the mouse brain. The hippocampus were washed with ice-cold physiological saline and then homogenized, centrifuged at 12000 rpm for 10 min at 4 °C. The supernatants were collected to detect 5-HT, GABA, DA, NA and Glu with ELISA kit (JianCheng, NanJing, China).

**Statistical analysis**

All data were expressed as mean ± S.D. One-way analysis of variance was shown for multiple comparisons. T-test was used to analyze the therapeutic effect. The value of $p < 0.05$ was considered statistically significant for analysis.

**Discussion**

*A. mellea* is an edible mushroom that is widely used in the world for its medicinal and health-promoting properties. In the modern research, the extracts from *A. mellea* was found to be having antitumor, anti-inflammatory, anti-radiation and
immunomodulation activities (Yu et al. 2001; Lin et al. 1988). The fermented mycelia extracts are used as dietary supplement products (Chi. et al. 2013; Chen et al. 2014; Heo et al. 2007). In this study, the effects of PSAM on antidepressant-like effects of mice were studied by using behavioral experimental mouse model. In the present study, the results revealed that PSAM exhibited antidepressant-like effects, whose sub-effective and effective dose is 0.1 mg/kg and 1 mg/kg, respectively. In order to exclude the ‘false’ positive effect in the behavior tests induced by the enhancing motor activity by drugs, the locomotor activity of PSAM was determined, and the result indicated that PSAM treatment did not change the motor activity. Many studies have shown that the concurrent treatment with commonly used antidepressant drugs may display a stronger effect or faster onset speed than treated alone (Yan et al. 2016).

Subsequently, the experiments explored the neural pathways and molecular mechanisms of the antidepressant-like activity of PSAM. We determined that the dopaminergic, GABAergic and glutamatergic systems were involved in the antidepressant-like effect of PSAM in the TST. The levels changes in the DA, GABA and Glu in the hippocampus of the mice also support it. The dopaminergic pathway is involved in the regulation of mood and behaviors and plays a role in the pathophysiology of depression (Dailly et al. 2004). The plasma levels of dopamine metabolites are significantly lower in the depressed patients indicating a diminished dopamine turnover in depressive illnesses (Mitani et al. 2006). It has been reported that the potentiation of dopaminergic neurotransmission contribute to the therapeutic effect of antidepressant treatments (D'Aquila et al. 2000; Papakostas. 2006). Moreover, clinical studies have also reported that dopamine D2 receptor agonists are efficacious for treating patients with depressive illnesses (Waehrens et al. 1981). In our research, haloperidol treatment (0.2 mg/kg) partially inhibited the decrease of the immobility time of mice in the TST caused by PSAM (1 mg/kg), and significantly reversed the increase of the DA level in hippocampus.
induced by PSAM (1 mg/kg), indicating that haloperidol (0.2 mg/kg) partially blocked the anti-depressant-like effect of PSAM in the TST. Since haloperidol (0.2 mg/kg) was a non-selective dopamine D$_2$ receptor antagonist, these results indicated that the antidepressant-like activity of PSAM was partially involved in the dopaminergic system.

The neurotransmitter γ-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the CNS of mammals, causing the inhibition of almost all the key neurons (Sherman et al. 1982). Considerable evidence implicates GABA in the biochemical pathophysiology of mood disorders. Animal models of depression show regional brain GABA deficits and GABA agonists have antidepressant activity in these models. Clinical data indicate that decreased GABA function accompanies depressed or manic mood states (Petty. 1995). GABA is significantly reduced in experimental animals in the chronic stress depression model, TST and other animal models; therefore, supplementation of GABA can alleviate depression-like behavior (Borsini et al. 1988; Gronli et al. 2007; Machado-Vieira et al. 2009; Sherman et al. 1980). In this study, experimental results showed that bicuculline (a competitive GABA antagonist) treatment relieved the changes in the immobility time and the hippocampus GABA level induced by PSAM (1 mg/kg). These results proved that PSAM participate in the GABAergic system.

Growing evidence suggests that glutamatergic system dysfunction is directly involved in mood disorders (Machado-Vieira et al. 2009). Currently, clinical studies mostly use ketamine (a noncompetitive high-potency NMDA antagonist) as an agent for rapid relief of depressive symptoms, new NMDA receptor antagonists (modulators) are continuously being introduced for rapid antidepressant action, especially for use in treatment-resistant patients (Pochwat et al. 2014). Interestingly, traditional monoaminergic-based antidepressants have been repeatedly shown to interfere with glutamate system function, starting with modulation of N-methyl-D-aspartate (NMDA) receptors. Subsequently, it has been shown that antidepressants reduce glutamate release and synaptic transmission. In particular, it was found antidepressants prevent the acute stress-induced enhancement of glutamate release.
(Musazzi et al. 2013). In this experiment, NMDA (75 mg/kg) significantly increased the immobility time of PSAM (1 mg/kg) treatment in the TST. Meanwhile, PSAM (1 mg/kg) significantly decreased the Glu level compared to that of the control. These results showed that the glutamate system participates in the antidepressant effect of PSAM.

Based on the above observations, PSAM (1 mg/kg, i.p.) exhibited markedly antidepressant-like activity, which might be mediated by the DAergic, GABAergic and Gluergic systems, and we believe that PSAM might be a potential material for developing as a drug and food supplement against depression.

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Figure S1. Protoilludane sesquiterpenoid aromatic esters in the PSAM.
Figure S2. Effect of PSAM on the spontaneous locomotor activity. The values were represented as the mean ± SD (n = 10 in each group). Data were analyzed by t-test, compared with the control group, *p < 0.05, **p < 0.01.

Figure S3. (A-B) showed the antidepressant-like effect of different doses of PSAM in the TST and FST. (C-D) showed the changes in the immobility time over different time periods in the TST and FST. These values were represented as the mean ± SD (n = 10 in each group). The data were analyzed by t-test, compared with the control group, *p < 0.05, **p < 0.01.
Figure S4. The effects of PSAM (0.1 mg/kg), PSAM (1 mg/kg) and FLU (20 mg/kg) on the changes of the time of movement (A), the distance of movement (B) and the central residence time (C) in the OFT. The values were represented as the mean ± SD (n = 10 in each group). Data were analyzed by t-test, compared with the control group, *p < 0.05, **p < 0.01.

Figure S5. (A) showed the antidepressant-like effect of the co-administration of fluoxetine (5 mg/kg) with PSAM (0.1 mg/kg) in the TST; (B) showed the antidepressant-like effect of the co-administration of reboxetine (2.5 mg/kg) with PSAM (0.1 mg/kg) in the TST. The values were represented as the mean ± SD (n = 10 in each group). Data were analyzed by t-test, compared with the control group, *p < 0.05, **p < 0.01.
Figure S6. The effects of pre-treatment with haloperidol (A), bicuculline (B) and NMDA (C) on the antidepressant-like effect induced by PSAM (1 mg/kg) in the TST. The values were represented as the mean ± SD (n = 10 in each group). Data were analyzed by t-test, compared with the control group, * p < 0.05, ** p < 0.01; compared with the PSAM group, # p < 0.05, ## p < 0.01.

Figure S7. The effects of PSAM, haloperidol, bicuculline, NMDA and combined administration group on the DA (A), GABA (B) and Glu (C) levels in the hippocampus of mice exposed to the TST. The values were represented as the mean ± SD (n = 10 in each group). Data were analyzed by t-test, compared with the control group, * p < 0.05, ** p < 0.01; compared with the PSAM group, # p < 0.05, ## p < 0.01.