Antidepressant-like effects of *Marasmius androsaceus* metabolic exopolysaccharides on chronic unpredictable mild stress-induced rat model

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Abstract. *Marasmius androsaceus (M. androsaceus)*, a medicinal fungus, has various pharmacological activities including antidepression. The present study investigated the effects of exopolysaccharides obtained during *M. androsaceus* submerged fermentation in a chronic unpredictable mild stress (CUMS)-induced depression rat model. Similar to fluoxetine (positive drug), 4-week administration of *M. androsaceus* exopolysaccharides (MEPS) at doses of 6, 30 and 150 mg/kg strongly enhanced bodyweight gain and sucrose consumption, and reduced the immobility time in forced swimming test and tail suspension test in CUMS rats. MEPS resulted in significant enhancement on the levels of noradrenalin, dopamine, 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid in the serum and hypothalamus of CUMS rats, as detected by ELISA. Western blotting results revealed that MEPS upregulated the protein expression levels of tyrosine hydroxylase in the hypothalamus of CUMS rats. In conclusion, these results confirmed the antidepressant-like effects of MEPS, and suggested that the monoamine neurotransmitter system is involved in its antidepressive effects in a CUMS rat model. The present study provided evidence for the clinical application of MEPS as an effective agent against depression.

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Introduction

The incidence of depression is usually based on a variety of negative signals received from bodies, which causes tremendous economic losses, and endanger social stability and security (1). Patients suffering with major depression are usually characterized by decreased interest, despair and other negative emotions, and is often accompanied with appetite disorder, social phobia, idiopathic pain, cognitive dysfunction and other mental or physical symptoms, and may even have severe suicidal thoughts or behavior (2).

The pathology of depression remains to be fully studied, although several hypotheses including monoamine neurotransmitter hypothesis have been suggested (3). In the monoamine neurotransmitter hypothesis, the incidence of depression is triggered by reduced levels of monoamine neurotransmitters such as 5-hydroxytryptamine (5-HT) (4). Conventional antidepressants can markedly increase the concentration of 5-HT, dopamine (DA) and other neurotransmitters in the synaptic gap after administration, which suggests that the generation of depression is associated with changes on the sensitivity of monoamine neurotransmitter receptors (5). However, depression is not only caused by one factor; an integrated action of multivariate amine neurotransmitters and their related receptors may be involved (6).

Drugs applied for depression treatment only affect 60% patients, and are required at for at least 6-9 weeks to achieve improved therapeutic effects (7). Furthermore, serious adverse effects including gastrointestinal reactions, and liver and kidney damage, have been observed in patients after long-term using of antidepressants (8). Identifying safe and effective antidepressant agents has become an important research field. Due to the complex pharmaceutical functions, previous research has sought to identify effective antidepressant agents from natural products. As reported previously, *Hypericum enshiense* exhibits beneficial effects to moderate depressive behaviors in animal models, as assessed from forced swimming test (FST) and tail suspension test (TST) (9). The stress-attenuating activities of *Panax ginseng* have been confirmed experimentally and clinically (10). *Marasmius androsaceus*, a medicinal fungus, is mainly used for treating traumatic injury, fracture pain,

sciatica, migraine, leprosy neuralgia and rheumatic arthritis in China (11). In China, 'An-Luo Tong', produced by submerged fermentation mycelium of *M. androsaceus,* has been used as a painkiller. The main chemical constituents of *M. androsaceus* mycelium are mannitol, cholesteryl acetate, amino acids, hydroxycinnamic acid, ergosterol, proteins and a large number of carbohydrates (12). Our previous study demonstrated that exopolysaccharides produced during *M. androsaceus* submerged fermentation exhibit antidepressant like effects, especially in 5-HT-induced head twitch and reserpine-induced hypothermia mouse models (13).

Compared with endopolysaccharides, exopolysaccharides, produced by microorganism secretion, have the advantages of efficient production time, convenient extraction and no geographical restrictions (14). Differing from our previous studies, the present study aimed to evaluate the antidepressant-like effects of *M. androsaceus* exopolysaccharides (MEPS) in a chronic unpredictable mild stress (CUMS)-induced rat model. Sugar consumption test, FST and TST were applied to test the anti-depression-like activities of MEPS. Its regulatory effects on 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), DA and norepinephrine (NE) in the serum and hypothalamus were further detected. Western blotting was used to confirm if the dopaminergic system was involved in this effect. As with our previous study (13), the present study aimed to systematically confirm the antidepressant-like effects of *M. androsaceus* exopolysaccharides, and to provide experimental evidence for its further investigation and/or clinical application.

Materials and methods

MEPS preparation. According to a protocol described in our previous study (13), after a 4-day submerged fermentation, the products were filtered with 120 mesh sieves and centrifuged at 4,500 x g for 10 min at 25˚C. Proteins existing in the supernatant were removed by the Sevag method (15) and then a 4-fold volume of alcohol was added. After 12 h, the precipitate was collected by centrifuging at 4,500 x g for 10 min at 25˚C. After dialyzing and lyophilizing, MEPS were stored in a vacuum drier for further experiments.

Development of the CUMS rat model. Male Sprague-Dawley rats (weight, 180-200 g; age, 8 weeks; n=60) of specific pathogen-free grade [SCXK (JI)-2013-0003] were purchased from the laboratory animal center of Jilin University (Changchun, China). The experimental protocol was approved by the Animal Ethics Committee of Jilin University. Rats were allowed to adapt to the new surroundings for 1 week and were housed at 22±2˚C and 50±10% moderate humidity with a 12-h light/dark cycle, and fed autoclaved standard chow and water *ad libitum*.

According to a chronicle stress procedure as previously described (16), 10 from the 60 rats were randomly chosen for the vehicle group. Another 50 rats were randomly exposed to different stimuli daily for 8 weeks (Fig. 1). These stimuli included 12-h food deprivation and 12-h water deprivation, forced swimming at 4˚C for 10 min, thermal stimulation at 45˚C for 5 min, overnight illumination, 24-h wet litter stimulation, tail suspension test for 10 min and foot shock (1 mA current per minute 10 sec shock, 30 times). During the 8-week

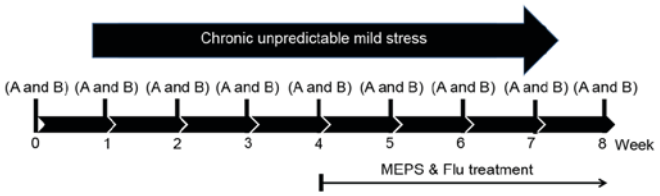

Figure 1. Experimental protocol for the development of a chronic unpredictable mild stress rat model and drug administration. MEPS, *M. androsaceus* exopolysaccharide, Flu, fluoxetine.

experimental process, all stimuli were applied individually and continuously, and applied preferably at the beginning of the light phase between 9:00 and 1:00 am. The vehicle rats (n=10) were housed in a separate room with no contact with the stressed groups. The changes in bodyweights and 1% sucrose consumption were detected every week to monitor the depression degree of rats.

Drug treatment procedure. Depressant-like rats were randomly divided into 5 groups (n=10 each), and orally treated with 2.0 ml/kg sterile saline (Model group), 6 mg/kg fluoxetine hydrochloride (Flu; Zhongxi Pharmaceutical Co., Ltd, Shanghai, China; Positive group), and MEPS at doses of 6, 30 or 150 mg/kg beginning on the day 28 of CUMS exposure, and lasting to day 56. Normal rats orally treated with 2.0 ml/kg sterile saline served as the vehicle group (n=10). Drug administration was 1 h prior to daily CUMS procedure.

Sucrose consumption test. A total of 2 days prior to testing, rats were adapted to sugar after overnight fasting and given only 1% sucrose solution for 48 h. Subsequently, the rats were fed normally for 3 days. After food and water deprivation for 12 h, a bottle of 1% sucrose water and a bottle of normal water were given to test rats; the consumption of 1% sucrose water was recorded within 1 h. The test was performed on days 1, 7, 14, 21, 28, 25, 42 and 49 (Fig. 1).

TST. A total of 30 mins after MEPS administration, rats were suspended by the tail to a horizontal wooden bar located inside a yellow plastic box (40x46x40 cm) with their heads 5 cm above the box bottom. Due to depression, rats failed to find a climbing site, which caused a low movement time record. Two blinded observers monitored the experimental rats for 6 min, starting from when the rat moved, and the immobility time within the last 5 min was calculated (17).

FST. A total of 30 min after MEPS administration, rats were placed in an open cylindrical container (30x32 cm) with 29-cm depth and 24±1˚C temperature water for 15 min to adapt. The behavior of the rats was monitored for 6 min, and the duration of immobility was recorded in the last 5 min by a blinded observer. The duration of immobility was defined as the cessation of all movements except for those necessary to keep its head above water (18).

Samples collection and ELISA assay. Following all testing experiments, blood (0.8-1.0 ml) was obtained from the caudal vein of each rat. Rats were then sacrificed, and the

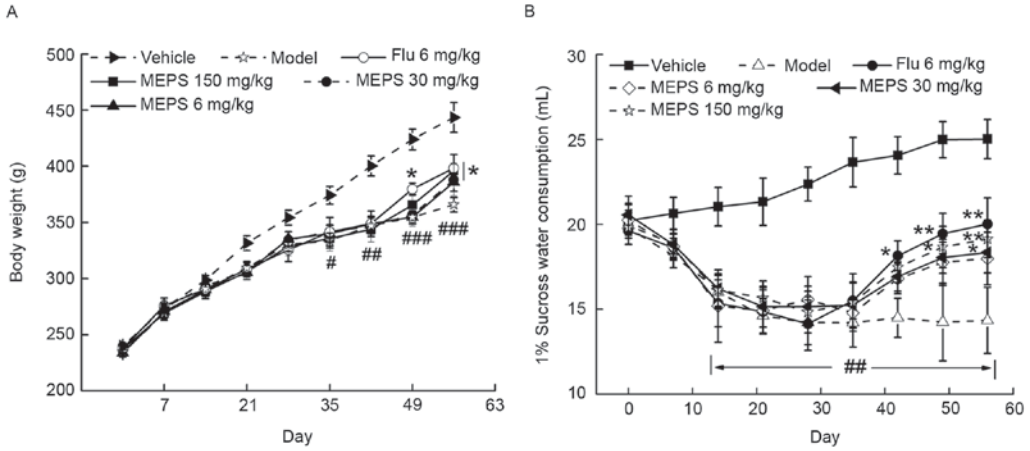

Figure 2. Effects of MEPS (6, 30 and 150 mg/kg) and Flu (6 mg/kg) on (A) body weight and (B) 1% sucrose consumption in chronic unpredictable mild stress rats. Data are expressed as the mean ± standard deviation (n=10/group). #P<0.05, ##P<0.01 and ###P<0.001 vs. Vehicle; *P<0.05 and **P<0.01 vs. Model. MEPS, *M. androsaceus* exopolysaccharide, Flu, fluoxetine.

hypothalamus was collected (19), weighed and stored at -80˚C for further experiments.

Half of the hypothalamus was lysed in double distilled water. The levels of DA, 5-HT, 5-HIAA and NE were measured using ELISA kits (CK-E30237, CK-E30326, CK-E92141R and CK-E30189R respectively; Shanghai Yuanye Bio-Technology Co., Ltd, Shanghai, China).

Western blot analysis. The other half of the hypothalamus was lysed using radio-immunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing 1% protease inhibitor cocktail. After analysis of protein concentration via the Bradford method, 30 μg proteins were separated by 10% SDS-PAGE. The electrophoretically transferred membranes (0.45 m; Bio Basic, Inc. USA) were incubated with 5% bovine serum albumin (Merck KGaA, Darmstadt, Germany) for 4 h at 4˚C, and incubated with anti-tyrosine hydroxylase (TH) (sc-14007) and anti-GAPDH (sc-2577) primary antibodies at 4˚C overnight at a dilution of 1:1,000 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (sc-3836) at 4˚C for 4 h at a dilution of 1:2,000 (Santa Cruz Biotechnology, Inc.) for 4 h at room temperature. After detecting via Enhanced Chemiluminescence detection kits (GE Healthcare Life Sciences, Little Chalfont, UK), the intensity of bands was quantified using ImageJ software version 1.51 (National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. All data are presented as the mean ± standard derivation. SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA) was used to analysis the data. Differences among experimental groups were determined by one-way analysis of variance followed by Dunn's post-hoc multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

Antidepressant-like effects of MEPS. CUMS for 8 weeks strongly reduced body weight gain beginning from day 49, which were strongly reversed by Flu (6 mg/kg) and MEPS (30 and 150 mg/kg) administration (P<0.05; Fig. 2A). The sucrose consumption test is a widely-accepted measure of depressant-like behavior (20). From day 14, 1% sucrose consumption was strongly reduced in CUMS rats (P<0.05; Fig. 2B). Comparatively, Flu (6 mg/kg) and MEPS at certain doses significantly enhanced the sucrose consumption, starting from day 49 of the whole experimental period (P<0.05; Fig. 2B).

FST is a useful screening test for antidepressant drugs, as it can measure depressive behaviors (21). Compared with non-treated CUMS rats, MEPS at 6, 30 and 150 mg/kg remarkably decreased the duration of immobility in the FST, which demonstrated similar effects as that of Flu (P<0.05; Fig. 3A).

Furthermore, 8-week CUMS caused a strong enhancement of the immobility time of each rat in TST. Similar to Flu (6 mg/kg), 4-week MEPS administration at 150 mg/kg reduced up to ~50% of immobility time compared with non-treated CUMS rats (P<0.001; Fig. 3B).

Effects of MEPS on monoaminergic neurotransmitter levels. CUMS procedures for 8 weeks caused a strong suppression of 5-HT and 5-HIAA levels in serum (P<0.05; Fig. 4A and B, respectively) and hypothalamus (P<0.05; Fig. 4C and D, respectively). Flu at 6 mg/kg strongly enhanced the serum and hypothalamus levels of 5-HT and 5-HIAA back to normal levels (P<0.05; Fig. 4). MEPS at certain doses exhibited similar effects as Flu, and resulted in >50% enhancement of 5-HT and 5-HIAA levels in the hypothalamus (P<0.05; Fig. 4C and D).

Furthermore, the abnormally low levels of NE in serum (Fig. 5A; P<0.05) and the hypothalamus (Fig. 5B; P<0.01), and of DA in serum (Fig. 5C; Fig. P<0.01) and the hypothalamus (Fig. 5D; P<0.001) in CUMS rats were strongly reversed by 4-week Flu and MEPS treatment (P<0.05; Fig. 5).

Effects of MEPS on TH expression. CUMS procedures for 8 weeks resulted in a strong reduction on TH protein expression levels in the hypothalamus (P<0.01; Fig. 6). Both Flu and MEPS exhibited beneficial effects on enhancing TH expression (Fig. 6). MEPS at 150 mg/kg enhanced nearly one-fold

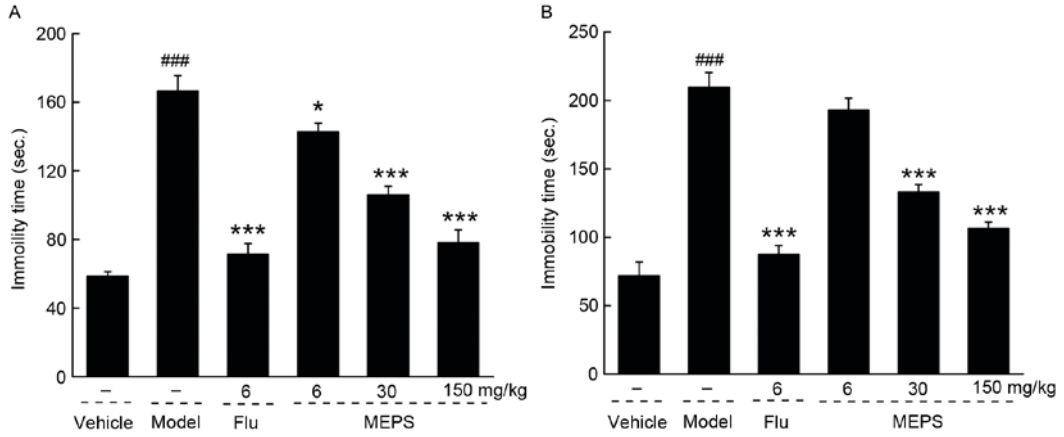

Figure 3. MEPS (6, 30 and 150 mg/kg) and fluoxetine (6 mg/kg) treatment for 4 weeks strongly reduces the immobility time of chronic unpredictable mild stress rats in (A) FST and (B) TST. Data are expressed as the mean ± standard deviation (n=10/group). $^{###}$P<0.001 vs. Vehicle; *P<0.05 and ***P<0.001 vs. Model. MEPS, *M. androsaceus* exopolysaccharide, Flu, fluoxetine.

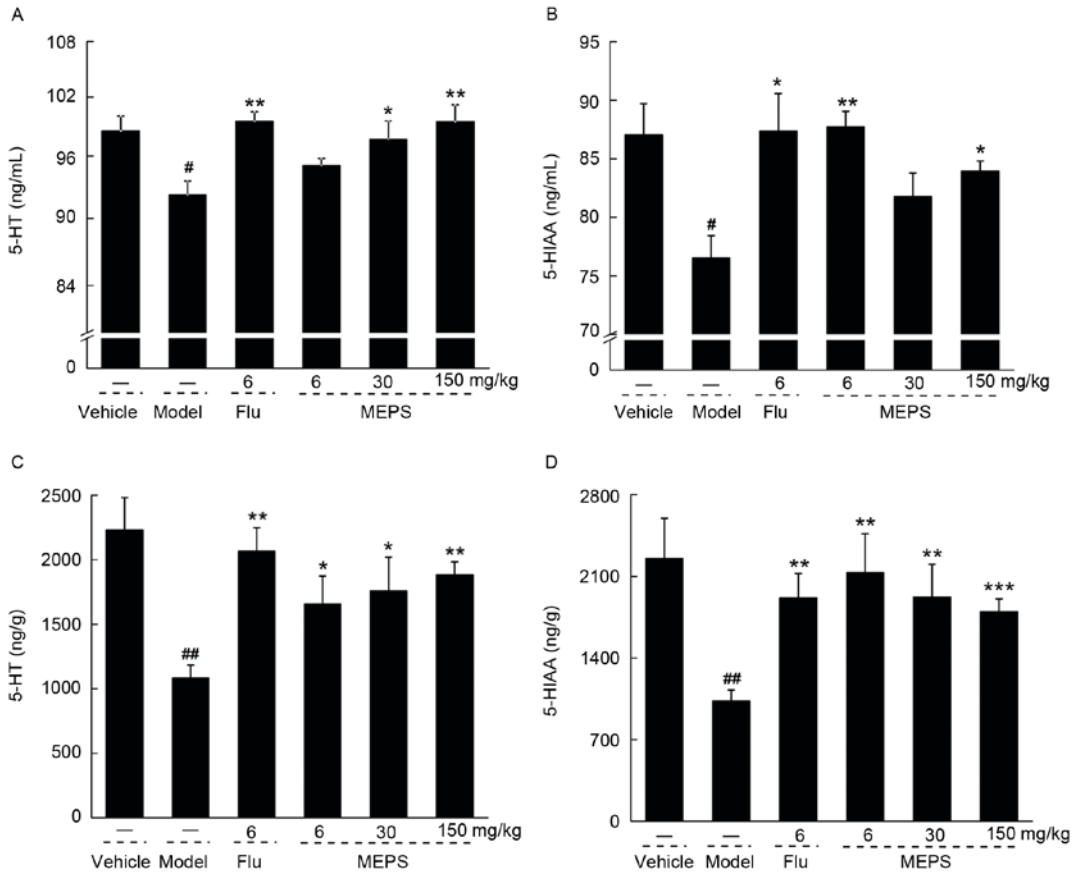

Figure 4. MEPS (6, 30 and 150 mg/kg) and fluoxetine (6 mg/kg) treatment for 4 weeks significantly enhances the levels of serum (A) 5-HT and (B) 5-HIAA, and hypothalamic (C) 5-HT and (D) 5-HIAA. Data are expressed as the mean ± standard deviation (n=10/group). $^{#}$P<0.05 and $^{##}$P<0.01 vs. Vehicle; *P<0.05, **P<0.01 and ***P<0.01 vs. Model. MEPS, *M. androsaceus* exopolysaccharide, Flu, fluoxetine; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid.

of TH expression in the hypothalamus compared with CUMS rats (P<0.01; Fig. 6).

Discussion

The current social climate, with humans being subjected to increasing pressure, has led to an increase in the incidence of depression (22). Patients with major depression can fail to take care of themselves properly, and may suffer from other serious physical dysfunction (23). Due to most antidepressants having varying degrees of toxicity and high drug resistance (24), many experiments have focused on searching for a more efficient antidepressant with low toxicity (19). CUMS, an experimental procedure with variable unpredictable stressors, is commonly

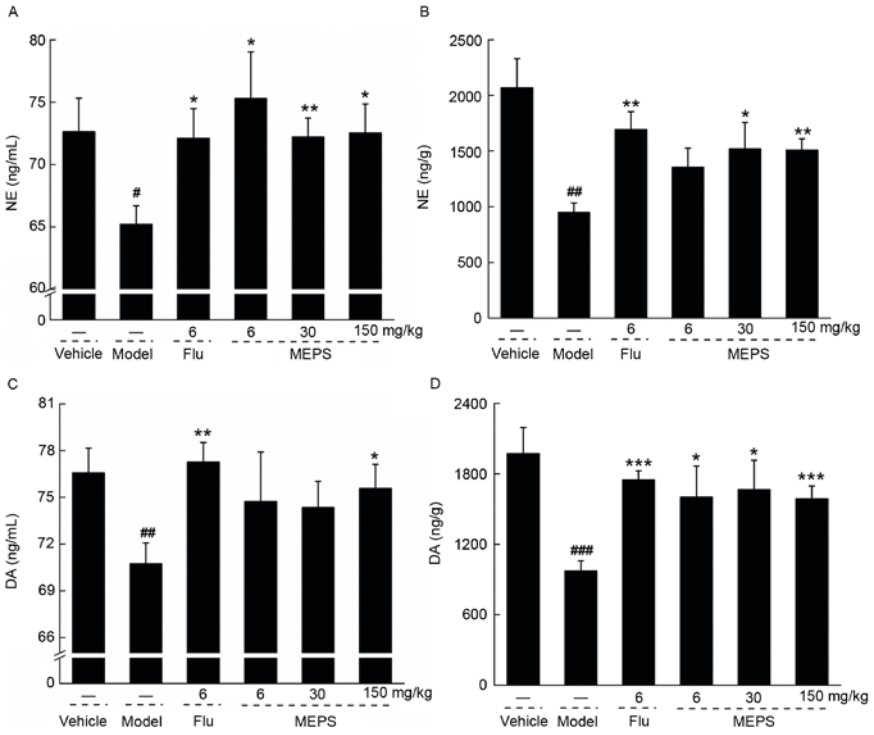

Figure 5. MEPS (6, 30 and 150 mg/kg) and fluoxetine (6 mg/kg) treatmentfor 4 weeks significantly enhances the levels of DA in (A) serum and (B) the hypothalamus, and NE in (C) serum and (D) the hypothalamus. Data are expressed as the mean ± standard deviation (n=10/group). $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. Vehicle; *P<0.05, **P<0.01 and ***P<0.01 vs. Model. MEPS, *M. androsaceus* exopolysaccharide, Flu, fluoxetine; NE, norepinephrine; DA, dopamine.

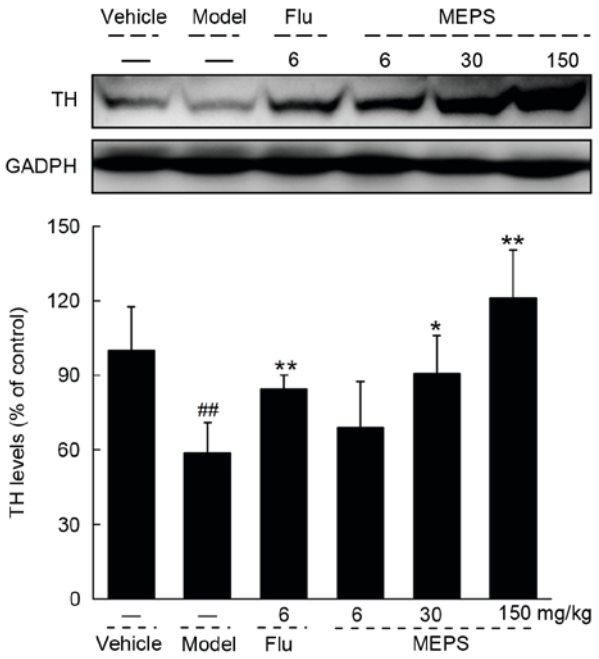

Figure 6. MEPS (6, 30 and 150 mg/kg) and fluoxetine (6 mg/kg) enhances the protein expression levels of TH in hypothalamus in chronic unpredictable mild stress rats, as assessed by western blotting. GAPDH served as an internal control. Data are expressed as the mean ± standard deviation (n=10/group). $^{\#\#}$P<0.01 vs. Vehicle; *P<0.05 and **P<0.01 vs. Model. MEPS, *M. androsaceus* exopolysaccharide, Flu, fluoxetine; TH, tyrosine hydroxylase.

used to develop depressant-like animal models, which was employed to evaluate the effects of MEPS in the present study. The results indicated that similar to antidepressants, oral administration of MEPS prevented the development of anxiety/depressant-like behaviors in CUMS rats. Bodyweight gain was used to examine the ability of free water and food intake in rats (25). TST and FST are widely used as a reliable animal model of depression to screen novel antidepressants (26). MEPS reduced the immobility time of CUMS rats in FST and TST without affecting spontaneous locomotor activity within certain doses, indicating its low toxicity. Sugar consumption, another method for the assessment of depressive-like behavior, has been strongly improved by MEPS. Unlike other antidepressants or candidates, MEPS contains multiple active ingredients that appear to 'systemically target' and eliminate depressant-like behaviors via multiple pathways, which suggests its efficacy and low adverse effects (27,28).

Based on neural monoamine hypothesis, depression is associated with the activities of DA and/or serotonin metabolic regulation (29). Results from the clinic have identified that the levels of 5-HT and 5-HIAA in platelets may serve as a supplementary biomarker for depression diagnoses (30). Exposure to stress increases the metabolism of 5-HT, and results in high levels of 5-HIAA in the hippocampus of mice (31), consistent with the results of the present study. 5-HT receptor agonist-induced head-twitch response provides a useful model for detecting the association the between 5-HTergic system and depression (32,33). Our previous studies demonstrated that MEPS increases head twitch in a 5-hydroxytryptophan-induced head-twitch mouse model (13), and that *Paecilomyces hepialid* possesses antidepressant-like effects via modulation of the dopaminergic system and 5-HT associated pathway (34). Notably, serotonin reuptake inhibitors enhance neurotransmission levels in cortical areas via blocking 5-HT_{2A}-mediated

responses, indicating its antidepressant efficacy (35). Although MEPS regulates 5-HT and 5-HIAA levels in the serum and hypothalamus, both present and previous experiments failed to detect the effects of MEPS on 5-HT receptors expression levels in brain areas in murine models. The results of the present study suggested that the 5-HTergic system may be involved in MEPS-mediated antidepression in CUMS rats; however, more studies on 5-HT receptors are required.

As previously reported, the 5-HTergic system influences the dopaminergic system, evidenced by 5-HT_{2A} receptors in the prefrontal cortex controlling neocortical DA release (36), and 5-HT depletion may reduce the ability of d-amphetamine ability to release DA from presynaptic terminals (37). Patients with major depression have a deficit of DA and/or DA metabolites in the brain (38). In the present study, MEPS enhanced the levles of DA and NE in the serum and hypothalamus of CUMS rats. A deficit of dopaminergic function in the limbic circuitry and striatum could be involved in the underlying mechanism of depression (39). Chronic stress produces changes on neurotransmitters levels, mainly exhibiting as a significant reduction in the levels of 5-HT and DA (40). This is consistent with the results of the present study, where MEPS enhanced the expression levels of TH in the hypothalamus of CUMS rats. TH is responsible for DA synthesis, and recognized as a rate-limiting step (41). Inhibition of TH activity halts catecholamine synthesis and produces depression in patients (42). The results of the present study suggested that the dopaminergic system may contribute to MEPS-mediated anti-depression in CUMS rats; however, the association between the 5-HTergic system and the dopaminergic system require further investigation.

In conclusion, MEPS has antidepressant-like effects verified by sucrose consumption test, TST and FST. Its regulation on 5-HT, 5-HIAA, DA and NE in the serum and hypothalamus suggested that the monoamine neurotransmitter system may be involved in its antidepressant-like effects in CUMS rat model. Therefore, these data provide evidence for the clinical application of MEPS as an effective agent against depression.

Acknowledgements

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