DOI: 10.31276/VJSTE.63(4).55-61

The antidepressant-like effects of an n-butanol fraction of *Ocimum sanctum* Linn. extract in unpredictable chronic mild stress-induced depression in mice

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Received 14 July 2021; accepted 8 September 2021

Abstract:

We previously reported that *Ocimum sanctum* Linn. (OS) ethanolic extract and its n-butanol fraction (OS-B) could improve depression-like behaviour in olfactory bulbectomized mice. The present study aims to clarify the antidepressant-like effects of OS-B and the possible mechanism of its action using mice subjected to unpredictable chronic mild stress (UCMS). UCMS mice were administered daily with OS-B (50 mg/kg, 100 mg/kg, p.o.) or imipramine (IMP, 8 mg/kg, i.p.), a reference drug. The UCMS-induced anhedonia in mice was analysed by the sucrose preference test, while behavioural despair was assessed using the tail suspension test (TST) and forced swimming test (FST). Locomotor activities and grooming behaviour of mice were elucidated using the open-field test (OFT). The UCMS procedure for 5 weeks induced anhedonia, and this symptom was significantly ameliorated by the administration of OS-B (100 mg/kg) as well as IMP during the UCMS period. Moreover, the OS-B and IMP treatment attenuated the UCMS-induced enhancement of behavioural despair in the TST and FST. In OFT, mice subjected to UCMS showed a decrease in grooming behaviour, and the effect of UCMS was reversed by OS-B and IMP administrations. No significant difference in locomotor activities between each animal group was observed. The amelioration effects of OS-B and IMP on UCMS-induced behavioural despair in the TST were abolished by administrating of ρ-chlorophenylalanine (PCPA, 80 mg/kg, i.p), a tryptophan hydroxylase inhibitor, and α-methyl-ρ-tyrosine (AMPT, 100 mg/kg), a tyrosine hydroxylase inhibitor. The present results suggest that OS-B attenuates UCMS-induced depression-like symptoms via monoaminergic systems including in the noradrenergic, dopaminergic, and serotonergic system.

Keywords: **antidepressant, monoaminergic system, *Ocimum sanctum*, serotonergic system, unpredictable chronic mild stress.**

Classification number: **3.3**

Introduction

Depression is currently one of the leading causes of the worldwide burden of disease. It is one of the most common psychiatric disorders characterised by psychological, behavioural, and physiological symptoms [1]. Lines of evidence have demonstrated that the monoaminergic-sympathetic nervous systems play essential roles in the depressive pathophysiology and antidepressant therapy [2]. The regulation of mood was significantly influenced by the monoaminergic system on the brain circuit especially serotonin, norepinephrine, and dopamine [3, 4]. In fact, prescription antidepressants such as tricyclics, monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, and selective noradrenaline reuptake inhibitors, almost solely focus on enhancing monoamine transmitter function [4]. However, the treatment of depression with conventional antidepressants has many adverse effects [5, 6]. Therefore, daily intake of herbs with the potential to affect monoaminergic neurotransmitter systems may offer an effective strategy for treating and preventing depressive disorders associated with various stressors.

OS has various medicinal properties in the traditional system of medicine. It is also used as herbal tea or spice in Southeast Asian countries, including Vietnam. Many studies reveal that OS has a unique combination of actions

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that include anti-inflammatory [7], anti-dementia [8, 9], anxiolytic and antidepressant [10], as well as antioxidant effects [11, 12], etc. Regarding its chemical properties, OS reportedly contains a volatile oil, flavonoids (luteolin, apigenin, orientin, vicenin, cirsimaritin, isothymusin, luteolin-7-*O*-glucuronide and apigenin-7-*O*-glucuronide) [11, 13, 14], glycoglycerolipids (ocimumoside A and B) [15], and triterpenoids (ursolic acid and oleanolic acid) [16]. In the previous study, we demonstrated that ethanolic extract of OS ameliorated cognitive dysfunction and neuro-histological damages in olfactory bulbectomized (OBX) mice via enhancing the central cholinergic systems and VEGF expression [17]. Moreover, it was found that the ethanolic extract of OS improved depression-like behaviour in OBX mice [18]. Our findings suggested that the anti-depression effect of the OS ethanol extract was due to chemical constituents fractionated by n-BuOH, but not n-hexane or ethyl acetate [19]. However, the mechanism underlying the antidepressant effect of the OS-B fraction is unclear.

Because the development of depression was closely related to socio-environmental chronic stressors, a variety of stress-based animal models have been used to explore the antidepressant potential of herbal medicines and their mechanisms of action. Based on the stress hypothesis of depression, the UCMS model has been employed as a useful model with predictive validity, face validity, and construct validity [20]. In UCMS-induced depression-like behaviour, dysfunction of monoaminergic systems is reportedly one of the most relevant biological factors [21, 22]. In the present work, we aimed to examine the antidepressant-like effect and the underlying mechanism of OS-B using a UCMS model of depression. Our results suggested that OS-B exhibits anti-depressive effects at least in part via noradrenergic, dopaminergic, and/or serotonergic systems.

Materials and methods

Animals

Male *Swiss albino* mice (5-6 weeks old) were supplied from the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. Animals were housed for at least one week before experiments. Mice had free access to tap water and conventional rodent food. Housing was kept under standardized conditions with temperature 25±1°C, humidity 65-75% and photoperiod 12:12 (lights on at 7:00 A.M.). All behavioural tests were performed in the light phase, between 9:00 and 18:00. The present studies were conducted in accordance with the Guiding principles for the care and use of animals (NIH publication #85-23, revised in 1985) and were approved by the Institutional Animal Use and Care Committees of the National Institute of Medicinal Materials (NIMM), Hanoi, Vietnam.

Preparation of plant extract

OS was collected in Hanoi city and identified by Dr. Pham Thanh Huyen (Department of Medicinal Plant Resources, NIMM, Vietnam). The voucher specimen (voucher #NIMM-16474B) was deposited at NIMM. The preparation of OS-B has been described previously [19]. Briefly, the aerial part of OS (4 kg) was extracted 3 times with 70% ethanol under reflux for 2 h. Each extract was combined, filtered using a filter paper (Whatman® qualitative filter paper, Grade 93, 580×580 cm, Merck, Darmstadt, Germany), and concentrated at 50°C under a vacuum yielding two litres of aqueous extract. The resultant extract was successively partitioned three times with n-hexane, ethyl acetate (EtOAc), and n-butanol (BuOH). Thereafter, the BuOH phase was evaporated and then dried in a vacuum oven at 50°C to obtain a 68-g n-BuOH fraction (OS-B). The chemical profile of OS-B was conducted using high-performance liquid chromatography (HPLC) as previous described [17]. The HPLC analysis exposed that the OS-B contained 5.82% luteolin-7-*O*-glucuronide, 2.26% apigenin-7-*O*-glucuronide, 0.15% apigenin, 0.45% luteolin, 1.00% ursolic acid, and 0.87% oleanolic acid (see supplemental data).

UCMS procedure and drug administration

The UCMS procedure reported by Mizuki, et al. (2014) [23] was used with minor modification. Mice were subjected to different unpredictable mild stressors such as: 18 h of isolation, food and water deprivation before sucrose preference test (SPT), 36 h of light exposure, 24 h of cage tilting at 45°, 24 h of exposure to an empty bottle, 3 h of confinement in a cramped jar for each mouse, a 24 h period of a wet cage (200 ml water/50 g rice husk), 3 h of a rat singing sound (playing a video record), and 24 h of paired caging. On average, the animals were exposed to two of these stressors at different times every day, which was randomly scheduled over 1 week. The stress process was repeated throughout 5 weeks before the behavioural tests. The UCMS was still in progress during the testing stages, excluding testing days to avoid affecting the test results. The non-stress control group was housed under normal conditions.

The aforementioned OS-B was freshly suspended in distilled water and administered at doses of 50 mg/kg/day (OS-B 50) and 100 mg/kg/day (OS-B 100) (p.o.). Imipramine (IMP; Sigma Chemical Co, St. Louis, MO, USA) was dissolved in 0.9% saline and administered at doses of 8.0 mg/kg/day (i.p.). Non-stressed and the UCMS control group mice were administered daily with tap water by oral route (p.o.). On the day of the behavioural tests, OS-B and IMP were administered for 60 and 30 min, respectively, before the test. This procedure is summarised in Fig. 1.

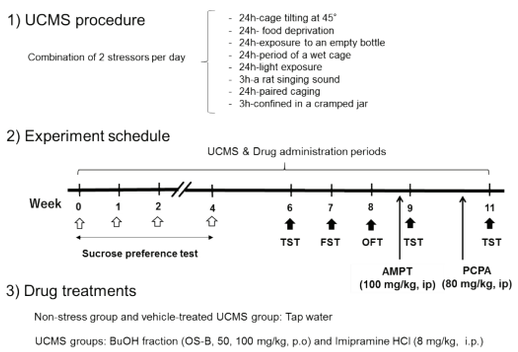

Fig. 1. Schematic drawing of the experimental protocol.

In week 0, *Swiss albino* mice were separated into non-stress and UCMS groups. The UCMS procedure was started from week 0 to week 11. The UCMS group was given tap water or butanol fraction of OS (OS-B, 50 and 100 mg/kg/day, p.o.) or imipramine HCl (IMP, 8 mg/kg, i.p.). The sucrose preference test was conducted weekly from week 0 to week 4 during the UCMS exposure. The depression-like behaviours were assessed using a TST, force swimming test (FST), and an open field test (OFT). 4 h before the drug administration on the week 9, OS-B 100- and IMP-treated group mice were pre-treated with a single dose of α-methyl-ρ-tyrosine (AMPT) (100 mg/kg, i.p.). Ten days after the AMPT treatment, the OS-B 100- and IMP-treated group mice were administered with ρ-chlorophenylalanine (PCPA) (80 mg/kg, i.p.) for 4 consecutive days before subjected to TST.

Behavioural performances

Sucrose preference test (SPT): SPT was used to assess as an index of anhedonia. The tests were conducted once before starting the UCMS procedure (week 0) and during UCMS induction (5 times in total) according to the previous report [24] with slight modification. The animals were trained to consume a 2% sucrose solution for a 48-h period without food and water before the first SPT at week 0. In the SPT, mice were separately placed in a cage and deprived of water and food for 18 h. The mice then received tap water or a 2% sucrose (w/v) solution from two drinking nozzles set at the cage for 1 h. The positions of the nozzles were randomly switched. The amounts of 2% sucrose solution and the weight of each mouse were recorded. The sucrose preference of each mouse was calculated with the following formula:

$$\text{Sucrose intake (g/kg b.w.)} = [0.02 \times V\,(\text{ml})]/[0.001 \times m\,(\text{g})]$$

TST: at week 6, TST was conducted as previously described [18, 23, 25]. Briefly, each mouse was suspended by adhesive tape, which was placed approximately 2 cm from the tip of the tail. The mouse's head was above the floor 40 cm. This short-term inescapable stress led to characteristic behaviour immobility. The animal behaviour was video-recorded during a 6-min period and then the mice were returned to the home cage. Immobility time was analysed for the last 5 min and defined as a state with a movement speed of no more than 0.05 cm^2/s using the Any-maze Video Tracking System (ver. 4.99, Stoelting Co., IL, USA).

FST: one week after the TST, mice were subjected to FST. In this test, each mouse was forced to swim in clear plexiglass cylinders (internal diameter: 12 cm, height: 24 cm) containing water (18 cm deep) at 20±1°C. The behaviours of each mouse were video-recorded from above for 6 min. The immobility duration was calculated using Any-maze Video Tracking System (ver. 4.99, Stoelting Co., IL, USA). Immobility was determined when the animals ceased struggling and showed floating motionless in the water with minimum movements to keep the nose above water surface [26].

OFT: spontaneous motor activity of animals was elucidated in the open field. The activity was video-recorded, and then the effects of test drugs were elucidated using the Digiscan Infrared Photocell system (Omnitech Electronics, Columbus, OH), which consisted of 40×40×50 cm plexiglass arenas with black floors fitted into infrared beams dividing the space into 16 equal squares. This system calculated the number of times that the animal interrupted infrared beams during a 10-min observation period. Measurement parameters were horizontal activity (units) and vertical activity (units). The vertical activity was defined as the number of times the animal interrupted the horizontal beam sensor. In addition, the total duration (seconds) that the animal spent grooming was scored as an index of UCMS-induced stress response by two experienced observers in a blind manner. The floor of the apparatus was cleaned with 70% ethanol before testing the next mouse.

Pharmacological treatments

α-methyl-ρ-tyrosine (AMPT, Sigma Chemical Co, St. Louis, MO, USA), an inhibitor of tyrosine hydroxylase, a rate-limiting enzyme for noradrenaline and dopamine synthesis [27] and DL-ρ-chlorophenyl alanine (PCPA, Acros Organics, Switzerland), an inhibitor of tryptophan hydroxylase (TPH) involved in the serotonin synthesis [28], were used to explore the role of serotonergic and noradrenergic system in the antidepressant-like actions of OS-B. AMPT and PCPA and were dissolved in saline with 1% Tween 80. On week 9, OS-B 100- and IMP-treated group mice were treated with a single dose of AMPT (100 mg/kg, i.p.) 4 h before the drug administration. Sixty minutes after the administration, the mice were subjected to TST. Ten days after AMPT treatment, the OS-B 100- and IMP-treated mice groups were administered with PCPA (80 mg/kg, i.p.) for 4 consecutive days. Thirty min after the last PCPA administration, mice were administrated the test drug treatment and, after 60 min, were submitted to the TST (Fig. 1).

Data analysis

All the results obtained in current study were represented as the mean ±S.E.M. The comparisons between all the groups were performed by one-way ANOVA followed by post hoc Student-Newman-Keuls test (SPSS 22.0). A p value of <0.05 was regarded significant.

Results

Effects of OS-B and IMP on UCMS-induced anhedonia using the sucrose preference test (SPT)

SPT was conducted weekly to assess anhedonia of UCMS-exposed mice. As shown in Fig. 2, no significant difference in sucrose consumption from week 0 to week 3 was found between each animal group. However, the sucrose consumption at week 4 markedly decreased in the vehicle-treated UCMS group as compared to non-stress group mice, indicating UCMS-induced anhedonia symptom. IMP treatment significantly suppressed the increase in sucrose intake at week 4 in UCMS-exposed groups. OS-B (50 and 100 mg/kg) also dose-dependently reversed UCMS-induced decrease in sucrose intake.

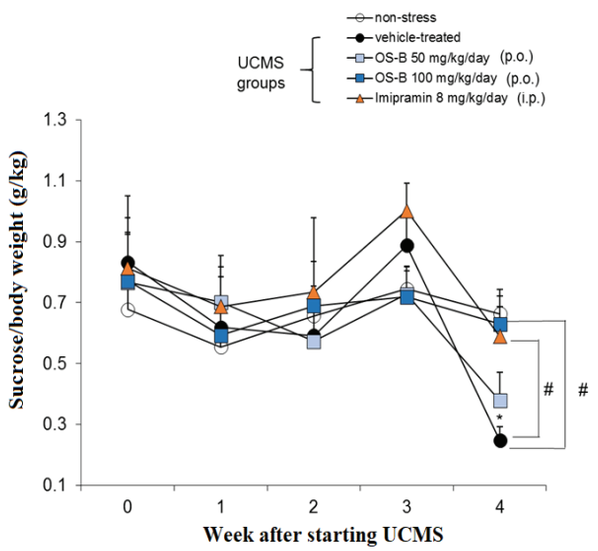

Fig. 2. The effects of OS-B (50 and 100 mg/kg) on UCMS-induced depression-like behaviours in the SPT. Sucrose preference of each animal group was weekly measured before and during 4-week UCMS procedure (n=9-10). *p<0.05 vs. non-stress group; #p<0.05 vs. UCMS control group.

Effects of OS-B and IMP on UCMS-induced depression-like behaviour in TST and FST

Depression-like behaviours of UCMS mice were analysed by the TST and FST. As shown in Fig. 3, the vehicle-treated UCMS group showed significantly longer immobility time than the non-stress group (p<0.01, p<0.05, respectively) in TST and FST, indicating a depression-like symptom. IMP treatment markedly decreased the immobility time of UCMS mice in both TST and FST. The OS-B (50 and 100 mg/kg)-treated UCMS group significantly and dose-dependently decreased the immobility time when compared with the UCMS control group mice.

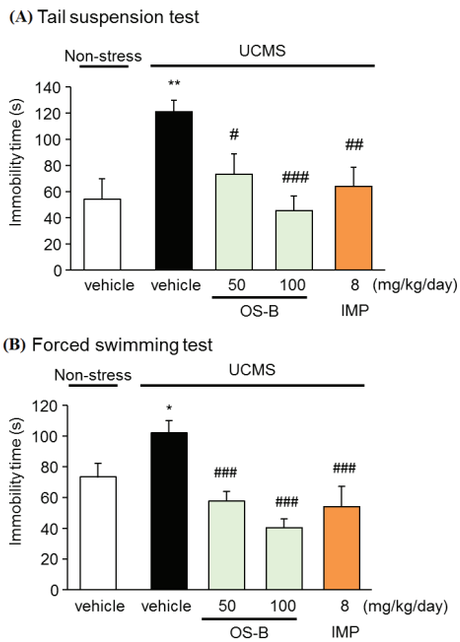

Fig. 3. The effects of OS-B (50 and 100 mg/kg) on UCMS-induced depression-like behaviour in the TST and the FST. The immobility time of each animal group in the TST **(A)** and FST **(B)** was calculated as an index of depression-like behaviour at weeks 6 and 7, respectively, during the UCMS procedure (n=9-10). *p<0.05, **p<0.01 vs. non-stress mice, #p<0.05, ##p<0.01 and ###p<0.001 vs. UCMS control group.

Effects of OS-B and IMP on locomotor activity and grooming behaviour of UCMS mice in the OFT

The OFT was conducted to determine the locomotor activity of the UCMS mice, so that false-positive results can be excluded. As shown in Fig. 4A1-2, no significant difference between each animal group in the horizontal and vertical activity, indicating that neither IMP nor OS-B affected the locomotor activity of UCMS mice.

Besides, the time the animals spent grooming was manually recorded since a decrease of grooming behaviour in the OFT is likely related to less motivation or interest in daily performance, such as the maintenance of minimal personal care, which is a symptom of depression [29]. As shown in Fig. 4B, mice subjected to UCMS treatment clearly showed decreased time spent grooming. Moreover, the administration of OS-B (50, 100 mg/kg) and imipramine significantly prolonged the time of self-grooming in UCMS-exposed mice.

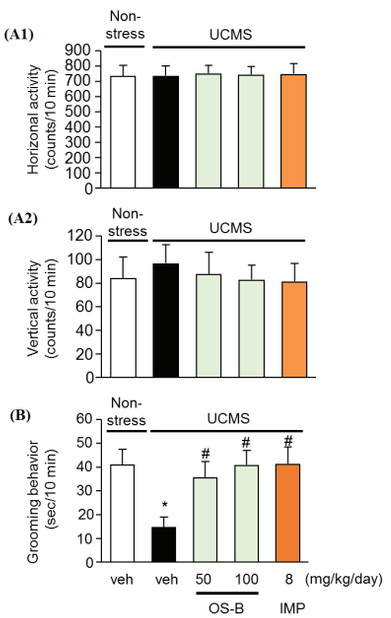

Fig. 4. The effects of OS-B (50 and 100 mg/kg) and IMP on locomotor activity and grooming behaviours of UCMS mice. Horizontal activity **(A1)** and vertical activity **(A2)**, **(B)** grooming behaviours expressed as a total accumulated time during a 10-min session (n=9-10), *p<0.05 vs. non-stress group, #p<0.05 vs. UCMS control group.

Involvement of monoaminergic system in the antidepressant-like effects of OS-B

AMPT, a tyrosine hydroxylase inhibitor, and PCPA, a tryptophan hydroxylase inhibitor, were used to clarify the implication of monoaminergic systems in the antidepressant-like effects of OS-B. The UCMS control group displayed remarkably longer immobility duration than the non-stress group (p<0.05) in the TST conducted at week 9 and 11 after starting the UCMS exposure. However, pre-treatment with AMPT (100 mg/kg, i.p.) completely abolished the suppressing effects of OS-B and IMP on the immobility of UCMS mice in the TST (Fig. 5A). The antidepressant-like effects of OS-B (100 mg/kg) and IMP on UCMS mice in the TST were also reversed by pre-treatment of UCMS mice with PCPA (80 mg/kg, i.p.) (Fig. 5B).

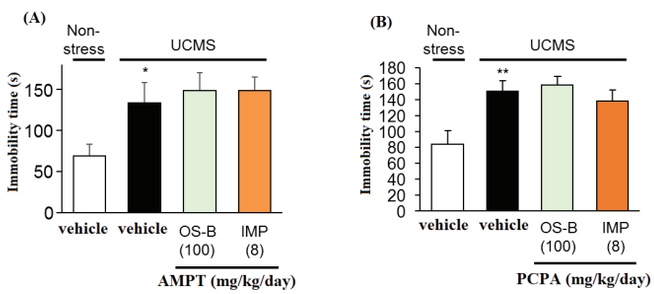

Fig. 5. The effect of the pre-treatment with α-methyl-ρ-tyrosine (AMPT, 100 mg/kg, i.p.) (A) and DL-ρ-chlorophenyl alanine (PCPA, 80 mg/kg, i.p.) (B) on OS-B induced antidepressant-like effects in the TST. n=9-10, *p<0.05, **p<0.01 vs. non-stress group. No significant difference between OS-B and IMP vs. non-stress group.

Discussion

The present study using a UCMS-exposed animal model of depression demonstrated that OS-B exhibited anti-depression-like effects the same as imipramine, a typical antidepressant drug, via the serotonergic and noradrenergic systems. Thus, it is likely that the daily OS administration is beneficial for preventing/improving core symptoms in patients with depressive disorders.

In this study, we employed the UCMS to induce depression-like behaviours in mice because the UCMS procedure, the UCMS-induced pathophysiological features, and the susceptibility to antidepressant drugs are associated with human depression. These features of the UCMS-exposed animal provide a rationale to use as an animal model with constructive, face, and predictive validities of depression. Using the UCMS model, we found that the daily treatment of the UCMS-exposed animals with OS-B (50 and 100 mg/kg) and imipramine dose-dependently reversed the UCMS-induced anhedonia. Indeed, anhedonia was detected as a gradual decrease in sucrose consumption in the animals exposed to the UCMS procedure. Anhedonia is one of the most prominent symptoms observed in humans with depressive illness [30] and is reportedly linked to dysfunctions in the reward-related processes. The dopaminergic system, in particular, plays an important role in predicting reward, generating motivation, and responding to conditioned incentive stimuli [31]. Downregulation of dopaminergic systems has been observed in animals subjected to chronic mild stress [32] and patients with depression [33]. Considering these findings, it is likely that OS-B can ameliorate anhedonia, probably, by affecting the dopaminergic systems implicated in the reward-related process in the brain.

The present study also revealed the antidepressant-like actions of OS-B in the TST and FST. As shown in Fig. 3, vehicle-treated UCMS mice showed a markedly exacerbated behavioural despair response measured as a prolonged immobility time in these tests, compared to non-stress mice. These data indicate that the UCMS-exposed mice are more susceptible to stressful stimuli than non-stressed control animals. Moreover, when administered daily during a UCMS exposing period, OS-B and imipramine almost completely prevented the exacerbation of behavioural despair responses to stressors in the TS and FST. These results are consistent with our previous study in which OS and OS-B ameliorated behavioural despairs exhibited by OBX animals, an animal model of dementia patients with depression [19]. Thus, our findings suggest that OS-B acts like an antidepressant-like drug and protects animals from being vulnerable to stressors.

Moreover, it should be noted that, in the present study,

the immobility time of 100 mg/kg/day OS-B- and 8.0 mg/kg/day imipramine-treated UCMS groups showed less than that of the non-stress group. These data raise the possibility that OS-B, at a daily dose of 100 mg/kg, may exhibit a psychostimulant activity in the UCMS animals, thereby apparently reducing the immobility time of UCMS animals in the TST and FST. However, this possibility seems to be little, if any, because OS-B (50-100 mg/kg/day, p.o.) had no effects on the spontaneous motor activity of the UCMS animals in the OFT. Rather, an interesting finding in the OFT is that the administration of OS-B and imipramine significantly reversed the USMC-induced decrease in the time animals spent grooming. Recent evidence indicates that a decrease in grooming behaviour in UCMS animals is associated with reduced motivation and a decrease in self stimulatory behaviour, or reduced sensitivity to pleasure, and is representative of anhedonia, a core symptom of depression [29]. Taken together, the findings in the OFT further support the idea that OS-B possesses the antidepressant activity in the UCMS animals like imipramine.

We also investigated whether monoaminergic systems are involved in the antidepressant-like effects of OS-B in UCMS animals because monoaminergic systems in the brain play an essential role in the clinical effects of various typical and atypical antidepressant drugs including imipramine. For this aim, we employed AMPT and PCPA, inhibitors for catecholamine and serotonin biosynthesis, respectively, because lines of evidence have demonstrated that depletion of monoamines by AMPT and PCPA antagonize the effects of antidepressant drugs on behavioural despairs in rodents [34-37]. The present results showed that the ameliorative effects of 100 mg/kg/day OS-B and 8 mg/kg/day imipramine on behavioural despairs detected in TST were completely abolished in the UCMS group treated with AMPT and PCPA. These findings strongly suggest that OS-B exerts the antidepressant-like action in UCMS animals in a manner depending on the levels of endogenous monoamines in the brain as imipramine does. This idea seems to explain the mechanism underlying the aforementioned ameliorative action of OS-B on anhedonia observed in UCMS animals. In addition, according to Richard, et al. (2016) [38], the anti-stress activity of *Ocimum sanctum* standardized extract (ociglycoside-I (>0.1% w/w), rosmarinic acid (>0.2% w/w), and triterpene acids such as oleanolic acid and ursolic acid (>2.5% w/w)) in chronic variable stress rats was contributed by inhibiting blood cortisol release, blocking corticotropin-releasing hormone receptor type 1, and obstructing activities of 11β-hydroxysteroid dehydrogenase type 1 and catechol-O-methyltransferase *in vitro*. Therefore, it cannot be denied that OS-B actions could be not only dependent on the monoaminergic system but also due to the other mechanisms.

It is still unclear whether the monoamine-dependent antidepressant-like effects of OS-B in the UCMS model of depression share the same mechanism as imipramine and other antidepressant drugs targeting monoaminergic systems and monoamine transporters in particular. In our preliminary study using an OBX model [19], we found that some major constituents of OS such apigenin, luteolin, and apigenin-7-*O*-β-D-glucuronide played a role in the antidepressant effects of OS-B. Moreover, several previous studies reported that apigenin possesses pharmacological and neurochemical effects associated with improvement of depression, such as increasing noradrenalin activity, inhibiting monoamine oxidase activities, and stimulating an L-tyrosine uptake [39]. In addition, luteolin reportedly exerts antidepressant-like effects via inhibiting and downregulating plasma membrane monoamine transporter [40]. Thus, it is likely that these chemical constituents of OS-B are involved in the monoamine-dependent antidepressant-like effects of OS-B. Nevertheless, further investigations are required to clarify the exact molecular mechanism(s) and chemical constituent(s) that account for OS-B's preventive and ameliorative effects on depression-related symptoms caused in the USCM animals.

Conclusions

In summary, the results of this study suggest that OS-B could prevent/ameliorate UCMS-induced depression-like symptoms and its actions are involved neurotransmitter systems included in the noradrenergic, dopaminergic and serotonergic systems.

ACKNOWLEDGEMENTS

This research is funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106-YS.05-2016.22.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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