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Original research article

Behavioral and biochemical evidences for antidepressant-like activity of palmatine in mice subjected to chronic unpredictable mild stress

Dinesh Dhingra *, Arun Bhankher

Department of Pharmaceutical Sciences, Guru Jambheshwar University of Science and Technology, Hisar, Haryana, India

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ABSTRACT

Background: In the present study, antidepressant-like activity of palmatine was evaluated in unstressed and stressed young male Swiss albino mice.
Methods: The animals were subjected to unpredictable mild stress daily for 21 successive days to induce depression-like behavior. Palmatine (0.25, 0.5, 1 mg/kg, *ip*) was administered for 21 successive days to unstressed and stressed mice. The antidepressant-like activity was evaluated using the tail suspension test, forced swim test and sucrose preference test.
Results: Palmatine (0.5 and 1 mg/kg, *ip*) significantly decreased immobility periods of unstressed and stressed mice in the forced swim test and tail suspension test, thus indicating its significant antidepressant-like activity. Only the highest dose (1 mg/kg) of palmatine significantly reversed the stress-induced decrease in sucrose preference. There was no significant effect on locomotor activity of the mice by palmatine and fluoxetine. The antidepressant-like activity of palmatine was found to be comparable to fluoxetine (10 mg/kg) administered for successive 21 days. Palmatine (0.5 and 1 mg/kg, *ip*) significantly reversed the stress-induced increase in brain catalase levels, MAO-A activity, lipid peroxidation, plasma nitrite and corticosterone levels.
Conclusions: Palmatine showed significant antidepressant-like activity in unstressed and stressed mice probably through inhibition of MAO-A activity, decrease in plasma nitrite levels and due to its antioxidant activity. In addition, palmatine also showed antidepressant-like activity in stressed mice probably through decrease in plasma corticosterone levels.

Introduction

Depression is one of the major mental disorders and involves a triad of symptoms with low or depressed mood, anhedonia and low energy or fatigue [5]. The primary causes of depression include depletion of monoamines, like serotonin, noradrenaline and dopamine by monoamine oxidase overactivation, oxidative stress and hyperactivity of HPA-axis. There was a strong relationship between MAO activity and the HPA axis function in depressed patients [28]. Stress has been observed to play an important role in the etiology of psychiatric disorders [9]. Stressful experiences have been reported to favor the development of depression in humans [18]. Animal stress models are widely used in pre-clinical evaluation of antidepressants [10]. In rats and mice, application of chronic unpredictable mild stress procedures resulted in a variety of behavioral, neurochemical, neuroendocrine and neuroimmune alterations, resembling some of the dysfunctions observed in human depression [35]. In rodents, CUMS elicits depression-like symptoms such as a lack of sucrose preference interpreted as anhedonia, a core symptom of major depression [37]. Animals exposed to CUMS show signs of increased activity of the HPA axis leading to hypersecretion of corticosterone [1]. St. John's wort, a clinically employed herbal antidepressant; and fluoxetine (a selective serotonin reuptake inhibitor) attenuated stress-induced increase in corticosterone [12]. Nitric oxide, an important neurotransmitter in the nervous system, regulates many behavioral, cognitive, and emotional processes, including depression [13]. Nitric oxide production is increased in depression [34]. Oxidative damage due to CUMS has been reported in rats [3]. Fluoxetine attenuated stress-induced increase in oxidative parameters [39]. There is a correlation of depressive disorders in humans with oxidative stress either in the brain or blood [4].

Palmatine is a quaternary protoberberine alkaloid. It is typically yellow in color and is an active constituent of a number of plants, such as *Coptidis rhizoma* [17]. Palmatine has been reported to possess sedative [15] and antioxidant [17] activities. It is also

Abbreviations: CUMS, chronic unpredictable mild stress; FST, forced swim test; HPA, hypothalamic–pituitary–adrenal axis; MAO, monoamine oxidase; TST, tail suspension test.

* Corresponding author.
E-mail addresses: din_dhingra@rediffmail.com, din_dhingra@yahoo.com (D. Dhingra).

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reported to be an inhibitor of acetylcholinesterase and butyrylcholinesterases; beta site APP cleaving enzyme 1 [17] and monoamine oxidase [21]. Since MAO inhibitors are classical antidepressant drugs, so palmatine has potential in the management of depression. Antidepressant-like activity of palmatine has not been reported in the literature. Therefore, the present study was designed to explore the antidepressant-like effect of palmatine in mice subjected to chronic unpredictable mild stress.

Materials and methods

Experimental animals

Male Swiss albino mice (3 months old, weighing around 20–25 g) were purchased from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar (Haryana, India). Since estrogens (female sex hormones) have been found to have antidepressant effect, so we excluded female mice and used only male mice for the study [23]. Animals were housed separately in groups of 10 per cage (polycarbonate cage size: 29 cm × 22 cm × 14 cm) under laboratory conditions with alternating light and dark cycle of 12 h each. The animals had free access to food and water. The animals were kept fasted 2 h before and 2 h after drug administration. The animals were acclimatized for at least five days before behavioral experiments which were carried out between 09:00 and 17:00 h. The experimental protocol was approved by Institutional Animal Ethics Committee and animal care was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forests, Government of India (Registration no. 0436).

Drugs and chemicals

Palmatine, fluoxetine hydrochloride (Sigma Aldrich, USA), sulfanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride, and meta-phosphoric acid (Hi-Media Laboratories Pvt., Ltd., Mumbai, India) were used in the present study. Fluoxetine hydrochloride was dissolved in normal saline (0.9% (w/v) sodium chloride).

Selection of doses

Doses of palmatine and fluoxetine hydrochloride (10 mg/kg) were selected on the basis of literature [15,20]. The volume of vehicle or drug solution administration was 10 ml/kg.

Chronic unpredictable mild stress procedure

The mice were subjected to chronic stress as described by Mao et al. [30] and Kumar et al. [20]. Animals were subjected to stress paradigm once a day over a period of 3 weeks between 09:00 and 14:00 h. The order of stressors used was as follows:

Weeks	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 1	I	E	F	O	T2	X	T1
Week 2	I	O	X	E	T2	C	F
Week 3	O	F	T1	S	C	I	F

I—Immobilization for 2 hours; E—Exposure to empty water bottles for 1 hour; F—Exposure to foreign object for 24 hours (e.g. piece of plastic); O—overnight illumination; T2—tail pinch (60 s); X—Tilted cage at 45 degree for 7 hours; T1—tail pinch (30 s).

Mice subjected to CUMS procedure were called as stressed mice. Unstressed mice were exposed to behavioral tests, and not subjected to CUMS procedure. Drugs were administered 30 min before CUMS procedure in case of stressed group. Behavioral testing was done in independent groups of mice on the 22nd day.

Laboratory models employed for evaluation of antidepressant-like activity

Forced swim test

This test was carried out on mice according to the method of Porsolt et al. [29] and as followed earlier in our laboratory [8]. Briefly, mice were individually forced to swim in an open glass chamber (25 cm × 15 cm × 25 cm) containing fresh water to a height of 15 cm and maintained at 26 ± 1 °C. Water in the chamber was changed after subjecting each animal to FST. Mice placed in the chamber for the first time were initially highly active, vigorously swimming in circles, trying to climb the wall or diving to the bottom. After 2 min, activity began to subside and to be interspersed with phases of immobility or floating of increasing length. The duration of immobility was manually recorded during the next 4 min of the total 6 min testing period. Mice were considered to be immobile when they ceased struggling and remained floating in water, making only those movements necessary to keep their head above water. Following the swimming session, mice were towel dried and returned to their housing conditions.

Tail suspension test

It is a commonly employed behavioral model for screening antidepressant-like activity in mice [33]. For the test, the mouse was individually suspended on the edge of a table, 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Each animal under test was both acoustically and visually isolated from other animals during test. The total period of immobility was recorded manually for 6 min. Animal was considered to be immobile when it did not show any body movement, hung passively and completely motionless. The test was conducted in a quiet room to avoid disturbances to animals.

Sucrose preference test

The sucrose preference test [37] was employed herein to determine anhedonia, one of the core symptoms of major depression in human. The procedure was composed of training and testing courses. After 1 week of acclimatization, mice were trained to consume 1% (w/v) sucrose solution before the start of the CUMS protocol. In training course, mice were deprived of food and water for 24 h and only exposed to 1% (w/v) sucrose solution. Three days later, after 23-h food and water deprivation, 1-h baseline test was performed, in which mice were housed in individual cages and were free to access two pre-weighted bottles, one with 1% (w/v) sucrose solution and the other with tap water. Then, the sucrose preference was calculated according to the following formula:

$$\text{Sucrose preference} = \frac{\text{sucrose solution intake (g)}}{\text{sucrose solution intake (g)} + \text{water intake (g)}} \times 100$$

The test was again performed on the 21st day to evaluate the effect of stress as well as drug treatment.

Measurement of locomotor activity

To rule out the effects of various drug treatments on locomotor activity, horizontal locomotor activities of control and test animals were recorded for a period of 5 min using photoactometer (INCO, Ambala, India). The locomotor activity was assessed 2 h after drug administration on day 22.

Biochemical estimations

After subjecting unstressed and stressed mice to FST on the 22nd day and 1 h after drug administration on the 23rd day, blood (0.5–0.8 ml) was withdrawn from retro-orbital plexus of mice. Plasma was separated using refrigerated centrifuge (Remi, Mumbai, India) at 2500 rpm for 10 min to separate the plasma. The plasma was used for estimation of nitrite and corticosterone levels. After collecting blood sample, mice were sacrificed by decapitation, and their brains were isolated. The collected brain samples were washed with cold 0.25 M sucrose–0.1 M Tris–0.02 M EDTA buffer (pH 7.4) and weighed. The buffer-washed brain sample was homogenized in 9 volumes of cold 0.25 M sucrose–0.1 M Tris–0.02 M EDTA buffer (pH 7.4) buffer and centrifuged twice at 2500 rpm for 10 min at 4 °C in a cooling centrifuge (Remi Instruments, Mumbai, India). The pellet was discarded. The supernatant was then centrifuged at 12,000 rpm for 20 min at 4 °C in a cooling centrifuge. This centrifuged supernatant was separated into two parts – Part I: the precipitates (mitochondrial fraction) were used for estimation of MAO-A activity. Part II: the remaining supernatant was used to assay lipid peroxidation, reduced glutathione and catalase levels.

Estimation of plasma nitrite levels

Plasma nitrite was measured by using the method of Green et al. [11]. A mixture of 1% (w/v) sulfanilamide in 5% aqueous solution of m-phosphoric acid (1 part) and 0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride (1 part) was prepared and kept at 0 °C for 60 min. 0.5 ml plasma was mixed with 0.5 ml of the above mixture and kept in dark for 10 min at room temperature. The absorbance was read at 546 nm using UV–visible spectrophotometer 2203 (Systronics, Ahmedabad).

Estimation of plasma corticosterone levels

The quantitative estimation of corticosterone level in the blood plasma was performed by the method of Bartos and Pesez [2]. To 1.0 ml of sample in ethanol, 0.50 ml of 0.10% solution of p-nitroso-N,N-dimethylaniline in ethanol was added and the tubes were immersed in ice water for 5 min, and then 0.50 ml of 0.10 N sodium hydroxide was added. The tubes were plugged with cotton-wool, and were let to stand at 0 °C for 5 h, protected against light. To the above solution, 2.0 ml of buffer pH 9.8, 5.0 ml of 0.10% solution of phenol in ethanol and 0.50 ml of 1.0% aqueous solution of potassium ferricyanide were added. The tubes were kept in a water bath at 20 ± 2 °C for 10 min. The absorbance was read at 650 nm using UV–visible spectrophotometer 2203 (Systronics, Ahmedabad).

Measurement of brain MAO-A activity

The MAO-A activity was assessed spectrophotometrically [6,26,31]. The mitochondrial fraction of brain was washed twice with about 100 ml of sucrose–Tris–EDTA buffer and suspended in 9 volumes of cold sodium phosphate buffer (10 mM, pH 7.4, containing 320 mM sucrose) and mingled well at 4 °C for 20 min. The mixture was then centrifuged at 15,000 rpm for 30 min at 0 °C and the pellets were re-suspended in cold sodium phosphate buffer. Then, 2.75 ml of sodium phosphate buffer (100 mM, pH 7.4) and 100 μl of 4 mM 5-hydroxytryptamine were mixed in a quartz cuvette which was placed in UV–visible spectrophotometer 2203 (Systronics, Ahmedabad). This was followed by the addition of 150 μl of the solution of mitochondrial fraction to initiate the enzymatic reaction and the change in absorbance was recorded at wavelength of 280 nm for 5 min against the blank containing sodium phosphate buffer and 5-HT.

Estimation of brain protein concentration

Total protein concentration was estimated in brain homogenate by using a total protein kit (Erba Chem, Mumbai, India), using semi-autoanalyzer (Model C-500, Logitech).

Estimation of brain lipid peroxidation

The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of Wills [38]. Briefly, 0.5 ml of postmitochondrial supernatant and 0.5 ml of Tris–HCl were incubated at 37 °C for 2 h. After incubation, 1 ml of 10% trichloroacetic acid was added and centrifuged at 1000 rpm for 10 min. To 1 ml of supernatant, 1 ml of 0.67% thiobarbituric acid was added, and the tubes were kept in boiling water for 10 min. After cooling, 1 ml of redistilled water was added, and absorbance was measured at 532 nm. Thiobarbituric acid-reactive substances were quantified using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as nanomole of malondialdehyde per milligram protein. Tissue protein was estimated using the biuret method, and the brain malondialdehyde content was expressed as nanomole of malondialdehyde per milligram of protein.

Estimation of brain reduced glutathione

Reduced glutathione (GSH) was assayed by the method of Jollow et al. [16]. Briefly, 1.0 ml of postmitochondrial supernatant (10%) was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4 °C for at least 1 h and then subjected to centrifugation at 1200 rpm for 15 min at 4 °C. The assay mixture contained 0.1 ml of supernatant, 2.7 ml of phosphate buffer (0.1 M, pH 7.4), and 0.2 ml of 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, 0.1 mM, pH 8.0) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm, and GSH levels were calculated using molar extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$) and expressed as micromole per milligram protein.

Estimation of brain catalase activity

Catalase activity was assayed by the method of Claiborne [7]. Briefly, the assay mixture consisted of 1.95 ml of phosphate buffer (0.05 M, pH 7.0), 1.0 ml of hydrogen peroxide (0.019 M), and 0.05 ml of postmitochondrial supernatant (10%) in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was quantified using the millimolar extinction coefficient of H_2O_2 (0.07 mM) and expressed as micromoles of H_2O_2 decomposed per minute per milligram protein.

Experimental protocol

The animals were divided in the following groups having 6–10 mice in each group.

Groups for locomotor activity and forced swim test (FST)

Group 1: control group (unstressed mice): 10% (v/v) Tween80 in normal saline (0.9%, w/v) was administered *ip* daily for 21 successive days to unstressed mice. Locomotor activity was measured by using photoactometer on the 22nd day. After 2 h of locomotor activity testing, FST was performed.

Groups 2–5: palmatine (0.25, 0.5, 1 mg/kg, *ip*) and fluoxetine (10 mg/kg, *ip*) respectively were administered daily for 21 successive days to unstressed mice. Locomotor activity was measured by using photoactometer on the 22nd day. After 2 h of locomotor activity testing, FST was performed.

Group 6: control group (stressed mice): 10% (v/v) Tween80 in normal saline (0.9%, w/v) *ip* was administered daily, 30 min before induction of stress to mice for 21 successive days. Locomotor activity was measured by using photoactometer on the 22nd day. After 2 h of locomotor activity testing, FST was performed.

Groups 7–10: palmatine (0.25, 0.5, 1 mg/kg, *ip*) and fluoxetine (10 mg/kg, *ip*) respectively were administered daily, 30 min before induction of stress to mice for 21 successive days. Locomotor activity was measured by using photoactometer on the 22nd day. After 2 h of locomotor activity testing, FST was performed.

Groups for tail suspension test (TST)

Groups 11–20: separate groups of mice were treated similarly as mentioned under groups 1–10 and immobility periods were recorded using TST on the 22nd day.

Groups for sucrose preference test

Groups 21–30: separate groups of mice were treated similarly as mentioned under groups 1–10 and sucrose preference test was performed on the 21st day.

Statistical analysis

All the results are expressed as the mean ± SEM. Data were analyzed by analysis of variance (ANOVA) followed by Tukey's *post hoc* test in Graph Pad Instat (GPIS) package, version 3.05. $p < 0.05$ was considered as significant.

Results

Effect of palmatine and fluoxetine on immobility periods of mice in TST

Chronic unpredictable mild stress significantly increased the immobility period as compared to vehicle-treated unstressed mice. Fluoxetine (10 mg/kg, *ip*) administered for 21 successive days significantly ($p < 0.001$) decreased the immobility period in unstressed and stressed mice as compared to the respective controls. Palmatine (0.25 mg/kg, *ip*) administered for 21 successive days to unstressed mice and stressed mice did not show any significant effect on immobility period. The higher doses (0.5 and 1 mg/kg, *ip*) of palmatine administered for 21 successive days significantly decreased ($p < 0.01$ and $p < 0.001$ respectively) the immobility period in stressed mice as compared to its vehicle-treated control, but only the highest dose (1 mg/kg) of palmatine significantly decreased ($p < 0.001$) the immobility period in unstressed mice as compared to its vehicle treated control (Fig. 1).

Effect of palmatine and fluoxetine on immobility periods of mice in FST

Chronic unpredictable mild stress significantly increased the immobility period as compared to control mice. Fluoxetine (10 mg/kg, *ip*) administered for 21 successive days significantly ($p < 0.001$) decreased the immobility period in unstressed and stressed mice as compared to the respective controls. Palmatine (0.25 mg/kg, *ip*) administered for 21 successive days to unstressed mice and stressed mice did not show any significant effect on immobility period. The higher doses (0.5 and 1 mg/kg, *ip*) of palmatine administered for 21 successive days significantly decreased ($p < 0.05$ and $p < 0.01$ respectively) the immobility period in stressed mice as compared to its vehicle treated control, but only the highest dose (1 mg/kg) of palmatine significantly decreased ($p < 0.001$) the immobility period in unstressed mice as compared to its vehicle treated control (Fig. 2).

Effect of palmatine and fluoxetine on sucrose preference

There was no significant difference in sucrose preference (%) among all the groups in the baseline test. Palmatine (0.25, 0.5 and 1 mg/kg) and fluoxetine (10 mg/kg) administered for 21 successive days did not show any significant change in sucrose preference by unstressed mice. Exposure of the mice to stress for 21 successive days significantly ($p < 0.05$) decreased sucrose preference (%) in vehicle-treated mice as compared to unstressed mice. The lower doses of palmatine (0.25 and 0.5 mg/kg) did not significantly reverse the reduced sucrose preference (%) in stressed mice, but the highest dose (1 mg/kg) of palmatine and fluoxetine significantly ($p < 0.001$) restored the reduced sucrose preference (%) in stressed mice as compared to its vehicle-treated control (Table 1).

Effect of palmatine and fluoxetine on locomotor activity

Various treatments did not significantly affect the spontaneous locomotor activity in unstressed and stressed mice as compared to their respective vehicle-treated controls (Fig. 3).

Effect of palmatine and fluoxetine on plasma nitrite levels

Plasma nitrite levels were significantly ($p < 0.001$) elevated in mice subjected to chronic unpredictable mild stress. The lowest dose of palmatine (0.25 mg/kg) administered for 21 successive days did not show any significant effect on plasma nitrite levels of unstressed and stressed mice. Palmatine (0.5 and 1 mg/kg) and fluoxetine (10 mg/kg) *per se* administered for 21 successive days significantly ($p < 0.01$, $p < 0.001$ and $p < 0.001$ respectively) decreased plasma nitrite levels in stressed mice as compared to vehicle-treated control for stressed mice. But only the highest dose (1 mg/kg) of palmatine significantly ($p < 0.05$) decreased plasma

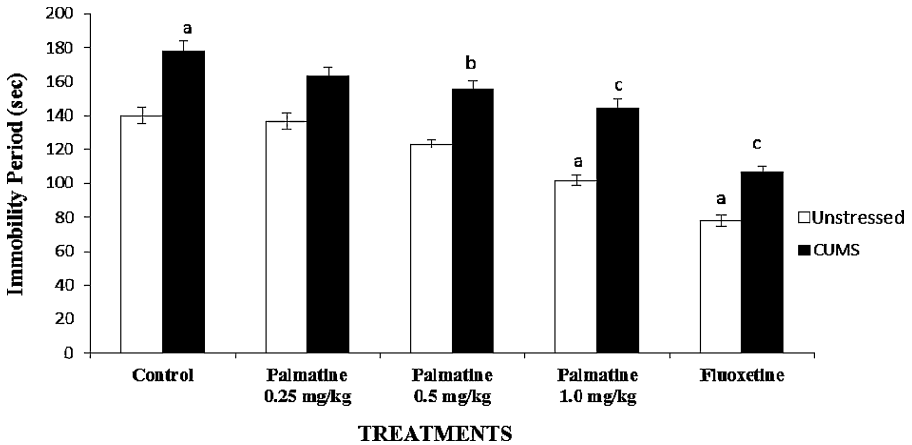

Fig. 1. Effect of palmatine and fluoxetine on immobility periods of mice in TST. $n = 10$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. CUMS – chronic unpredictable mild stress. $F(9, 90) = 41.868$; $p < 0.0001$. $^{a}p < 0.001$ as compared to vehicle-treated unstressed mice. $^{b,c}p < 0.01$ and $p < 0.001$ as compared to vehicle-treated stressed mice.

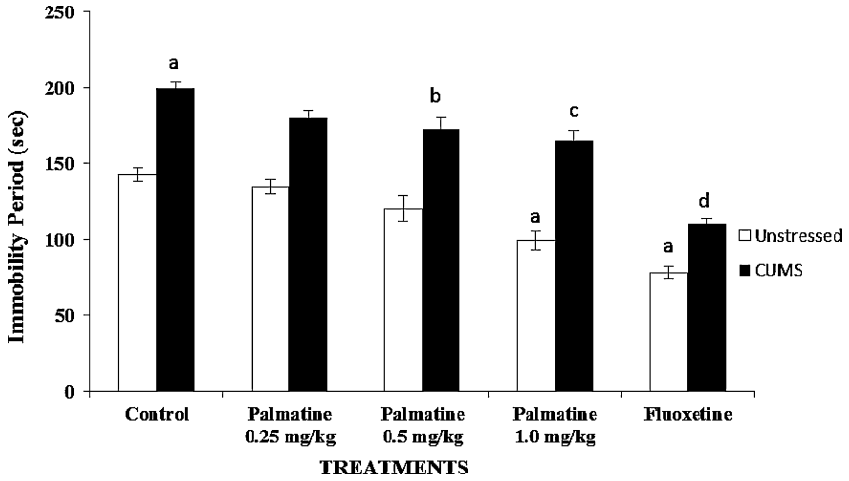

Fig. 2. Effect of palmatine and fluoxetine on immobility periods of mice in FST. $n = 9$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. CUMS – chronic unpredictable mild stress. $F(9, 81) = 52.823$; $p < 0.0001$. [a]$p < 0.001$ as compared to vehicle-treated unstressed mice. [b,c,d]$p < 0.05$, $p < 0.01$ and $p < 0.001$ as compared to vehicle-treated stressed mice.

Table 1
Effect of palmatine and fluoxetine on sucrose preference (%).

S. no.	Treatment for 21 days	Dose (mg/kg)	Sucrose preference (%) Baseline values	Sucrose preference (%) After 21 days
1	Vehicle (10% Tween80)	–	37.20 ± 3.95	28.85 ± 2.92
2	Palmatine (U)	0.25	38.21 ± 5.27	17.16 ± 2.99
3	Palmatine (U)	0.5	36.85 ± 2.91	42.71 ± 3.88
4	Palmatine (U)	1.0	38.24 ± 2.85	48.85 ± 1.94
5	Fluoxetine (U)	10	42.27 ± 3.69	51.88 ± 9.73
6	Vehicle (10% Tween80) + UCMS	–	37.83 ± 4.55	5.20 ± 1.44[a]
7	Palmatine (CUMS)	0.25	38.58 ± 5.44	20.66 ± 5.27
8	Palmatine (CUMS)	0.5	35.26 ± 3.23	26.27 ± 3.63
9	Palmatine (CUMS)	1.0	32.31 ± 4.42	46.61 ± 2.81[b]
10	Fluoxetine (CUMS)	10	44.08 ± 4.81	37.08 ± 5.49[b]

U, unstressed mice; CUMS, chronic unpredictable mild stress; $n = 10$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $F(9, 90) = 11.216$; $p < 0.0001$.
[a] $p < 0.05$ as compared to vehicle-treated unstressed mice.
[b] $p < 0.001$ as compared to vehicle-treated stressed mice.

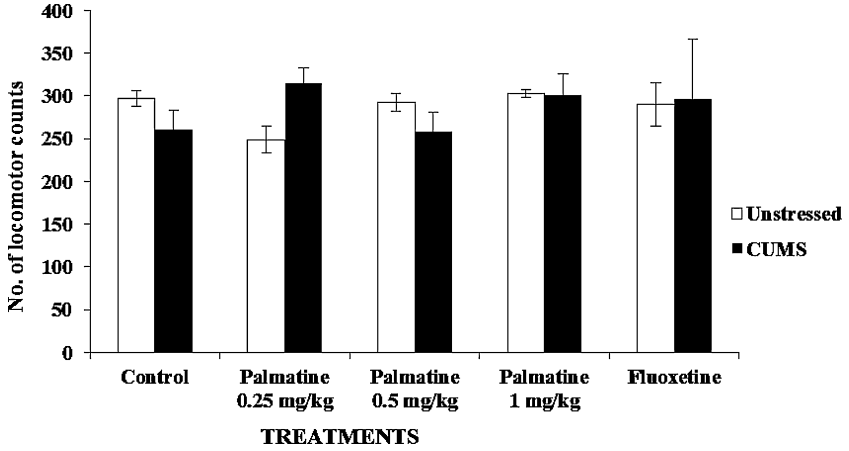

Fig. 3. Effect of palmatine and fluoxetine on locomotor activity of mice. $n = 6$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. CUMS – chronic unpredictable mild stress. $F(9, 64) = 0.7908$; $p > 0.05$.

nitrite levels in unstressed mice as compared to its vehicle-treated control (Fig. 4).

Effect of palmatine and fluoxetine on plasma corticosterone levels

There was no significant effect of palmatine and fluoxetine on plasma corticosterone levels in unstressed mice. Chronic unpredictable mild stress significantly ($p < 0.001$) increased plasma corticosterone content in stressed mice as compared to vehicle-treated unstressed mice. Fluoxetine, palmatine (0.5 and 1 mg/kg) *per se* administered for 21 successive days significantly ($p < 0.001$, $p < 0.01$ and $p < 0.001$ respectively) lowered the corticosterone contents in stressed mice as compared to vehicle-treated stressed mice, but the lowest dose of palmatine (0.25 mg/kg) administered

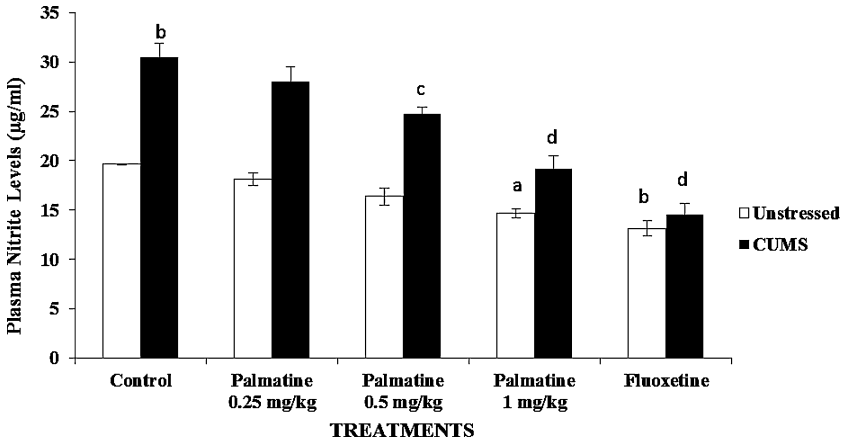

Fig. 4. Effect of palmatine and fluoxetine on plasma nitrite levels. $n = 9$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. CUMS – chronic unpredictable mild stress. $F(9, 80) = 37.638$; $p < 0.0001$. $^{a,b}p < 0.05$ and $p < 0.001$ respectively as compared to vehicle-treated unstressed mice. $^{c,d}p < 0.01$ and $p < 0.001$ respectively as compared to vehicle-treated stressed mice.

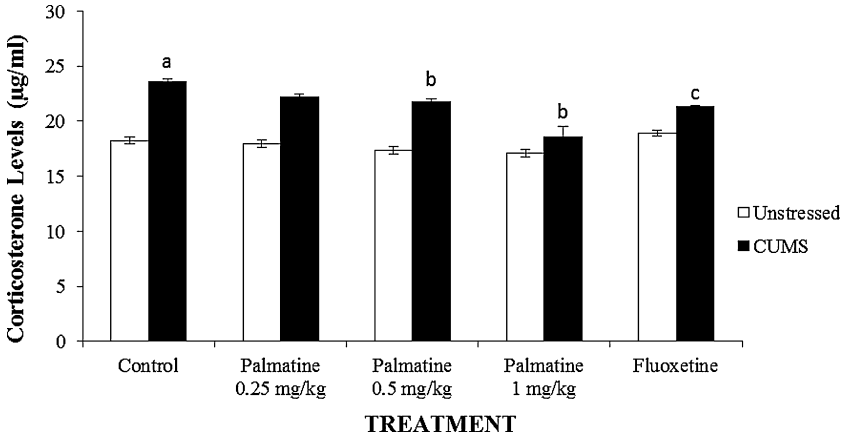

Fig. 5. Effect of palmatine and fluoxetine on corticosterone levels. $n = 10$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. CUMS – chronic unpredictable mild stress. $F(9, 90) = 48.668$; $p < 0.0001$. $^{a}p < 0.001$ as compared to vehicle-treated unstressed mice. $^{b,c}p < 0.01$ and $p < 0.001$ as compared to vehicle-treated stressed mice.

for 21 successive days did not significantly decrease plasma corticosterone levels in stressed mice (Fig. 5).

Effect of palmatine and fluoxetine on brain MAO-A activity

Chronic unpredictable mild stress resulted in significant ($p < 0.001$) increase in brain MAO-A activity as compared to vehicle-treated unstressed mice. Palmatine (0.5 and 1 mg/kg) and fluoxetine (10 mg/kg) *per se* administered for 21 successive days significantly reduced MAO-A activity in unstressed ($p < 0.05$, $p < 0.001$ and $p < 0.001$ respectively) and stressed ($p < 0.01$, $p < 0.001$ and $p < 0.001$ respectively) mice as compared to respective vehicle-treated controls. However, the lowest dose (0.25 mg/kg) of palmatine did not significantly decrease MAO-A activity in unstressed mice, but significantly ($p < 0.05$) decreased MAO-A activity in stressed mice as compared to their respective control groups (Fig. 6).

Effect of palmatine and fluoxetine on brain malondialdehyde levels

Malondialdehyde levels were increased significantly ($p < 0.001$) in the brains of chronically stressed mice as compared to control mice. Chronic treatment with palmatine (0.5 and 1 mg/kg) and fluoxetine (10 mg/kg) *per se* produced a significant ($p < 0.001$) reduction in malondialdehyde levels in the brain of stressed mice as compared to the respective controls, but fluoxetine and palmatine *per se* did not significantly decrease malondialdehyde levels in unstressed mice as compared to its control group (Fig. 7).

Effect of palmatine and fluoxetine on brain reduced glutathione levels

GSH levels were significantly ($p < 0.001$) decreased in brains of stressed mice as compared to respective vehicle-treated control. Palmatine (0.25, 0.5 and 1 mg/kg) and fluoxetine (10 mg/kg) *per se* produced a significant ($p < 0.01$, $p < 0.001$, $p < 0.001$ and $p < 0.001$ respectively) increase in GSH levels in unstressed mice as compared to its control. However, palmatine and fluoxetine did not significantly increase GSH levels in stressed mice as compared to its control (Fig. 8).

Effect of palmatine and fluoxetine on brain catalase levels

Catalase levels were significantly ($p < 0.001$) increased in brains of stressed mice as compared to respective vehicle-treated control. Palmatine (0.25, 0.5 and 1 mg/kg) and fluoxetine (10 mg/kg) *per se* produced a significant ($p < 0.001$, $p < 0.01$, $p < 0.001$, $p < 0.001$ respectively) decrease in the catalase levels in stressed mice as compared to respective controls, but only the highest dose (1 mg/kg) of palmatine and fluoxetine *per se* significantly ($p < 0.001$) decreased the catalase levels in unstressed mice as compared to its control (Fig. 9).

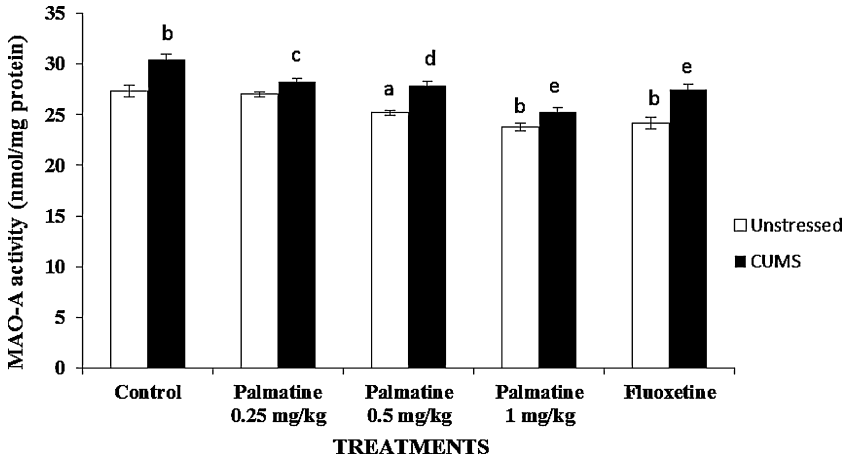

Fig. 6. Effect of palmatine and fluoxetine on brain MAO-A activity. $n = 10$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. CUMS – chronic unpredictable mild stress. $F(9, 90) = 22.120$; $p < 0.0001$. $^{a,b}p < 0.05$ and $p < 0.001$ respectively as compared to vehicle-treated unstressed mice. $^{c,d,e}p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively as compared to vehicle-treated stressed mice.

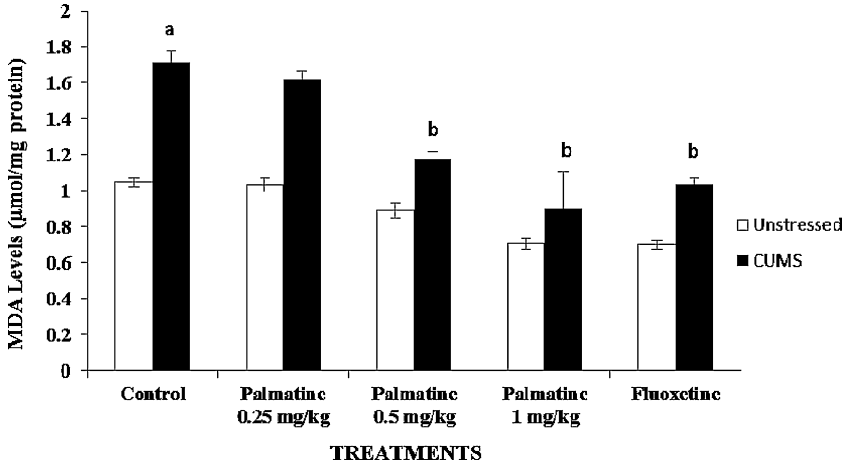

Fig. 7. Effect of palmatine and fluoxetine on brain malondialdehyde (MDA) levels. $n = 10$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. CUMS – chronic unpredictable mild stress. $F(9, 90) = 20.318$; $p < 0.0001$. $^{a}p < 0.001$ as compared to vehicle-treated unstressed mice. $^{b}p < 0.001$ as compared to vehicle-treated stressed mice.

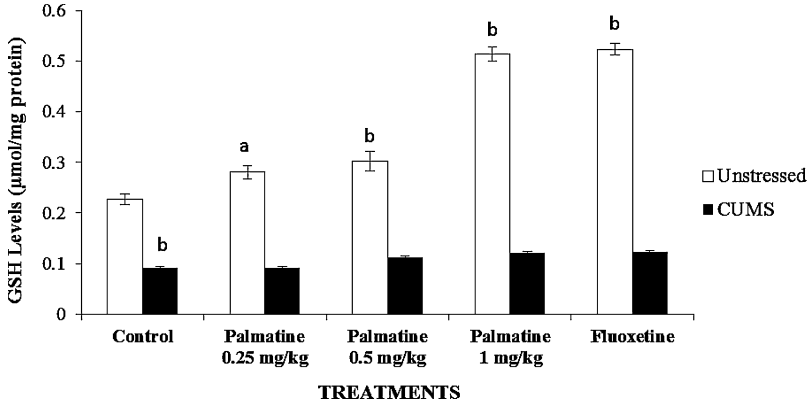

Fig. 8. Effect of palmatine and fluoxetine on brain reduced glutathione levels (GSH) in unstressed and stressed mice. $n = 10$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. CUMS – chronic unpredictable mild stress. $F(9, 90) = 275.23$; $p < 0.0001$. $^{a,b}p < 0.01$ and $p < 0.001$ respectively as compared to vehicle-treated unstressed mice.

Discussion

In the present study, palmatine administered for 21 successive days showed a significant antidepressant-like activity in unstressed and chronic unpredictable mild stress-exposed mice. This is the first study showing antidepressant-like activity of palmatine. CUMS-induced depression model can be used for evaluating the potential antidepressants by employing behavioral tests like FST, TST and sucrose preference test [20,22,32]. FST and TST are commonly used behavioral despair models in rodents to predict

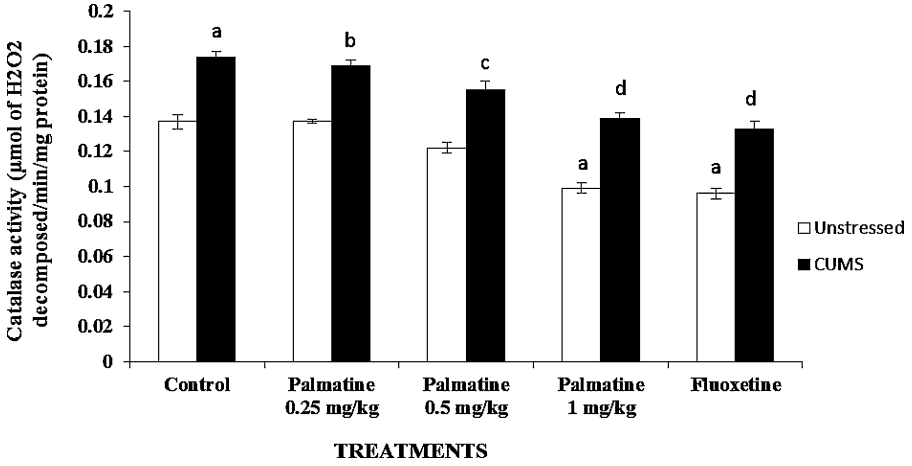

Fig. 9. Effect of palmatine and fluoxetine on brain catalase levels in unstressed and stressed mice. $n = 10$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. CUMS – chronic unpredictable mild stress. $F(9, 90) = 167.58$; $p < 0.0001$. $^{a}p < 0.001$ as compared to vehicle-treated unstressed mice. $^{b,c,d}p < 0.001$, $p < 0.01$ and $p < 0.001$ as compared to vehicle-treated stressed mice.

antidepressant potential by measuring the decrease in immobility periods [29,33]. Induction of depression using chronic unpredictable mild stress is considered as the most valid animal model of depressive behavior observed in humans after a long-term exposure to multiple stressors [36]. In the present study, mice that were exposed to chronic stress, exhibited greater immobility periods in FST and TST as compared to control animals, thus showed depression-like behavior. Chronic treatment with fluoxetine (10 mg/kg, *ip*) or palmatine (1 mg/kg, *ip*) produced a significant decrease in immobility periods of unstressed and stressed mice in FST and TST, indicating significant antidepressant-like activity. Since palmatine or fluoxetine did not affect the locomotor activity of the unstressed and stressed mice as compared to their respective controls, so this indicated that palmatine did not show CNS stimulant activity. Thus, antidepressant-like activity of palmatine in unstressed and stressed mice is specific and not false positive. Moreover, we employed another model, sucrose preference test for evaluation of antidepressant-like activity of palmatine in stressed mice. This test is an indicator of anhedonia-like behavioral change, indicating loss of interest or pleasure. Anhedonia, a main symptom of human major depression, was modeled by inducing a decrease in responsiveness to rewards reflected by a reduced consumption and/or preference of sweetened solutions [35,36]. In the present study, stressed mice showed a decrease in sucrose preference compared with unstressed mice. Sucrose preference was significantly restored in stressed mice by the chronic administration of fluoxetine (10 mg/kg) or palmatine (1 mg/kg, *ip*) which suggested their antidepressant-like actions. Thus, the results obtained from behavioral studies indicated that palmatine significantly produced antidepressant-like action in mice exposed to CUMS.

It is well known that HPA axis is activated in response to stress, with a resultant increase in circulating glucocorticoids, such as corticosterone in rodents or cortisol in primates. Sustained activation of HPA axis is associated with an abnormally high blood glucocorticoid levels, which may eventually lead to depression [27]. It has been reported that antidepressants produce their therapeutic effect through the reduction in hyperactivity of HPA axis in depressed patients [14]. Thus, the restoration of a normal functional status of HPA axis may be critically involved in the treatment of clinical depression. Our finding of CUMS-induced hyperactivity of HPA axis, as indicated by an increased serum corticosterone level, is supported by observations from other studies [25]. The antidepressant-like activity of palmatine in mice subjected to CUMS was also supported by a significant reduction of plasma corticosterone levels by it in stressed mice. There was no significant effect on plasma corticosterone levels in unstressed mice, indicating that hyperactivity of HPA axis is observed only in stressful conditions.

Reactive oxygen species play a role in the pathogenesis of neuropsychiatric disorders [4]. Excessive reactive oxygen species production can cause oxidative damage to macromolecules including lipids, proteins and DNA, leading to neuronal dysfunction and depression [24]. Lipid peroxidation and antioxidant enzymes may be markers of major depression because they returned to normal levels after treatment with antidepressants [4]. Chronic unpredictable mild stress was found to impair the antioxidant status of brain tissue, presumably through production of excessive reactive oxygen species [20]. In the present study, 21 days of exposure to different stressors resulted in increased lipid peroxidation and nitrite levels and decreased endogenous antioxidant activity in mice. Chronic administration of palmatine showed a significant decrease in lipid peroxidation in stressed mice, and increase in GSH in unstressed mice and decrease in catalase in brains of both unstressed and stressed mice. Thus, palmatine showed a significant antioxidant activity in mice. This is also supported by an earlier study [17]. Stressful situations in rats have also been reported to significantly increase plasma nitrite levels [22]. Palmatine (1 mg/kg) significantly reduced nitrosative stress as indicated by reduction of the plasma nitrite levels of unstressed and stressed mice as compared to their respective control groups. Thus, palmatine showed a strong protective effect against oxidative stress that plays a key role in chronic unpredictable mild stress-induced depression.

Monoamine oxidase is an enzyme which causes metabolic degradation of catecholamines, serotonin and other endogenous amines in the central nervous system. In the case of depression, the levels of MAO in the brain were increased, which in turn reduced the levels of monoamines. It exists in two similar molecular forms – A and B. MAO-A has substrate preference for serotonin and is the main target for the antidepressant monoamine oxidase inhibitors. MAO-B has substrate preference for phenylethyl amine. Both enzymes act on nor-adrenaline and dopamine. The type B is selectively inhibited by selegiline, which is used in treatment of Parkinsonism. Therefore, we measured only MAO-A in the present study. Experimentally, selective MAO-A inhibitors (clorgyline, moclobemide) are found to be more effective in treating major depression than MAO-B inhibitors like selegiline [19]. Chronic exposure to different stressors led to increased activity of MAO-A. Palmatine (0.5 and 1 mg/kg) administered for 21 successive days

significantly inhibited brain MAO-A activity in both unstressed and stressed mice. This is also supported by earlier study where palmatine showed monoamine oxidase inhibiting activity [21]. Thus, antidepressant-like activity of palmatine in unstressed mice and chronic unpredictable mild stress-exposed mice may also be attributed to its MAO-A inhibitory activity.

In conclusion, palmatine showed significant antidepressant-like activity in unstressed and chronically stressed mice probably through inhibition of MAO-A activity, decrease in plasma nitrite levels and due to its antioxidant activity. In addition, palmatine also showed antidepressant-like activity in chronically stressed mice probably through a decrease in plasma corticosterone levels.

Conflict of interest

No conflict of interest.

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