Punarnavine, an alkaloid isolated from ethanolic extract of *Boerhaavia diffusa* Linn. reverses depression-like behaviour in mice subjected to chronic unpredictable mild stress

Dinesh Dhingra* & Rekha Valecha
Department of Pharmaceutical Sciences,
Guru Jambheshwar University of Science and Technology, Hisar 125 001, India

Received 22 July 2013; revised 13 May 2014

Punarnavine (20 and 40 mg/kg) and fluoxetine (20 mg/kg) per se administered orally for 14 successive days significantly decreased immobility periods of both unstressed and stressed mice in forced swim test. These drugs also significantly decreased sucrose preference in both stressed and unstressed mice as compared to their respective controls, indicating significant antidepressant-like activity. The drugs did not show any significant effect on locomotor activity of mice. The alkaloid also significantly decreased monoamine oxidase (MAO-A) activity, malondialdehyde levels in both unstressed and stressed mice; and significantly reversed the stress-induced decrease in reduced glutathione and catalase activity. It also significantly attenuated the stress-induced increase in plasma nitrite and corticosterone levels. Thus, punarnavine showed antidepressant-like activity in unstressed and stressed mice probably through inhibition of brain MAO-A activity, decrease in plasma nitrite levels and due to its antioxidant activity. In addition, punarnavine also showed antidepressant-like activity in stressed mice possibly through decrease in plasma corticosterone levels.

**Keywords:** *Boerhaavia diffusa*, Depression, Forced swim test, Punarnavine, Sucrose preference test, Chronic unpredictable mild stress

Depression is a significant contributor to the global burden of disease and affects people in all communities across the world. In modern medicine, a large number of antidepressant drugs are available for the treatment of depression. Although these drugs provide improvement in the clinical condition of patient, they possess a variety of adverse effects. This is further complicated by the fact that, approximately 30% of patients may not respond to drug therapy. Therefore, the search for newer drugs for the treatment of major depression is still required.

Chronic unpredictable mild stress is commonly employed animal model for evaluation of antidepressant drugs. In this model, animals are subjected to chronic, continuous low-grade stressors analogous to those associated with depression in humans. With exposure to chronic unpredictable mild stress (CUMS), animals appeared to exhibit behavioural deficits such as anhedonia and behavioural despair. All these behavioural deficits may be assessed by sucrose preference test and forced swim test.

*Boerhaavia diffusa* Linn. (Family: Nyctaginaceae), commonly known as Punarnava is a herbaceous perennial plant. It is widely distributed in the tropics and subtropics, where it was used for centuries as a medicinal plant by indigenous populations and in Ayurvedic or natural herbal medicines. Punarnava roots possess laxative, diuretic and stomachic properties. These are employed for many purposes including treatment of liver, heart, gallbladder, kidney, renal and urinary disorders. Ethanolic extract of the plant has been reported to possess antidiabetic, antioxidant and antistress activities. Punarnavine, isolated from ethanolic extract of *B. diffusa* has antimetastatic and immunomodulatory activity. It also showed protective effect in immobilization stress-induced gastric ulceration in rats.

Antidepressant-like activity of aqueous extract and ethanolic extract of *B. diffusa* and punarnavine has been reported in mice. Although possible involvement of monoaminergic and GABAergic systems for antidepressant-like activity of ethanolic extract of *B. diffusa* and punarnavine in unstressed mice has been suggested, antidepressant-like activity...
of punarnavine in mice subjected to CUMS has not been explored. Therefore, the present study has been aimed to evaluate effect of punarnavine in mice subjected to chronic unpredictable mild stress. The effects of punarnavine on plasma corticosterone and nitrite levels, brain lipid peroxidation, MAO-A, glutathione and catalase activities have also been studied.

Materials and Methods

Collection of plant material—The dried roots of *Boerhaavia diffusa* were purchased from the commercial market, New Delhi and were authenticated as *Boerhaavia diffusa* Linn. from Raw Materials, Herbarium and Museum Division, CSIR-National Institute of Science Communication and Information Resources, New Delhi (Ref. No. NISCAIR/RHMD/Consult/2009-10/1205/09).

Isolation of punarnavine—The alkaloid punarnavine was isolated from *B. diffusa* according to the procedure of Manu and Kuttan. About 5 kg of powdered drug was extracted with ethanol (95%) using Soxhlet apparatus at 70 °C till siphoning solution became colourless. The solvent was recovered by distillation and the extract was dried by using water bath at 50-60 °C. The extract was concentrated to 20% of its volume, and boerhaavic acid was filtered off. The filtrate was concentrated to dryness leaving sticky material. This was extracted with hot water and concentrated to yield potassium nitrate. It was filtered and the filtrate was made ammonical and extracted repeatedly with chloroform. The chloroform extract was evaporated and the residue from evaporation was macerated with diethyl ether. It was then evaporated and gave amorphous Punarnavine. A further amount of Punarnavine was obtained from the sticky material, remaining from the water extraction by extraction with dilute HCl. Amorphous punarnavine was then crystallized from a small volume of ethyl alcohol and was purified again by recrystallization. The overall yield of punarnavine was 0.016%. The melting point of crystallized punarnavine is 235 °C. The thin layer chromatography for crystallized punarnavine was performed using solvent system diethyl amine: cyclohexane (30:70). It showed a single band (R_f = 0.87), which confirmed the presence of single constituent, punarnavine. R_f value was calculated by using the formula:

\[ R_f = \frac{\text{Distance travelled by solute front}}{\text{Distance travelled by solvent front}} \]

Experimental animals—Swiss albino mice of either sex, weighing around 20-25 g were purchased from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar (India). Animals were housed separately in groups of 10 per cage (polycarbonate cage size: 29×22×14 cm) under laboratory conditions with alternating light and dark cycle of 12 h each having 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 5 days before behavioural experiments which were carried out between 0900 and 1700 hrs. The experimental protocol was approved by IAEC and animal care was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India (Registration No.0436).

Drugs and chemicals—Fluoxetine hydrochloride (Ranbaxy laboratories, Gurgaon, India), p-nitroso-N,N-dimethylaniline (Sigma-Aldrich, St. Louis, USA), sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, tris, EDTA disodium salt AR, sucrose, 5-hydroxy tryptamine creatinine sulphate monohydrate (Hi-Media Laboratories Pvt. Ltd., Mumbai, India), acetic acid, boric acid, hydrochloric acid, potassium hydroxide, sodium hydroxide (CDH Lab Reagents, New Delhi), total protein measurement kit (Coral Industries Ltd., India) were used in this study.

Vehicles—Fluoxetine was dissolved in normal saline (0.9% NaCl). The isolated punarnavine was dissolved in double distilled water each time before administration. Doses of punarnavine were selected based on the literature.

Chronic unpredictable mild stress (CUMS)—The mice were subjected to chronic unpredictable mild stress as reported earlier with some modifications. Animals were subjected to stress paradigm once a day over a period of two weeks between 0900 and 1700 hrs. The order of stressors used was as follows:

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C = cage tilting at 45° for 7 h; E = exposure to empty water bottles; I = immobilization for 2 h; T = tail pinch (60 sec); F = exposure to foreign object (24 h); O = overnight illumination; T1 = tail pinch (30 sec).

Mice subjected to CUMS procedure were called as stressed mice. Unstressed mice were exposed to behavioural tests, and not subjected to CUMS procedure.

**Behavioural tests**—Forced swim test (FST): FST is a most frequently used behavioural model for screening of antidepressant activity in rodents. The procedure was same as previously followed in the laboratory.

Sucrose preference test: Sucrose preference test was employed to determine anhedonia, one of the core symptoms of major depression in humans. The procedure was composed of training and testing sessions. After 1 week of acclimatization, mice were trained to consume 1% (w/v) sucrose solution before the start of the CUMS protocol. In training session, mice were deprived of food and water for 48 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:

\[
\text{Sucrose preference} = \frac{\text{sucrose solution intake (g)}}{\text{sucrose solution intake (g) + water intake (g)}}
\]

The test was again performed on the 14th day to evaluate the effect of stress as well as drug treatment.

Measurement of locomotor activity: To rule out the effects of the extract on immobility period, horizontal locomotor activities of control and test animals were recorded. Vehicle and punarnavine (20 and 40 mg/kg, po) were administered consecutively for 14 days and locomotor activity was measured on 14th day for a period of 10 min using Photoactometer (INCO, Ambala, India).

**Biochemical estimations**—Animals after subjecting to FST/sucrose preference test on 14th day, were tested for locomotor activity on 15th day. One hour after testing for locomotor activity on 15th day, animals were sacrificed by cervical dislocation, and immediately brain samples were collected on ice plate and washed with phosphate buffer saline. The brain samples were analyzed for monoamine oxidase-A (MAO-A), malondialdehyde (MDA), protein and reduced glutathione levels; and catalase activity. At the same time, blood samples were collected by carotid bleeding and centrifuged (Remi Centrifuge, Mumbai, India) at 2500 rpm for 10 min to separate plasma followed by estimation of corticosterone and nitrite levels.

**Biochemical estimations in plasma**—Estimation of corticosterone levels: The quantitative estimation of corticosterone level in the blood plasma was performed using UV-Visible-NIR Spectrophotometer (Varian Cary-5000, Christ, Netherland) using the procedure of Bartos and Pesez.

Estimation of nitrite levels: Plasma nitrite levels were measured by using the method of Green et al.

**Biochemical estimations in brain**—Mouse brain mitochondrial fraction was prepared following the procedure described elsewhere. Briefly, the brain samples were collected immediately on an ice plate. Mouse brain mitochondrial fraction were prepared by cutting the brain sample into small pieces and rinsed in cold 0.25 M sucrose 0.1 M tris 0.02 M EDTA (pH 7.4) to remove blood. The pieces were homogenized for 45 sec in a homogenizer with 400 mL of the same medium. The homogenate was centrifuged (Remi Centrifuge, Mumbai, India) at 800 rpm for 10 min at 4 °C and the pellets were discarded. The supernatant was then centrifuged at 12,000 rpm for 20 min in the same medium. 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.

**Measurement of MAO-A**: MAO activity was assessed spectrophotometrically as described previously. The precipitate (Part 1) was washed twice more with 100 mL of sucrose-tris-EDTA buffer and resuspended in 50 mL of the medium. The assay mixture contained 100 µL of 4 mM 5-hydroxytryptamine as the specific substrate for MAO-A, 250 µL solution of mitochondrial fraction and 100 mM sodium phosphate buffer (pH 7.4) up to a final volume of 1mL. The reaction was allowed to proceed at 37 °C for 20 min, and stopped by adding 200 µL of 1M HCl, the reaction product was extracted with 5 mL of butyl acetate for MAO-A assay. The absorbance of the organic phase was measured at a wavelength of 280 nm using UV-Visible-NIR Spectrophotometer (Varian Cary-5000, Christ,
Netherland). Blank samples were prepared by adding 100 µL of 4 mM 5-hydroxytryptamine and 100 mM sodium phosphate buffer (pH 7.4) up to a final volume of 1 mL and worked up subsequently in the same manner.

Estimation of protein content—Total protein was estimated in brain homogenate by using Erba total protein kit from Coral Industries Ltd., India using colorimeter (Digital Photocolorimeter, Biomed).

Estimation of brain malondialdehyde levels—The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances. The brain malondialdehyde content was expressed as nanomole of malondialdehyde/mg of protein.

Estimation of brain reduced glutathione levels—Reduced glutathione was assayed by the method of Jollow et al. Reduced glutathione were calculated using molar extinction coefficient of 1.36×10⁴ M⁻¹ cm⁻¹ and expressed as micromole/mg protein.

Estimation of brain catalase activity—Catalase activity was assayed by the method of Claiborne. Catalase activity was quantified using the millimolar extinction coefficient of H₂O₂ (0.07 mM) and expressed as micromoles of H₂O₂ decomposed/ min/ mg protein.

Experimental protocol

Animals were divided into following 16 groups of 10 each

Groups for forced swim test

Groups 1, 2, 3 and 4: Vehicle (double distilled water), fluoxetine (20 mg/kg) and punarnavine (20 and 40 mg/kg) respectively were administered orally for 14 consecutive days and on 14th day, 60 min after the administration; immobility period of mice were recorded using FST.

Group 5, 6, 7 and 8: Vehicle (double distilled water), fluoxetine (20 mg/kg) and punarnavine (20 and 40 mg/kg) respectively were administered orally for 14 consecutive days 30 min before the induction of stress. On 14th day, 60 min after the administration; immobility period of mice were recorded using FST.

Groups for sucrose preference test

Groups 9 to 16 were similar as mentioned under FST (Group 1 to 8) except the antidepressant activity was assessed using sucrose preference test.

Groups for measurement of locomotor activity

Animals in groups 9 to 16 after subjecting to sucrose preference test on 14th day were assessed for locomotor activity on 15th day to rule out any effect on locomotion by the drugs.

Statistical analysis—All the results were expressed as mean±SE. The data of all the groups were analyzed using one-way ANOVA followed by Tukey’s post-hoc test using the software Graphpad Instat. In all the tests, the criterion for statistical significance was P<0.05.

Results

Effect of punarnavine and fluoxetine on immobility periods of mice in FST—Chronic unpredictable mild stress did not significantly increase the immobility period of mice in FST as compared to control unstressed mice. Fluoxetine (20 mg/kg, po) and punarnavine (20 and 40 mg/kg, po) per se administered for 14 successive days decreased the immobility period in both unstressed and stressed mice as compared to the respective vehicle treated controls (Fig. 1).

Effect of punarnavine and fluoxetine on sucrose preference test—There was no significant difference in sucrose preference (%) among all the groups in the baseline test. Punarnavine (20 and 40 mg/kg) and fluoxetine (20 mg/kg) per se administered for 14 successive days showed significant increase in sucrose preference (%) by unstressed mice. Exposure of the mice to stress for 14 successive days significantly decreased sucrose preference (%) in control stressed mice as compared to control unstressed mice. Reduced sucrose preference (%) in stressed mice was
significantly restored by the administration of fluoxetine (20 mg/kg) or punarnavine (20 and 40 mg/kg) for 14 successive days (Table 1).

**Effect of punarnavine and fluoxetine on locomotor activity**—Punarnavine (20 and 40 mg/kg, po) and fluoxetine per se administered for 14 successive days did not show any significant change in the locomotor function of mice as compared to the vehicle treated group in both stressed and unstressed mice (Table 2).

**Effect of punarnavine and fluoxetine on plasma corticosterone levels**—Punarnavine (20 and 40 mg/kg, po) administered for 14 consecutive days to mice, did not significantly reduce corticosterone content as compared to the respective vehicle treated group in unstressed mice. But CUMS-induced increase in corticosterone levels were significantly decreased by pretreatment with fluoxetine or punarnavine (20 mg/kg) (Fig. 2).

**Effect of punarnavine and fluoxetine on plasma nitrite levels**—Plasma nitrite levels were significantly elevated in mice subjected to CUMS. Lower dose of punarnavine (20 mg/kg) and fluoxetine (20 mg/kg) per se administered for 14 successive days significantly decreased plasma nitrite levels in unstressed and stressed mice as compared to respective control mice. The higher dose of punarnavine (40 mg/kg) administered for 14 successive days did not show any significant effect on plasma nitrite levels of unstressed mice but significantly decreased the plasma nitrite levels in stressed mice as compared to respective control mice (Fig. 3).

**Effect of punarnavine and fluoxetine on brain MAO-A activity**—Punarnavine (20 and 40 mg/kg) and fluoxetine (20 mg/kg) per se administered for 14 consecutive days to mice, significantly reduced the brain MAO-A levels as compared to the respective vehicle treated group in both stressed and unstressed mice. The efficacy of punarnavine was found to be comparable to fluoxetine (Fig. 4).
Effect of punarnavine and fluoxetine on brain malondialdehyde levels—Malondialdehyde levels were not significantly increased in the brains of chronically stressed mice as compared to vehicle treated group. Chronic treatment with lower dose of punarnavine (20 mg/kg) and fluoxetine (20 mg/kg) per se produced a significant reduction in malondialdehyde levels in both unstressed and stressed mice as compared to the respective vehicle treated groups. The higher dose (40 mg/kg) of punarnavine did not significantly decrease malondialdehyde levels in unstressed mice as compared to the respective vehicle treated group but significantly decreased the same in stressed mice as compared to respective control mice (Fig. 5).

Effect of punarnavine and fluoxetine on brain reduced glutathione and catalase activity—Reduced glutathione content and catalase activity were significantly decreased in brains of stressed mice as compared to respective vehicle treated control mice. Fluoxetine (20 mg/kg) produced a significant increase in the reduced glutathione in both unstressed and stressed mice as compared to the respective controls. Punarnavine did not significantly increase the reduced glutathione levels in unstressed mice as compared to respective control mice. However, punarnavine at both the doses produced a significant increase in the reduced glutathione in stressed mice as compared to control mice. Punarnavine (20 and 40 mg/kg) and fluoxetine (20 mg/kg) per se produced a significant increase in the catalase activity in both unstressed and stressed mice as compared to their respective controls (Figs 6 and 7).

Discussion
In the present study, mice that were exposed to chronic unpredictable mild stress exhibited greater immobility period in FST as compared to control animals and thus showed depression-like behaviour. This is also supported by an earlier study. Punarnavine (20 and 40 mg/kg, po) administered for 14 successive days to mice produced significantly decrease in the immobility period of both stressed and unstressed mice in FST, thus showed significant antidepressant-like effect. The efficacy of punarnavine was found to be comparable to fluoxetine.
FST is a commonly used behavioural despair model of depression. This model is widely employed in rodents to predict antidepressant potential by decrease of immobility period produced by several different classes of antidepressant drugs. Moreover, another model, sucrose preference test was employed to evaluate antidepressant-like activity of punarnavine. This test is an indicator of anhedonia-like behavioural change, indicating loss of interest or pleasure. Anhedonia is a main symptom of major human depression. In the present study, when CUMS was applied, there was a decrease in sucrose preference as compared to vehicle treated unstressed mice. Punarnavine (20 and 40 mg/kg) and fluoxetine (20 mg/kg) per se restored the decreased sucrose preference in both stressed as well as unstressed mice.

In sucrose preference test, both the doses of punarnavine were equally effective in unstressed (P<0.05) and stressed (P<0.001) mice. But in FST, lower dose (20 mg/kg) of punarnavine was more effective (P<0.001) than 40 mg/kg (P<0.01) in unstressed mice; and both the doses were equally effective (P<0.001) in stressed mice. Punarnavine produced maximum effect at lower dose (20 mg/kg), so there might be saturation of receptors on increasing the dose of punarnavine. This might be the reason for lack of dose-dependent effect of punarnavine in the behavioural tests performed.

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Further, punarnavine (20 and 40 mg/kg, po) did not show any significant change in locomotor functions of unstressed and stressed mice as compared to the respective vehicle-treated control mice, so it did not produce any overt motor effects. Thus, antidepressant-like activity of punarnavine in CUMS model of depression in mice is specific and not false positive. Moreover, punarnavine reduced the mouse whole brain MAO-A activity as compared to respective controls in both stressed and unstressed mice, so it indicated that punarnavine inhibited the metabolism of monoamines, particularly serotonin and noradrenaline. Thus, punarnavine showed antidepressant-like activity probably by selectively inhibiting MAO-A activity. Levels of monoamines like norepinephrine and serotonin are decreased in depression, so drugs like tricyclic antidepressants and MAO inhibitors, which enhance the levels of these monoamines, have been used as antidepressant drugs.
Hypersecretion of glucocorticoids and dysregulation of glucocorticoid receptor function are involved in the pathogenesis of depression\(^3\). Experimental data show that the elevated levels of corticosterone and/or CRH induce or aggravate most behavioural, biochemical and morphological changes similar to those found in depressed patients. The present results demonstrated that pretreatment of mice with punarnavine (20 mg/kg) and fluoxetine (20 mg/kg) per se significantly reduced this elevation in stressed mice. There was no significant effect on plasma corticosterone levels in unstressed mice, indicating that hyperactivity of hypothalamic pituitary adrenal axis is observed only in stressful conditions. This result indicates that the antidepressant mechanism of punarnavine involves attenuation of stress-induced increase of corticosterone levels.

Reactive oxygen species (ROS) also play a role in the pathogenesis of neuropsychiatric disorders\(^3,35\). Excessive ROS production can cause oxidative damage to macromolecules including lipids, proteins, and DNA\(^3,36\), culminating in neuronal dysfunction and depression\(^37\). CUMS was found to impair the antioxidant status of brain tissue, presumably through production of excessive ROS. This observation is proved by results of other studies\(^18\). Lipid peroxidation and antioxidant enzymes may be state markers of major depression because they returned to normal ranges after antidepressant treatment\(^17\). In the present study, 14 days of exposure to different stressors resulted in increase in malondialdehyde and nitrite levels and decreased endogenous antioxidant activity in mice. Chronic administration of punarnavine showed antioxidant activity in both unstressed and stressed mice, as indicated by decrease in malondialdehyde levels; and increase in reduced glutathione and catalase activity in mice brains. The antioxidant activity of punarnavine has already been reported\(^12\). Depressed patients show an enhanced expression of NOS in the hippocampus, thus implicating the role of nitrosative stress in the neurobiology of stress and depression\(^38\). Stressful situations in mice have also been reported to significantly increase plasma nitrite levels\(^17\). Punarnavine significantly reduced nitrosative stress as indicated by reduction in the plasma nitrite levels of both unstressed and stressed mice as compared to their respective vehicle-treated controls. Thus, punarnavine showed a strong protection against oxidative stress that plays key role in chronic stress-induced depression.

In conclusion, the results of the present study indicate that punarnavine showed antidepressant-like activity in unstressed and stressed mice probably through inhibition of brain MAO-A activity, decrease in plasma nitrite levels and due to its antioxidant activity. In addition, punarnavine also showed antidepressant-like activity in stressed mice possibly through decrease in plasma corticosterone levels. Thus punarnavine may be explored further for its therapeutic role in treatment of depression.

Acknowledgement
Authors are thankful to Ranbaxy Research Laboratories, Gurgaon (India) for providing gift sample of fluoxetine.

References


