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Evaluation of anti-anxiety effects of the hydromethanolic extract of *Boerhaavia diffusa* L. roots in mice exposed to unpredictable chronic mild stress

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Stress and anxiety are common psychiatric manifestations seen because of modern-day living especially expressed during demanding situations. *Boerhaavia diffusa* is an important medicinal plant widely used in the northeastern part of India. This study aimed to investigate the antioxidant and anti-anxiety activities of the hydro-methanolic extract of *B. diffusa* roots. The antioxidant property of the hydromethanolic extract of *B.diffusa* was determined by *in vitro* antioxidant assays such as DPPH scavenging activity. The cytotoxicity of the extract was investigated using SH-SY5Y cell lines and the anxiolytic activity was evaluated using the elevated plus maze test and open field tests. The results imply that the hydromethanolic extract of *B. diffusa* showed excellent antioxidant activity. The extract did not show any significant toxic effect on SH-SY5Y cells. The hydro-methanolic extract of *B. diffusa* also demonstrated significant anxiolytic activity at 100 and 200 mg/kg doses. Thus, it can be concluded that the hydromethanolic extract of *B. diffusa* possesses anxiolytic activity and antioxidant properties, proving its therapeutic usefulness in the treatment of anxiety disorders.

Keywords: Antioxidant, Anxiolytic activity, *Boerhaavia diffusa* L., Phytochemical screening, Stress

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Introduction

In the face of demanding situations such as a pandemic, people are forced to face certain challenges that can be stressful and cause strong emotional changes in adults as well as children. The necessary precautions like social distancing can make an individual feel isolated and lonely which increases stress and anxiety. Anxiety is an uncomfortable emotional condition that is associated with uneasiness and worries or fear about some unknown potential danger[1]. The signs and symptoms can be mild to severe and are more often chronic than episodic. These disorders affect about one-quarter of all adults at some stage in their lives and are extremely common among teenagers and young adults[1]. The symptoms of anxiety include heart palpitations, exhaustion, nausea, and shortness of breath, which is a common response to stress. Anxiety disorders are a category of psychiatric disorders marked by feelings of stress and terror. These diseases include panic disorder, generalized anxiety disorder (GAD), phobias, social anxiety, post-traumatic stress disorder (PTSD) and obsessive-compulsive disorder (OCD), according to the World Health Organization[2].

The neurotransmitters synthesized by the brain such as gamma amino butyric acid, serotonin, norepinephrine, acetylcholine, adrenaline, and dopamine play special roles in the neurophysiology of anxiety. Anxiety disorders have centred on the Gamma amino butyric acid mechanisms[1]. Serotonergic mechanism[2,3] and Noradrenergic mechanisms[1]. GABA regulates neuronal excitability, working as a 'brake' on the neuronal circuitry under duress thereby acting as the brain's natural stress reliever. Serotonin plays an important role in sleep, mood and temperature regulation and pain perception. This neurotransmitter is involved in regulating emotional status. The increased levels of nor-epinephrine are beneficial during emergencies or in the fight-or-flight response, but continuously elevated levels, even in the absence of danger or stress, create negative emotions such as fear, irritability, and anxiety. Anxiety is a highly prevalent mental condition, affecting about one-eighth of the population and has been a major focus area in the current psychopharmacological studies. Although there are several medications available for anxiety, they are usually associated with numerous side effects. Thus, coping with stress in a healthy way isan important aspect of the current scenario.

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Boerhaavia diffusa is a perennial herb of the Nyctaginaceae family, found across the tropics and subtropics. It has a long history of use among indigenous people as well as in Ayurvedic or natural herbal medicine[4]. This plant has recently piqued the curiosity of experts because of its alleged medicinal properties such as anti-diabetic, anti-bacterial, anti-inflammatory, immunomodulatory, anti-viral, anticonvulsant, anti-fibrinolytic, hepatoprotective, and anti-tumour activities. *B. diffusa* contains a huge number of phytoconstituents such as alkaloids, flavonoids, steroids, lignins, lipids, triterpenoids, glycoproteins, and carbohydrates[5-7]. According to CharakaSamhita and SushritaSamhita, the ayurvedic preparations made from punarnava were used for the treatment of various ailments. It is a diuretic that is used to treat renal ailments as well as high blood pressure. The flowers and seeds are used as a contraceptive and the root juice is used in treating asthma, scanty urine and internal inflammatory disorders. The anti-diabetic property of *B. diffusa* leaf extract has been reported by Satheesh *et al*. and Pari *et al*.[8,9]. The immunomodulatory property of ethanolic extract of *B. diffusa* roots was observed by Mehrotra *et al*.[10]. The anti-lymphoproliferative and anticarcinogenic activity in the ethanolic extract of *B. diffusa* roots was also reported by the same group. Agrawal *et al.*, have screened the antifungal activity in petroleum ether, chloroform, ethyl acetate and ethyl alcohol fractions of aerial and root parts of *B. diffusa*[11]. The anxiolytic property has been reported by Malik *et al*., in the hydro-alcoholic extract of *B. diffusa* leaves[6]. However, regarding the anti-anxiety activity of the *B. diffusa* roots, no reports are available. Thus, the aim of the present investigation is to investigate the antioxidative and anti-anxiety activities of the hydromethanolic extract of *B. diffusa* roots.

Materials and Methods

Reagents and chemicals

All chemicals used in this study were of the highest-grade purity available. *B. diffusa* fine root powder was procured from HM Herbals.1,1-Diphenyl-2-picrylhydrazyl (DPPH), Methanol, sodium hydroxide, sulfuric acid, ascorbic acid, Tween20, and hydrogen peroxide were purchased from Fine Chemicals.

Extract preparation

The extraction of the phytochemicals from the dried root powder was carried out by successive cold maceration using solvents according to polarity. Hydromethanolic extract showed the optimum yield when compared to the other solvents used for extraction. Briefly, 500 g fine root powder was mixed with 80% methanol and 20% water and kept for 7 days at room temperature for cold maceration. After the 7th day, the extract was filtered and the filtrate was concentrated by evaporating the solvent using a rotary evaporator under reduced pressure[12]. The crude extracts obtained were dried under hot-air dryer to produce powdery hydromethanolic extracts. The extracts were stored in air-tight jars at room temperature for further use.

Preliminary phytochemical screening of hydro-methanolic extract of *B. diffusa* roots (BDM)

The percentage yield of the hydro-methanolic root extract of *B. diffusa* was calculated using the formula[13]:

$$\%\ Yield = \frac{Weight\ of\ extract\ obtained}{Weight\ of\ powder\ taken} X\ 100$$

Preliminary phytochemical screening of the hydromethanolic extract was done to detect the presence (+) or absence (-) of certain phytoconstituents. Standard phytochemical screening procedures were followed for the qualitative identification of plant constituents such as alkaloids (Mayer test), carbohydrates (Molisch test), glycosides (Keller-Kilian test), flavonoids (Shinoda test), phenols(Ferric chloride test), saponins (Foam test), sterols (Liebermann-Burchard test), tannins (Braymer test) and terpenoids (Salkowskitest)[14].

Determination of anti-oxidative property

The antioxidant activity of the BDM extract was determined by DPPH free-radical scavenging activity. The stock solution of the extract was prepared in methanol at a concentration of 1 mL/mL. Further, it was diluted to obtain sample solutions of varying concentrations 25, 50, 100, 150, 200 and 250 µg/mL. 1 mL each of the test samples of various concentrations were mixed with 2 mL of methanolic DPPH solution. The mixture was incubated for 30 min in darkness at room temperature and absorbance was measured at 517 nm. The solution of 1 mL methanol and 2 mL DPPH was taken as control. Ascorbic acid solutions at different concentrations (corresponding to the concentration of the extract) were used as the reference[15]. The per cent of DPPH scavenging was calculated by using the following formula:

$$\%\ Inhibition = \left\{\frac{Ac - As}{Ac}\right\} X100$$

Where Ac is the absorbance of the control and As is the absorbance of the sample.

From the obtained values, the IC_{50} (the concentration of extract having 50% of maximum scavenging activity) was calculated.

Cell viability assay

The human neuroblastoma cells (SH-SY5Y cells) at a density of 1.0×10^4 cells per well were plated in 96 well plates and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetalcalf serum. The cells were grown at 37°C in a humidified 5% CO_2 incubator. After 24-48 h, the medium was removed, and the cells were washed and incubated in complete medium with increasing concentrations of BDM (1-100 µg/mL) for 48 h. 10 µL of 5 mg/mL MTT (3(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide in phosphate-buffered saline was further added to each well, 4 h before the completion of incubation. The plate was centrifuged, the supernatant was removed and 100 µL of dimethyl sulfoxide (DMSO) was further added to dissolve the formazan formed. The absorbance was read at 530 nm in a microplate reader[16].

Experimental design and unpredictable chronic mild stress induction procedure

Disease-free Swiss albino mice weighing (25-30 g), procured from the animal house of Birla Institute of Technology, Mesra, Ranchi, (1968/PO/Re/S/17/CPC-SEA) were housed under standard conditions of temperature (23±1)°C and relative humidity of 45 to 55% under 12 h light: 12 h dark cycle. The mice were given a standard pellet mealand free access to water. All the animals were allowed to acclimatize two weeks prior to the commencement of experiments. The experiments were designed and conducted in accordance with guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional animal ethical committee (Approval No. 1972/ PH/BIT/ 116/21/IAEC).

Mice were divided randomly into five groups (n=6), i.e., the Control group with no stress (treated with saline); UCMS group (exposed to chronic mild stress and treated with saline only); UCMS + BDM extract 100 mg/kg; UCMS + BDM extract 200 mg/kg; UCMS + Diazepam 2 mg/kg.

Further, the control mice were kept unstressed and mice in the UCMS and drug-treated groups were exposed to a variety of mild environmental or psychosocial stressors including food deprivation, restrain stress, forced swimming, social defeat stress and loss of consciousness.

Restraint stress

One of the most widely used techniques to cause stress-related behavioural, biochemical, and physiological changes in laboratory animals is restraint stress and immobilisation. Animals were restrained for 120 min to induce immobilisation stress by gently not moving their upper limbs, lower limbs, and neck by placing the animals inside mice restrainers. This technique was used to trigger chronic stress till 21 days[17].

Swimming stress

It was carried out in a clear glass tank (25×25× 60 cm) containing 39 cm of clean water at 26°C. The apparatus was cleaned thoroughly, and water was changed individually for each mouse. Mice were kept inside the glass tank for 10 min. The mice were dried and kept warm for 30 min under a heating bulb before being returned to their cages[18,19].

Social defeat stress

The social defeat stress (SDS) protocol consists of the introduction of a single mouse (intruder) in the home cage of a resident male mouse (aggressor). During the test, the behaviours of the intruder mouse and resident aggressor mouse were recorded for 30 min. The time spent by an intruder mouse in a social defeat posture induced by the presence of an aggressor was observed. This procedure was used in acute or chronic stress protocols[20].

Loss of consciousness

It was carried out in a clear glass tank (25×25× 60 cm) containing a piece of cotton inside the tank. The apparatus was cleaned thoroughly, and diethyl ether solvent was used for loss of consciousness. A piece of cotton dipped in the solvent was kept inside the tank. After 30 sec of solvent saturation inside the tank, mice were placed inside for about 20 sec.

Evaluation of anxiolytic activity of BDM extract

For the evaluation of the anxiolytic activity of BDM extract, stressed mice were subjected to the administration of BDM extract at a low and high dose of 100 and 200 mg/kg, respectively. The doses were administered orally for 7 days. The control group received normal saline (10 mL/kg) and standard groups of mice were treated with Diazepam (2 mg/kg) orally for 7 days. On the 7th day, experiments were performed 30 min after the last dose administration.

Elevated plus maze

The animal behaviour was observed through elevated plus-maze (EPM). The elevated plus-maze apparatus was made of wood and consisted of two

opposed open arms (50×10 cm), two opposed enclosed arms with no roof (50×10×40 cm), and an open square (10×10 cm) in the centre. The maze was elevated 50 cm above the floor[21,22].

One hour after the oral administration of drugs, the mice were placed at the centre of the maze, facing one closed arm. During the entire experiment, mice were allowed to socialize. Other than the height of the plus-maze, every effort was taken to ensure that no external cues might cause anxiety. During the 5 min experiment, the number of entries into the open and closed arms along with the time spent in the open and closed armswere recorded. Before each animal was placed, the arena was cleaned with 5% alcohol to remove any potential bias caused by the preceding animal's odour[22].

Open field test

On the 7th day, 60 min after administration of the vehicle, standard and test extracts, each mouse was placed in the centre of open field arena, and the following parameters were recorded during a test session of 5 min[22]. Ambulation: Measured in terms of the number of squares crossed by the animal, rearing: Number of times the animal stood on its hind limbs, self-grooming: Number of times the animal groomed facial region and licked/washed/scratched various parts of its body and faecal droppings: Number of faecal droppings excreted during the period.

Results and Discussion

Phytochemical screening of BDM

The percentage yield of the hydro-methanolicroot extract of *B. diffusa* was 4.5% (w/w). Phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, terpenoids and saponin in various concentrations with alkaloids and flavonoids showing greater occurrence (Table 1). Literature reports also confirm the presence of these phytochemicals in the methanolic root extract of the plant. Thus, it may be incurred that these phytochemicals could be responsible for its antioxidant and anxiolytic activity but further studies are warranted.

Antioxidant activity of BDM Extract

The results of DPPH scavenging activity revealed significant antioxidative activity of BDM extract that was comparable to the standard (ascorbic acid) (Table 2). An increase in extract concentration increased the proportion of free radical inhibition. The maximum free radical scavenging activity was seen with 250 µg of the extract which was expressed as % DPPH scavenging activity. 51% inhibition was seen with the maximum concentration of the extract in comparison to 79% inhibition of ascorbic acid (control). The antioxidant property of BDM extract may be due to its phytoconstituents such as alkaloids and flavonoids. These constituents are known for their significant free radical scavenging property that has extensively been studied with regard to numerous diseases. Several works of literature have previously reported the anti-oxidant potential of *B. diffusa* in illnesses such as diabetes[23] and renal impairment[24].

Cell viability assay

The results of the cell viability assay conducted using varying concentrations of the plant extract demonstrated the highest cell death inhibition at 10 µg/mL of the extract (Fig. 1). Neuronal protection

Table 1 — Phytochemical screening of *Boerhaavia diffusa* root extract

Phytochemicals	*B. diffusa* extract
Alkaloids	+++
Flavonoids	+++
Tannin	++
Saponin	+
Carbohydrates	+
Protein	+
Terpenoids	++

KEY: +++ = highly present, ++ = moderately present, + = present in trace amount, - = absent.

Table 2 — Free radical scavenging activity of *Boerhaavia diffusa* root extract

Concentration (mcg/mL)	% Scavenging of DPPH	
	Ascorbic acid	*B. diffusa* extract
25	48	20
50	56	25
100	69	38
150	71	41
200	76	43
250	79	51

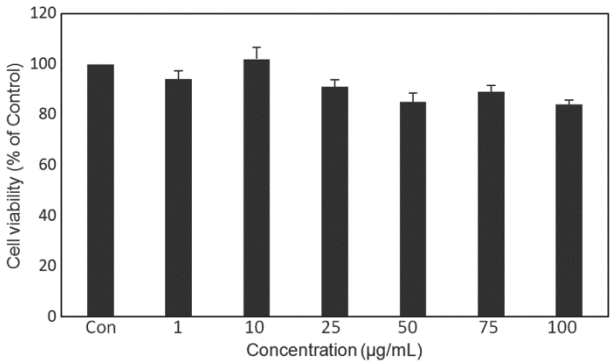

Fig. 1 — Effect of *Boerhaavia diffusa* root extract on the viability of human neuroblastoma cells (SH-SY5Y cells).

Table 3 — Effect of *Boerhaavia diffusa* extraction elevated plus maze behaviour of mice

Gr	Treatment	Dose (mg/kg)	No of entries (5 min)		Time spent (s)	
			Open arm	Closed arm	Open arm	Closed arm
I	Control	-	11.5±5.20	2.45±0.61	402±3.15	108±2.50
	UCMS	-	3.22±0.31	11.39±0.38	170±2.70	310±1.80
II	UCMS + BDM	100	5.98±0.87	06±0.18	210±2.40	297±1.70
III	UCMS + BDM	200	7.38±0.28	05.23±0.86	260±2.10	220±1.20
IV	UCMS + Dz	2	12.2±0.72	2.99±0.27	380±1.40	110±2.00

Values are expressed as Mean±SEM, (n=6).

Table 4 — Effect of *Boerhaavia diffusa* extract on open field test in mice

Gr	Treatment	Dose (mg/kg)	No. of line crossed	Grooming time(s)	Time spent at the Centre(s)	Fecal drops
I	Control	-	49.12±5.10	16.40±9.31	65.27±3.15	1.2±0.50
	UCMS	-	34±5.10	86.54±12.61	5.70±1.35	10±1.50
II	UCMS + BDM	100	48.12±2.23	56.32±13.16	41.21±5.60	4.2±0.80
III	UCMS + BDM	200	47.21±3.55	34.20±6.15	55.50±4.03	3.3±1.10
IV	UCMS + Dz	2	51.40±4.31	10.70±8.55	62.0±18.12	0.5±1.20

Values are expressed as Mean±SEM, (n=6).

against cell death of human neuroblastoma cells could be beneficial in protecting the cells against cellular stressors, necessary for anxiolytic activity and protection against psychiatric disorders.

Anxiolytic activity of BDM extract

The elevated plus maze test is the most used behavioural test in mice for detecting anxiolytic behaviour. Anxiolytics work by promoting the opening of GABA-A-activated chloride channels, which improves the response to GABA. The results demonstrate that BDM extract has significantly increased the time spent in open arms and the number of entries in open arms while time spent in closed arms decreased significantly indicating that the plant showed anti-anxiety activity (Table 3).

The open-field method was also used in the present study, which provides a clearer indication of the animal's emotional state. The results imply that administration of the BDM extract and diazepam produced a significant reduction in grooming time and an increase in time spent at the centre of the field. Both the parameters, grooming behaviour after stress and an increase in time spent at the centre of the field clearly suggest that the plant extract has an anxiolytic function. The results also demonstrate that administration of BDM extract increased the time spent in the centre of the arena as well as the number of squares crossed and decreased the number of faecal drops as compared with control animals (Table 4). In the present study, BDM extract at both the dose levels (100 & 200 mg/kg) showed anxiolytic activity by the elevated plus maze and open field apparatus test.

Conclusion

The extracts of medicinal plants containing flavonoids and tannins are known to possess significant anti-stress activities. Alkaloids and flavonoids are important secondary metabolites in many plants that are responsible for their anxiolytic actions. The results of the present study reveal that the extract of *Boerhaavia diffusa* roots possesses anxiolytic and antidepressant activities in unpredictable chronic mild stress-induced depression in mice which is also comparable with the standard drug diazepam. Phytochemical screening of the hydromethanolic extract of the roots showed the presence of flavonoids, tannins and alkaloids that may contribute directly to antioxidant activity and play a key role in the free radical scavenging activity. The restoration of the antioxidant system may contribute to its anxiolytic and antidepressant activities. This study, therefore, confirms that BDM extract may be used for treating depression and related psychiatric disorders. Future studies related to its neurobiological mechanisms of action are warranted.

Conflict of interest

The authors present no conflict of interest.

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