European Journal of Medicinal Plants

Volume 35, Issue 6, Page 200-210, 2024; Article no.EJMP.123927
ISSN: 2231-0894, NLM ID: 101583475

Evaluation of Antioxidant, Anticancer, Anthelmintic, Neuropharmacological Activity of *Boerhaavia diffusa* Linn. (Family: Nyctaginaceae)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/ejmp/2024/v35i61219

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/123927

Original Research Article

Received: 05/08/2024
Accepted: 09/10/2024
Published: 24/10/2024

ABSTRACT

Aims: *Boerhaavia diffusa Linn* belongs to the family Nyctaginaceae has been documented for its antioxidant, anticancer, anthelmintic & neuropharmacological effects. Literature review of the plant revealed that some research works are performed during this plant. That's why; this study was performed to gauge the antioxidant, anticancer, anthelmintic & neuropharmacological activities of the methanolic extract from the leaves of *Boerhaavia diffusa* (MEBD).

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Cite as: *Dina, Jesmin Aktar, Nazmun Nahar, Esha Tamanna Sathi, Mst Nasrin Khatun, and Afrin Jahan. 2024. "Evaluation of Antioxidant, Anticancer, Anthelmintic, Neuropharmacological Activity of Boerhaavia Diffusa Linn. (Family: Nyctaginaceae)". European Journal of Medicinal Plants 35 (6):200-210. https://doi.org/10.9734/ejmp/2024/v35i61219.*

Method and Results: Antioxidant activity was evaluated using DPPH free radical scavenging assay using serial dilution concentration and the IC_{50} was 1.51. Anticancer test was done using cell viability assay. During anticancer test in the concentration of 1000µg/mL the percent of inhibition was 29.16%. Anthelmintic activity was investigated using earthworms (*Pheretima posthuma*) and fresh leaf juice of the plant. In anthelmintic activity, the time of paralysis for fresh leaf juice was begin from 64 min 55 sec and end to 23 min 19 sec while the time for tested drug Albendazole was started from 76 min 09 sec and end to 30 min 43 sec. Besides, the time of death start from 71 min 09 sec and end to 29 min 54 sec for leaf juice and for Albendazole the time of death start from 82 min 19 sec and end to 38 min 18 sec respectively at several doses which suggest considerable anthelmintic activity of the plant. Neuropharmacological activity was performed by hole cross and light/dark box tests at the doses of 200 mg/kg and 400 mg/kg in Swiss albino mice contrast to the test drug Diazepam (2mg/kg). The extract significantly decreases the locomotor activity as shown by the results of hole cross test which indicate significant antidepressant property. The results also matching the activity in light/dark box test.
Conclusion: Thus, the obtained results in the research work provide a support for the utilization of this plant for medicinal purposes and encourage further investigations for more fruitful results.

Keywords: Boerhaavia diffusa; antioxidant; anticancer; anthelmintic & neuropharmacological.

1. INTRODUCTION

Medicinal plants have played a vital role in healthcare throughout history, thanks to their healing properties and essential components that have greatly contributed to the advancement of modern medicine [1]. The plant-derived phytochemicals are abundant in biologically active compounds that display a variety of pharmacological activities, such as antioxidant, anticancer, anthelmintic, and neuropharmacological effects [2]. The presence of compounds like phenols, flavonoids, and tannins in medicinal plants is primarily responsible for their antioxidant activity. Antioxidants are crucial in counteracting harmful reactive oxygen species and safeguarding against oxidative stress, linked to various chronic diseases such as cancer and neurodegenerative disorders [3]. The importance of these plant-based antioxidants in modern medicine is highlighted by their effectiveness in fighting oxidative stress [4]. The medicinal properties of plants that fight against cancer are believed to result from their capacity to trigger apoptosis, hinder cell growth, and impede the formation of blood vessels in tumors, among other mechanisms [5]. Several plant compounds, such as curcumin, resveratrol, and quercetin, have shown potential in fighting cancer, which has led to their consideration in the field of oncology [6]. The anthelmintic activity is an important aspect of how medicinal plants contribute to modern medicine. Several plant compounds, including alkaloids, terpenoids, and saponins, have been found to possess strong anthelmintic properties. These natural substances provide a cost-effective alternative to synthetic anthelmintic drugs [7]. The medicinal properties of plants have great potential in the field of neuropharmacology. Phytochemicals such as alkaloids, flavonoids, and terpenoids have demonstrated promising effects in treating neurological disorders, including Alzheimer's, Parkinson's, and depression [8]. These findings emphasize the therapeutic possibilities of plant-derived compounds in the field of neurology and mental health. Ultimately, the impact of medicinal plants on modern medicine is vast and diverse [9]. The various plant-derived compounds have a wide array of pharmacological effects that play a crucial role in preventing and treating different illnesses. As research progresses in understanding the intricacies of these natural compounds, their incorporation into modern pharmacology is poised to gain more prominence [10].

Boerhaavia diffusa L. is a perennial herb that belongs to the Nyctaginaceae family. It has a broad distribution in tropical and subtropical regions. The plant exhibits long-petioled broadly ovate leaves, greenish purple. Its stems are stiff and cylindrical, while its small white flowers bloom on long stalks [11]. It boasts a rich historical background, with extensive utilization by indigenous and tribal communities, as well as in Ayurvedic or natural herbal remedies [12]. The genus Boerhaavia consists of 40 species that can be found in various regions across the globe, including tropical, subtropical, and temperate areas. It can be found in various countries around the world, including Australia, China, Egypt, Pakistan, Sudan, Sri Lanka, South Africa,

the USA, and several countries in the Middle East. *B. diffusa* is also native to India. This plant species is commonly found in the warmer regions of the country, reaching altitudes of up to 2000 m in the Himalayan region. The plant is also grown to some extent in West Bengal. The plant is found throughout Bangladesh [13]. It possesses numerous ethnobotanical applications, such as the utilization of its leaves as a vegetable and the extraction of its root juice for the treatment of asthma, urinary disorders, leucorrhoea, rheumatism, and encephalitis. Additionally, it is employed medicinally within the traditional Ayurvedic system. Punarnava is a highly beneficial herb renowned for its effectiveness in treating kidney diseases [14].

This study centered around examining the medicinal properties of a methanol extract derived from an entire plant. The extract was evaluated for its potential as an anticancer, antioxidant, anthelmintic, and neuropharmacological agent.

2. METHODS

2.1 Plant Materials

A specimen of *B. diffusa* was collected from Ramna Park, Dhaka, Bangladesh in October 2023, during daylight hours. Scientists from the Bangladesh National Herbarium in Mirpur, Dhaka, were able to identify the plant. The accession number (DACB: 41278) has been assigned to it and a voucher specimen has been placed in the Pharmacy Department at Stamford University Bangladesh.

2.2 Plant Extraction

The dried leaves were meticulously pulverized into a fine powder using a state-of-the-art commercial grinder. Then, around 170 g of each powdered substance was immersed in 900 mL of methanol in a beaker, maintaining a temperature of 23 ± 2°C for 72 hours. The mixture was stirred at regular intervals using a sterile glass rod. There are different chemical classes present in the methanol used for extracting plant bioactive components, including alkaloids, glycosides, tannins, flavonoids, steroids, and other compounds. Compared to other organic solvents, methanol boasts a lower boiling point and a polarity index of 5.1. The entire mixture was filtered using a sterile, white cotton filter cloth, just as a meticulous expert would do. We utilized the Whatman 102 filter paper for their collection [15]. The filtrates were evaporated using a rotary evaporator from a reputable manufacturer (BC-R 201). From 171 g of powder after the drying process, we obtained a dried extract weighing 27.6 g, resulting in a yield of 19.79%. Experiments were conducted on the methanol extract of *B. diffusa* using the maceration method to study its antioxidant, anticancer, anthelmintic, and neuropharmacological activity.

2.3 Antioxidant Test

2.3.1 DPPH free radical scavenging assay

The DPPH radical scavenging capacity was determined using the procedure outlined previously, with some slight modifications [10]. For this investigation, different concentrations of MEBD extracts were utilized, along with the standard ascorbic acid at various concentrations (500, 250, 125, 62.5, 31.25, 15.62, and 7.81 µg/mL). The concentrations varied across a range of levels. Here is the formula for calculating the percentage of the inhibited free radical [16,17]. The formula for calculating the percentage of inhibition is as follows:

$$\%\text{Inhibition} = \frac{(\text{Absorbance of blank} - \text{Absorbance of Test sample})}{\text{Absorbance of blank}} \times 100$$

2.4 Anticancer Test

2.4.1 Cell viability assay

Cells were cultured in their respective media in 96-well plates until they reached approximately 70% confluence. Subsequently, the cells were exposed to various concentrations of the extract, as well as a control containing the vehicle/DMSO, for approximately 24 hours. Afterward, the media was removed to cleanse the cells using phosphate buffer saline (PBS). A solution of MTT at a concentration of 0.5 mg/ml was added to each well, and the plate was incubated at 37 °C for 4 hours in the absence of light. After incubation, the MTT solution was replaced with 200 µl of DMSO. The plate was agitated at 150 rpm for 5 minutes and the optical density was measured at 490 nm using a plate reader (ELx 800; Biotek, Winooski, VT, USA). The experiment was conducted multiple times to ensure accurate data for graph plotting [18].

2.4.2 Morphology study

Cells were plated in 24-well plates and exposed to DMSO or extract (at IC-50 concentration) for

24 hours. Following the treatment, the image was captured using phase contrast microscopy.

2.5 Anthelmintic Test

2.5.1 Experimental animals

Adult earthworms (*Pheretima posthuma)* were gathered from the damp soil of a private location in Bangladesh. Earthworms were rinsed with normal saline to eliminate any fecal residue before being utilized for the anthelmintic study. Earthworms measuring 3–8 cm in length and 0.1–0.2 cm in width were utilized for all experimental protocols.

2.5.2 Anthelmintic assay

The leaves were carefully weighed (75 g) and then blended into a solution using 250 ml of water. The mixture was then spun at 100 revolutions per minute. The supernatant was carefully filtered using sterile filter paper into a conical flask. Therefore, the freshly extracted juice of the plant was prepared to test its anthelmintic activity [19]. The fresh juice extract and methanol extract of *B. diffusa* were dissolved in a small amount of DMF and the volume was adjusted to 10mL with saline water, just like a pharmacologist would do. All medications and extract solutions were prepared just prior to commencing the experiment. For each trial, 5 earthworms were introduced into 10ml of different formulations. These included a vehicle solution (5% DMF in normal saline), Albendazole at various concentrations (5 mg/mL, 10 mg/mL, 20 mg/mL, 50 mg/mL, and 100 mg/mL), and fresh juice of *B. diffusa* in a normal saline solution with 5% DMF, matching the concentration of Albendazole. An observation was conducted to measure the duration of paralysis and death in each individual worm. Paralysis was observed when the worms were unable to move, even in a saline solution.

2.6 Neuropharmacological Test

2.6.1 Experimental mice

The male *Swiss albino* mice, weighing 20-25 g, were kept in individual cages and maintained under 12/12 h light/dark cycles at room temperature (22-25 °C). The subjects were given a generous amount of commercially available diet and water.

2.6.2 Hole cross test

In this research, a cage with the dimensions of 0.30 m x 0.20 m x 0.14 m was used, following the method from previous research [20]. A partition was added to divide the area. Located at a height of 0.075 m, precisely in the centre of the framework, a hole measuring 0.03 m in diameter was carefully crafted. The animals used in the experiments were housed in one section of the cage and administered either a placebo, a standard, or a test sample. After the administration of the control, standard, and experimental extracts, the number of mice passing through the opening from one chamber to the other was counted for 5 minutes at 30, 60, 90, and 120 minutes. The participants in Group I were given a placebo, which was distilled water, while those in Group II were administered the standard medication, diazepam. Groups III and IV were administered MEBD orally at doses of 200 and 400 mg/kg of body weight, respectively.

2.6.3 Forced swimming test

The method used by previous research. After making some adjustments, we conducted the forced swimming test by dividing the mice into five groups, each containing five mice. Each group was administered with a control, Imipramine hydrochloride, and three different doses of the extract. A glass cylinder measuring 45 cm in height and 20 cm in diameter was utilized for the experiment. It was filled with water at a temperature of 25±1ºC, up to a depth of 17 cm. A mouse is deemed immobile if it stays afloat in the water, with minimal movement to keep its head above the surface. The experiment was conducted between 1 P.M. and 3 P.M. for 5 minutes [21].

2.7 Statistical Analysis

The experimental data was replicated three times, and the mean and standard deviation were used to represent the results. Excel is commonly utilized for conducting statistical analyses.

3. RESULTS

3.1 Antioxidant Activity

The extent of DPPH inhibition was also determined. The results showed that there was a correlation between the concentration and the increase in DPPH radical scavenging activity. The inhibition of DPPH ranged from 8% to 74% at concentrations of 7–500 µg/mL, as shown in Table 1. Ascorbic acid (used as a reference) demonstrated a 50% inhibition of the DPPH radical at a concentration of 21.86 µg/ml, whereas MEBD showed a 50% inhibition at a concentration of 1.51 µg/mL.

Table 1. *In vitro* free radical scavenging effect of MEBD

Samples	Concentrations	% of Inhibition	IC_{50} in DPPH radical scavenging analysis (µg/mL)
Ascorbic Acid	7.81	55.72	
	15.625	59.67	
	31.25	70.57	21.86
	62.5	73.70	
	125	81.60	
	250	88.55	
	500	93.86	
MEBD	7.81	48.21	
	15.625	52.49	
	31.25	62.56	1.51
	62.5	65.47	
	125	74.83	
	250	82.71	
	500	92.81	

3.2 Anticancer Activity

The alcoholic extract (MEBD) of the plant materials was standardized using established methods, and its potential as an anticancer agent was evaluated on HeLa cell lines. The methanolic extract from the *B. diffusa* plant showed promising results, as indicated in Table 2.

Table 2. Anticancer activity of MEBD

Concentration (µm/mL)	Survival of the cell (%)	% of Inhibition
125	85.69	14.36
250	76.94	24.06
500	69.63	19.37
1000	60.84	29.16

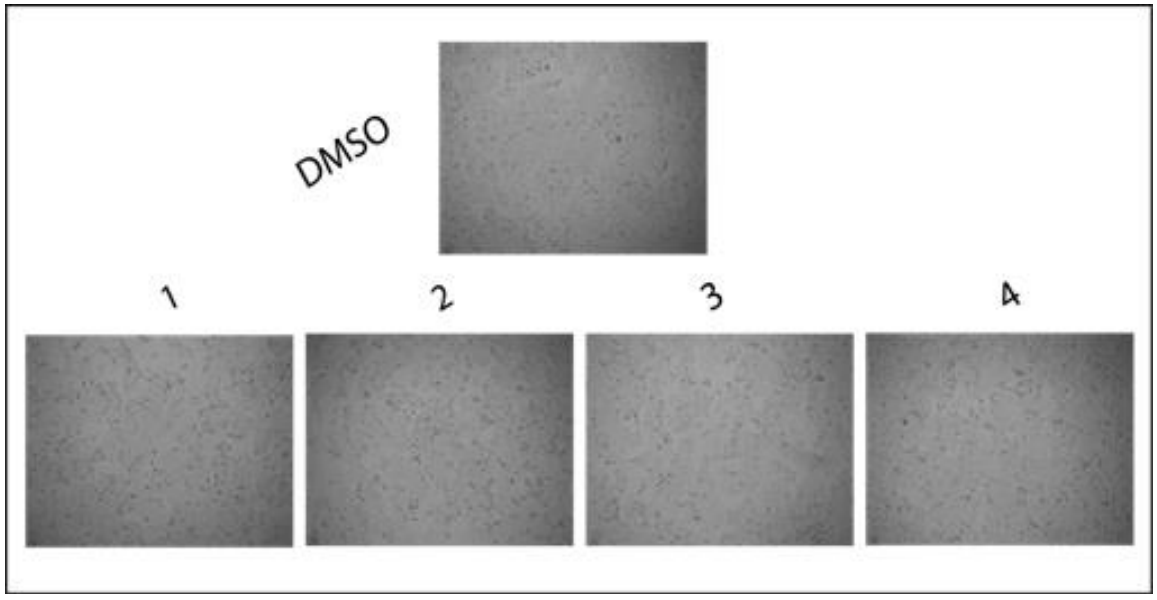

Fig. 1. Displays a phase contrast image that reveals notable and distinct morphological alterations. The serial 1,2,3,4 indicates the concentration of MEPH ranging from 125 to 1000 µm/mL, as presented in Table 1

3.3 Anthelmintic Activity

To assess the anthelmintic effectiveness of fresh leaf juice, various concentrations of the liquid (5mg/mL, 10mg/mL, 20mg/mL, 50mg/mL, and 100mg/mL) were administered to earthworms. In line with the standard medication albendazole, the leaf extract demonstrated significant anthelmintic activity (Table 3).

3.4 Neuropharmacological Activity

3.4.1 Hole cross test

The extract considerably reduced the locomotor activity as shown by the results of the hole-cross test. The locomotor activity lowering effect was evident for the both doses of 200mg / kg and 400mg / kg weight at the 2nd observation (30 min) and continued up to 3rd and 4th observation (60 and 90 min) period (Table-4). Moreover, the validation of hysteria was administered by measuring external signs, through hole-cross test.

3.4.2 Forced swimming test

In comparison to the control group, the immobility time was significantly prolonged by the administration of *B. diffusa* extract at doses of 200 and 400 mg/kg. In the mouse model, the standard medication diazepam (1 mg/kg, i.p.) greatly increased the immobility time. The most significant depressive effect of *B. diffusa* was observed at a dose of 200 mg/kg (Table 5).

4. DISCUSSION

Punarnava, also known as *Boerhaavia diffusa*, has a long-standing reputation in the field of traditional medicine. This plant contains a wealth of phytochemicals that have strong antioxidant properties, which contribute to its potential therapeutic benefits [22].

Table 3. Anthelmintic activity of MEBD

Test sample	Concentrations	Time taken for paralysis	Time taken for death
MEBD	5	64 min 55 sec	71 min 09 sec
	10	53 min 22 sec	61 min 53 sec
	20	51 min 26 sec	57 min 33 sec
	50	34 min 47 sec	38 min 47 sec
	100	23 min 19 sec	29 min 54 sec
Albendazole	5	76 min 09 sec	82 min 19 sec
	10	62 min 47 sec	72 min 32 sec
	20	57 min 52 sec	69 min 37 sec
	50	41 min 03 sec	47 min 12 sec
	100	30 min 43 sec	38 min 18 sec

Table 4. Effect of *Boerhaavia diffusa* on hole-cross test

Group	Route of Administration	Observation				
		0 min	30 min	60 min	90 min	120 min
Control	Oral	15.2±0.075	9±0.036	11.6±0.82	10.7±0.22	5±0.67
Positive Control	Oral	8.2±0.31	11±0.251	6.3±0.032	4.5±0.045	3±0
Group I	Oral	7.7±0.17	6±0.345	3.3±0.089	2.9±0.045	1.5±0.07
Group II	Oral	1.4±1.3	1.7±0.047	2±0.031	2.3±0.017	1.3±0.05

Table 5. Effect of MEBD on forced swimming test

Treatment	Dose (mg/kg)	mobility time (s)
Control	0.1mL/mice	24.60±1.23
Diazepam	1	91.60±1.32
MEBD	200	61.00±1.31
MEBD	400	75.43±0.7

The antioxidant properties of *B. diffusa* are mainly due to the presence of phenolic compounds, such as flavonoids and tannins. These compounds can neutralize free radicals and protect against oxidative damage [23]. According to previous researcher have found that certain compounds in *B. diffusa*, such as flavonoid quercetin and tannin punarnavine, play a significant role in its antioxidant activity [12].

The mechanism of action of these plant compounds involves multiple pathways. For instance, Quercetin is highly effective at neutralizing reactive oxygen species (ROS), which can be detrimental byproducts of cellular metabolism. This process occurs by providing a hydrogen atom to the ROS, which effectively neutralizes them and safeguards cells and tissues against oxidative damage [24]. In contrast, Punarnavine has been demonstrated to enhance the function of important antioxidant enzymes like superoxide dismutase and catalase. These enzymes are vital for protecting the body against oxidative stress [25]. Through its ability to boost enzyme activity, punarnavine plays a crucial role in preserving the body's antioxidant defense system, shielding it from harmful oxidative damage. *Boerhaavia diffusa* also has therapeutic potential, including anticancer activity attributed to its abundant phytochemical profile [22]. In B. diffusa, there are certain phytochemicals like eupalitin and boeravinones that have been found to possess anticancer properties. Eupalitin, a flavonoid, demonstrates promising anticancer activity through various mechanisms. It triggers apoptosis in cancer cells, a process of programmed cell death, by activating caspase enzymes that break down the cell internally [26]. In addition, eupalitin has the ability to interrupt the cell cycle, specifically during the G_2/M phase, which effectively prevents the growth of cancer cells [14].

Boeravinones, which are derivatives of rotenoids, are a group of potent compounds that play a significant role in the potential of *B. diffusa* to combat cancer. Boeravinones A and B have been found to demonstrate potent cytotoxic effects on cancer cells. They work by triggering apoptosis, a process of programmed cell death, and also by inhibiting the proliferation of cancer cells [27]. They have also demonstrated the ability to hinder angiogenesis, the vital process of forming new blood vessels that is necessary for tumor growth and metastasis [25]. In addition, the lignan compounds discovered in *B. diffusa* have shown remarkable cytotoxic effects on various cancer cell lines, including breast and ovarian cancers. These compounds have the ability to induce apoptosis by altering the Bax/Bcl-2 ratio, leading to the release of cytochrome c. This release is a crucial step in the intrinsic pathway of apoptosis, as explained in previous research [28].

It is established that some key phytochemicals are responsible for its therapeutic efficacy, precisely for its efficacy against helminth infections. It has been found that an earthworm assay, more precisely, conducted on a species like *Pheretima posthuma*, acts as an effective model in understanding the anthelmetic action of *Boerhaavia diffusa* since these worms model the human intestinally lived helminths. Phyto-constituent presence in *Boerhaavia diffusa* includes alkaloids, flavonoids, saponins, and tannins-all of which have been implicated in their anthelmintic action. Alkaloids act by neuromuscular paralysis on the helminths, inhibiting their movement and finally leading to their death. Examples include punarnavine. Antioxidant properties of flavonoids include the promotion of general health in the host while at the same time having adverse effects on the parasite by interfering with its metabolic processes. Such is the case with flavonoids present in *Boerhaavia diffusa*. Moreover, saponins in *Boerhaavia diffusa* have been known to form pore-forming effects in the helminth cell membranes that result in cell lysis and death [29]. The earthworm assay is most relevant in assessing the anthelmintic potency of *Boerhaavia diffusa* since it is an easy system to work with and the physiology of earthworms is close enough to that of human intestinal helminths. As such, different concentrations of extracts from *Boerhaavia diffusa* are used in this assay on earthworms and observe the action on motility, paralysis, and mortality [30]. The results have always yielded that the extracts elicit a significant anthelmintic action, which is dose-dependent. Therefore, with the increase in the extract concentrations, a reduced time for paralysis and mortality of the earthworms is recorded, thus evidence that *Boerhaavia diffusa* is effective against helminths [31].

The mechanism of action was not only limited to direct toxicity but also involved neuromuscular junction interactions. The active phytoconstituents, mainly alkaloids, have been considered to block neuromuscular activity of the

parasites. Thereafter, it is suggested that such compounds interfere with neurotransmitter release, resulting in disrupted coordinated movement of the helminths and their eventual paralysis. This neuromuscular blockade enables the expulsion of the parasites out of the host system successfully [32]. Some of the phytochemicals present in this plant, such as alkaloids, flavonoids, and phenolic compounds, play a crucial role in the regulation of neurological functions. Traditional experiments with *Boerhaavia diffusa* for the treatment or mitigation of symptoms related to depression and anxiety have mostly been through behavioral studies conducted via the Hole Cross method and the Forced Swimming test, respectively. These methods are quite indicative of the plant's pharmacological effect on animal models through conditions of despair and exploration that can be related to neurobehavioral disorders [33]. The Hole Cross method is widely used in studies for the measurement of exploratory behavior and anxiety levels in rodents. In studies, *Boerhaavia diffusa* extracts have shown a significant increase in the number of crossings, which signifies lower anxiety levels in the animals treated with the extract compared to controls. It shows that flavonoids and alkaloids enhance serotonergic and dopaminergic activities, which plays a crucial role in maintaining mood regulation along with reducing anxiety [34]. Punarnavine is one such type of alkaloid extracted from *Boerhaavia diffusa*, which has neuroprotective action mode through neurotransmitter level modulation activities [32].

On the other hand, the Forced Swimming Test examines depressive-like behavior with a measure of the time of immobility of the subjects. A significant decrease in the time of immobility after treatment with *Boerhaavia diffusa* extracts might be indicative of its probable antidepressant action. Its bioactive principles might act on monoaminergic systems, particularly on norepinephrine and serotonin pathways. This modulation may be beneficial for increased physical activities and improved mood in treated subjects, reflecting the efficacy of *Boerhaavia diffusa* as a natural remedy for depression [35]. Further, other studies have identified that aqueous and ethanolic extracts have antioxidant activities, which can be utilized against oxidative stress implicated in neurodegenerative and psychiatric disorders. Antioxidant activity of *Boerhaavia diffusa* has been ascribed to flavonoid content that helps in scavenging free radicals and protection of neuronal cells [36]. Moreover, the synergistic mode of action exerted by these phytochemicals in *Boerhaavia diffusa* has tempted researchers to make the proposal that such efficacy evidenced in neuropharmacological assays may, in fact, be related to this synergistic effect where several compounds act together and complement each other's actions to afford improved therapeutic benefits. However, in order for *Boerhaavia diffusa* to be considered a potential medicine in contemporary medicine, further studies will be required to elucidate the establishment of actual concentrations, synergism, and pathways involved in its neuropharmacological action.

5. CONCLUSION

The present study on *Boerhaavia diffusa* Linn. indicates its immense potentiality regarding its multi-beneficial medicinal values, featuring a wide range of biological activities such as antioxidant, anticancer, anthelmintic, and neuropharmacological properties. Its high phytochemical content, containing active principles like polyphenols and flavonoids, also makes it very active against oxidative stress and thus helps maintain overall health. The anticancer activities observed in various studies further explain the plant's ability to inhibit tumor growth and induce apoptosis in tumor cells, which designates it as a promising candidate for developing natural cancer therapies. Additionally, efficacy against helminthic infections established the application of *Boerhaavia diffusa* in parasitic treatment methods, especially in areas where such diseases usually dominate. It thus exhibits neuropharmacological effects: the ability to improve mood and cognition, hence a role in mental health interventions. In summary, *Boerhaavia diffusa* wide assessment does call for further research into its action mechanisms and therapeutic use for its full incorporation into modern medicine.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This research followed all rules set forth by the US Food and Drug Administration, the Declaration of Helsinki, and the International Conference on Harmonization. Stamford University Bangladesh's Faculty of Science examined and accepted the research procedure and written consent form (reference number: SUB/ERC/202402).

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to the Laboratory of Pharmacology at the Stamford University Bangladesh and the Laboratories of Bangladesh Council of Scientific and Industrial Research for their invaluable assistance, allocation of resources, and provision of adequate infrastructure that were crucial for the successful implementation of this research trial.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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