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Pharmacological Investigations of *Boerhaavia diffusa Linn.* (Family: Nyctaginaceae)

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Abstract

Boerhaavia diffusa belonging to the family of the *Nyctaginaceae* is one of the renowned medicinal plants used to treat large number of human ailments. The present study deals with the investigations of phytochemical analysis and evaluation of the cytotoxocity, anthelmintic and analgesic activities of methanol extract of the plant. The results of preliminary phytochemical screenings revealed that the extract contains alkaloids, steroids, flavonoids, tannins, gums, carbohydrates and glycosides etc. The cytotoxic activity of plant extract on brine shrimp napulii increase in mortality with increased concentration which suggests the presence of possible antitumor, antibacterial and pesticidal agents. The anthelmintic activity was performed by observing the time of paralysis and the time of death of earthworms *Pheretima posthuma* compared with the standard drug, albendazole which confirmed the presence of significant anthelmintic activity of *B. diffusa*. The analgesic potential of the extract was evaluated using acetic acid induced writhing response, tail immersion and hot plate test to understand the central analgesic activity in mice (Swiss albino mice) at 200 mg/kg and 400 mg/kg doses. The results of these tests showed potential analgesic activity of the extract which is comparable to the standard drug, diclofenac Na. Thus, the obtained results provide a support for the use of this plant for medicinal purposes.

Keywords

Boerhaavia diffusa, Albendazole, Diclofenac Na, Cytotoxic Activity, Anthelmintic Activity, Analgesic Activity

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1. Introduction

Boerhaavia diffusa L. is an herbaceous perennial member of the family Nyctaginaceae. It is widely distributed in the tropics and subtropics. It has long-petioled broadly ovalate leaves, greenish purple, stiff, cylindrical stems and small white flowers on long stalks [1]. It has a long history of uses by indigenous and tribal people and in Ayurvedic or natural herbal medicines. The genus Boerhaavia has 40 species and is distributed in the tropical, subtropical, and temperate regions of the world. It is found in Australia, China, Egypt, Pakistan, Sudan, Sri Lanka, South Africa, USA and in several countries of the Middle East. *B. diffusa* is also indigenous to India. It is found throughout the warmer parts of the country upto an altitude of 2000 m in the Himalayan region. The plant is also cultivated to some extent in West Bengal. In Bangladesh the plant grows all over the country. It has many ethno-botanical uses (the leaves are used as vegetable; the root juice is used to cure asthma, urinary disorders, leucorrhoea, rheumatism and encephalitis) and is medicinally used in the traditional Ayurvedic system. Punarnava is an herb which is very useful for curing kidney diseases [2].

Various parts of the plant contain two quinolizidine alkaloids, punarnovine I and punarnovine II, xanthine derivatives, squalene, phytol, sitosterol, myricyl alcohol, ursolic, myristic and oxalic acids, tannins and potassium nitrate. Roots contain an anti-fibrinolytic agent, hentriacontane, ursolic acid, beta-sitosterol, aglycoside, hypoxanthine-9-L-arabinoside,

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stigmasterol, amino acids, a dihydro-isofuranoxanthrone, boerhavine and some rotenoids, boerhavinones A, B, C, D, E and F, repenone and repenol [3].

2. Materials and Methods

2.1. Chemicals

The following drugs and chemicals were used in the current study: Diclofenac Na (Square Pharmaceuticals Ltd., Bangladesh), Vincristine sulphate (Gedeon Richter), Albendazole (SK+F), Acetic acid, Methanol, DMSO (Merck, Germany) and Distilled water.

2.2. Plant Materials and Extraction

The fresh plants were collected from Dhaka, Bangladesh during September, 2014 and identified by the taxonomist of the Bangladesh National Herbarium, Mirpur, Dhaka. An accession number was given from there and a voucher specimen (DACB: 41278) has been deposited in the herbarium for future reference. The shade dried and powdered plant material (100gm) was soaked in 300ml of methanol for 10 days and then the extract was filtered through a cotton plug followed by Whatman filter paper number 1 and then concentrated by using a rotary evaporator at low temperature (40-50°C) and reduced pressure.

2.3. Animals

Artemia salina leach (brine shrimp eggs) were collected from an animal shop of Katabon, Dhaka. For anthelmintic test, *Pheretima posthuma* were collected from damp soil, Dhaka. Swiss albino mice (20-25g) were collected from Animal Resources Branch of the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). The animals were maintained under standard environmental conditions (temperature: 24.0±1.0°C, relative humidity: 55-65% and 12 hrs light /12 hrs dark cycle). The animals were fasted overnight before the experiments. All experimental protocols were approved by the Institutional Ethics Committee (SUB/TAEC/11.01) [4].

2.4. Drugs and Treatments

In brine shrimp lethality bioassay, vincristine sulphate and in anthelmintic activity test, albendazole is used as standard drug. For analgesic activity test, diclofenac Na (5mg/kg) in writhing test, diclofenac sodium (5mg/kg) in tail immersion and hot plate test is used as standard drug. Diclofenac Na was orally administered intraperitonially to the mice. The mice in control group received vehicle (water) at the dose of 0.1 ml/mouse 30minutes before experiments. Methanol extract of the plant was administered to the test groups 30 minutes before experiments at the doses of 200 and 400 mg/kg body weight in all cases for the determination of analgesic activities.

2.5. Phytochemical Screening

The methanolic extract of *B. diffusa* were tested for the detection of alkaloids, glycosides, carbohydrates, proteins, flavonoids, saponins, tannins and steroids with standard procedures [5].

Table 1. Procedure of Chemical Group Test.

Sample	Test solution	Observation
Test for Alkaloids:		
2ml solution of the extract and 0.2ml of dilute hydrochloric acid	0.1ml of Mayer's reagent.	Yellowish buff colored precipitate was obtained.
2ml solution of the extract and 0.2ml of dilute hydrochloric acid.	0.1ml of Dragendroff's reagent.	Orange brown precipitate was observed.
2ml solution of the extract and 0.2 ml of dilute hydrochloric acid.	0.1ml of iodine solution (Wagner's reagent).	Reddish brown precipitate was obtained.
2ml solution of the extract and 0.2ml of dilute hydrochloric acid.	0.1ml of picric acid solution (Hager's reagent).	Yellowish precipitate was obtained.
Test for Steroids: 10mg extract dissolved in 1ml chloroform.	1ml sulphuric acid.	Chloroform layer acquired reddish brown color and acid layer showed green fluorescence.
Tests for Flavonoids:		
10ml of solution of extract hydrolyzed with10% sulphuric acid. This was extracted with ether and divided into three portions.	a) 1ml dilute Ammonia solution. b) 1ml dilute sodium carbonate solution. c) 1ml dilute sodium hydroxide solution.	a) Greenish yellow color was obtained. b) Pale yellow color was obtained. c) Yellow color was obtained.
Tests for Reducing sugars: 5ml solution of extract.	5ml Fehling's A and B solution. Boiled for 5minutes on a boiling water bath.	Brick red colored precipitate was not observed.
5ml solution of extract.	5ml Benedict's reagent and boiled for 5minutes on a boiling water bath.	Brick red colored precipitate was not observed.
5ml solution of extract.	2drops of 5% alpha-Naphthol solution (Freshly prepared and added 1ml o f sulphuric acid on the sides of the test tube.)	Violet colored ring was not formed at the junction of two liquids.
Tests for Tannins: 5ml solution of extract.	1ml of 10% Lead acetate solution.	Yellow precipitate.
5ml solution of extract	1ml of 10%potassium dichromate solution.	Yellowish brown precipitate was not obtained.

Sample	Test solution	Observation
Test for Glycosides: Small amount of extract.	1ml of water and a few drops of sodium hydroxide solution	A yellow color was formed.
Test for Saponins: 1ml solution of the extract	Diluted to 20ml with distilled water	Shake during 15minutes and form 1centimeter foam layer.
Test for Carbohydrate: 2ml of extract	2 ml of conc. Sulphuric acid	Red or reddish violet ring was formed.

2.6. Pharmacological Investigations of Methanol Extract of *Boerhaavia diffusa*

2.6.1. Brine Shrimp Lethality Bioassay

Brine shrimp lethality bioassay technique stands superior to other cytotoxicity testing procedures because it is rapid in process, inexpensive and requires no special equipment or aseptic technique. It utilizes a large number of organisms for statistical validation and a relatively small amount of sample. Furthermore, unlike other methods, it does not require animal serum [6].

Brine shrimp eggs are hatched in simulated sea water to get nauplii. Test samples are prepared by dissolving in DMSO and by the addition of calculated amount of DMSO, desired concentration of the test sample is prepared. The nauplii are counted by visual inspection and are taken in vials containing 5ml of simulated sea water. Then samples of different concentrations are added to the remarked vials through micropipette. The vials are then left for 24 hours and then the nauplli are counted again to find out the cytotoxicity of the test agents.

2.6.2. Anthelmintic Activity Test

Fresh juice extract of B. *diffusa* were dissolved in minimum amount of DMF and the volume was adjusted to 10 ml with saline water. All drugs and extract solutions were freshly prepared before starting the experiment. In each case, 6 earthworms released into 10 ml of desired formulations as follows:

Vehicle (5% DMF in normal saline), Albendazole (25 mg/ml, 50 mg/ml, 100 mg/ml), Fresh juice extract of *B. diffusa* in normal saline solution containing 5% DMF (25 mg/ml, 50 mg/ml, 100 mg/ml). Observation was made for the time taken to paralysis and death of individual worm. Paralysis was said to occur when the worms were not able to move even in saline solution [7].

2.6.3. Acetic Acid-induced Writhing Test

Pain has been officially defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. Pain acts as a warning signal against disturbances of the body and has a proactive function [8].

The acetic acid induced writhing method is an analgesic behavioural observation assessment method that demonstrates a noxious stimulation in mice. The test consists of injecting the 0.7% acetic acid solution intraperitoneally and then observing the animal for specific contraction of body referred as 'writhing'. A comparison of writhing was made between standard (diclofenac Na), control and test sample given orally 30 minutes prior to acetic acid injection. Five minutes after the injection of acetic acid, the mice were observed and the number of writhing was counted for 10 minutes. If the sample possesses analgesic activity, the animal that received the sample, will give lower number of writhing than the control, i.e. the sample having analgesic activity will inhibit writhing [9].

2.6.4. Tail Immersion Test

The animals of the control, standard and test groups were treated with diclofenac Na (5 mg/kg body weight), water (0.10 ml/mouse) and test samples at the doses of 200 and 400 mg/kg body weight respectively. 1 to 2 cm of the tail of mice was immersed in warm water kept constant at 55°C. The reaction time was the time taken by the mice to deflect their tails. The latent period of the tail-flick response was taken as the index of antinociception and was determined at 0, 30, 60, 90 and 120 min after the administration of the test drugs and standard [10].

2.6.5. Hot Plate Test

The paws of mice are very sensitive to temperature at 55±0.5°C, which are not damaging to the skin. The animals were placed on Eddy's hot plate kept at a temperature of 55±0.5°C. A cut off period of 15 sec was observed to avoid damage to the paw. Reaction time was recorded when animals licked their fore or hind paws, or jumped at 0, 30, 60, 90 and 120 min after oral administration of the samples. The animals of test groups received test samples at the doses of 200 and 400 mg/kg body weight. Positive control group and vehicle control group were treated with diclofenac Na (5 mg/kg b.w.) and 1 in water (0.1 ml/mouse) respectively [11].

3. Results and Discussion

3.1. Phytochemical Screening

Preliminary phytochemical screening revealed that, the methanol extract of the whole plant contains alkaloids, tannins, glycosides, saponins and carbohydrates.

Table 2. Results of Different Chemical Group Test of the methanol extract of *B. diffusa.*

Extract	Chemical Groups	Results
Methanol extract of *Boerhaavia diffusa*	Alkaloids	+
	Steroids	-
	Flavonoids	-
	Reducing sugars	-
	Tannins	+
	Glycosides	+
	Saponins	+
	Carbohydrates	+

(+)=Present, (-) =Absent

3.2. Brine Shrimp Lethality Bioassay

Following the procedure of the lethality, the Methanol extract of *B. diffusa* to brine shrimp was determined and the summary expressed in a Table 3.

Table 3. Effects of vincristine sulphate and methanol plant extract on brine shrimp nauplii.

Conc.(C) (µg/ml)	LogC	%Mortality Methanol extract	LC_{50}(µg/ml) Methanol extract	Vincristine Sulfate			
				Conc.(C)(µg/ml)	LogC	%Mortality	LC_{50}(µg/ml)
400	2.602	100	0.25	40	1.602	100	0.812
200	2.301	100		20	1.301	100	
100	2	90		10	1.000	90	
50	1.699	90		5	0.698	80	
25	1.398	80		2.5	0.397	70	
12.5	1.097	70		1.25	0.096	50	
6.25	0.796	70		0.625	-0.204	40	
3.125	0.495	60		0.312	-0.505	30	
1.562	0.194	50		0.156	-0.806	30	
0.781	-0.107	30		0.078	-1.107	20	

The plant extract of *B. diffusa* showed potency to toxic activity against brine shrimp nauplli with LC_{50} value of 0.25 µg/ml when compared with the standard vincristine sulphate (0.812 µg/ml). Comparison with positive control vincristine sulphate the plant extract signifies potent cytotoxicity. The lethal concentration LC_{50} of the test samples after 24 hr. was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from data by means of regression analysis. The regression analysis data is given in the table 4.

Table 4. Regression analysis data for vincristine sulphate and plant extract.

Sample	LC_{50}(µg/ml)	Regression equation	R^2
Vincristine sulphate (Standard)	0.812	Y=33.219x+52.781	0.9717
Methanol extract of *B. diffusa*	0.25	Y=24.16x+43.85	0.940

3.3. Anthelmintic Activity Test

The results of the present study clearly indicated that *B. diffusa* produced anthelmintic activity against earthworm *P. posthuma*. The plant possesses moderate anthelmintic activity at 100 mg/ml concentration measured by time taken for paralyze/death of the earth worms.

Table 5. *In vitro* anthelmintic activity of *B. diffusa.*

Test Samples	Conc. (mg/ml)	Time Taken for Paralysis (minutes)	Time Taken for Death(minutes)
Albendazole	25	6	14
	50	5	11
	100	3	10
Fresh juice extract of leaves	25	14	18
	50	10	13
	100	5	8

3.4. Acetic Acid-induced Writhing Test

The analgesic effect of *B. diffusa* on acetic acid-induced writhing in mice was determined. The extract significantly inhibited writhing response in a dose dependent manner and was comparable to the reference drug, diclofenac Na.

Table 6. Effect of methanol extract of *B. diffusa* on acetic acid induced writhing test.

Treatment	Dose (mg/kg)	Mean±SE	%Writhing	%Inhibition
Control	0.1ml/mouse	40.60±2.130	100	0
Diclofenac Na	5	24.30±1.347	59.85	40.15
Group-1	200	31.60±1.427	77.83	22.17
Group-2	400	13.60±4.328	33.59	66.50

Values are expressed as Mean±SEM (n=5); [Control (water, 0.1 ml/mouse), Standard (diclofenac Na, 5mg/kg), Group1=200 mg/kg, Group2=400 mg/kg.]

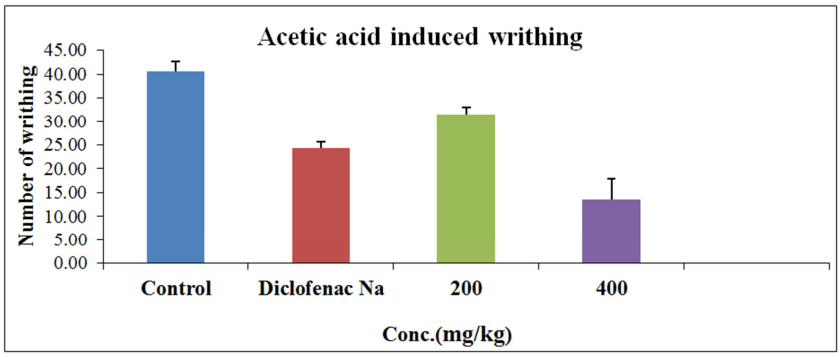

Figure 1. Bar diagram showing the result of writhing test for methanol extract of *B. diffusa*.

A dose dependent reduction in the number of abdominal constriction was observed in animals treated with different concentration of methanol extract of *B. diffusa* at the dose of 200 mg/kg and 400 mg/kg, inhibition of writhing response was observed 22.17% and 66.50% respectively for plant extract.

3.5. Tail Immersion Test

The analgesic activity test was carried out in the laboratory on five groups of mice by tail-immersion method. Time interval for the test was 30 minutes. The tail withdrawal reflex time after administration of the *B. diffusa* was found to increase with increasing dose of the extract.

Table 7. Effect of *B. diffusa* on tail withdrawal reflex in mice.

Treatment	Dose	Response Times (in Seconds)				
		0min	30min	60min	90min	120min
Control	0.1ml/mouse	4.60±0.40	4.40±0.40	6.40±2.462	3.60±0.60	4.60±1.536
Diclofenac Na	5mg/kg	1.60±0.40	2.80±0.735	5.00±1.761	2.00±0.316	7.60±2.960
Group-1	200mg/kg	1.80±0.583	3.00±1.761	1.60±0.40	1.80±0.374	2.00±0.775
Group-2	400mg/kg	1.60±0.4	3.60±0.510	5.20±1.908	2.80±0.583	2.80±0.583

Values are expressed as Mean± SEM (n=5); [Control (water, 0.1 ml/mouse), Standard (diclofenac Na, 5 mg/kg), Group 1=200 mg/kg, Group 2=400 mg/kg.]

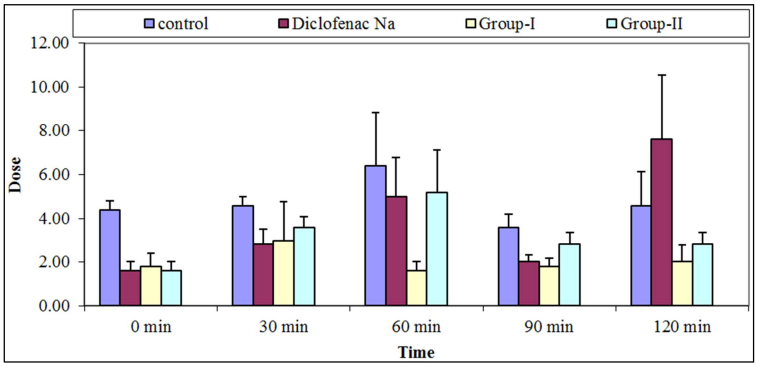

Figure 2. Effect of *B. diffusa* on tail withdrawal reflex in mice.

The tail-immersion test was widely used for assessing analgesic activity. In this experiment, *B. diffusa* exhibited higher analgesic activity in tail-immersion test. It seems possible that the higher doses of the extract have more potent analgesic effect.

3.6. Hot Plate Test

Table 8. Effect of *B. diffusa* on hot plate test.

Treatment	Dose	Response Times (in seconds)				
		0min	30min	60min	90min	120min
Control	0.1ml/mouse	5.60±0.245	7.00±1.095	6.80±2.396	7.40±2.293	5.60±1.435
Diclofenac Na	5mg/kg	8.20±1.428	3.80±0.917	5.00±1.761	4.40±0.872	7.20±2.059
Group-1	200mg/kg	6.60±1.60	5.60±0.678	5.20±1.150	5.40±1.661	5.40±0.748
Group-2	400mg/kg	5.60±0.927	6.00±0.548	5.60±0.872	7.60±2.015	5.40±1.536

Values are expressed as Mean±SEM (n=5); [Control (Water, 0.1 ml/mouse), Standard (diclofenac Na,5 mg/kg), Group1=200 mg/kg, Group2=400 mg/kg].

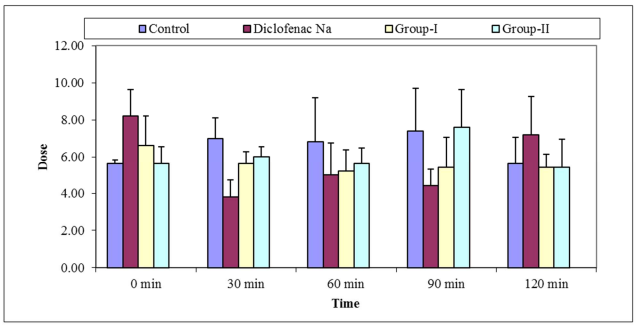

Figure 3. Effect of *B. diffusa* on hot plate test in mice.

The hot plate test was widely used for assessing central antinociceptic activity. In this experiment, *B. diffusa* exhibited higher analgesic activity than diclofenac Na in hot plate test. It seems possible that the higher doses of the extract have more potent analgesic effect.

4. Conclusion

Based on the result of the present study, it can be concluded that the crude plant extracts of *B. diffusa* possesses cytotoxic effect, anthelmintic activity and remarkable analgesic potential. Various phytochemical constituents like glycoside, alkaloid, tannin, saponin and carbohydrate present in the plant, as evident from phytochemical analysis. Significant cytotoxic activity can be suggested from the results of brine shrimp lethality bioassay. Anthelmintic activity test showed potent anthelmintic effect of the plant extract. The plant extract showed satisfactory analgesic properties compared to respective standard drugs. At higher dose, notable analgesic activity was observed from acetic acid induced writhing test, hot plate and tail immersion test. Dose dependant activity was also identified by all the performed pharmacological investigations.

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