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Phytochemical screening and *in vitro* bioactivities of the extracts of aerial part of *Boerhavia diffusa* Linn.

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ABSTRACT

Objective: To investigate the bioactivities of crude *n*-hexane, ethyl acetate and methanol extracts of aerial part of *Boerhavia diffusa* Linn. (*B. diffusa*) and its phytochemical analysis. **Methods:** The identification of phytoconstituents and assay of antioxidant, thrombolytic, cytotoxic, antimicrobial activities were conducted using specific standard *in vitro* procedures. **Results:** The results showed that the plant extracts were a rich source of phytoconstituents. Methanol extract showed higher antioxidant, thrombolytic activity and less cytotoxic activity than those of *n*-hexane and ethyl acetate extracts of *B. diffusa*. Among the bioactivities, antioxidant activity was the most notable compared to the positive control and thus could be a potential rich source of natural antioxidant. In case of antimicrobial screening, crude extracts of the plant showed remarkable antibacterial activity against tested microorganisms. All the extracts showed significant inhibitory activity against *Candida albicuns*, at a concentration of 1000 μg/disc. **Conclusions:** The present findings suggest that, the plant widely available in Bangladesh, could be a prominent source of medicinally important natural compounds.

1. Introduction

Boerhavia is a genus of 40 species[1], almost all of which are widely distributed in tropical and sub-tropical areas of Asia, Africa, America and Australia[2]. Among those species, *Boerhavia diffusa* Linn. (Synonym: *Boerhavia* glabrata Blume; Family: Nyctaginaceae) (*B. diffusa*) is a most widely studied plant and has a long history of uses by the indigenous & tribal people and in Ayurvedic and Unani medicines. In Ayurvedic and Unani, the miracle medicinal plant finds to use as a cure for 22 ailments. In Brazilian pharmacopeia, 23 uses have been described for the plant, while in Africa and Middle East, the plant is prescribed for 14 ailments[1].

The vernacular names of *B. diffusa* include Gondhapurna, Punarnava (Bengali, Sanskrit); Pigweed, Spreading hog-weed (English), *etc.*[2]. The plant is an abundant perennial creeping or climbing herb that exhibits somewhat periodic efficacy, with its maximum activity being noticed in the month of May (Summer)[3].

The root and the whole plant of *B. diffusa* are used in traditional medicine for the treatment of diabetes, stress, dyspepsia, abdominal pain, inflammation, jaundice, enlargement of spleen, heart diseases, bacterial infections[4,5] and impotence[2]. It has also been reported to be useful in the treatment of elephantiasis, night blindness, corneal ulcers, various hepatic disorders and as an antiviral agent[5,6]. In Nigerian folk medicine it has been widely used for the treatment of epilepsy[7], infertility and menstrual pain[8].

Pharmacological studies have demonstrated that *B. diffusa* known to possess anticonvulsant[4], diuretic, anti-inflammatory, antifibrinolytic[9], antibacterial[10], anti-hepatotoxic, anthelmintic, febrifuge, anti-leprosy, antiasthmatic, antiurethritis, antilymphoproliferative[11], antimetastatic[12], immunosuppressive[13], antidiabetic, antioxidant[14], immune-modulation[15], hepatoprotective[16], anti-nociceptive, nephroprotective[17], bacteria induced ulcer & diarrhea[18] and antiurolithiatic[19] activities.

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The main chemical ingredients of this plant include alkaloids (punarnavine), rotenoids (boeravinones A to J) and flavones[20].

A large number of research works on the phytochemistry, pharmacology and several other aspects have been conducted, but there have been no report on phytochemical screening and *in vitro* bioactivities of *B. diffusa*. collected from Bangladesh. So the present investigations were carried out to study the phytoconstituents and *in vitro* antioxidant, thrombolytic, cytotoxic and antimicrobial activities of *n*-hexane, ethyl acetate and methanol extracts of aerial part of *B. diffusa* available in Bangladesh.

2. Materials and methods

2.1. Plant collection and identification

The fresh aerial parts of the plant were collected from the surrounding of Sher-e-Bangla Agricultural University, Dhaka, Bangladesh during January, 2010 and identified by the taxonomist of the Bangladesh National Herbarium, Mirpur, Dhaka as *B. diffusa* Linn. A voucher specimen of the plant has been deposited (Accession No.: DACB 35440) in the herbarium for further reference.

2.2. Extraction of the plant material

Shade-dried and pulverized plant material (150 g × 3) was successively extracted with *n*-hexane (BDHE), ethyl acetate (BDEA) and methanol (BDME) by continuous hot extraction using Soxhlet apparatus at a temperature for 6 hours not exceeding the boiling points of the solvents. The extracts were concentrated with a rotary evaporator (IKA, Germany) at low temperature (40-50 ℃) and reduced pressure. The extracts (BDHE: 6.40 g, BDEA: 7.49 g, BDME: 8.83 g) were stored at 4 ℃ until used.

2.3. Phytochemical screening

The freshly prepared crude extracts of *B. diffusa* (BDHE, BDEA and BDME) were qualitatively tested for the presence of Alkaloids (Hager′s test), Flavonoids (Modified Ammonia test), Steroids (Salkowski test), Terpenoids (Modified Salkowski test), Reducing sugars (Fehling′s test), Saponins (Frothing test), Tannins ($FeCl_3$ test), Cardiac glycosides (Killer-Killani′s test) and Anthraquinones (Chloroform layer test)[21].

2.4. Determination of total phenolic content

The total phenolic content of the extracts were determined by using Folin-Ciocalteu reagent[22] using gallic acid as standard. The extracts were oxidized with 10% Folin-Ciocalteu reagent (Merck, Germany), and were neutralized with 700 mM sodium carbonate solution. The absorbance of the resulting blue color was measured at 765 nm after 60 minutes using UV-VIS spectrophotometer (Shimadzu, Japan). The total phenolic contents were determined from a standard curve prepared with gallic acid. The estimation of the phenolic compounds were carried out in triplicate and the results were expressed as mean±SD.

2.5. DPPH radical scavenging activity

The free-radical scavenging activity of *B. diffusa* extracts were measured by decrease in the absorbance of methanol solution of DPPH (2,2-Diphenyl-1-picrylhydrazyl)[23]. A stock solution of DPPH (400 μg/mL) (Sigma-Aldrich, USA) was prepared in methanol, which gave initial absorbance of 0.197, and 100 μL of this stock solution was added to 5 mL of solutions of *B. diffusa* extracts of different concentrations (20-100 μg/mL). The solutions were then mixed properly and kept in dark for 20 minutes and the absorbances weremeasured at 517 nm. Scavenging activity was expressed as the percentage inhibition calculated using the following formula:

$$\%\ \text{free radical scvanging activity} = \frac{\text{Absorbance of contrl} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Then % inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated. Ascorbic acid, a potential antioxidant was used as positive control.

2.6. Nitric oxide scavenging assay

Sodium nitroprusside (SNP) (5 mM) in phosphate buffer saline (PBS) was mixed with different concentration of extracts (5-200 μg/mL) of the plant dissolved in ethanol and incubated in dark at room temperature for 2 hours. 2 mL solution was withdrawn from the mixture and mixed with 1.2 mL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride and 2% *o*-phosphoric acid) and the absorbances of the solutions were measured at 546 nm using UV-VIS spectrophotometer against blank. Ascorbic acid was used as a positive control and was treated in the same way with Griess reagent[24, 25].

$$\text{Nitric oxide scavenged}\ (\%) = \frac{\text{Absorbance of contrl} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

SNP in aqueous solution at physiological pH (7.2) spontaneously generates $NO^{\bullet}$[24, 25], which interacts with oxygen to produce nitrate and nitrite ions that can be estimated by the use of Griess reagent. Antioxidants (scavengers of $NO^{\bullet}$) compete with oxygen leading to reduced production of nitrate and nitrite ions and a pink color chromophore is formed[24, 25].

2.7. In vitro thrombolytic activity

3 mL venous blood drawn from healthy volunteer was transferred to 5 pre-weighed sterile eppendorf tubes (500 μL/tube) and incubated at 37 ℃ for 45 minutes. After clot formation, serum was completely removed without disturbing the clot formed. Each tube having clot was again weighed to determine the clot weight (Clot weight = weight of clot containing tube − weight of tube alone). Each eppendorf tubes containing clot was properly labeled and 100 μL of BDHE, BDEA and BDME extracts (100 mg/mL) was added to the tubes separately[26, 27]. As a positive control, 100 μL of streptokinase (CSL Behring GmbH, Germany) (3000 000 IU/mL) and as a negative control, 100 μL of normal saline (0.9% NaCl) were separately added to the control tubes. All the tubes were then incubated at 37 ℃ for 90 minutes and observed for clot lysis. After incubation, fluid obtained was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was conducted on the blood samples of 10 volunteers (male=5; female=5) without a history of oral contraceptive or anticoagulant therapy[26, 27] since two weeks.

2.8. Cytotoxic activity

Brine shrimp lethality bioassay[28] was used for testing cytotoxic potential of the extracts. The eggs of Brine shrimp (*Artemia salina* Leach) were collected and hatched in a tank at a temperature ~37 ℃ and pH at 8.4 with continuous oxygen supply[29]. Two days were allowed to hatch and mature the nauplii. Stock solutions of the samples were prepared by dissolving required amount of extracts in specific volume of pure DMSO: dimethyl sulfoxide (Merck, Germany). 4 mL of seawater was given to each of the vials. Then specific volume of sample was transferred from the stock solution to the vials to get final sample concentrations of 50 to 400 μg/mL. In the control vials same volumes of DMSO (as in the sample vials) were taken as negative control and solutions of different concentrations of potassium dichromate was used as positive control[30]. Using a Pasteur pipette 10 living nauplii were put to each of the vials. After 24 h the vials were observed and the number of nauplii survived in each vial was counted. After that, the percentage of lethality of brine shrimp nauplii was calculated for each concentration of the extracts.

2.9. Antimicrobial assay

The antibacterial activity was carried out by the disc diffusion method[31] using 100 μL of suspension containing ~10^3 CFU/mL of microorganism spread on nutrient agar medium (Himedia, India). Dried and sterilized filter paper discs (6 mm diameter), impregnated with 500 and 1 000 μg of BDHE, BDEA and BDME extracts, were placed gently on the previously marked zones in the agar plates. Standard disc (Himedia, India) of ciprofloxacin (5 μg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. After incubation at 37 ℃ for 24 hours, the antimicrobial activity of the test agents were determined by measuring the diameter of zone of inhibition expressed in mm.

2.10. Statistical analysis

Statistical comparisons were performed with Student's *t* tests using Microsoft Excel 2007. A P value of 0.05 and 0.001 or less was considered to be significant. Mean values ± S.D. were calculated for the parameters where applicable.

3. Results

3.1. Phytochemical screening

Preliminary phytochemical screening showed (Table 1) the presence or absence of alkaloids, flavonoids, steroids, terpenoids, reducing sugars, saponins, tannins, cardiac glycosides, anthraquinones in varying amount in the *B. diffusa* extracts.

Table 1
Phytochemical compositions of *B. diffusa* extracts.

Phytoconstituents	Name of the test	BDHE	BDEA	BDME
Alkaloids	Hager's test	+++	+	+++
Anthraquinones	Chloroform layer test	++	++	+++
Cardiac glycosides	Killer-Killani's test	+	−	−
Flavonoids	Ammonia test (modified)	++	+	+++
Reducing sugars	Fehling's test	−	−	++
Saponins	Frothing test	+++	+++	−
Steroids	Salkowski test	++	−	−
Tannins	$FeCl_3$ test	−	+++	++
Terpenoids	Salkowski test (modified)	++	−	++

+++: highly present; ++: moderately present; +: slightly present; −: absent.

3.2. Total phenolic content

The total phenolic content of the *B. diffusa* extracts were expressed as gallic acid equivalent and are presented in Table 2. Among the three extracts, the ethyl acetate extract showed the highest amount of phenolic compounds followed by the methanol extract and the *n*-hexane extract.

Table 2
Total phenolic content of extracts of *B. diffusa* (in mg/g, Gallic acid equivalents).

Extracts	Total phenolic content
BDHE	37.32±4.98
BDEA	192.67±2.52
BDME	163.14±1.95

Values are represented as mean±SD with triplicate estimation.

3.3. DPPH radical scavenging activity

From the analyses of Figure 1, it can be concluded that the scavenging effect of *B. diffusa* extracts increases as the concentration increases. The methanol extract of *B. diffusa* (BDME) showed (Figure 1 & Table 3) highest radical scavenging capacity followed by *n*-hexane (BDHE) and ethyl acetate (BDEA) extracts.

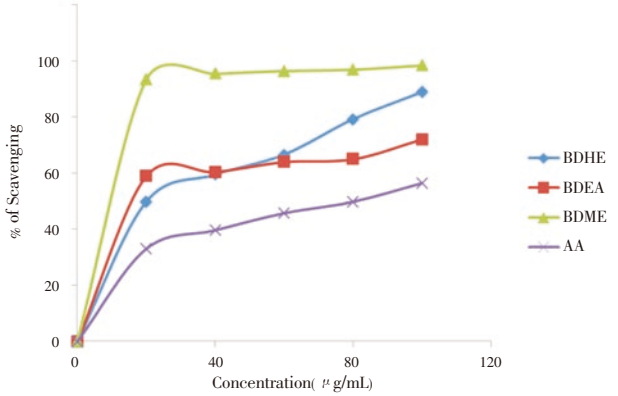

Figure 1. DPPH scavenging activity of the BDHE, BDEA and BDME extracts of *B. diffusa*.

Table 3
Antioxidant activity of the *B. diffusa* extracts (BDHE, BDEA and BDME) and ascorbic acid (AA).

Extracts/Positive control	IC_{50} (μg/mL)	
	DPPH radical scavenging activity	Nitric oxide scavenging assay
BDHE	40.61	132.47
BDEA	43.81	102.30
BDME	8.18	95.16
AA	76.11	34.06

3.4. Nitric oxide scavenging assay

The scavenging of $NO^{\bullet}$ by the extracts was increased in dose dependent manner (Figure 2). Table 3 illustrates a significant $NO^{\bullet}$ scavenging ability of extracts and ascorbic acid. The *n*-hexane, ethyl acetate and methanol extracts showed maximum activity of 67.71%, 76.00% and 71.08% respectively at 200 μg/mL, where as ascorbic acid at the same concentration exhibited 84.38% inhibition (Figure 2).

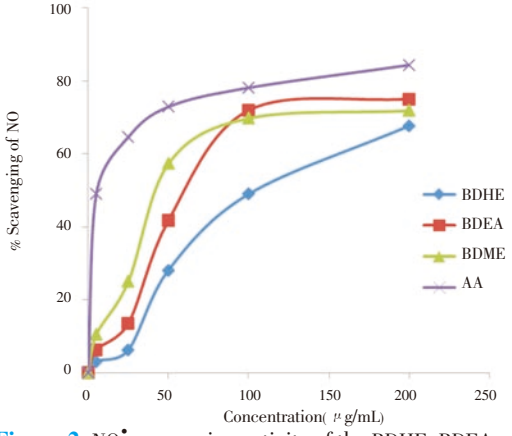

Figure 2. $NO^{\bullet}$ scavenging activity of the BDHE, BDEA and BDME extracts of *B. diffusa*.

3.5. Thrombolytic activity

Percent clot lysis obtained after treating the clots with different extracts and controls is shown in Table 4. In this study, BDME and streptokinase (positive control) exerts statistically significant thrombolytic activity ($P<0.001$) compared to negative control (normal saline). Thrombolytic activity showed by BDME and streptokinase were 10.26% and 40.40% respectively. After treatment of clots with 100 μL of BDHE and BDEA negligible clot lysis were obtained but mean of percentage of clot lysis was higher than negative control.

Table 4
Effect of *B. diffusa* extracts and controls on *in vitro* clot lysis.

Extracts/Control	Mean±SD (% Clot lysis)
BDHE	6.60±2.37**
BDEA	7.12±2.39**
BDME	10.26±2.06***
Streptokinase	40.40±5.32***
Negative control (normal saline)	4.58±0.83

Values are expressed in mean±SD ($n=10$). ***$P<0.001$ and **$P<0.05$ when compared with negative control (normal saline).

3.6. Cytotoxic activity

In brine shrimp lethality bioassay, percentage of mortality increased gradually with the increase in concentration of the test samples. From the results (Table 5) it was revealed that BDHE gave maximum cytotoxicity with the lower LC_{50} value of 140.55 μg/mL. In comparison to positive control (KDC: Potassium dichromate), the cytotoxic potentiality exhibited by *B. diffusa* extracts were very negligible.

Table 5
LC_{50} value of extracts of aerial part of *B. diffusa* and potassium dichromate (KDC).

Samples	LC_{50} (μg/mL)	95% Confidence interval	
		Upper limit	Lower limit
BDHE	140.55±2.08	142.90	138.20
BDEA	141.89±1.16	143.20	140.57
BDME	163.75±2.20	166.24	161.27
KDC	13.23±0.87	14.22	12.24

Values are represented as mean±SD of three observations in each experimental set.

3.7. Antimicrobial assay

Antimicrobial activities of the *B. diffusa* extracts were tested against three pathogenic organisms and the results are presented in Table 6. In the antimicrobial screening, the extracts showed average zone of inhibition ranging from 0-9.77 mm at concentration 500 μg/disc to 1 000 μg/disc. No zone was noticed against the growth of tested microorganisms at concentration 500 μg/disc except BDME against *S. aureus*.

Table 6
Zone of inhibition of *n*-hexane (BDHE), ethyl acetate (BDEA), methanol (BDME) extracts of *B. diffusa* aerial part, positive control ciprofloxacin (CP) and negative control (respective solvents) (*n*=3).

Name of microorganisms	Types	Negative control	BDHE		BDEA		BDME		CP
			500 μg/disc	1000 μg/disc	500 μg/disc	1000 μg/disc	500 μg/disc	1000 μg/disc	(5 μg/disc)
S. aureus	Bacteria: gram(+)ve	–	–	–	–	–	7.47±0.15	9.77±0.25	42.67±2.31
S. dysenteriae	Bacteria: gram(–)ve	–	–	–	–	–	–	7.43±0.25	38.67±2.52
C. albicans	Fungus	–	–	7.33±0.15	–	7.70±0.20	–	7.90±0.10	49.33±1.53

Values are expressed as mean±SD. " – " Indicates no zone of inhibition.

The concentration at 1000 μg/disc, showed remarkable effects against all of the tested microorganisms.

4. Discussion

Previous studies reported the presence of alkaloids, tannins, carbohydrates, saponins, glycosides, proteins and amino acids, phytosterols, phenolic compounds, flavonoids and terpenoids in *B. diffusa*.[5, 18] The present study also correlated with the aforesaid studies. Presence of varieties of chemical compounds impart significant amount of biological activities of *B. diffusa* extracts.

Based on the scavenging capacity of the free radicals (DPPH, $NO^{\bullet}$), the highest antioxidant activity was found in BDME. This is due to the presence of flavonoids, which are polyphenolic compounds[32, 33], in the methanol extract of the plant. The results agree with the findings of the other researchers[34] in which the antioxidant activity of methanol extract of *B. diffusa* was reported due to the presence of rotenoids (boeravinone G, H and D). Among the rotenoids, boeravinone G plays the major role which is also a polyphenolic compound[34-39].

Although *B. diffusa* contains antifibrinolytic punarnavoside[9], methanol extract of the plant showed significant ($P<0.001$) thrombolytic activity compared negative control. The phytochemical analysis showed that the BDME was a rich source of alkaloids, flavonoids, tannins and anthraquinones. These compounds could participate for its clot lysis activity[26].

From the results (higher LC_{50} values than 100 μg/mL) of brine shrimp lethality bioassay, it can be concluded that the extracts of *B. diffusa* did not show any apparent *in vitro* toxicity compared to positive control[26]. It supports the use of the plant as a non-toxic leafy vegetable by the tribes[40]. Moreover, the vegetable was found as a rich source of some macro and micronutrients by the researchers[40]. Large amount of alkaloids present in the plant are also non-carcinogenic.

The antimicrobial study indicated that the crude extracts of *B. diffusa* showed better antibacterial activities at higher concentrations against the tested microorganism. This may be due to the insufficient concentration of antimicrobial constituents in the solvent extracts. The finding of zone of inhibition was found higher than a study reported by other researchers[5]. Probably it is the result of the variation of collection place & time and extraction process of the plant. *B. diffusa* extracts showed significant antifungal activity, which may be related with the presence of anthraquinones. Anthraquinone and its derivatives were reported to have antifungal activities[41].

The organic solvent extracts of aerial part of *B. diffusa* available in Bangladesh is a very good source of phytochemicals and showed marked *in vitro* bioactivities which can offer remedies to some of the common ailments ranging from common cold to complex pathological disorders. Studies are in progress in the laboratory to unveil the active components responsible for the bioactivities of the plant with high medicinal value.

Conflict of interest statement

We declare that we have no conflict of interest.

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