

Phytochemical Screening and Biological Studies of *Boerhavia Diffusa* Linn

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Abstract

Hexane, ethyl acetate and methanol extracts of whole plant of *Boerhavia diffusa* were screened for phytochemical and biological activities. Qualitative phytochemical screening via colorimetric method and the quantitative estimation of phenolic and flavonoid content were performed. Antioxidant assay using DPPH scavenging method was studied. Antimicrobial screening of plant extracts was done by cup diffusion technique. Cytotoxic activity of *B. diffusa* was studied by brine shrimp bioassay and anthelmintic activity was evaluated in vitro in *Pheretima posthuma*. This study revealed *B. diffusa* as a source of various phyto-constituents such as alkaloids, glycosides, saponins, tannins, carbohydrates, cardiac glycosides, flavonoids and terpenoids. Quantitative estimation of total phenol was found to be maximum in BEE i.e. 29.73±0.88, BME 19.8±2.02 and in BHE 9.15±0.304 mgGAE/g. Similarly, the total flavonoid content was found to be 17.44±0.75 in BEE, 14.43±0.23 in BHE and 3.678 mg QE/g in BME. Ethyl acetate extract showed its antibacterial activity against all tested pathogens except *Escherichia coli* whereas *Staphylococcus aureus* and *Salmonella Typhi* were resistant to methanol and hexane extract. The zone of inhibition (ZOI) of ethyl acetate extract against *S. Typhi* and *B. cereus* was found to be 18 mm and 14 mm respectively. The MIC value of BEE in *S. Typhi* was 3.125 µg/ml and in *B. cereus* was 12.5 µg/ml. The preliminary screening of anticancer property of *B. diffusa* i.e. BSLT in methanol was found to be 165.19 µg/ml. *B. diffusa* was also found to contain anthelmintic property. The study helped in further exploration of medicinal properties of *B. diffusa* by phytochemical screening and biological activities paving the path for study and investigation in this plant.

Keywords: *Boerhavia Diffusa*, DPPH, Qualitative Phytochemical Screening, Brine Shrimp Bioassay, Anthelmintic Activity

1. Introduction

Plants and plant-based medicaments are the basis for modern pharmaceuticals used today for various ailments. Pharmacotherapy started with the use of plant in the treatment of various diseases^[1]. Generally, the herbal formulations use fresh or dried plant parts. One fourth of the world population is dependent on the traditional medicines, particularly plant drug for curing the ailments. According to WHO, approximately 80% of the

world's population rely on traditional medicine, and aromatic plants (MAPs)^[2].

B. diffusa also known as red spiderling, has a long history in traditional medicine. It is used to cure broad range of ailments. Major parts of traditional therapy involve the use of the plant extracts and their active chemical constituents. The exploration of chemical constituents, pharmacological and phytochemical screening of the plant extract would provide a basis for the development of the new lead molecules in the strategic favor, for the discovery of drugs^[3]. In addition, the knowledge about the chemical constituents would be valuable in discovering the actual value of folkloric remedies. The in vivo lethality in a simple zoological organism, such as the brine shrimp, can be used as a simple tool for the screening and fractionation of physiologically active plant extracts^[4]. Toxicology can simply be understood as pharmacology at a higher dose, thus a toxic compound with low toxic dose might elicit a useful phar-

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macological, perturbation in physiological system^[5]. Excessive formation of free radicals, either reactive oxygen species or reactive nitrogen species plays a crucial role in ageing, neuron-degeneration, and damage in bio-molecules like DNA, lipids and proteins resulting in various pathological conditions^[6].

Chemical control of helminthes coupled with improved management has been an important worm control strategy throughout the world. However, development of resistance in GI nematodes and helminthes against conventional/allopathic anti-helminthic drug is a foremost problem in treatment of helminthic diseases. Therefore, it is important to study the alternative strategies against gastrointestinal nematodes. In addition, allopathic drugs are likely to possess side effects, toxicity, increased cost^[7] and non-adaptability, which have increased the need to evaluate the natural products that can replace current strategies to control GI nematodes^[8]. Despite of tremendous advancement in human medicine, infectious diseases caused by bacteria, fungi, and parasites have been major threat to public health. The efficacy of antibiotics and other chemotherapeutics agent which is discovered till date is being threatened by the emergence of multidrug resistance microbes^[9]. Antibiotic resistance has been a major concern between scientist and clinician worldwide. The failure of antibiotic treatment against the microbes has led to the screening and the discovery of new antimicrobial compounds from medicinal plants with diverse chemical structure due to their potential antimicrobial activity. Therefore, there is constant need for new products to manage numerous infections^[10].

2. Materials and Methods

2.1. Sample Collection and Extraction

The plant was collected from Saljhandi VDC, Rupen-dehi district, Nepal. It was cut into pieces and shade dried at room temperature. Dried sample was crushed into powder by electric blender. *B. diffusa* was extracted at 40°C in n-hexane, 40-45°C in ethyl acetate and 40°C in methanol. The extract was evaporated in rotatory evaporator at 40°C under the vacuum; a solid mass was obtained and refrigerated at 4°C for further analysis. Total ash value and percentage yield of extract was calculated to determine the purity and quality of crude drug.

2.2. Test Animals and Pathogens

Laboratory-breed of *Artemia nauplii* was used for Brine shrimp assay. Locally available *P. posthuma* (earthworm) was used for antihelmenthics test. *S. aureus* (ATCC 25923), *B. cereus* (clinical isolation), *S. Typhi* (clinical isolation) and *E. coli* (ATCC 25922) were used for antimicrobial test. Antimicrobial testing was done using cup diffusion technique^[11].

2.3. Chemicals, Reagents and Standard Drugs

DPPH (1, 1-Diphenyl-2-picrylhydrazyl) from Sigma Chemical Co. Ltd, (St. Louis, MO, USA), Albendazole and 50 µg/mL ofloxacin were used (Time Pharmaceuticals, Nepal). All the chemicals and reagents used were of analytical grade.

2.4. Phytochemical Screening

The qualitative phytochemical screening was done to characterize the main groups of chemical constituents present in different extracts of *B. diffusa* by their color reactions with different color reagents. Total phenolic content was studied using Folin–Ciocalteu procedure^[12]. TPC values were determined from a calibration curve prepared with a series of Gallic acid standards. Total flavonoid content was measured using Aluminium Trichloride colorimetric assay^[13]. The flavonoid concentration was expressed in milligrams of Quercetin equivalent per gram of dry weight (mg of QE/g) of extract.

2.5. Biological Studies

The plant extract were studied for antioxidant property, anti-microbial activity, and brine shrimp lethality bioassay and anthelmintics activity.

2.6. Antioxidant Activity Test

Stable free radical DPPH(1, 1-Diphenyl-2-picrylhydrazyl) scavenging method was applied for the estimation of antioxidant activity of the hexane, ethyl acetate and methanol plant extract^[11]. The following equation was used to calculate the capability to scavenge the DPPH radical.

$$\text{Percentage scavenging} = \frac{(A_o - A_s)}{A_o} \times 100$$

A_o = absorbance of DPPH radical.

A_s = absorbance of test or reference sample.

The percentage scavenging was then plotted against concentration and regression equation was obtained to calculate IC₅₀ (micro molar concentration required to inhibit DPPH radical formation by 50%). The experiment was conducted in triplicate.

2.7. Minimum Inhibitory Concentration (MIC) Determination

Lowest concentration of the antibiotic that inhibits the growth of organism is known as MIC. Tube dilution technique was used to determine MIC values^[14]. 50 ml of nutrient broth was prepared in distilled water. 1 ml of nutrient broth was added to 11 test-tube and sterilized by autoclaving. Test samples were prepared by adding dilutions of the ethyl acetate extract (100 mg/mL) to the series of broth. MIC values were determined on *S. Typhi* and *B. cereus*. A positive control with 1ml of nutrient broth and organism and a negative control with only 1 ml of nutrient broth were taken. 1 ml of the test extract was added to test-tube labeled as 1 and thoroughly mixed with nutrient broth present in it. From test tube number 1 ml of solution was drawn and added to test tube labelled as 2. Similar dilution was performed up to tube no 11 and remaining 1 ml was discarded. 50 µL of organisms was added to each test tube except for negative control. All the test-tubes were incubated at 37°C for 24 hours. After incubation all, the tubes were observed for growth/ turbidity. MIC value was then determined.

2.8. Brine Shrimp Bioassay

The brine shrimp bioassay is based on the determination of the concentration of sample, which kills 50% of laboratory-breed of *A. nauplii* within 24 hours under a specified condition. This concentration is known as LC₅₀ and is expressed in µg/mL^[4].

The percent (%) mortality of brine shrimp nauplii was calculated using the following formula:

$$\text{Mortality} = \frac{N_t}{N_o} \times 100$$

Where, N_t = Number of killed nauplii after 24 hrs. of incubation, N_o = Number of total nauplii transferred i.e. 10. The LC₅₀ (Median lethal concentration) was then determined using regression analysis. LC₅₀ values > 1000 µg/mL (non-toxic), $\geq 500 \leq 1000$ µg/mL (weak toxicity) and < 500 µg/mL (toxic)^[4].

2.9. Anti – helminthic Activity

Anti-helminthic activity of plant extract was determined by experimenting in *P. posthuma* found in moist soil^[15,16]. Albendazole sample was prepared in DMSO solvent in three different concentrations (25 mg/mL, 50 mg/mL and 100 mg/mL) and administered to earthworm placed in petri plates. The plant extracts was also prepared using DMSO solvents of three different concentrations as albendazole. The duplicate test was carried out. The hexane, ethyl acetate, methanol extracts of *B. diffusa* and albendazole were tested in 3 doses (25 mg/mL, 50 mg/mL and 100 mg/mL) in each group. Test was carried by forming four groups including one control and three dosage groups. Approximately equal weight of earthworm in each group was used in this study. Time taken for the paralysis and death of individual earthworm up to four hours of test period was observed. Time of paralysis and death were noted when there was no movement after vigorous shake or poking by sharp object.

3. Results and Discussion

After extraction, 2.6%, 8.35% and 7.98% yield value was obtained with hexane, ethyl acetate and methanol solvents respectively. Total ash value of plant material was found to be 11%.

3.1. Qualitative Phytochemical Screening

The phytochemical screening of hexane extract of *B. diffusa* showed the presence of alkaloids, flavonoids, carbohydrates and cardiac glycosides and ethyl acetate extract have alkaloids, tannins, saponins, carbohydrate and cardiac glycoside (Table 1). The methanol extract showed the maximum number of phyto-constituents such as alkaloid, terpenoids, tannins, glycosides, cardiac glycosides and carbohydrates. Protein was absent in all extracts in this study. These differences can be attributed to the solubility of the bioactive substances in different solvent and degree of polarity^[17]. The literature review of preliminary phytochemical screening of alcoholic aerial and roots extracts of *B. diffusa* showed the presence of proteins, saponins, alkaloids, flavonoids, carbohydrates and tannins^[18]. However, the compounds in different solvent extract varied from the previous study. This may be due to geographical and climatic variations. The phytochemical test, executed in this study has

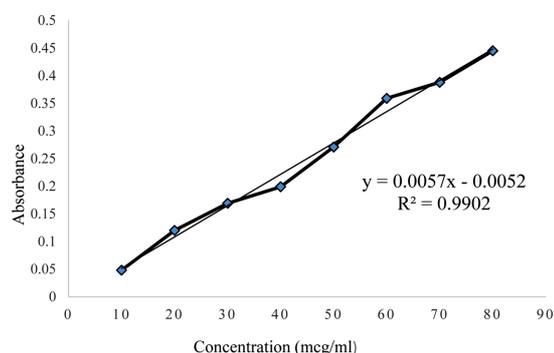
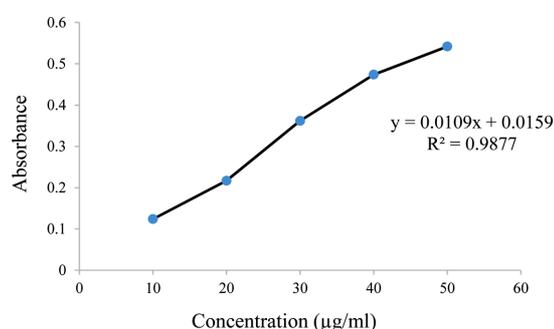
Table 1. Phytochemical screening of extracts of *Boerhavia diffusa* (+ = presence, - = absence)

| Phytochemicals | Hexane extract | Ethyl acetate extract | Methanol extract |
|--------------------|----------------|-----------------------|------------------|
| Alkaloids | + | + | + |
| Tannins | - | + | + |
| Saponins | - | + | - |
| Glycosides | - | - | + |
| Terpenoids | - | - | + |
| Flavonoids | + | + | + |
| Carbohydrate | + | + | + |
| Cardiac glycosides | + | + | + |

comprehensively validated its therapeutic importance. Various secondary metabolites have been implicated to exhibit a wide range of biological activities such as alkaloids exhibit antibacterial property, saponin and glycoside exhibit cytotoxic effect^[19].

3.2. Total Polyphenol and Flavonoid Content Determination

Calculation of total polyphenol content of the extracts was done by using the calibration curve of standard gallic acid (Fig. 1). Similarly, total flavonoid content was determined by using standard quercetin calibration curve (Fig. 2) and Table 2 represents total flavonoid content and total phenolic content of three different extracts. Phenolic and flavonoid compounds are in unique category of phytochemicals especially in terms of their vast health benefiting properties^[20]. The amount of total phenol of *B. diffusa* was measured by FC reagents and UV-spectrophotometer. The content varied in different extracts and ranged from 9.15±0.304 in hexane, 19.8±2.02 in methanol and 29.73±0.88 mgGAE/g in ethyl acetate. Similarly the amount of total flavonoids also varied in different extract and ranged from 17.44±0.75 in ethyl acetate, 14.43±0.23 in hexane, and 3.678±0 mgQE/g in methanol. The TPC and TFC were

**Fig. 1.** Calibration curve of Gallic acid.**Fig. 2.** Calibration curve of Quercetin.

found to be higher in ethyl acetate extract compared to hexane and methanol extract. TPC and TFC determination of whole plant of *B. diffusa* in previous study was not found but the study of different parts of plant such as leaves, stem and root as well as aerial part of *B. diffusa* were found. Hexane, ethyl acetate and methanol extract of aerial part of test plant TPC was 37.32, 192.67±2.52 and 163.14±1.95 respectively^[19]. Maximum TPC and TFC were recorded in methanol leaves extract and lowest TPC and TFC in methanol root extract^[21].

3.3. Antioxidant Activity Test

The IC₅₀ value of ethyl acetate extract was lowest

Table 2. TPC, TFC and IC₅₀ value of different extract of *Boerhavia diffusa*

| Parameters | Methanol extract | Hexane extract | Ethyl acetate extract |
|-----------------------------------|------------------|----------------|-----------------------|
| Total phenolic content (mg GAE/g) | 19.8±2.02 | 9.15±0.304 | 29.73±0.88 |
| Total flavonoid content (mg QE/g) | 3.678±0.00 | 14.43±0.23 | 17.44±0.75 |
| IC ₅₀ (µg/ml) | 235.47 | 224.68 | 198.07 |

compared to other extracts in this study i.e. the radical scavenging property is higher in crude ethyl acetate extract of *B. diffusa*. The IC_{50} value of ethyl acetate, hexane and methanol was found to be 198.07 $\mu\text{g/mL}$, 224.68 $\mu\text{g/mL}$ and 235.47 $\mu\text{g/mL}$ respectively (Table 2). Antioxidant properties of whole plant of *B. diffusa* was not found in previous literature, but the test on methanol extract of different parts of plant such as leaves, stem and root was found to be carried out separately. Antioxidant activity decreased from stem to root i.e. stem possess highest antioxidant activity ($90.8 \pm 2.275 \mu\text{g/mL}$) while roots showed lowest antioxidant activity of $398.03 \pm 43 \mu\text{g/mL}$ ^[21].

3.4 Antimicrobial Activity and MIC Determination

The result of antimicrobial activity was measured in terms of the zone of inhibition in millimeters (mm). The maximum zone of inhibition was seen in *S. Typhi* i.e. 18mm (subtracting diameter of well) by crude ethyl acetate extract. The zone of inhibition was significant in other pathogens as well. The antimicrobial activity of aerial and roots of *B. diffusa* was tested in 4 different pathogens (Table 3). Better zone of inhibition by ethyl acetate extract was obtained in *S. Typhi* i.e. 18 mm. The extracts were not effective against *E. coli*. Ethyl acetate extract showed comparable zone of inhibition with positive control against all *S. Typhi*, *B.cereus* and *S. aureus* though the extract was taken in higher concentration. In the previous research work, the aerial parts of plant material showed no zone of inhibition in 0.5 mg/ml and 1 mg/ml in ethyl acetate extract. Thus, the study indicated the crude extracts of *B. diffusa* have a better antibacterial activity against the tested microorganisms. Aqueous extracts of aerial part of *B. diffusa* showed no activity in *S. aureus*, *S. Typhi*, *B. cereus*, *E. coli* i.e. aqueous extract was not indicative for antibacterial properties^[22]. The MIC on *S. Typhi* and *B. cereus* were determined to be 3.125 mg/mL and 12.5 mg/mL respectively.

Table 3. Antimicrobial activity of different extracts *Boerhavia diffusa*

| Organisms | Zone of inhibition (mm) | | | Positive control |
|------------------------------|-------------------------|----------------|-----------------------|------------------|
| | Methanol extract | Hexane extract | Ethyl acetate extract | |
| <i>Staphylococcus aureus</i> | 13 | 12 | 11 | 19 |
| <i>Bacillus cereus</i> | 9 | 11 | 14 | 16 |
| <i>Salmonella typhi</i> | 16 | 14 | 18 | 18 |
| <i>Escherichia coli</i> | 0 | 0 | 0 | 19 |

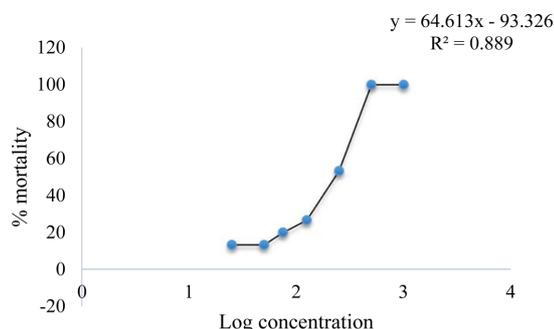


Fig. 3. Brine shrimp lethality assay of crude methanol extract of *Boerhavia diffusa*.

3.5. Brine Shrimp Bioassay

The brine shrimp lethality assay was carried out in methanol extract (Fig. 3) to study cytotoxic effect of *B. diffusa*. The LC_{50} was found to be 165.19 $\mu\text{g/mL}$. Cytotoxicity was evaluated in terms of LC_{50} value^[17]. The 50% lethality of the crude plant extract was greater than 100 ppm to Brine shrimp. Thus, its use as a traditional medicine in alleviating cancers treatment such as in abdominal cancer is justified. The LC_{50} value of methanol extract in aerial part was found to be $163.75 \pm 2.20 \mu\text{g/mL}$ ^[19]. The result obtained is similar from the previous study.

3.6. Evaluation of Anti-helminthic Activity

The anti-helminthic activity of different extract of *B. diffusa* was measured by paralysis and death time. It was found to be dose dependent. The ethyl acetate extract showed potent anthelmintic activity showing least time for paralysis and death of earthworm followed by methanol extract and hexane extract. Fig. 4 shows paralysis time (pt) and death time (dt) (in minutes) of different extract of *B.diffusa* and albendazole at 25, 50, and 100 mg/mL of concentration. The data obtained revealed that the ethyl acetate extract showed significant anthelmintic activity at all the doses tested (25 mg/mL, 50 mg/mL, and 100 mg/mL). Ethyl acetate

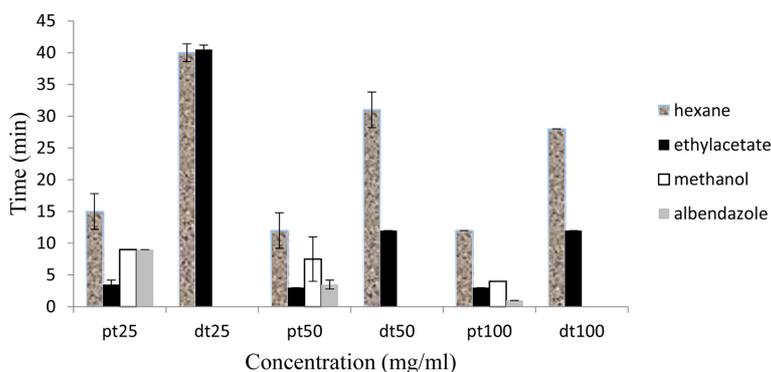


Fig. 4. Anthelmintic activity of *Boerhavia diffusa* at different concentration.

extract shows potent anthelmintic activity with least paralysis and death time i.e. paralysis time in 3.5 ± 0.7 , 3 and 3 minutes with 40.5, 12 and 12 minutes death time at 25, 50, and 100 mg/mL respectively. The anthelmintic activity of ethyl acetate extract was remarkable followed by methanol and hexane extract with least time for paralysis of worms. The paralysis time of earthworm in albendazole was found to be 9 ± 0 , 3.5 ± 0.7 , 1 minute in 25, 50, and 100 mg/mL of doses. Since, standard drug (albendazole) was insoluble in solvent DMSO in which it was prepared and drug was not evenly distributed during test, the data obtained from anthelmintic test of standard drug could not be used to compare with extracts. As well as, in the 3 hours of our observation period earthworm in methanolic extract and albendazole were paralyzed in all doses. However, between 3-12 hours the test organism were dead which was beyond our observation period. Therefore, the lethal/death time in methanol extract and albendazole could not be recorded. Overall, previous research work^[16] as well as the current investigation justifies the traditional use of plant in worm infestations which leads to conclusion that *B. diffusa* possess promising bioactive compounds that might be useful in the control of helminthic infections

4. Conclusion

The methanolic extract of *B. diffusa* contained maximum amount of phyto-constituents. The ethyl acetate extract was found to be effective as antimicrobial, anthelmintic activity followed by methanol and hexane extract. *E. coli* was found to be resistant to all of the

extracts. *S. aureus* and *S. Typhi* were resistant to hexane and methanol. The present study gave the preliminary scientific evidence for its traditional use as antibacterial and anthelmintic. Different from the previous literature, the result obtained in antioxidant activity test was not significant in this study. In the brine shrimp bioassay, crude methanol extract of *B. diffusa* showed moderate toxicity. Thus, it has laid sufficient background for the further study in future for identification, subsequent purification and isolation of compounds for the antibacterial and anthelmintic activities. This study has helped in establishing scientific evidence in rationality of traditional use of plants.

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Abbreviations

| | |
|-----|---|
| AA | Ascorbic Acid |
| BEE | <i>Boerhavia diffusa</i> Ethylacetate Extract |

| | |
|------|---|
| BHE | <i>Boerhavia diffusa</i> Hexane Extract |
| BME | <i>Boerhavia diffusa</i> Methanol Extract |
| BSLT | Brine Shrimp Lethality Test |
| DMSO | Dimethyl Sulfoxide |
| DPPH | 1,1-diphenyl-2-picrylhydrazyl |
| dt | Death time |
| FC | Folin-Ciocalteu |
| GAE | Gallic Acid Equivalent |
| IC | Inhibitory Concentration |
| LC | Lethal Concentration |
| MAPs | Medicinal and Aromatic Plants |
| MIC | Minimum Inhibitory Concentration |
| NS | Normal Saline |
| pt | Paralysis time |
| QE | Quercetin Equivalent |
| TM | Traditional Medicine |
| TFC | Total Flavonoid Content |
| TPC | Total Phenolic Content |
| WHO | World Health Organization |
| ZOI | Zone Of Inhibition |