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Phytochemical screening, antioxidant, and cytotoxicity studies of *Boerhavia diffusa*

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Abstract

Cervical cancer occurs more in females than males and needs more attention to treat the disease. Owing to the side effects by treatments in current use, scientists are working effortlessly in search of alternative medicines. Plants are a rich source of medicinally active substances that can perform significant pharmacological activities. So we aimed to analyse *Boerhavia diffusa* (BD) plant for its medicinal uses. Crude ethanolic extract of BD was analysed for phytochemical screening, antioxidant, and cytotoxicity assays. Phytochemical screening showed the presence of flavonoids, tannins, saponins, glycosides, amino acids, terpenoids, phenols, anthraquinones, steroids, alkaloids, and carbohydrates. *In vitro* antioxidant activity by DPPH assay at varying concentrations of 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, and 100 µg/ml showed percentage inhibition values of 8.4%, 13.8%, 26.1%, 30.7%, and 41%, respectively. Similarly, the reducing power assay revealed the percentage inhibition values as 25.8%, 48.3%, 66.7%, 75.8%, and 87.8% for the corresponding concentrations. Comparison of the IC_{50} values of BD extract obtained from both the assays (DPPH-19.1 µg/ml; reducing power-55µg/ml) revealed its strong antioxidant property as that of the standard (DPPH-21.3 µg/ml; reducing power-57.5 µg/ml). Cytotoxicity tests by MTT assay with various concentrations against SiHa cell line showed the best cell viability value of 47.1 % at 125 µg/ml concentration. The phytochemicals present in BD are responsible for imparting antioxidant and cytotoxic properties. Further studies might be useful to obtain key insights for exploring this plant as a contemporary medicine against cervical cancer.

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Introduction

Cervical cancer, the most recurrent disease, is seen in females (Adesina, 1979). It is the second leading disease among women across the globe, accounting for an expected 529,409 cases newly diagnosed and 274,883 deaths in 2008 (Adeyemi *et al.*, 2008). It is found to be the most recurrent malignancy that is seen in multiple regions of India in women (Ahmed-Belkacem *et al.*, 2007). India accounts for a fifth of the 500,000 additional cervical cancer incidences observed throughout each year (Alvarez-Salas and DiPaolo, 2007). This condition is caused by a virus named Human papillomavirus more specifically by HPV 16 and HPV 18 (Jeena *et al.*, 2023).

Evaluating for and eliminating precancerous lesions and immunisations can help reduce high death rates (Aviello *et al.*, 2011). Typical treatments, including chemotherapy and radiation, can harm both malignant cells and healthy cells, leading to worse reactions such as vomiting, complications in bone marrow, diminished hair growth, and lack of energy (Baskaran *et al.*, 2011).

Preventive treatments are yet to be discovered to overcome these situations. Herbal remedies are being investigated for treatment but need to demonstrate outcomes in laboratory and clinical studies. Plants containing active compounds are a significant source of healthcare benefits.

Boerhavia diffusa L., otherwise known as punarnava, is used as medicine widely in India. Punarnava encompasses punarnavoside, which displays antibacterial effects (Berrington and Lall, 2012), and anticonvulsant (Bhalla *et al.*, 1968) and antifibrinolytic (Bhalodiya *et al.*, 2020) properties. Researchers have proved that this particular plant extract has the ability to mitigate pain and reduce inflammation (Desai *et al.*, 2008; Feresin *et al.*, 2002), safe-guard the liver (Flay and Matthews, 1995), and regulate the immune system (Gacche and Dhole, 2006). Boeravinone G and boeravinone H, two rotenoids found in BD, have been shown to effectively block the expulsion potential of a multidrug transporter, which is BCRP/ABCG2 (commonly known as breast cancer resistance protein), that causes cancer cells to resist chemotherapy (Ghani, 2003).

The present research is aimed at identifying the presence of phytochemical substances present in the ethanolic extract of *Boerhavia diffusa* and assessing its antioxidant property by using DPPH and reducing power assays. Further, to explore the cytotoxic property of the plant, an MTT assay against the SiHa cervical cancer cell line was carried out.

Materials and methods

Collection and extraction of plant

The fresh plants of *Boerhavia diffusa* were obtained from an agricultural land in Salem, Tamil Nadu. The whole plant was used for the study. The plant was authenticated by the Senior Agricultural Officer, Dr. V. Balasubramanian, at TANU in Coimbatore, Tamil Nadu.

The plants were cleaned and dried in the dark for a couple of weeks and were ground to a fine powder. Using ethanol as a solvent, the crude extract of *B. diffusa* ET (BD ET) was prepared by using a hot continuous Soxhlet apparatus. The filtered extract was subjected to evaporation in a rotary evaporator and stored in a refrigerator.

Phytochemical screening

The freshly prepared crude extract of *B. diffusa* ET was screened qualitatively for the presence of tannins, alkaloids, saponins, flavonoids, terpenoids, anthraquinones, steroids, glycosides, amino acids, phenols, and carbohydrates (Hiruma-Lima *et al.*, 2000).

Antioxidant assay

BD extracts' free-radical scavenging ability was measured by reducing the absorbance of a methanol solution containing DPPH (2,2-Diphenyl-1-picrylhydrazyl) (Jain and Khanna, 1989). To the microtiter plate, an IO ET extraction sample of 100 µL was placed and 100 µL of 0.1% methanolic DPPH was introduced and kept for half an hour. Strong and

weak positive results were interpreted based on the discolouration (purple, yellow, and pale pink). With the help of an ELISA plate reader, the absorbance of the sample was monitored at 490 nm. (Reference: ascorbic acid). The whole process was conducted thrice, and the obtained values were calculated. The IC_{50} value was computed.

DPPH scavenging (in percentage) = [(blank's absorbance–sample's absorbance)/(blank's absorbance)] × 100.

Reducing power assay

The protocol followed by Oyaizu (1986) was used to determine the reducing power in the sample (Beegum *et al.*, 2014). The extract was treated with 2.5 ml of each 1% K_3 [Fe (CN) $_6$] and Na_3PO_4 buffer (200 mmol/l). After 20 minutes, 10% TCA (w/v) (2.5 ml) was added to the solution and centrifuged.

Next, to the top layer, ferric chloride was added with distilled water. The optical density of the preparation was read (700 nm). Increased reducing power was indicated by increased absorption. The tests were conducted in triplicate, with ascorbic acid serving as the standard. The IC_{50} value was calculated for each concentration.

Percentage of reducing power = [(Sample absorbance–Blank absorbance)/ (Blank absorbance)] × 100.

Cell viability assay

The MTT assay was employed to study the effect of ethanolic extract of BD on SiHa cells.

Initially, SiHa cell lines were cultivated in DMEM together with 10% FBS, 100 µg/ml penicillin, and 100 µg/ml streptomycin. Starting with, the cells were maintained in specific conditions (37°C, 5% CO_2), trypsinised and mixed. 200 microlitres of 1×10^5 cells/mL were kept in a 96-well microplate and incubated for 24 hours at 37°C in 5% CO_2. The cells were treated with BD ethanolic extracts at various concentrations of 20 µg/ml to 100 µg/ml for 48 hrs. Then the medium was aspirated, and the MTT solution (220 µL) was mixed and kept for 4 hours at 37°C in CO_2 incubator. Then washed cells with 1X PBS (200 µL) after discarding the MTT solution. The crystals were then dissolved with 100 µL of DMSO. The optical density of the test was found (570 nm) by means of a microplate reader. The percentage cell inhibition and half-maximal inhibitory concentration (IC_{50}) values were derived (Kumar and Pandey, 2013).

Cell inhibition (%) = (Control's O.D.-Test's O.D./ Control's O.D.) × 100

Results

Qualitative phytochemical analysis of our study involving ethanolic extract of *B. diffusa* resulted in the presence of all the phytoconstituents under study, namely tannins, alkaloids, saponins, flavonoids, terpenoids, anthraquinones, steroids, glycosides, amino acids, phenols, and carbohydrates, as shown in Table 1.

Table 1. Preliminary phytochemical screening analysis of *Boerhavia diffusa*

Sl	Test	Ethanol extract of *B. diffusa*
1.	Tannins	Positive
2.	Alkaloids	Positive
3.	Saponins	Positive
4.	Flavonoids	Positive
5.	Terpenoids	Positive
6.	Anthraquinones	Positive
7.	Steroids	Positive
8.	Glycosides	Positive
9.	Amino Acids	Positive
10.	Phenols	Positive
11.	Carbohydrates	Positive

In the present analysis, the in vitro antioxidant activity of the plant extract by the DPPH assay that was carried out in varying concentrations that ranged from 20 µg/ml to 100 µg/ml showed the percentage inhibition values of 8.4%, 13.8%, 26.1%, 30.7%, and 41%, respectively, which were nearly equal to the standard inhibition values as shown in Table 2. The scavenging action of BD extract increased with its concentration. The results proved the radical scavenging efficiency of the plant extract was similar to that of the standard.

Table 2. *In vitro* antioxidant activity of *B. diffusa* by DPPH assay

Test	Concentration µg/ml	% Inhibition of Extract	% Inhibition of standard
DPPH	20	8.4	8.7
	40	13.8	14.2
	60	26.1	26.4
	80	30.7	31.0
	100	41.0	43.7

In vitro reducing power assay of *B. diffusa* ET extract with concentrations ranging from 20 µg/ml to 100 µg/ml showed the percentage inhibition values of 25.8%, 48.3%, 66.7%, 75.8%, and 87.8%, which were comparable with standard inhibition values (26.7%, 49.4%, 69.8%, 76.7%, and 88.6%, respectively). The results are presented in Table 3. The high antioxidant activity was due to the presence of phytochemical compounds present in the ethanol extract of the plant.

Table 3. *In vitro* antioxidant activity of *B. diffusa* by reducing power assay

Test	Concentration µg/ml	% Inhibition of Extract	% Inhibition of standard
Reducing power assay	20	25.8	26.7
	40	48.3	49.4
	60	66.7	69.8
	80	75.8	76.7
	100	87.8	88.6

In comparison with the standard IC_{50} value of 19.1 µg/ml, the DPPH assay of the *B. diffusa* ethanolic extract showed IC_{50} of 21.3 µg/ml. Similarly, in comparison with the standard IC_{50} value of 55 µg/ml, reducing power assay showed IC_{50} value of 57.5 µg/ml. The results are given in Table 4.

Table 4. Antioxidant IC_{50} values of *B. diffusa* and standard

Sl Test	IC_{50} Value µg/ml	
	Extract	Standard
1. DPPH	19.1	21.3
2. Reducing power assay	55	57.5

In the present in vitro anticancer research, varying amounts of BD ethanolic extract that ranged between 1000 µg/ml and 7.8 µg/ml showed cell viability of 3.7%, 20.7%, 35.8%, 47.1%, 62.2%, 75.4%, 86.7%, and 94.3% for respective concentrations (Table 5).

Table 5. Percentage cell inhibition of BD ET extract against SiHa cell line

Sl	Concentration (µg/ml)	Dilutions	Absorbance (O.D.)	Cell viability (%)
1.	1000	Neat	0.02	3.7
2.	500	1:1	0.11	20.7
3.	250	1:2	0.19	35.8
4.	125	1:4	0.25	47.1
5.	62.5	1:8	0.33	62.2
6.	31.2	1:16	0.40	75.4
7.	15.6	1:32	0.46	86.7
8.	7.8	1:64	0.50	94.3
9.	Cell control	-	0.53	100

Discussion

Cancer therapies that are in current use have dismal results that necessitate the identification of the best solutions. Scientists are always working to produce effective chemotherapies. The plants' secondary metabolites have the potential to function as potent medicinal substances (Leyon *et al.*, 2005).

Medicinal plants include a variety of pharmacologically active secondary metabolites, including polyphenolic chemicals like flavonoids, biflavonols, and phenols, as well as nitrogen compounds like alkaloids, which have been shown to have substantial antioxidant action (Liu *et al.*, 1997). A study of phytochemicals on *B. diffusa* by Rakhi Srivastava *et al.*, 2011 detected the presence of phenolic chemicals, specifically alkaloids and amino acids, which have been shown to have substantial antioxidant capabilities. It also has quinolizidine alkaloids and potassium salts (Manikandan *et al.*, 2016).

According to Velu *et al.*, 2018 phytochemicals are responsible for the plant's pharmacological actions (Manu and Kuttan, 2007). Flavonoids can reduce reactive oxygen species and prevent oxidative stress due to their high antioxidant activity (Mehrotra *et al.*, 2002). Anjali Jeena *et al.,* 2023 investigated phytochemicals in the leaves of methanolic extract of *B. diffusa* and inferred that the flavonoids and phenols as major constituents (Mehrotra *et al.*, 2002). Bhalodiya *et al.,* 2020 reported the presence of phenols, flavonoids, and tannins in various extracts of *B. diffusa* (Mfengwana *et al.,* 2019).

Many investigators isolated phenols, carbohydrates, saponins, phytosterols, tannins, proteins, alkaloids, terpenoids and glycosides in *B. diffusa* plant extracts (Middleton *et al.*, 2000; Mosmann, 1983).

A research study that performed qualitative phytochemical screening using ethanolic extract of *B. diffusa* showed the presence of phenols, tannins, saponins, steroids, cardiac glycosides, alkaloids, terpenoids, and flavonoids (Olukoya *et al.*, 1993).

Ethanolic extracts were commonly employed for anticancer screening because conventional practitioners believed that polar chemicals were primarily responsible for the reported anticancer effects. Mehrotra *et al.,* 2002 found that the ethanolic extract of *B. diffusa* has substantial antiproliferative and immunosuppressive action (Oseni *et al.*, 2024).

It is well recognised that a wide range of clinical symptoms are caused by free radicals produced during metabolic activities. Natural antioxidants are highly powerful at halting the negative effects of free radicals, whether they are present as raw extracts or as their chemical constituents (Oyaizu, 1986). Our body's chemical reactions produce oxygen free radicals that destroy cellular DNA and biological membranes, leading to growing older and persistent degenerative illnesses. Oxidative stress, caused by an imbalance between prooxidants and antioxidants, might impact the use of dietary or pharmacological supplements.

Substances that can function against free radicals lessen the risk of long-term illnesses including cancer (Pandey and Shukla, 2008). Three benzene rings and an unpaired electron of centred nitrogen make up the synthetic free radical known as DPPH that has the highest UV uptake. Antioxidants with reducing power interact with an unpaired electron of DPPH and turn colour from purple to yellow. This results in lowered absorbance at 517 nm (Priyadarsini *et al.*, 2009).

Earlier studies confirmed that the presence of flavonoids and other bioactive compounds in the plant is responsible for high antioxidant activity (Rachh *et al.*, 2009; Srivastava *et al.*, 2011; Rawat *et al.*, 1997). Anjali Jeena *et al.,* 2023 demonstrated the strong antioxidant activity of *B. diffusa* in methanolic leaf extract by Ferric reducing power assay and Ascorbate-Iron (III)-catalyzed phospholipids peroxidation (Mehrotra *et al.*, 2002). In recent years, the antioxidant potential was investigated by applying DPPH, FRAP, and ABTS assays on BD of various extracts, and the methanolic extract had the maximum antioxidant capacity (Mfengwana *et al.*, 2019). The researchers found a strong connection between antioxidant activity and phenolic levels.

Juna Beegum *et al.*, 2014 also examined the antioxidant property of various extracts of BD and showed remarkable radical scavenging power by ethanolic extract by comparing it with other extracts and standard (Oyaizu, 1986). Gacche and Dhole (2006) showed the exhibition of IC_{50} value of 0.21 mg/mL by a 50% ethanolic extract of *B. diffusa*. A research study demonstrated the ability of different concentrations of *B. diffusa* extract to search for free radicals and degrade it's function along with enhanced reducing power in the presence of BHA. Ethanolic preparations of *B. diffusa* were found to be lethal (50 µg/mL) versus Vero cell lines (Sharma, 2001). Rachh *et al.*, 2009 highlighted the excellent antioxidant characteristics of alcoholic extracts of BD roots when compared to ascorbic acid (Sharma *et al.*, 2009).

Assays inferring the number of viable cells by compounds present in extracts are important to gain insights into the performance of cells. In the MTT assay, active cells convert water-soluble MTT to an insoluble purple formazan (Sreeja and Sreeja, 2009). The cytotoxic activity of ethanolic root extract of BD and its purified portion (BDF 5) was examined against U 87 (Glioma), Hep 3B, HCT 15, and NIH 3T3 cell lines. The extract was found to be highly effective against the HeLa cell. Mehrotra *et al.,* showed the reduction in PBMC proliferation by BD ethanolic root extract when exposed to PHA, Con-A, and PPD antigens (Oseni *et al.*, 2024).

The scientists also identified that the inhibition of multiple cell lines such as lymphoma and leukaemia in both mice and humans. Srivastava *et al.*, 2011 used bioassays to the fractionation of a 95% alcoholic extract of *B. diffusa* root, resulting in the lethality of 30% of HeLa cells (Sridhar, 2001).

Purification by column chromatography yielded a more effective portion, generating 85% and 55% cell lethal in a period of 72 and 24 hours, respectively, at 300 µg/mL. Sreeja and Sreeja (2009) highlighted that BD plant methanolic extract lowered the longevity of cells by 46.8% in 48 hours at 320µg/mL, indicating antiproliferative and antiestrogenic properties.

The researchers have proved that *B. diffusa* methanol extract can block the cancerous progression in B16F10 melanoma cells (Stanley, 2010). An alkaloid, Punarnavine, produced from BD, has been demonstrated to increase the immune reaction in response to metastatic spread of melanoma cells (B16F-10) in mice (Gnanavel *et al.*, 2018). Eupalitin-3-O-β-d-galactopyranoside (Bd-1) obtained and purified from the leaves of BD ethanolic extract shows a selective immunosuppressive effect (Zhang and Tsao, 2016).

Conclusion

Cervical cancer is one of the most common prevalence cancer in women. Plants remain an extensive source for treating a wide array of diseases, including cancer. Here, the ethanol extract of *B. diffusa* is enriched with various biochemical compounds that can exert pharmacological properties. The plant extract showed potent antioxidant activity, possessing significant radical scavenging power against free radicals causing oxidative damage.

Furthermore, the present study suggests the potent cytotoxic effect of the plant *B. diffusa* ET extract against the SiHa cell line that can be attributed to the biochemical compounds present in it. Further studies involving characterization of the determined compounds and in vivo studies are essential to use this medicinal plant against cervical cancer.

Recommendation

A study can be done on the cytotoxic activities of the ethanolic extract of *Boerhavia diffusa* against the MCF-7 and MDA-MB-231 cell lines of breast cancer.

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