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Original Article

Validation of the Impact of Polyphenol-Rich Extracts on the Pro-Oxidant, Antibiotic Modulating, and Urobactericidal Properties of *Boerhavia diffusa* L. Root: A Uroprotective Ethnomedicine

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ABSTRACT

Background and Aim: The *Boerhavia diffusa* L. root is traditionally used by tribal communities in Odisha, particularly in the Ganjam district, to manage urinary tract infections, liver ailments, and inflammatory conditions. However, its therapeutic potential remains underexplored scientifically. This study aims to validate the antioxidant potential of different solvent (n-hexane, chloroform, acetone, and methanol) extracts of *B. diffusa* root, mainly focusing on the urobactericidal activity of the polyphenol-rich methanolic extract of *B. diffusa* root (BDR-ME) against three uropathogenic bacteria (*Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus*) isolated from patients with urinary tract infection in Ganjam district, thereby validating its local ethnomedicinal use. **Materials and Methods:** Polyphenolic compound assessment and antioxidant assays were performed, and the results were used for Principal Component Analysis (PCA) and correlation analysis. The results revealed that the methanolic fraction had a higher antioxidant potential than the other extracts. Hence, further antibacterial tests were conducted with BDR-ME. **Results:** The polyphenol-rich BDR-ME has a significant correlation between antioxidant and urobactericidal activity. Though BDR-ME was effective against all three urobacterial strains, it had the highest growth inhibitory effect against *P. aeruginosa* in multiple assays. *P. aeruginosa* was resistant to Ampicillin (AMP), Cefixime (CFM), and Nitrofurantoin (NIT), but BDR-ME exhibited complete synergism with these antibiotics, resulting in a substantial effect against *P. aeruginosa*. **Conclusion:** The above findings suggest that the *B. diffusa* root has substantial potential in combination therapy against multidrug-resistant bacteria. Therefore, it can be proposed that the roots of *B. diffusa* may be prescribed as a suitable nutraceutical and biopharmaceutical source of natural antioxidants and antimicrobial agents for augmenting urinary and renal wellbeing in humans.

Keywords: Antibiotic modulatory activity, Antioxidant, Phytochemical screening, Urobactericidal activity, Uropathogenic bacteria, Urinary tract infections.

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INTRODUCTION

Infectious diseases are substantially contributing to mortality and morbidity globally. Pathogens are becoming resistant to conventional treatments, which requires effective, alternative, and complementary future therapeutics (Alaoui Mdarhri *et al*., 2022). The Aboriginal people in developing countries, often located far from modern healthcare facilities, frequently face challenges in combating such threats and frequently seek help from traditional remedies. Although multiple local remedies are available and used to address such conditions, their scientific validation often remains scarce and is often undermined. Plant-based remedies have tremendous healing potential among all natural therapies, as they have abundant, multifarious antimicrobial chemical agents (Cheesman *et al*., 2017). Medicinal plants offer promising, accessible, and culturally acceptable alternatives for managing infectious diseases.

Urinary Tract Infections (UTIs) are highly prevalent in Odisha. UTI cases were reported to be 45.2% among rural females, 41.74% overall in a tertiary hospital, and 47% among pregnant women, highlighting a significant regional health burden (Dash *et al*., 2013). Both gram-positive and gram-negative bacteria are involved in causing the infections. Among them, widely known Gram-negative strains, such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Citrobacter freundii, Enterobacter aerogenes*, and *Acinetobacter*

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baumannii, are major contributors. A few Gram-positive strains, including *Staphylococcus aureus* and *Enterococcus faecalis*, also serve as disease-causing agents. UTIs are often self-limiting, yet can lead to serious health complications if left untreated. A neglected UTI can lead to sepsis, a life-threatening sequel of postinfection. Sepsis can cause tissue damage, organ failure, and death if not promptly treated. Individuals may experience rapid heart rate, confusion, and high fever (Flores-Mireles *et al*., 2015). The bacteria can also ascend from the bladder to the kidney and impair or damage renal function. Selective antibiotics such as Fosfomycin, Nitrofurantoin, and Fluoroquinolones (e.g., Ciprofloxacin, Levofloxacin) are effective in treating the diseases as they damage bacterial DNA or inhibit cell wall synthesis, or inhibit DNA gyrase and topoisomerase IV (Gardiner *et al*., 2019). Overuse of antibiotics can lead to the development of antibiotic-resistant bacteria. This resistance makes infection more complex to treat and increases the risk of complications. Here comes the role of naturally available compounds in increasing the potency of relevant drugs.

Natural products play a crucial role in the development of therapeutic drugs for treating various human ailments. New drug development is a time-consuming, expensive, and complex process in pharmaceutics (Katiyar *et al*., 2012). However, plant-based drugs are most acclaimed and used because of their abundance, diversity, low cost, and easy accessibility. For generations, the plant parts have been used as food and medicines (Chaachouay and Zidane, 2024). Roots especially have special demands, being the main reservoir of bioactive phytoconstituents. Buried underground, these are least affected by environmental parameters, except for soil conditions, and contain notable phytocompounds that can act against pathogens. They are often loaded with multiple therapeutic components and comparatively more trustworthy as nutraceuticals.

Boerhavia diffusa L., commonly known as *Punarnava* in Sanskrit, is a traditional medicinal plant widely recognised for its uroprotective and renoprotective activities. Ethnomedicinal reports document its extensive use in managing renal and urinary disorders. According to ancient Ayurvedic literature, Charaka, the eminent Indian physician, prescribed the root decoction of *B. diffusa* for the treatment of oedema and kidney stones. In Unani (Tibbi) medicine, it has been used to treat urethritis. The alkaloids of *B. diffusa* constitute its major active principles, which are potent diuretics. They act on the renal epithelium and glomeruli to enhance urine flow. Traditionally, the root paste and oil have also been applied externally for wound healing and the treatment of infections (Nadkarni, 1954). The aborigines of Brazil used the root decoction as a diuretic, for urinary disorders like nephritis, polyuria, albuminuria, gallstones, and urinary retention (Cruz, 1995). Hence, the present study was undertaken to explore its urobactericidal activity.

The roots of *B. diffusa* are rich in diverse phytochemicals with varied pharmacological activities and are widely used in Ayurveda and other traditional systems of medicine (Mishra *et al*., 2014). In the southern region of Odisha, this plant is popularly known as "*Atikapodi*," and its roots are traditionally used by aboriginal communities for their diverse therapeutic benefits. This plant is often used in traditional medicine for the treatment of bacterial infections, dyspepsia, abdominal pain, inflammation (Bairwa and Jachak, 2015), spleen enlargement, cardiac diseases (Prathapan *et al*., 2017), stress (Desai *et al*., 2011), diabetes (Pari and Satheesh, 2004), and impotence due to its multifaceted phytoconstituents (Gaur *et al*., 2022). The rotenoid derivatives, such as Boerhavinone G, have shown potent antioxidant and genoprotective effects, including inhibition of ROS formation and protection against DNA damage (Akhter *et al*., 2013; Aviello *et al*., 2011). *B. diffusa* root extract exhibited antibacterial activity against uropathogenic strains (Ilangage *et al*., 2024). However, no such implication of polyphenolic compounds found in sequentially extracted solvents was analysed, which are responsible for curing UTIs. Plant-based bioactive compounds are used as alternatives to conventional antimicrobial agents. Different solvent extractions have revealed varying levels of efficacy in isolating biologically active compounds, with methanol and water often yielding promising results (Kaviya *et al*., 2022). *B. diffusa* roots are traditionally used in Odisha for urinary and liver issues, but lack scientific validation. This study investigates the effectiveness of root extracts collected from Ganjam, Odisha, against local urobacterial strains isolated from patients visiting Maharaja Krushna Chandra Gajapati (MKCG) Hospital, providing scientific evidence for their traditional use.

While the pharmacological and phytochemical benefits of *B. diffusa* have been widely studied, the present study offers a methodical approach by employing a modified retrospective extraction method to systematically compare its solvent fractions, ranging from non-polar to polar. Emphasis was placed on the final methanolic polar fraction (rich in polyphenols), assessing its antioxidant properties that lead to urobactericidal potential. This focused analysis, especially in the context of microbial resistance and oxidative stress within the Ganjam district ecosystem, provides new insights into the therapeutic relevance of *B. diffusa* root compounds.

MATERIALS AND METHODS

Description of the study area

Ganjam is one of the districts in South Odisha, about 142 km south of Bhubaneswar, Odisha. It has the latitude and longitude of 19º17 53.00" N and 84º52'40.00" E with an elevation between 380-383 meters (800 feet) above sea level.

Plant sample collection and authentication

The plant roots were collected from the areas where they grew luxuriously in the Ganjam district, especially from the Berhampur

University campus area, during October to December 2022, following the specific guidelines (Wondafrash, 2008). A plant specimen was identified by Prof. Malaya Kumar Mishra (senior taxonomist) and deposited in the herbarium collection of the Department of Botany, Berhampur University, Odisha, bearing voucher no. BOTBU2307.

Extraction of phytoconstituents

Fresh roots of *Boerhavia diffusa* were collected, thoroughly washed with tap water to remove adhering soil and debris, and shade-dried at room temperature (24-30℃) for 10-12 days until a constant weight was achieved. The air-dried roots of *B. diffusa* (1.5 kg) were ground using a mechanical grinder and passed through a 40-mesh sieve (particle size ~0.4 mm). Then, powdered samples were successively extracted with n-hexane, chloroform, acetone, and methanol (400 mL × 3, 72 hr each) using the Soxhlet apparatus, with the solvents chosen to reflect increasing polarity, abbreviated as BDR-HE, BDR-CE, BDR-AE, and BDR-ME, respectively. Each fraction of the plant extracts was then concentrated using a rotary evaporator under reduced pressure, scraped, and dried in a warm metal mantle using a desiccator to remove the solvent residue. The dried crude extracts were stored at 4°C in a desiccator until further use.

Estimation of yield percentage

The crude extract's final percentage yield was calculated using a modified formula for yield calculation, with minor adjustments to the standard formula.

$$\text{Yield (\%)} = F/S \times 100,$$

Where F=The weight of the extract in grams

S=The weight of the initial dried sample in grams

Experimental design

The investigation evaluated the phytochemical constituents and antioxidant activity of different solvent extracts of *B. diffusa* root (BDR-HE, BDR-CE, BDR-AE, and BDR-ME), urobactericidal and antibiotic modulatory efficacies of BDR-ME against clinically isolated uropathogens. The experiments were conducted in a systematic manner involving multiple stages as described below:

Preparation of the sample for phytochemical tests

These crude extracts were dissolved in lukewarm water or methanol to prepare the required working concentrations of the different extracts, depending on the type of study (phytochemical/antibacterial tests).

Preliminary qualitative phytochemical screening

Preliminary phytochemical qualitative analysis of the extract was carried out for the different solvent fractions of *B. diffusa* root and screened for the presence or absence of biologically active compounds using standard lab procedure (Harborne, 1988; Sofowora, 1993; Trease and Evans, 1983) to ascertain the availability of the primary as well as secondary metabolites like alkaloids (Mayer's test, Wagner's test), anthocyanin, anthraquinones, carbohydrates (Benedict's test, Fehling's Test, Molisch Test), coumarin, emodin, flavonoids, glycosides (Liebermann's test, Acetic acid Test), leucoanthocyanin, phenol compounds ($FeCl_3$ test, Lead acetate test, Potassium dichromate test), protein (Biuret test, Conc. HNO_3 test, Ninhydrin solution test), Saponin (Foam test with water and $NaHCO_3$), steroid, tannin, and terpenoid tests.

Quantification tests for polyphenolic compounds

Estimation of Total Phenolic Contents (TPC)

The Folin-Ciocalteu (FC) method was used to quantify the TP content of different solvent extracts of the BD root (Singleton *et al.*, 1999). Briefly, 200 µL of crude extract (1 mg/mL) was added to 3 mL of distilled water, followed by 0.2 mL of FC-reagent, and the mixture was mixed thoroughly for 8 min. Then, 0.6 mL of 10% Na_2CO_3 was added and incubated overnight in the dark. The OD was recorded at 765 nm for triplicate sets of each sample. The test findings were represented as milligrams of gallic acid equivalent per gram dry weight (GAE/g DW) of different extracts, and the TPC was calculated from the standard gallic acid curve.

Estimation of Total Flavonoid Contents (TFC)

The TFC was determined using a modified colorimetric assay (Lamaison and Carnat, 1990; Quettier-Deleu *et al.*, 2000). Plant extracts (1000 µg/mL) were reacted with methanol and a 2% $AlCl_3$ solution, and then incubated in the dark for 60 min. Absorbance was measured at 415 nm, and TFC was expressed as milligrams of Rutin Equivalent per gram of extract Dry Weight (RUE/g DW).

UV-Spectroscopic screening of phytocompounds

Different samples were placed in a quartz cuvette and scanned from 200 to 1000 nm using a double-beam UV-visible spectrophotometer (model UV2700, Thermo Fisher Scientific India Pvt. Ltd., Pune, India). The analysis of the absorption peaks was conducted and compared with those of specific bioactive compounds.

LC-HRMS analysis of BDR-ME for major compounds

The separation and identification of BDR-ME compounds were performed using a Waters Acquity UPLC system coupled to a Waters Xevo G2-XS QT of high-resolution mass spectrometer (HRMS). The analysis was carried out in positive electrospray ionisation (ESI) mode. For chromatographic separation, an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm) was employed (Wolfender *et al.*, 2015). The mobile phase consisted of solvent I (water with 0.1% formic acid) and solvent II (acetonitrile mixed with 0.1% formic acid), with a gradient elution starting at 5% II and linearly increasing to 95% II over 15 min. LC-HRMS was

performed with a flow rate of 0.3 mL/min, a column temperature of 40°C, and an injection volume of 5 µL. Source parameters included 120°C temperature, 3 kV capillary voltage, and 30 V cone voltage. Nitrogen was used as the desolvation gas (800 L/hr, 350°C). Data were acquired in the m/z range 50-1200 Da in MS and DIA modes.

Antioxidant tests of plant samples through different methods

Determination of 1, 1 Diphenyl-2-Picrylhydrazyl Radical (DPPH) scavenging assay

Using the DPPH assay, the extract's capacity to scavenge free radicals was assessed (Apu *et al.*, 2012)**.** Variable concentrations (10-500 µL) of plant extracts (1000 µg/mL) were taken in a series of test tubes, and the volume was made up to 3 mL by the addition of methanol, which was then combined with 1 mL of the methanolic solution of 0.1 mM DPPH. After 30 minutes of vigorous shaking and standing, absorbance was measured at 517 nm. Ascorbic acid was used as a standard. The following formula was used to determine the sample's percentage of DPPH decolorization

$$\% \text{ Decolourization} = \frac{C_0 - S_t}{C_0} \times 100$$

Where C_0; Absorbance of Control, S_t; Absorbance of test sample

Determination of Hydroxyl Radical Scavenging Assay (HRSA)

The previously described method was followed (Smirnoff and Cumbes, 1989). The reaction mixture (3 mL) contained 1 mL $FeSO_4$ (1.5 mM), 0.7 mL hydrogen peroxide (6 mM), 0.3 mL of 10% sodium salicylate, and extract concentrations ranging from 10 to 500 µg/mL. The absorbance of the hydroxylated salicylated complex was measured at 562 nm after 1 hr of incubation at 37°C. Ascorbic acid was employed as the standard. The percentage scavenging effect was calculated as

$$\% \text{ scavenging activity} = 1 - \frac{A_1 - A_2}{A_0} \times 100$$

Where A_0 represented the absorbance of the control (without extract), A_1 represented the absorbance when sodium salicylate was present in the extract, and A_2 represented the absorbance when sodium salicylate was absent.

Determination of superoxide radical scavenging assay

Superoxide dismutase or SOD is a well-known antioxidant enzyme responsible for scavenging $O^{2\bullet}$ radicals. The superoxide anion scavenging activity was measured based on the previously described method (Robak and Gryglewski, 1988). According to this, the reduction of Nitroblue Tetrazolium (NBT) in the presence of reduced Nicotinamide Adenine Dinucleotide (NADH) and Phenazine Methosulfate (PMS) under aerobic conditions constituted the basis for this assay. Sodium phosphate buffer (3 mL, 100 mM, pH 7.4) containing 1 mL NBT (150 µM) solution, 1 mL NADH (468µM) solution, and an extract sample solution (10-500 µg/mL) in distilled water was combined. The reaction began when 1 mL of PMS solution (60µM) was added to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured against the equivalent blank samples. Ascorbic acid was utilised as a reference. The reaction mixture's lower absorbance suggested improved superoxide anion scavenging activity.

Determination of Nitric Oxide ($NO^{\bullet}$) scavenging assay

Different concentrations of the plant's extracts (10-500 µg/mL) were dissolved in ethanol and combined with Sodium Nitroprusside (SNP) (5 mM) in Phosphate Buffer Saline (PBS), and the mixture was then incubated for 2 hr at room temperature in the dark (Mfotie Njoya *et al.*, 2017). Nitric oxide scavenging was evaluated using the Griess reagent. SNP in phosphate buffer (pH 7.2) generated NO, which formed nitrite, detected by a pink chromophore at 546 nm. Antioxidants reduced nitrite formation by competing with oxygen. Ascorbic acid was used as a positive control.

Bacterial samples used for antibacterial tests

The clinically isolated bacteria, *Escherichia coli* (BUMCC002), *Pseudomonas aeruginosa* (BUMCC004), and *Staphylococcus aureus* (BUMCC005) were received from the Department of Microbiology, MKCG Hospital, Berhampur, Odisha. The identification of clinical isolates was done by 16S rDNA sequencing and BLAST analysis. The bacterial strains were regularly maintained on Nutrient Agar (NA) or Luria-Bertani Agar (LBA) plates. For long-term storage, glycerol stocks were prepared by following the standard protocol.

Determination of Minimum Bactericidal Concentration (MBC) and Minimum Inhibitory Concentration (MIC) of methanolic extract of *B. diffusa* root (BDR-ME)

Based on phytochemical and antioxidant assays, the Methanolic Extract (BDR-ME) was selected for further antibacterial studies. The Minimum Bactericidal Concentration (MBC) and Minimum Inhibitory Concentration (MIC) were determined using the microtiter plate method (Klančnik *et al.*, 2010), with modifications according to CLSI guidelines (Andrews, 2002). A stock solution of the extract (250 mg/mL) was prepared in Muller-Hinton (MH) broth. The bacterial inoculum (1×10^6 CFU/mL) was obtained by adding 10 µL of culture to 5 mL of sterile broth. For the assay, 100 µL of inoculum was added to each well. Ciprofloxacin (5 µg/200 µL) served as the positive control. Serial dilutions of the extract (starting from 5 mg in 200 µL) were prepared in duplicate. Wells without bacteria served as blanks. Plates were sealed with parafilm and incubated at 37°C for 20-24 hr.

For MBC, loopfuls from each well were streaked on agar and incubated overnight. The lowest concentration with no growth was recorded as MBC. For MIC, 40 µL of MTT (0.2 mg/mL) was added, incubated for 30 min at room temperature, and absorbance was measured at 595 nm. MIC was defined as the lowest concentration showing minimal purple color.

Inhibition zone calculation in the disc diffusion method

Discs of 6 mm diameter were prepared from Whatman No. 3 filter paper. It was loaded with 250, 500, 750, and 1000 µg/disc of BDR-ME and left to dry. A negative control was also included. Muller Hinton agar (MHA) plates were set up and swabbed with sterilized cotton swabs containing specific bacterial culture. The extract-treated discs were aseptically positioned on the inoculated plates and incubated overnight at 37°C. ZOI (Zone of Inhibition) in mm was measured, which depicts the growth inhibitory activity of the extract.

Bactericidal efficacy tested by agar well diffusion

The antibacterial activity of the methanolic crude extract was tested using both the swabbing and pour plate methods. Activated bacterial cultures were inoculated onto or into nutrient agar plates, and wells were loaded with extract concentrations of 500, 1000, 1500, and 2000 µg. Ciprofloxacin served as the positive control, and zones of inhibition were measured after overnight incubation at 37°C.

Determination of CFU/mL in control and BDR-ME-treated culture

BDR-ME at 500 µg/mL (low) and 2500 µg/mL (high) was added to MH broth containing 10 µL of activated bacterial culture. A positive control (culture without extract) was included. Tubes were incubated at 37°C for 4 hr (early log phase) and 48 hr (late death phase). Post-incubation, 10 µL of culture was serially diluted 10^4-fold using sterile distilled water. From the final dilution, 20 µL was plated on MHA plates, spread with a sterile L-rod, and incubated overnight at 37°C. CFU/mL was calculated based on colony counts and the dilution factor. Percent and log reductions in CFU/mL were determined to assess the inhibition of bacterial growth (Devries and Hamilton, 1999).

Log reduction calculation

Log reduction in bacterial count was calculated to determine the bactericidal effect of the extract using the formula:

$$\text{Log reduction} = \log_{10}\frac{N_0}{N}$$

Where N_0 is CFU/mL of the control culture (untreated bacteria or wild), and N is CFU/mL of the treated bacterial culture.

Determination of antibiotic modulation activity

The standard antibiotic discs of AMP 10-Ampicillin 10 µg, NIT 300-Nitrofurantoin 300 µg, CIP 5-Ciprofloxacin 5 µg, CFM 5-Cefixime 5 µg, S10-Streptomycin 10 µg, GEN 10-Gentamycin 10 µg, C 30-Chloroamphenicol 30 µg and AK 30-Amikacin 30 µg were aseptically placed on three test bacteria swabbed nutrient agar plates, incubated overnight, and the inhibitory zone was measured in millimeters to determine the bacterial susceptibility. These ZOIs were used as a reference for comparison with another set of plates containing antibiotics supplemented with BDR-ME at a concentration of 500 µg/disc (Tripathy *et al.*, 2023). The variations in the inhibition zones indicated the extent to which the methanolic extracts enhanced or augmented the antibiotic discs' inhibitory capacity.

Statistical Analysis

Statistical analyses were conducted to evaluate the Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant activity of *B. diffusa* root fractions in comparison with standard compounds. Data from spectrophotometric and antibacterial assays were expressed as Mean±Standard Deviation (SD) based on triplicate measurements (n=3). Statistical significance was determined at $p<0.05$ and $p<0.01$. One-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test was used to analyze the antibacterial activity data. These tests were performed using Microsoft Excel and GraphPad Prism version 8. Principal Component Analysis (PCA) was carried out to assess the variation among different solvent extracts by including TPC, TFC, and antioxidant activity results, following the method described earlier (Sinan *et al.*, 2021). PCA and correlation analyses were conducted using R software version 4.4.2.

RESULTS

Boerhavia diffusa root extract preparation and yield percentage (%) calculation

The estimated yield percentages of *B. diffusa* roots were noted to be 3.90±0.017%, 8.92±0.01%, 5.76±0.02%, and 23.05±0.01% in n-hexane (BDR-HE), chloroform (BDR-CE), acetone (BDR-AE), and methanol (BDR-ME), respectively.

Preliminary analysis of phytocompounds in different solvent extracts of *B. diffusa* root

Phytochemical screening of *B. diffusa* root extracts revealed notable variation in bioactive compounds depending on solvent polarity. BDR-HE (non-polar) was rich in lipophilic compounds like alkaloids, terpenoids, phenols, saponins, steroids, and coumarins, but lacked proteins and polar compounds. BDR-CE (moderately polar) contained alkaloids, terpenoids, saponins, proteins, steroids, glycosides, and reducing sugars, but no phenols, tannins, flavonoids, or coumarins. BDR-AE exhibited the richest profile, extracting terpenoids, saponins, proteins,

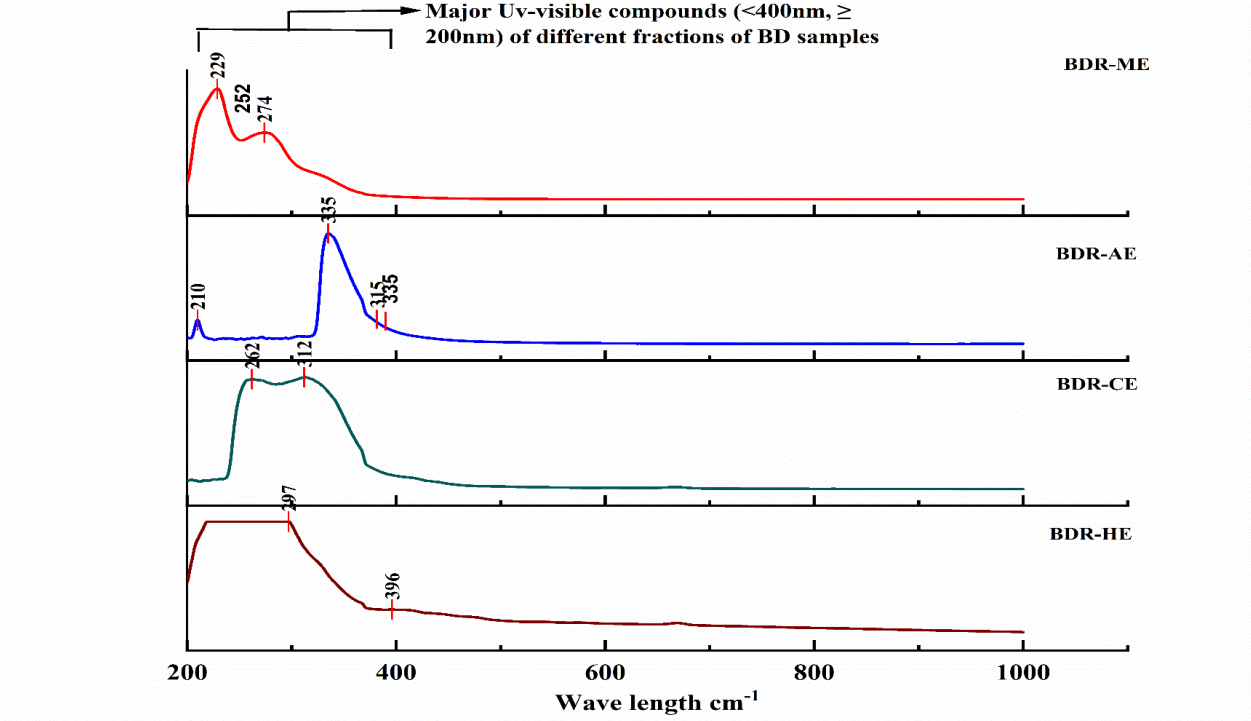


Figure 1: UV-visible spectrophotometric analysis of four different solvents (hexane, chloroform, acetone, and methanol) fractions of *B. diffusa* root extract (BDR-HE, BDR-CE, BDR-AE, and BDR-ME).

steroids, anthocyanins, coumarins, glycosides, and flavonoids. BDR-ME (highly polar) was rich in alkaloids, terpenoids, phenols, saponins, proteins, and flavonoids, but lacked steroids, glycosides, and anthocyanins. Leucoanthocyanins and phlobatannins were undetected in all extracts. Overall, acetone and methanol extracts excelled in extracting polar and semi-polar compounds, while hexane and chloroform were better for non-polar and moderately polar constituents.

Quantitative analysis of different solvent extracts of *B. diffusa* root

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of samples

The total phenolic contents of BDR-HE, BDR-CE, BDR-AE, and BDR-ME samples were calculated to be 23.91±3.34, 33.57±2.25, 56.09±12.39, and 369.30±2.682 mg gallic acid equivalent per gram Dry Weight (GAE/g DW) as determined from the calibration curve (R^2=0.95) and straight-line equation:

$$y=0.0009x+0.0619$$

The TFC content of samples BDR-HE, BDR-CE, BDR-AE, and BDR-ME was observed as 5.56±1.10, 16.74±4.25, 22.16 ±0.45, and 130.90±3.96 mg rutin equivalent per gram dry weight (RUE/g DW), respectively. These were determined from the calibration curve (R^2=0.99) and straight-line equation:

$$y=0.0013x+0.2521$$

UV-visible spectrophotometric analysis of different solvent extracts of *B. diffusa* root

UV-vis spectral analysis of *B. diffusa* root extracts revealed multiple absorption maxima (λ_{max}), indicating various conjugated phytocompounds, especially phenolics and flavonoids. BDR-ME showed peaks at 229, 252, and 274 nm (phenolic acids) and at 210, 315, and 335 nm, with the latter two suggesting flavonoids like quercetin. BDR-CE exhibited peaks at 262 and 312 nm, indicating flavonoid and phenylpropanoid derivatives. BDR-HE had peaks at 297 and 396 nm, with the 396 nm peak hinting at highly conjugated chromophores (e.g., oxidised flavonoids or anthocyanins). Common peaks at 229-274 nm across extracts suggest widespread low molecular weight phenolics, while 312, 315, 335, and 396 nm peaks reflect more complex flavonoids. Overall, these UV-Vis profiles highlight a diverse phytochemical composition in the *B. diffusa* root extracts. The spectra of different extracts are presented in Figure 1.

LCHR-MS analysis of BDR-ME

LC-HRMS analysis of *B. diffusa* root methanolic extract revealed a rich profile of polyphenolic compounds alongside other bioactives. Major flavonoids such as kaempferol, quercetin, luteolin, and their glycosides were detected at RT 2-4 min with characteristic fragments (m/z 153, 179, 285). Boeravinones B-E, polyphenolic rotenoids, were identified at RT 24-26 min with m/z 303 and 285 fragments. Phenolic acids like caffeic and ferulic acids were also present. The extract also showed alkaloids, triterpenoid saponins, and phytosterols, confirming its high polyphenol

content and phytochemical diversity. The summarised data are presented in Table 1.

Antioxidant activity

DPPH free radical scavenging assay

The DPPH radical scavenging activity of *Boerhavia diffusa* root extracts showed that BDR-ME had the highest activity, followed by BDR-AE, BDR-CE, and BDR-HE. The IC_{50} values were 50.43±0.86 µg/mL for ascorbic acid, 64.65±1.97 µg/mL for BDR-HE, 57.52±1.60 µg/mL for BDR-CE, 55.49±1.51 µg/mL for BDR-AE, and 51.03±2.31 µg/mL for BDR-ME, indicating their relative antioxidant potential.

The Hydroxyl Radical Scavenging Assay (HRSA)

The antioxidant potential of *B. diffusa* root extracts were evaluated based on their hydroxyl radical scavenging activity. BDR-ME exhibited the highest activity, followed by BDR-AE, BDR-CE, and BDR-HE shown in Figure 3b. The IC_{50} values were 65.76 ± 1.64 µg/mL for ascorbic acid, 95.92±1.20 µg/mL for BDR-HE, 100.39±1.00 µg/mL for BDR-CE, 97.43±0.84 µg/mL for BDR-AE, and 75.77±1.58 µg/mL for BDR-ME.

Superoxide radical scavenging assay

The superoxide radical scavenging activity of *B. diffusa* root extracts increased significantly with rising concentrations (10-500 µg/mL) across all solvent fractions. BDR-ME showed the highest activity, followed by BDR-AE, BDR-CE, and BDR-HE. The IC_{50} values were 64.15±1.58 µg/mL for ascorbic acid, 98.02±0.42 µg/mL for BDR-HE, 142.16±2.49 µg/mL for BDR-CE, 96.98±1.42 µg/mL for BDR-AE, and 77.23±2.16 µg/mL for BDR-ME.

Nitric oxide scavenging assay

The nitric oxide ($NO^{\bullet}$) scavenging activity of *B. diffusa* root extracts was evaluated. The methanolic extract (BDR-ME) showed the highest activity, followed by the hexane (BDR-HE), chloroform (BDR-CE), and acetone (BDR-AE) extracts. The IC_{50} values were 70.65±1.42 µg/mL for ascorbic acid, 98.08±1.46 µg/mL for BDR-HE, 106.65±4.03 µg/mL for BDR-CE, 93.83±3.25 µg/mL for BDR-AE, and 81.54±3.97 µg/mL for BDR-ME.

The antioxidant potential of different solvent extracts of *B. diffusa* roots was compiled and presented in Figure 2. All the values and test results of the different antioxidant assays conducted in the present study are compiled and presented in Table 2.

Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was used to examine the relationships among different antioxidant parameters (DPPH, nitric oxide, superoxide, hydroxyl radical scavenging, TPC, and TFC) across the different extracts, as presented in Figure 3. The scree plot (Figure 3a) showed that PC1 (55%) and PC2 (39.6%) together explained 94.6% of the variance. The correlation

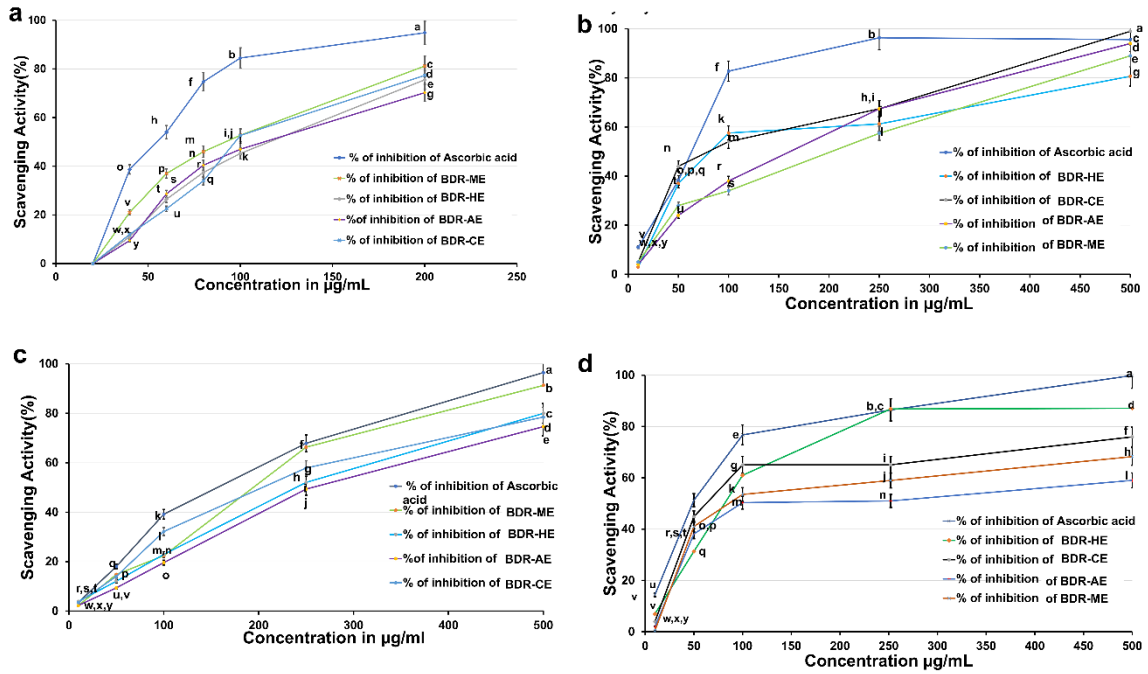
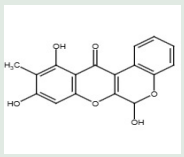
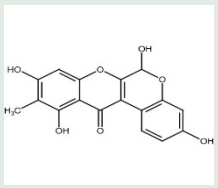
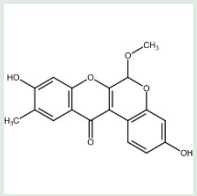
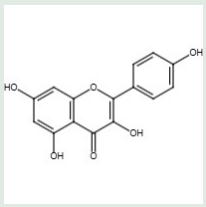
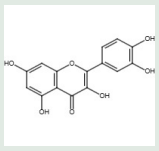
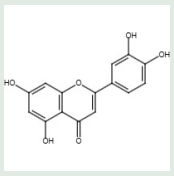
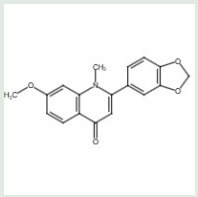
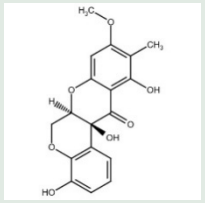
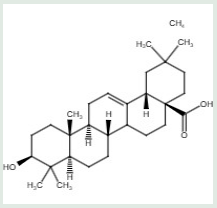
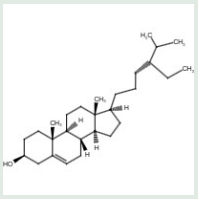
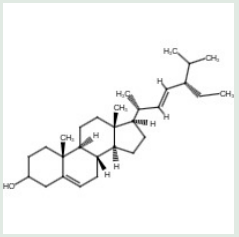
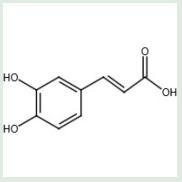
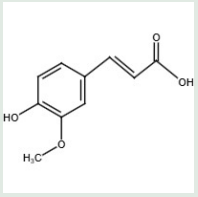
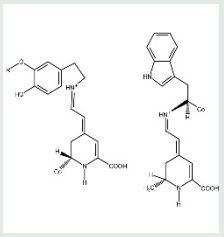
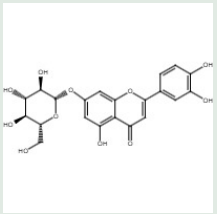
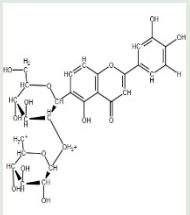
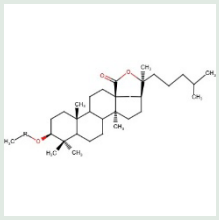


Figure 2: Antioxidant activity of different solvent fractions of *Boerhavia diffusa* root was evaluated using four distinct assays: (a) DPPH radical scavenging assay, (b) hydroxyl radical scavenging assay, (c) Superoxide radical scavenging assay, and (d) Nitric oxide scavenging assay. For each assay, the activity was measured at various concentrations (10-500 µg/mL), and results were expressed as % radical scavenging activity. Values represent the Mean±SD of three replicates. Different letters (a-y) indicate statistically significant differences between means ($p<0.05$), with 'a' representing the highest scavenging activity and subsequent letters indicating decreasing activity.

Table 1: Putative phytochemicals identified in the methanolic root extract of *Boerhavia diffusa* L. by LC-HRMS.

Peak no.	Rtention Time	m/z (Observed)	Molecular Formula	Putative Compounds	Fragment Ions (m/z)	Chemical structure	References/Note
1	24.4	446.23	$C_{21}H_{18}O_{11}$	Boeravinone B	303.05, 285.04		
2	24.4	477	$C_{22}H_{20}O_{11}$	Boeravinone E	303.05, 285.04		
3	26.01	462-468	$C_{22}H_{18}O_{10}$	Boeravinone D	303.05, 285.04		
4	3.29	303.24	$C_{15}H_{10}O_6$	Kaempferol	153.01, 179.03		(Patil and Bhalsing, 2016; Sinan *et al.*, 2021)Polyphenol study in *B. diffusa*; RT ~3 min confirmed
5	3.37	303.05	$C_{15}H_{10}O_7$	Quercetin	153.01, 179.03		
6	3.21	285	$C_{15}H_{10}O_6$	Luteolin	153.01, 179.03		
7	5.85	368.23	$C_{18}H_{20}NO_7$	Punarnavine	152.05, 181.06		The isoquinoline core present in *Punarnava*
8	24.09	462-468	$C_{22}H_{18}O_{10}$	Boeravinone C	303.05, 285.04		

9	27.14	727-993	C_{36}-$C_{53}H_{60-88}O_{15-26}$	Oleanolic acid glycosides	Sugar losses (-162, -146 Da)		
10	21.3	414.38	$C_{29}H_{50}O$	β-sitosterol	396.37, 381.34		
11	20.56	412.4	$C_{29}H_{48}O$	Stigmasterol	394.11, 379.09		
12	2.09	179.03	$C_9H_8O_4$	Caffeic acid	135.01, 161.02		
13	2.84	193.05	$C_{10}H_{10}O_4$	Ferulic acid	149.06, 178.04		
14	3.19	554.1	$C_{24}H_{26}N_2O_{13}$	Betacyanin derivatives	389, 329		
15	3.96	447.09	$C_{21}H_{20}O_{11}$	Luteolin-7-O-glucoside	285.04(luteolin core), 162.05 loss		
16	3.03	274.27	$C_{15}H_{10}O_6$	Flavonoid glycoside fragment	153.01, 179.03		Common aglycone fragment from glycosides

17	27-28	727-993	C_{36}-$C_{53}H_{60-88}O_{15-26}$	Triterpenoid saponins (various)	Sequential sugar losses (162, 146 Da)		High m/z range typical for saponins in *B. diffusa* root

circle (Figure 3b) indicated that DPPH and TFC were strongly associated with PC1, while nitric oxide and superoxide scavenging were linked to PC2. TPC and hydroxyl radical scavenging had moderate associations with both components, and DPPH and TFC showed a strong positive correlation.

The PCA biplot (Figure 3c) revealed distinct clustering of extract groups. BDR-ME strongly correlated with DPPH and TFC, highlighting its antioxidant potential. BDR-CE was associated with nitric oxide and superoxide scavenging, while BDR-AE (acetone extract) correlated with TPC and hydroxyl radical scavenging. BDR-HE showed no clear association. Overall, PCA effectively distinguished the extracts, emphasizing the superior antioxidant profile of the BDR-ME.

Correlation among TPC, TFC, and antioxidant assays

Additionally, a correlation heatmap (Figure 3d) further supported these findings. TPC showed a strong negative correlation with both DPPH (r=-0.87) and hydroxyl radical scavenging (r=-0.87), as well as moderate negative correlations with superoxide (r=-0.70) and nitric oxide (r=-0.68). TFC demonstrated weaker correlations across all antioxidant parameters, with a slight positive correlation with DPPH (r=0.11) and moderate negative correlations with the rest, shown in Figure 3d.

These results indicate that TPC plays a more significant role in antioxidant activity than TFC, particularly in relation to DPPH and hydroxyl radical scavenging, while methanolic extracts (BDR-ME) stand out as the most antioxidant-rich fraction.

Antibacterial assays

Growth inhibitory effect in the disc diffusion method

The antibacterial activity of the test extract at four different concentrations (250, 500,750, and 1000 µg) was evaluated against *Escherichia coli, Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The antibacterial activity of the extract increased with dose, showing the highest zones of inhibition and percentage inhibition at 1000 µg for all bacterial strains tested. *P. aeruginosa* showed moderate inhibition (27.59%), while *S. aureus* and *E. coli* showed substantial inhibition (50.46% and 60.34%, respectively). Overall, the extract was most effective against *E. coli* at the highest dose. To assess the significance of these findings, a one-way ANOVA was conducted for each strain, comparing the inhibition zones across all extract doses and ciprofloxacin. For *E. coli*, the analysis revealed a highly significant difference among treatments (p<0.0001). Post-hoc Tukey's HSD (Honestly Significant Difference) test showed that the 1mg extract had significantly higher inhibition than the lower doses. For *S. aureus*, the extract showed moderate activity at 250 µg and 500 µg, but significant differences were observed at higher concentrations (p<0.0001), with 1mg showing the highest inhibition. For *P. aeruginosa*, the extract displayed modest but statistically significant differences across doses (p=0.003). The 1mg dose was notably more effective than the 500 and 750 µg doses.

Growth inhibition analysis in agar well diffusion test

Both Swabbing (SM) and Pour Plate (PPM) methods demonstrated a dose-dependent increase in the zones of inhibition for *P. aeruginosa, E. coli*, and *S. aureus. P. aeruginosa* exhibited the highest inhibition zone at 2000 µg in SM (31.17 mm) and PPM (29.17 mm). *S. aureus* showed higher inhibition in PPM (26.67 mm) than in SM (20.83 mm). Overall, PPM produced consistently larger zones of inhibition at higher doses for all three bacterial strains, indicating its greater sensitivity in detecting antibacterial activity. The results are presented in Table 3.

Bacterial cell viability or CFU/mL determination assay

The plant extract treatment significantly reduced the bacterial count of *E. coli, S. aureus*, and *P. aeruginosa* over time and in a dose-dependent manner. After 48 hr, the high dose showed the greatest reduction for all strains, with *P. aeruginosa* showing the most pronounced effect (92.58% reduction; 1.13 log reduction). *E. coli* and *S. aureus* also showed substantial reductions of 72.10% (0.55 log) and 71.42% (0.54 log), respectively. Results are shown in Table 4.

Calculation of IC_{50} and MIC/MBC value of BDR-ME

BDR-ME showed the highest antibacterial activity against *P. aeruginosa* with the lowest MIC (625 µg/mL) and IC_{50} (75.11±6 µg/mL), followed by *S. aureus* (same MIC but IC_{50} of 139.4±6 µg/mL). *E. coli* had the highest MIC (1250 µg/mL) and IC_{50} (446.2±6 µg/mL). MBC results indicated concentration-dependent bactericidal activity, with the lowest MBC (1250 µg/mL) for *P. aeruginosa* and *S. aureus*, and a higher MBC (2500 µg/mL) for *E. coli*. The antibiogram (microtiter assay) is shown in Figure 4.

Antibiotic modulatory effects of the methanolic extract of *Boerhavia diffusa* root

The antibiotic modulatory activity of BDR-ME (500 µg/disc) was assessed by measuring the differences in the zone of inhibition (in mm) and calculating the percentage of increased or decreased inhibition against *E. coli, P. aeruginosa*, and *S. aureus*. In the presence of BDR-ME, AMP10 showed enhanced activity against all tested strains, with the highest increase of 100% in *P. aeruginosa* and moderate increases of 8.60% and 7.38% in *E. coli* and *S. aureus*, respectively. Similarly, CFM5 exhibited increased activity against *P. aeruginosa* (100%) and *S. aureus* (35.98%) but a slight decrease (−3.31%) against *E. coli*. NIT300 displayed enhanced activity against *P. aeruginosa* (100%) but significant inhibition reduction in *E. coli* (−66.56%). S10+BDR-ME showed an increase (115%) against *E. coli* but decreased activity in *P. aeruginosa* (−59.76%) and *S. aureus* (-21.09%). CIP5 demonstrated the highest increase in *E. coli* (78.51%), a minor increase in *S. aureus* (0.84%), but reduced activity in *P. aeruginosa* (−64.33%). Conversely, GEN10, C30, and AK30 combinations generally showed reduced antibiotic activity in all tested bacteria. Overall,

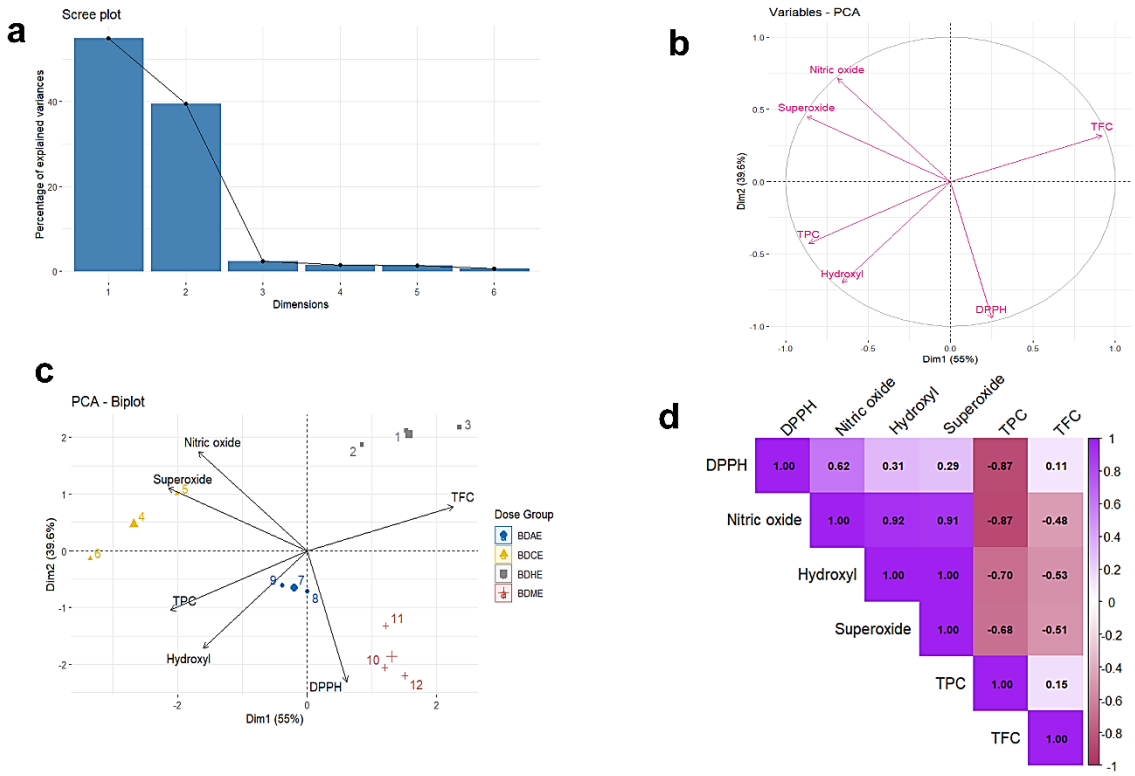


Figure 3: Principal Component Analysis (PCA) and variable contribution summary. (a) Scree plot (b)Variables in PCA (c) PCA biplot representing various antioxidant activities (DPPH, HRSA, Superoxide, and Nitric oxide antioxidant capacity) and phytochemical content (TPC and TFC) (d) Correlation matrix between Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant parameters (DPPH, hydroxyl, superoxide, and nitric oxide scavenging). The color intensity and values represent Pearson correlation coefficients. of *B. diffusa* root solvent fractions.

Table 2: Antioxidant activity of different solvent extracts of *B. diffusa* root and their IC_{50} values

Extract/positive control	IC_{50} (µg/mL)			
	DPPH radical scavenging activity	Hydroxyl radical scavenging activity	Superoxide radical scavenging activity	Nitric oxide radical scavenging activity
Ascorbic acid	50.43±0.86^{d}	65.76±1.64^{d}	64.15±1.58^{d}	70.65±1.42^{d}
BDR-HE	64.65±1.97^{a}	95.92±1.20^{b}	98.02±0.42^{b}	98.08±1.46^{b}
BDR-CE	57.52±1.60^{b}	100.39±1.18^{a}	142.16±2.49^{a}	106.65±4.03^{a}
BDR-AE	55.49±1.51^{bc}	97.43±0.84^{ab}	96.98±1.42^{b}	93.83±3.25^{b}
BDR-ME	51.03±2.31^{cd}	75.77±1.58^{c}	77.23±2.16^{c}	81.54±3.97^{c}

Note: The results are displayed as mean±standard deviation (n=3). a-d indicates that the means in a row are different at the significant level of (p<0.05) using One-Way Analysis of Variance (ANOVA) and Tukey's test. DPPH: 2,2′ -diphenyl-1-picrylhydrazyl; Hydroxyl radical scavenging assay (OH^{-}); Superoxide radical scavenging assay(O^{2-}); Nitric oxide radical scavenging assay (NO^{-})

Table 3: Inhibition zone calculation of BDR-ME against uropathogens in agar well diffusion methods

Organisms	Methods	Concentration in µg			
		500	1000	1500	2000
Pseudomonas aeruginosa (BUMCC004)	SM	7.17±1.00 [a]	12.50±2.12 [b]	22.50±1.25 [c]	31.17±0.76 [d]
	PPM	12.50±1.50 [a]	15.67±2.08 [b]	27.17±2.02 [c]	29.17±0.76 [d]
Escherichia coli (BUMCC002)	SM	7.17±0.29 [a]	11.17±1.04 [b]	11.83±0.76 [b]	19.17±2.52 [c]
	PPM	9.40±1.40 [a]	12.00±2.00 [b]	20.00±2.65 [c]	24.33±0.58 [d]
Staphylococcus aureus (BUMCC005)	SM	10.17±0.76 [a]	13.13±1.02 [b]	16.83±0.76 [c]	20.83±0.76 [d]
	PPM	11.20±2.56 [a]	12.70±0.80 [a]	17.33±2.52 [b]	26.67±1.53 [c]

Note: SM- Swabbing method, **PPM**- Pour plate method, **a-d**- for significance level difference

BDR-ME demonstrated strain-specific and antibiotic-dependent effects, enhancing or suppressing antibiotic activity. The synergistic interactions, particularly with *P. aeruginosa* and *E. coli*, suggest BDR-ME's potential as an antibiotic adjuvant, though antagonistic effects underscore the need for careful combination. Results are presented in Figure 5.

DISCUSSION

Plants with a long history of use in traditional medicine represent a vast resource for discovering and investigating new remedies in pharmaceutical sciences. Plants are considered the richest natural sources for screening potential antimicrobial compounds. For example, *B. diffusa* has been used extensively in traditional medicine in India and Southeast Asia.

Correlation of phytoconstituents with antioxidant potential of *B. diffusa* root extracts

Medicinal plants possess numerous secondary metabolites, but proper identification and scientific validation are essential for their prospective pharmaceutical use. The rise of antibiotic-resistant microorganisms has given extra impetus to the search for novel antibacterial compounds. Numerous alkaloids, flavonoids, glycosides, terpenes, tannins, and polyphenols from plant origins have been shown to exhibit antibacterial activity. Many have also exhibited synergistic effects with existing antimicrobial drugs (Tan and Lim, 2015). Several studies have reported the biological activities of different parts of *B. diffusa*; however, there are limited reports on the bioactivities of *B. diffusa* root extracted with different solvents. Hence, the current study aimed to explore and compare the biological activities of different solvent extracts of *B. diffusa* roots and examine their use as a potential natural curative as well as complementary treatment to existing remedial measures. Since polar solvents are better at extracting bioactive compounds, in the present study, the biopotentiality of methanolic solvent extract was compared with other moderately polar and non-polar solvents.

The roots of *B. diffusa* are rich in biologically active compounds with multifarious potentiality against different ailments**.** In this study, hexane, chloroform, acetone, and methanol are used to extract the phytocompounds present in the roots of *B. diffusa.* Among the solvent fractions, the methanolic fraction had a higher yield percentage (23.05%) than the other three fractions, possibly due to the presence of more polar compounds in *B. diffusa* roots. The spectrophotometric analysis of the different solvent fractions of root samples resulted in different spectral patterns corresponding to the specific absorption profiles under UV and visible wavelengths. A strong absorption peak at 229-274 nm was consistently observed across all extracts, but specifically in BDR-ME, which is characteristic of $\pi \rightarrow \pi^*$ transitions in aromatic rings and phenolic structures, including hydroxybenzoic acids and gallic acid derivatives. The peak at 262 nm, along with a moderate band at 297 nm, suggests the presence of phenylpropanoid derivatives such as caffeic acid, ferulic acid, or other cinnamic acid-based compounds in BDR-ME (Figure 1).

LC-HRMS results of BDR-ME showed the presence of phenolic acids, like caffeic and ferulic acids. The extract also showed alkaloids, triterpenoids, saponins, and phytosterols (Table 1), confirming its high polyphenolic content. These compounds are known for their antioxidant potential and are commonly found in medicinal plants. The presence of these compounds in BDR-ME explains the reason behind its significant antioxidant activity in all the different types of free radical scavenging assays carried out in the present study.

B. diffusa has been suggested to treat several pathophysiological conditions, including diabetes, inflammation, and cancer (Dhingra and Valecha, 2014; Gunaseelan *et al.*, 2022). As reported earlier, several plant bioactive compounds have shown a relationship between the antioxidant activity and

polyphenolic content (Apu *et al.*, 2012; Sharma *et al.*, 2023). This study determined the antioxidant potential of different solvent fractions of *B. diffusa* roots by measuring ROS and RONS (reactive oxygen and nitrogen species) scavenging activity (Table 2; Figures 2a-d). Kaviya *et al.*, (2022) estimated that the flavonoid contents are 60±0.00, 50±0.01, and 50±0.01 mg QE/g, and phenolic compounds were also measured to be 14±0.2 and 105±0.57 GAE/g DW in *B. diffusa* decoction and aqueous extract that gave IC_{50} of 498 µM and 645 µM in DPPH assay (Kaviya *et al.*, 2022). Flavonoids inhibit the generation of reactive oxygen species (ROS) and effectively scavenge ROS once they are formed, contributing to their strong antioxidant potential (Agati *et al.*, 2012). In this study, the TPC and TFC were found to be 369.30±2.682 mg GAE/g DW and 130.90±3.96 mg RUE/g DW in the methanolic fraction, which is the highest among other solvent extracts, so that it showed a high IC_{50} of 51.49±2.3 µg/mL in BDR-ME. The findings clearly indicate that the BDR-ME exhibited comparatively higher polyphenolic content in both quantitative tests, possibly due to environmental factors and its geographical position. This probably led to the higher traditional uses in this region.

The reliability and depth of antioxidant profiling are enhanced by using multiple methods, as each reactive species behaves differently and interacts uniquely with various phytochemicals (Bhalodiya *et al.*, 2020). The free radical scavenging study of the *B. diffusa* root sample showed an IC_{50} value of 258.40±11.73 µg/mL in the DPPH assay (Tacchini *et al.*, 2015). The methanol extract showed higher OH^- and superoxide radical (O^{2-}) scavenging activity than chloroform and hexane extracts (Apu *et al.*, 2012; Patel, 2014). Several studies revealed correlations between some plant extracts' antioxidant activity and reducing power. This multi-assay approach not only mimics the complexity of oxidative stress conditions in biological systems but also enables a comparative analysis of the extract's efficacy against specific radicals. Collectively, the results provide a comprehensive overview of the antioxidant behavior of *B. diffusa* root in different solvent fractions and highlight its potential as a natural therapeutic agent against oxidative stress-related bactericidal activity. The antioxidant potential of *B. diffusa* root extract was evaluated by

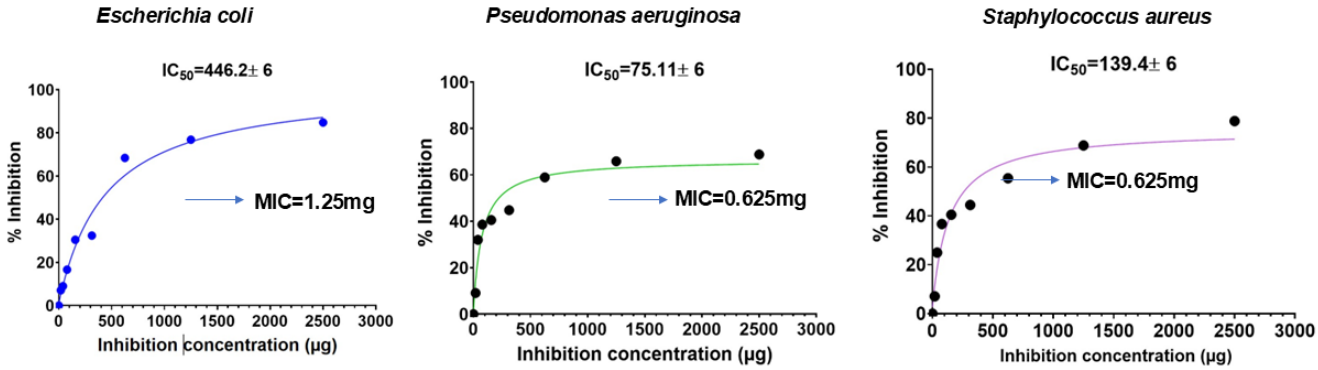


Figure 4: Estimation of IC_{50} and MIC of the methanolic fraction of *B. diffusa* root.

Table 4: Tabular presentation of the percentage of reduction of colony-forming units by treating with BDR-ME

Bacteria	Time	Wild (CFU/mL)	After treatment, the count of bacterial cells			
			Low Dose CFU/mL (%Reduction)	Log Reduction	High Dose CFU/mL (%Reduction)	Log reduction
Escherichia coli (BUMCC002)	4 hr	9.3×10^7	6.2×10^7 (33.33)	0.18	6.1×10^7 (33.40)	0.18
	48 hr	4.3×10^7	2.2×10^7 (48.83)	0.29	1.2×10^7 (72.10)	0.55
Staphylococcus aureus (BUMCC005)	4 hr	6.1×10^7	5.3×10^7 (13.11)	0.06	4.9×10^7 (19.67)	0.10
	48 hr	3.5×10^7	1.3×10^7 (62.85)	0.43	1.0×10^7 (71.42)	0.54
Pseudomonas aeruginosa (BUMCC004)	4 hr	3.6×10^7	2.9×10^7 (19.44)	0.10	2.4×10^7 (33.33)	0.18
	48 hr	3.1×10^7	1.2×10^7 (61.29)	0.41	0.23×10^7 (92.58)	1.13

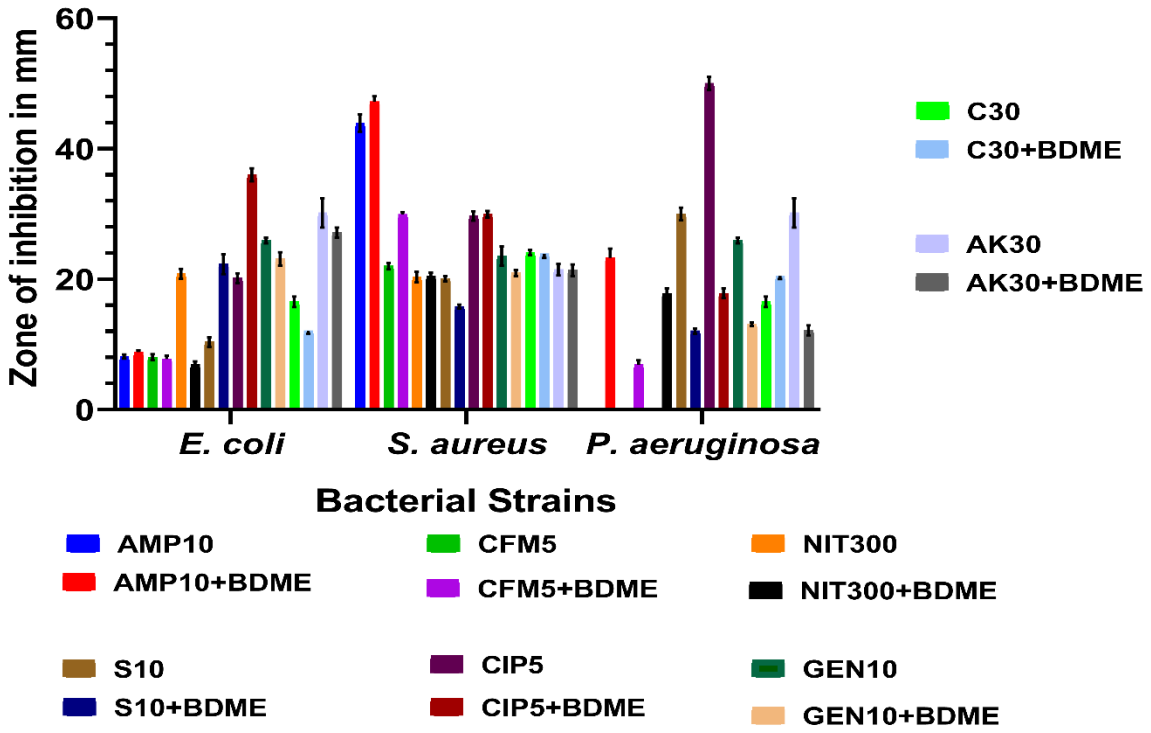


Figure 5: Antibiotic modulatory effect of methanolic fraction of B. diffusa root fraction. All experiments were performed in triplicate, and data were expressed as Mean±Standard Deviation (SD). One-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test was used to determine statistical significance between treatment groups. A *p*-value of <0.05 was considered statistically significant.

using DPPH, OH•, O_2•, and NO scavenging assays. These assays target different reactive species, offering a comprehensive view of antioxidant activity. Such antioxidants may enhance antibacterial effects by inducing oxidative stress in bacterial cells (Zahra *et al.*, 2024).

In previous studies, it has already been documented that antibacterial evaluation depends on the comparatively higher yield, broader phytochemical diversity, and strongest antioxidant activity among all tested solvent extracts. PCA results effectively differentiated the extract types based on their antioxidant profiles, highlighting the BDR-ME as the most potent due to its association with multiple antioxidant parameters (Figures 3a-c). Correlation matrix results indicate that TPC plays a more significant role in antioxidant activity than TFC, particularly with DPPH and hydroxyl radical scavenging, while BDR-ME stands out as the most antioxidant-rich fraction (Figure 3d). Phenolics act as effective hydrogen or electron donors, directly neutralising free radicals. In contrast, TFC showed weak to moderate correlations, suggesting a lesser role for flavonoids in the overall antioxidant potential. The high antioxidant potential of the methanolic fraction is therefore attributed to its rich phenolic content. These plant extracts exert their action by inhibiting the generation of free radicals during cellular metabolism. This net difference in low scavenging activity results in a flux of free radicals into the bacterial cellular matrix, leading to increased cell damage. The scavenging mechanisms of polyphenolic compounds play an important role in protecting humans against degenerative and infectious diseases (Reznick *et al.*, 2006). Hydroxyl groups at 3′, 4′, and 5′of ring B (a pyrogallol group) and the double bond between carbon-2 and carbon-3 conjugated with the 4-oxo (=O) and 3-hydroxyl (-OH) group in ring C, enhance the radical scavenging activity of flavonoids (Balasundram *et al.*, 2006). Flavonoids are a group of phenolic substances that have a C_6-C_3-C_6 carbon structure (Phenyl benzopyran). These core structures, supported by a functional group of hydroxyls, have a major role in determining the antibacterial potential of the substances (De Rossi *et al.*, 2025; Mandal and Domb, 2024).

Urobactericidal and antibiotic modulatory activity of phytoconstituents present in the methanolic extract of *B. diffusa* root

In the literature, it is more common to see both antioxidant and antibacterial activities reported concurrently, particularly when bioactivity-driven solvent extracts are involved. It is, therefore, similarly crucial that the methods used for antibacterial testing also be standardised and optimised to ensure the accuracy of reports. Among such studies, the root decoction of *B. diffusa* showed a 7±0 mm ZOI against *P. aeruginosa* bacteria at 200 µg, and the ZOI of ethanol extract ranged from 7±0 to 8±0 mm. The inhibition zone showed 8±1, 9±0, 8±0, and 17±2 mm at 200 µg in the decoction, aqueous, chloroform, and ethanol forms of the extract against *S. aureus*, respectively (Kaviya *et al.*, 2022). In other

studies, Adeku *et al*., (2022) found that the ethanolic extract of *B. diffusa* had a 7 mm inhibition zone against *E. coli*. In this study, BDR-ME exhibited significant activity against all three bacterial strains, with the maximum inhibition observed against *S. aureus* in the disc diffusion assay. However, in the agar well diffusion assay, particularly using the swabbing method, *P. aeruginosa* was found to be significantly inhibited (Table 3). Log reduction analysis supports that *B. diffusa* root extract possesses moderate to strong antibacterial activity, with notable efficacy against *P. aeruginosa* at higher concentrations. These findings suggest the extract's potential as a natural antimicrobial agent, particularly when applied at optimal doses and over sufficient exposure time, as mentioned in Table 4. Though all three urobacterial strains were significantly inhibited by BDR-ME, the degree of their sensitivity differed in different assays, possibly due to variable interaction of phytoconstituents and the bacterial strains in different assay methods, like solid agar or broth cultures. However, BDR-ME had the highest growth inhibitory effect against *P. aeruginosa,* which justifies the use of *B. diffusa* roots for UTIs caused by this bacterium.

The results of the MIC assay highlight the antimicrobial potential of *B. diffusa* root extracts. In this study, the methanolic extract exhibited inhibitory activity against *P. aeruginosa* and *S. aureus*, with MIC values of 625 µg/mL; however, for *E. coli*, the MIC value was found to be 1250 µg/mL. The MBC values were determined to be 1250 µg/mL against *P. aeruginosa* and *S. aureus* and 2500 µg/mL for *E. coli* (Figure 4). These findings indicate a moderate antibacterial effect, particularly against *E. coli*. Comparatively, Kaviya *et al*., (2022) reported complete growth inhibition of the same bacterial strains at a much lower concentration (50 µg/mL) using an ethanolic extract. The variation may be due to differences in solvent polarity, as methanol and ethanol extract different phytocompounds. Differences in plant source or experimental methods may also affect results. The higher MIC values suggest the methanolic extract is active but may need purification or combination with other agents for improved efficacy.

The secondary metabolites contained in several medicinal dietary plants are known for their pharmacological activities, including antibacterial properties. Following established cutoff points, a botanical is considered significantly active when MIC< 625 µg/mL, moderately active when 625 ≤ MIC ≤ 1250 µg/mL, and poorly active when MIC > 1250 µg/mL. Based on these cutoff points, BDR-ME can be considered significantly active, with a MIC value of 625 µg/mL against *P. aeruginosa* and *S. aureus*. The phytochemical composition of BDR-ME can be responsible for the antibacterial activity of the samples, since flavonoids, phenols, and alkaloids (reported in this fraction) are known to have antimicrobial and curative properties against several pathogens (Ambadiang *et al*., 2020).

B. diffusa showed significant antibacterial activity against uropathogens isolated from clinical samples of female patients and can be used as an alternative or complementary treatment for UTIs (Prasad and Mishra, 2024). In *E. coli*, BDR-ME enhanced the inhibitory effects of ampicillin, ciprofloxacin, and particularly streptomycin, indicating potential synergism. However, the combination with nitrofurantoin resulted in a marked antagonistic interaction. For *P. aeruginosa*, strong synergistic effects were observed with ampicillin, cefixime, and nitrofurantoin. In contrast, combinations with gentamycin, chloramphenicol, amikacin, and ciprofloxacin showed reduced efficacy, suggesting antagonism. In *S. aureus*, the combination of BDR-ME with cefixime led to the most significant enhancement, followed by ciprofloxacin, indicating a synergistic effect. Conversely, the combinations with streptomycin and gentamycin displayed antagonistic responses (Figure 5). These findings underscore the complexity of plant extract-antibiotic interactions and highlight the importance of considering both bacterial species and antibiotic class when evaluating combination therapies. The observed synergistic effects suggest that BDR-ME could enhance antibiotic efficacy in specific contexts, while its antagonistic actions warrant caution in others.

Phytochemicals like flavonoids and phenolics can disrupt bacterial membranes by increasing permeability, leading to cell lysis (Nouir *et al*., 2023). Some also induce reactive oxygen species (ROS) accumulation, causing oxidative damage to DNA, proteins, and lipids (El-Sherbiny *et al*., 2024). Additionally, antioxidants may enhance antibiotic efficacy by inhibiting efflux pumps or increasing membrane permeability (Sobi *et al*., 2022). Plant-derived compounds, particularly polyphenols, flavonoids, and alkaloids, can synergise with antibiotics, offering a promising strategy against resistant bacterial strains and supporting combination therapy (Hemaiswarya *et al*., 2008).

Antibiotics generally penetrate the cells without damaging cell walls and target the collapse of specific cellular machinery, such as inhibition of biosynthesis of peptidoglycan, inhibition of protein biosynthesis, and breaking of double-stranded DNA (Al-Mamun *et al*., 2016; Hancock and Rozek, 2002; Nawrot *et al*., 2014). Polyphenols like quercetin and gallic acid disrupt bacterial antioxidant enzymes (e.g., catalase, superoxide dismutase), making them more susceptible to oxidative damage caused by antibiotics. Besides the secondary metabolite, scientists have focused on plant compounds that exhibit antagonistic action against various microbes. These phyto-derived compounds can be considered potential agents for overcoming drug resistance due to differences in the mode of action from conventional antibiotics. The present study demonstrates that BDR-ME exhibits differential modulatory effects on antibiotic activity, which vary depending on the bacterial species and the antibiotic used.

CONCLUSION

The findings of the current study indicate that the polyphenol-rich methanolic fraction of *B. diffusa* root exhibits potent antioxidant and antibacterial properties and can synergise with antibiotics against major uropathogens. The synergistic interaction observed with the conventional antibiotics suggests that the flavonoid- and phenolic-enriched fraction may interfere with bacterial redox balance and membrane permeability, thereby augmenting drug susceptibility. These findings validate the ethnopharmacological potential in urinary disorders and suggest it as a possible natural adjuvant in managing antibiotic-resistant UTIs. Future work should focus on isolating the active principles, characterising their molecular mechanism, and evaluating their *in vivo* efficacy and safety for potential development into a complementary phytopharmaceutical formulation.

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ABBREVIATIONS

BDR-HE: Hexane fraction of *Boerhavia diffusa*; **BDR-CE:** Chloroform fraction of *B. diffusa*; **BDR-AE:** Acetone fraction of *B. diffusa*; **BDR-ME:** Methanolic fraction of *B. diffusa*; **ºC:** Degree Celsius; **AK:** Amikacin; **AMP:** Ampicillin; **NIT:** Nitrofurantoin; **CFM:** Cefixime; **CIP:** Ciprofloxacin; **S:** Streptomycin; **GEN:** Gentamicin; **C:** Chloramphenicol; **UTI:** Urinary Tract Infection; **MIC:** Minimum Inhibitory Concentration; **FC Reagent:** Folin-Ciocalteu's Phenolic Reagent; **$FeCl_3$:** Ferric Chloride; **g:** Gram; **L:** Litre; **TPC:** Total Phenolic Content; **TFC:** Total Flavonoid Content; **DW:** Dry Weight; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **MDR:** Multi Drug Resistant; **mg:** Milligram; **Min:** Minute; **mL:** Millilitre; **µL:** Microlitre; **MS Excel:** Microsoft Excel; **LB:** Luria Bertani Agar; **MHA:** Mueller Hinton Agar; **MHB:** Mueller Hinton Broth; **OD:** Optical Density; **Sp.:** Species; **Temp.:** Temperature; **ZOI:** Zone of Inhibition; **CFU:** Colony Forming Unit; **SD:** Standard Deviation; **SM:** Secondary Metabolite; **ROS:** Reactive Oxygen Species; **RONS:** Reactive Oxygen and Nitrogen Species; **PCA:** Principal Component Analysis; **HRSA:** Hydroxyl Radical Scavenging Assay; **NBT:** Nitroblue Tetrazolium; **NADH:** Reduced Nicotinamide Adenine Dinucleotide; **PMS:** Phenazine Methosulfate; **SNP:** Sodium Nitroprusside; **PBS:** Phosphate Buffer Saline; **LBA:** Luria-Bertani Agar; **NA:** Nutrient Agar; **GAE:** Gallic Acid Equivalent; **RUE:** Rutin Equivalent; **SM:** Swabbing Method; **PPM:** Pour Plate Method; **ANOVA:** Analysis of Variance; **OD:** Optical Density.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS' CONTRIBUTIONS

Conceptualisation: SD; Methodology and Investigation: SA and JB; Data analysis: SD and SA; Writing-Original Draft: SA and JB; Writing-review and editing: SD, SA, JB and Supervision: SD. All authors read and approved the final version of the manuscript.

SUMMARY

Boerhavia diffusa root is used against urological diseases by aboriginals, but lacks evidence of sequentially extracted methanolic fraction on *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*.

- First report on comparative urobactericidal and antibiotic-modulating potential of *B. diffusa* polyphenol-rich methanolic fraction.
- LC-HRMS analysis found 17 bioactive compounds, among them majorly polyphenols, steroids, and alkaloids.
- Methanolic fraction showed a strong correlation among antioxidant, antibacterial, and antibiotic modulation by analyzing the synergistic effect.
- Provides scientific validation for the traditional uroprotective use of *B. diffusa* roots.

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