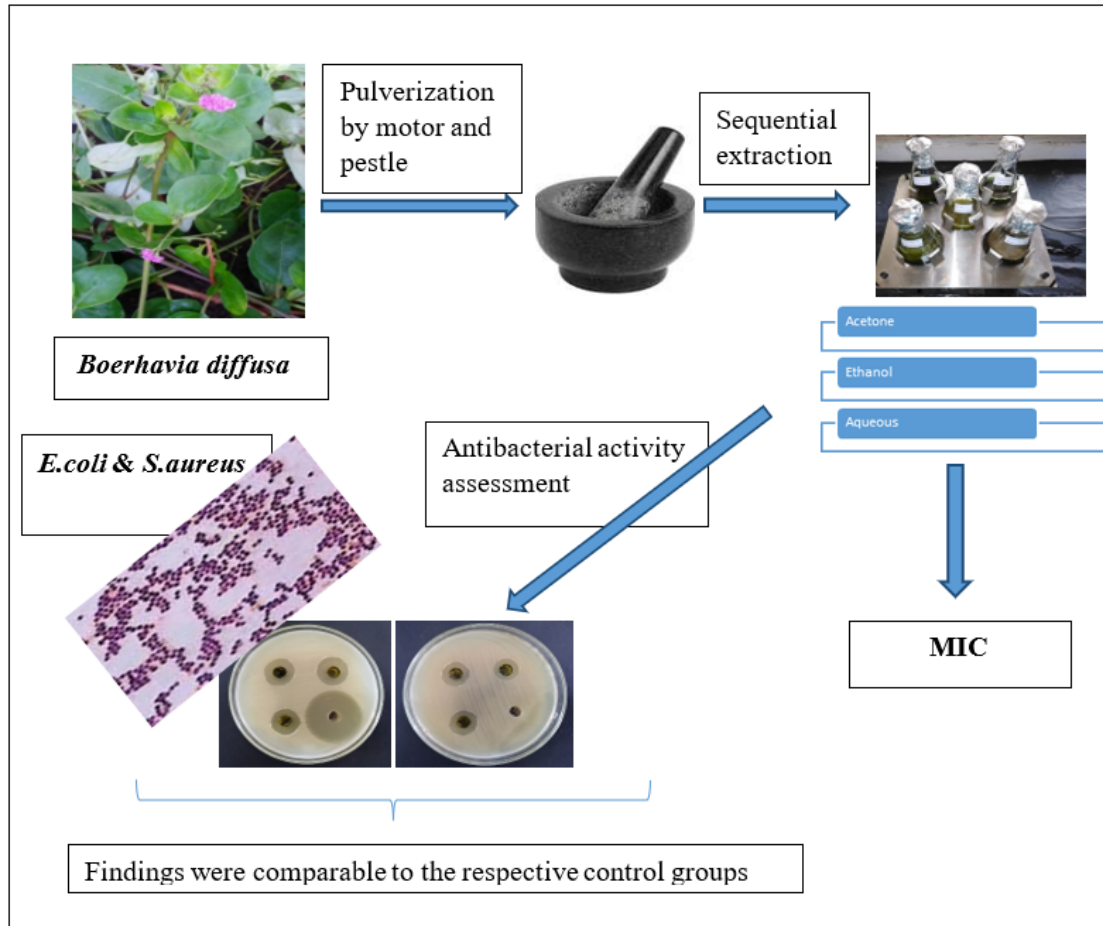


RESEARCH ARTICLE

In-vitro* antibacterial activity of *Boerhavia diffusa* leaf extract against *Staphylococcus aureus* and *Escherichia coli

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**Highlights**

- *Boerhavia diffusa* is a medicinal plant used to treat infectious diseases.
- Antibacterial activity and Minimum Inhibitory Concentration (MIC) values of plant extracts were evaluated by the agar-well diffusion method.
- The leaves of *B. diffusa* potentially contain concentration-dependent inhibitory activity against *Escherichia coli* and *Staphylococcus aureus*.

RESEARCH ARTICLE

In-vitro* antibacterial activity of *Boerhavia diffusa* leaf extract against *Staphylococcus aureus* and *Escherichia coli

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Abstract: Plants are playing a significant role in maintaining human health and improving the wealth of human life and are a better alternative to synthetic drugs that display negative side effects, such as sensitization reactions, and disruption of the metabolic processes in the body *via* interaction with the body system. The main objective of this study was to evaluate the *in-vitro* antibacterial activity of Green Leafy Vegetable (GLVs), namely *Boerhavia diffusa*. Fully matured fresh leaf samples were collected from different localities in the Western Province, Sri Lanka. The acetone, ethanol and aqueous extracts of leaf samples were tested for antibacterial activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), by using agar well diffusion method at the test concentrations of 0.25, 0.50 and 0.75 mg/ μ L. The maximum inhibitory effect was observed in the ethanol extracts of leaf samples against both bacterial strains at 0.75 mg/ μ L concentration. Ethanol extract of *B. diffusa* showed the maximum inhibition effect (17.8 \pm 0.3 mm) against *S. aureus* and it was the most susceptible bacteria than *E. coli* in plant extracts of all three solvents. All three extracts of *B. diffusa* showed most effective antibacterial activity against both bacterial strains, tested. The MIC values obtained from plants exhibited antibacterial activity ranged between 0.0625 and 0.25 mg/ μ L. The lowest MIC values were given by the ethanol extracts. Thus, the ethanol extract of this plant leaf sample was the most effective extract, which exhibited strong antimicrobial activity on both bacterial strains. Presence of bioactive compounds in these plant extracts could be the reason to indicate high antibacterial activity.

Keywords: *Boerhavia diffusa*; Antibacterial activity; *Escherichia coli*; *Staphylococcus aureus*

INTRODUCTION

Sri Lanka is rich in all three levels of biodiversity, as species, ecosystem and genetic diversity (De Zoysa et al., 2019). Green Leafy Vegetables (GLVs) play a significant role in maintaining human health and improving the wealth of human life (Hossaini et al., 2020). In order to reduce the burden of infectious diseases worldwide, antimicrobial drugs are crucial (Manandhar et al., 2019). The majority of the population, especially those living in rural areas depends mainly on medicinal plants to treat diseases. Plants have been used for centuries to treat infectious diseases and are considered an important source of new antimicrobial agents (Bereksi et al., 2018).

Pharmacological industries have produced various new antibiotics ever since, but microorganisms have slowly

developed resistance to these drugs because bacteria have the genetic capability to transmit and acquire resistance to these drugs (Abubakar et al., 2015). This has created immense clinical problems in the treatment of infectious diseases. Hence antimicrobial agents from plants are more reliable and effective sources to fight against these microorganisms without developing resistance. Antimicrobial agents in plants are secondary metabolites and are constantly present in active forms in all plants (Bereksi et al., 2018). Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant (Hussain et al., 2010). Plants have fantastic ability to produce a wide variety of secondary metabolites, like alkaloids, glycosides, terpenoids, saponins, steroids, flavonoids, tannins, quinones and coumarins (Manandhar et al., 2019). These biomolecules are the source of plant-derived antimicrobial substances (Nasrullah et al., 2012). Antimicrobial agents from plants target and destroy biochemical and morphological components of microorganisms not found in host cells (Bereksi et al., 2018). The use of plant compounds for pharmaceutical purposes has gradually increased (Nasrullah et al., 2012). Usually 70-80% of people worldwide rely chiefly on traditional, largely herbal medicine to meet their primary healthcare needs. It is further indicated that herbal medicine is still the mainstay of about 75-80% of the world population, mainly in the developing countries for primary healthcare. This is primarily due to the general belief of less or no side effects besides being cheap and locally available of plants (De Zoysa et al., 2019). Therefore, this study was carried out to evaluate the *in-vitro* antibacterial activity of *Boerhavia diffusa* that were collected from the Gampaha district, Sri Lanka.

METHODOLOGY

Selection of plant species

Boerhavia diffusa (In Sinhala: *Pita sudu sarana*, In Tamil: *Mukkarattai keerai*) was used in this study for the evaluation of the antibacterial activity. This plant has a history of using in traditional medicine.

Collection of leaf samples

The fully matured fresh leaves of selected plants without

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infection of insect pests and diseases were collected from the home garden and surrounding areas of Western Province, Sri Lanka in September 2019 and packed on the same day to bring to the Department of Botany, Eastern University, Sri Lanka.

Preparation of leaf extracts

The fresh leaves were washed three to four times thoroughly using running tap water to remove associated dust and debris. The collected samples were then air dried under the shade condition at room temperature to obtain a constant weight. Completely dried leaf samples were pulverized to produce a fine powder by using motor and pestle. The resulted powders were extracted by using three solvents like acetone, ethanol and water respectively.

Preparation of crude extracts

Ethanol and acetone extractions:

Ten grams of each finely powdered leaf samples was dissolved in 100 mL of ethanol separately in airtight conical flasks. The conical flasks were covered with cotton wool and foil paper to prevent contamination and obtain higher concentrations. The contents were kept on a mechanical orbital shaker for three days (72 hours) at room temperature and extracts were filtered through a cotton plug/double-layered Muslin cloth followed by Whatman No. 1 filter paper to remove free extractable substances. The filtrates were collected into airtight bottles. The similar process was repeated twice with fresh ethanol and the filtrates were pooled together. Finally, the solvent, ethanol was evaporated from the filtrates by keeping it in an oven at 40 °C. The thick pastes obtained are known as crude extracts. Then the dried and concentrated crude extracts were stored in sterile bottles at 4 °C until used for further study. The similar procedure was followed to obtain the acetone extract as well (Jeyaseelan et al., 2012; Nasrullah et al., 2012).

Aqueous extraction:

Ten grams of powdered plant material was dissolved in 100 mL of sterilized distilled water in a conical flask and allowed to stand for 24 hours covered with cotton wool and foil paper to prevent contamination and obtain a higher concentration. The mixture was filtered after shaken continuously for three days, and then the filtrate was concentrated to get the water extract (Aernan et al., 2016). The extraction was stored in the refrigerator at 4 °C in airtight bottles for further studies.

Antibacterial susceptibility test

Preparation of leaf extract concentrations:

Three test concentrations, 0.25 mg/μL, 0.50 mg/μL and 0.75 mg/μL of each plant extract were prepared. This weighed 250, 500, and 750 mg of crude samples, which were dissolved in 1 mL of each solvent at room temperature to prepare three concentrations.

Culture media and test microorganisms used for antibacterial activity evaluation:

Nutrient Agar (NA) media was used as the culture media. Two bacterial strains gram-negative *Escherichia coli* and

gram-positive *Staphylococcus aureus* were used to evaluate the antibacterial activity of crude extracts of selected plant leaves. Standard bacterial cultures were obtained from the Microbiology lab, Teaching hospital, Batticaloa. These bacterial strains were reactivated by sub-culturing on nutrient agar slope and maintained in a refrigerator at 4 °C for until used.

Assessment of antibacterial activity:

Agar well diffusion method was used to evaluate the antibacterial activities of different plant leaves extracts. Bacteria cell suspensions were adjusted to turbidity standards to prepare 1×10^8 bacteria cells/mL inoculum from serial dilution by measuring bacterial count using a hemocytometer. Then 0.1 mL of each bacterial suspension was inoculated on the sterilized nutrient agar medium in petri dishes through the spread plate technique by using a sterilized glass spreader. This suspension was used to inoculate on the agar medium within 15 minutes of preparation.

Wells of 8 mm in diameter and about 2 cm apart were made in the culture media using sterile cork borer to make four uniform wells in each petri dish. A drop of molten nutrient agar was used to seal the base of each well. For each treatment level six replicates were maintained. Amoxicillin (0.25 mg/μL) was used as positive control and 100 μL of each solvent was used as negative control respectively.

Antibacterial activity by well diffusion technique was identified after incubation for 24 hours at 37 °C in the incubator and the results were obtained by measuring the zone of inhibition of growth around the well in mm.

Determination of Minimum Inhibitory Concentrations (MIC values) of the plant extracts

MIC is defined as the lowest concentration of the antibacterial agent that inhibits the microbial growth after 24 hours of incubation. Different concentrations of the plant extracts (0.03125, 0.0625 and 0.125 mg/μL) were prepared separately by serial dilutions of the original extracts using the same solvent and MIC was determined by well diffusion method (Mostafa et al., 2018).

Data analysis

Results obtained in this study were expressed as mean inhibition zone diameter (mm) ± SD of six replicates. The data were analyzed by one way analysis of variance (ANOVA, P value < 0.05) using statistical software, MINITAB 14 system.

RESULTS

Antibacterial activity evaluation

The antibacterial activity of three solvent extracts of both leaf samples showed concentration-dependent inhibitory activity against both bacterial strains, with varying degrees of potency. Both leaf extracts of all three solvents exhibited a significant difference in the inhibitory activity ($P < 0.05$) against both bacterial strains.

As per the results shown in the Table 1, growth of *E. coli* and *S. aureus* were effectively inhibited by acetone and ethanol extracts of *B. diffusa* even at 0.25 mg/μL concentration

level. Acetone extract of *B. diffusa* showed prominent inhibitory action (16.4 ± 0.3 mm) at 0.75 mg/ μ L against *S. aureus*. The ethanol extract of *B. diffusa* showed better antibacterial activity than the acetone and aqueous crude extracts against both bacterial strains. Maximum inhibitory action was observed in ethanol extract of *B. diffusa* against *S. aureus* (17.8 ± 0.3 mm) at 0.75 mg/ μ L. *B. diffusa* did not show an inhibitory effect at the concentration of 0.25 mg/ μ L of aqueous against both bacterial strains tested, whereas 0.75 mg/ μ L of the aqueous extracts showed an inhibitory effect on both bacteria, with different degrees of potency (Table 1).

Minimum Inhibitory Concentrations (MIC) of the plant extracts

In this case, there was no inhibitory activity recorded at 0.25 mg/ μ L against both bacterial strains in the aqueous extract (Table 1). So, the MIC values of plant extracts were 0.75

and 0.50 mg/ μ L against *E. coli* and *S. aureus*, respectively. There was effective inhibitory activity observed in acetone and ethanol extracts against both bacterial strains at 0.25 mg/ μ L. So, it was serially diluted to prepare 0.125 and 0.0625 mg/ μ L concentrations to determine the MIC.

As per the results shown in Table 2, the MIC values obtained from plant extracts exhibited the antibacterial activity ranged between 0.0625 and 0.25 mg/ μ L. The MIC values of acetone extract of *B. diffusa* leaves were 0.25 mg/ μ L and 0.125 mg/ μ L against *E. coli* and *S. aureus*, respectively, whereas that of ethanol extract of *B. diffusa* leaves against *E. coli* and *S. aureus* were 0.125 mg/ μ L and 0.0625 mg/ μ L, respectively. However, the lowest MIC values were given in the ethanol extracts (Table 2). Thus, these results revealed that the ethanol extracts of these plant samples have more antimicrobial activity.

Table 1: Mean diameter of inhibition zone (mm), caused by acetone, ethanol and aqueous extracts of *Boerhavia diffusa* leaf samples against *Escherichia coli* and *Staphylococcus aureus* at different test concentrations

Solvent extract	Inhibition zone in mm [Test concentration(mg/ μ l)]					
	E. coli			S. aureus		
	0.25	0.50	0.75	0.25	0.50	0.75
Acetone	10.2 ± 0.2	11.6 ± 0.1	15.3 ± 0.3	11.6 ± 0.2	13.2 ± 0.2	16.4 ± 0.3
Ethanol	11.6 ± 0.2	14.2 ± 0.2	17.3 ± 0.2	12.0 ± 0.0	14.3 ± 0.3	17.8 ± 0.3
Aqueous	-	-	11.5 ± 0.5	-	9.6 ± 0.3	11.2 ± 0.3
Amoxicillin (0.25 μ g/ μ l)			24.6 ± 0.3			34.8 ± 0.2

Values are diameter of inhibition zone in mm (Mean \pm SD), (-) indicates no activity, values are significantly ($P < 0.05$) different.

Table 2: MIC values of *Boerhavia diffusa* leaf extracts against *Escherichia coli* and *Staphylococcus aureus*.

Plant species	MIC values (mg/ μ L) of extracts in different solvents					
	E. coli			S. aureus		
	Acetone	Ethanol	Aqueous	Acetone	Ethanol	Aqueous
<i>Boerhavia diffusa</i>	0.25	0.125	0.75	0.125	0.0625	0.50

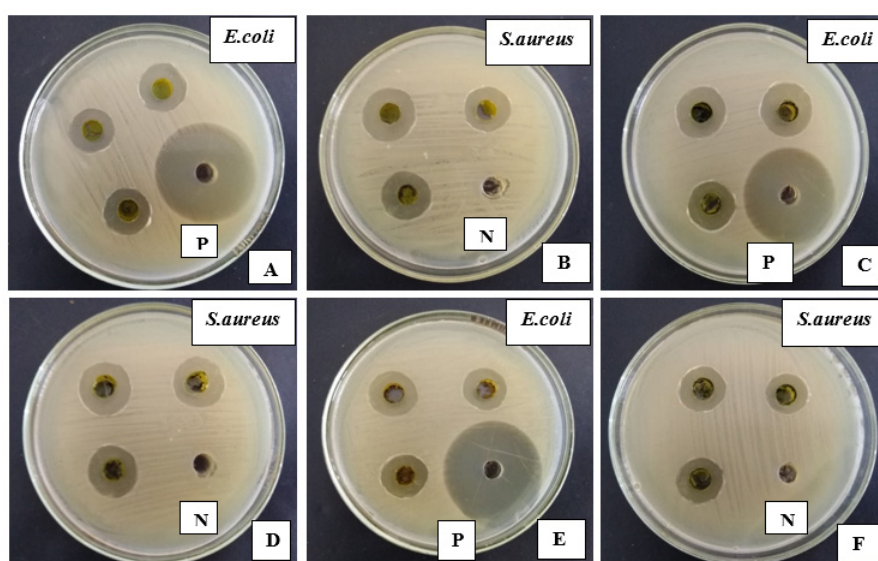


Figure 1: Antibacterial activity of *Boerhavia diffusa* leaf extracts, acetone (A, B), ethanol (C, D) and aqueous (E, F) against *Escherichia coli* and *Staphylococcus aureus* at 0.75 mg/ μ l with their respective positive (P) and negative (N) controls.

DISCUSSION

Bereksi et al., (2018) stated that the antibacterial activity of *B. diffusa* leaves was found in methanol and ethanol extracts against gram-positive and gram-negative bacteria at 0.75 mg/ μ L. Kaviya et al., (2022) also revealed that the ethanol extracts of *B. diffusa* roots inhibited the growth of *Pseudomonas aeruginosa* and *S. aureus* with zones of inhibition of about 8 mm and 20 mm at 200 μ g concentration, respectively. This is in agreement with the present study and however, the aqueous extract of the leaves of this plant also showed the inhibition of growth of both bacteria tested in the study.

Boerhavia diffusa showed effective antibacterial activity in all three solvent extracts against both bacteria. *S. aureus* was inhibited even at low concentrations of the acetone and ethanol plant extracts by exhibiting remarkable susceptibility. Single species from each of the Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*S. aureus*) were selected to study their inhibition effects against the selected plants extracts. The *E. coli* is easily cultured in the laboratory, well characterized, found in a wide range of hosts and acquires resistance to antimicrobial agents easily (Welch, 2006). Similarly, the *S. aureus* was chosen because it is a commensal in humans and develops resistance to antibiotics easily (Rasigade & Vandenesch, 2013).

Most plant antimicrobial and bioactive compounds extracted during the extraction process mainly depend on the type of solvent. Solvents are chosen based on the yield of extracts, rate of extraction and ease of evaporation at low heat (Ncube et al., 2008). Aqueous extracts are the most commonly used primary solvent in traditional medicine because water is a universal solvent. These observations can be due to the polarity of the compounds which were extracted by each solvent. Presence of bioactive compounds in plant extracts may contribute to the observed antibacterial activity of the leaves.

CONCLUSION

The leaf extracts of *Boerhavia diffusa* demonstrated an antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. The antibacterial activity of leaf extracts of all three solvents showed concentration-dependent activity against both bacterial strains.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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