# Potency of *Rhizophora stylosa* Leaves Ethanol Extract from Bama Beach, Baluran National Park in Inhibiting the Growth of Pathogenic Bacteria

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**Abstract.** *Rhizophora stylosa* leaf extract contains bioactive compounds with potential antibacterial activity, yet its effectiveness across bacterial species and concentration ranges remains insufficiently characterized. This study evaluates the antibacterial performance of ethanol-based extracts at concentrations of 10, 50, 100, 150, and 200 mg/mL against Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Vibrio cholerae, Bacillus cereus ATCC 14579, and Staphylococcus aureus ATCC 25923. Antibacterial activity was assessed using disc diffusion to measure inhibition zones and dilution assays to determine MIC and MBC values. The extract at 50 mg/mL produced the strongest inhibition against V. cholerae (9.14 ± 0.19 mm). MIC–MBC results showed E. coli with a MIC of 25 mg/mL and MBC of 100 mg/mL; B. cereus with MIC ≥1.56 mg/mL and MBC >200 mg/mL; V. cholerae with MIC 3.75 mg/mL and MBC 6.25 mg/mL. The MBC for S. aureus and P. aeruginosa was >200 mg/mL, indicating resistance. These findings demonstrate that ethanol extracts of R. stylosa leaves possess antibacterial activity, particularly against V. cholerae, and may contribute to mitigating antibiotic resistance. This work supports Sustainable Development Goal 3 by exploring natural antibacterial alternatives to enhance global health resilience.

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# 1 Introduction

Pathogenic bacteria can negatively impact health and are responsible for causing various diseases. According to Chokshi et al., in 2019, bacterial infections were considered a significant contributor to human disease in both developed and developing countries. Factors such as health, genetic makeup, and age influence a person's susceptibility to bacterial infections [1]. In 2019, more than 1 million deaths were caused by Staphylococcus aureus bacteria, and more than 500,000 deaths were caused *by Escherichia coli, Streptococcus pneumoniae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* bacteria [2].

According to the World Health Organization (WHO) in 2019, more than 4.9 million people died in 204 countries due to antibiotic-resistant bacterial infections [3]. One way to overcome antibiotic resistance is to utilize natural ingredients, such as plants. Indonesia has many islands and places with various benefits, one of which is Baluran National Park. Baluran National Park is an ecosystem with high biodiversity. Mangroves have been a source of several bioactive compounds, and several secondary metabolites, such as tannins, alkaloids, steroids, flavonoids, naphthoquinones, glycosides, and terpenoids, have been found in mangrove leaf extracts [4]. Farhadi *et al*. in 2019 stated that flavonoids can prevent bacterial division and form complex compounds that can combat extracellular proteins, thereby disrupting cell membrane integrity [5].

This study will test the antibacterial activity of ethanol extracts of *R. stylosa* leaves from Bama Beach, Baluran National Park, against several pathogenic bacteria using dilution and diffusion methods. In this study, the Minimum Inhibitor Concentration (MIC), Minimum Bactericidal Concentration (MBC) and inhibition zone values will be obtained in disc diffusion. Research in exploring the potential of ethanol extract of *R. stylosa* leaves from Bama Beach, Baluran National Park in inhibiting the growth of pathogenic bacteria has not previously been evaluated in this context, so this study is the first study in examining the use of *R. stylosa* leaves in Bama Beach, Baluran National Park to inhibit the growth of pathogenic bacteria, especially Gram-negative test bacteria (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *V. cholerae*) and Gram-positive bacteria (*B. cereus* ATCC 14579 and *S. aureus* ATCC 25923). This study is also expected to help minimize antibiotic resistance that occurs.

# 2 Materials and Methods

## 2.1 Materials

Gram-negative test bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Vibrio cholerae*) and Gram-positive bacteria (*Bacillus cereus* ATCC 14579 and *Staphylococcus aureus* ATCC 25923) were obtained from the Center for Biomolecular Engineering Research (Biome) of Airlangga University, Indonesia. The experimental material consisted of ethanol extract of *R. stylosa* leaves obtained from Exploration and Synthesis of Bioactive Compounds (ESBC) research group, PUI-PT Center for Engineering and Technology Research, Airlangga University.

## 2.2 Antimicrobial activity assay

### *2.2.1 Preparation of agar medium*

Nutrient agar (NA), Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS), Mueller-Hinton Broth (MHB), and Mueller-Hinton Agar (MHA) were prepared according to manufacturer's

instructions (Oxoid, UK). Media concentrations were calculated based on the recommended formula (e.g., 28 g/L for NA, 38 g/L for MHA, 30 g/L for MHB, and 89 g/L for TCBS) and sterilized at 121 °C for 15 minutes in an autoclave, except TCBS which was prepared without autoclaving because its selective components (sucrose, bile salts, and citrate) are heat-labile and may degrade under high temperature, thereby reducing the medium's selectivity. The prepared media were poured into sterile Petri dishes (25 mL per plate) and solidified at room temperature prior to use.

### 2.2.2 *Preparation of inoculum*

NA and TCBS media were prepared as described and used for inoculation. Fresh bacterial colonies from stock cultures were aseptically transferred using a sterile inoculating loop onto the agar plates, which were then incubated at 37 °C for 24 h. NA media was used to grow *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 14579 and *Staphylococcus aureus* ATCC 25923 bacteria. Meanwhile, TCSBS media was used to grow *Vibrio cholerae* bacteria. Next, all NA and TCSBS media were scratched in a zigzag manner using an Ose needle. Then, the media were incubated for 24 hours at 37°C.

### 2.2.3 *Preparation of microbiological suspension*

Using a sterile tube needle, bacterial inoculum was transferred and suspended in MHB within a sterile test tube. The microbial density was then adjusted using a spectrophotometer (Genesys 50 UV-VIS, Thermo Scientific) set at 600 nm wavelength, targeting an optical density (OD) of 0.1 for standardization.

### 2.2.4 *Preparation of Rhizophora stylosa Leaf Extract Solution*

The obtained herbal extract was weighed according to the target concentration and dissolved in sterile distilled water to prepare test solutions of 1%, 5%, 10%, 15%, and 20% (w/v), each in a final volume of 1 mL. The 20% solution was used as a stock solution and serially diluted to prepare lower concentrations (10%, 5%, and 1%). All dilutions were performed under aseptic conditions and vortexed to ensure homogeneity prior to microbiological testing.

### 2.2.5 *Antimicrobial screening*

A suspension of Gram-negative test bacteria (*E coli* ATCC 25922, *P.aeruginosa* ATCC 27853, and *V.cholerae*) and Gram-positive bacteria (*B.cereus* ATCC 14579 and *S.aureus* ATCC 25923) were prepared and adjusted to an optical density (OD) of 0.1. A volume of 100 µL of each bacterial suspension was evenly spread onto MHA plates using sterile cotton swabs with a continuous streaking technique to ensure uniform distribution. Sterile paper discs were placed on the surface of the agar using sterile tweezers. Each disc was carefully loaded with 20 µL of *R.stylosa* extract solutions at concentrations of 10, 50, 100, 150, and 200 mg/mL. A negative control (distilled water) and a positive control (Gentamicin) were included for bacterial comparison. All treatments were performed in duplo. The plates were then incubated at 37°C for 18–24 hours. After incubation, the diameter of the inhibition zone surrounding each disc was measured using a digital caliper to assess antibacterial activity.

### 2.3 MIC and MBC assay

All tests were performed in Mueller-Hinton medium. MIC and MBC testing were performed using the CLSI microdilution method (2012). The MIC assay was conducted in a 96-well microtiter plate using serial two-fold dilutions of the herbal extract. Each well was filled with 50 μL of Mueller-Hinton Broth (MHB) and 50 μL of extract solution, resulting in final concentrations ranging from 200 mg/mL to 0.39 mg/mL. Bacterial suspensions of *E. coli* ATCC 25922, *P.aeruginosa* ATCC 27853, *V.cholerae*, *S.aureus* ATCC 25923, and *B.cereus* ATCC 14579 were prepared and added at 5 μL per well. Negative control wells contained only MHB and bacterial culture, while positive control wells were treated with gentamicin. All treatments were performed in triplicate. The plates were incubated at 37°C for 18–24 hours. After incubation, absorbance readings were taken at 630 nm using a microplate reader. The MIC was defined as the lowest concentration of the extract that showed no visible turbidity and minimal OD value, indicating inhibition of bacterial growth. MBC determination was performed by subculturing wells with no visible growth onto agar plates and observing for colony formation. A decrease in OD and color change in indicator wells confirmed microbial inhibition and extract activity.

## 3 Result and Discussion

As presented in Table 1 and Figure 1, the Gram-positive bacterium *Bacillus cereus* exhibited a significantly larger inhibition zone diameter than *Staphylococcus aureus* when treated with ethanol extract of *Rhizophora stylosa* leaves. At a concentration of 200 mg/mL, *B. cereus* produced an inhibition zone of 9.1 ± 1.0 mm, while *S. aureus* only reached 7.5 ± 0.5 mm, indicating that *B. cereus* is more sensitive to the bioactive compounds in the extract. For Gram-negative bacteria, namely *Escherichia coli*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*, the inhibition zone diameters for *E. coli* and *P. aeruginosa* remained constant at 6.00 ± 0.00 mm across all tested concentrations, except for *E. coli* at 100 mg/mL, which showed a slight increase to 6.6 ± 0.3 mm. These results suggest that the ethanol leaf extract of *R. stylosa* exhibited weak antibacterial activity against *E. coli* and *P. aeruginosa*. In contrast, *V. cholerae* showed a larger inhibition zone (8.06 ± 0.02 mm at 10 mg/mL), which decreased at higher concentrations (7.63 ± 0.11 mm at 200 mg/mL). Such variations may reflect differences in the activity of specific bioactive compounds within the extractthas very weak antibacterial activity against both *E. coli* and *P. aeruginosa*. Interestingly, *V. cholerae* demonstrated a larger inhibition zone of 8.06 ± 0.02 mm at a low concentration of 10 mg/mL, but this decreased to 7.63 ± 0.11 mm at 200 mg/mL. The variation in inhibition zone diameters may be influenced by the specific bioactive compounds present in the extract.

Phytochemical analysis revealed that the ethanol extract of *R. stylosa* leaves from Baluran National Park contains alkaloids, phenolics, and flavonoids, with the highest total flavonoid and phenolic content among 17 mangrove species—346.02 ± 4.48 mg QE/g for flavonoids and 64.66 ± 1.72 mg GAE/g for phenolics [7]. These compounds are known to contribute to antibacterial activity, which may explain the sensitivity observed in *B. cereus* and *V. cholerae*.

Comparative studies further support these findings. Gopal et al. (2019) investigated *R. stylosa* leaf extracts using methanol, chloroform, and petroleum ether solvents at concentrations of 12.5, 25, and 50 mg/mL via the well diffusion method. Their results showed inhibition zones ranging from 8–12 mm for Gram-positive bacteria (*S. aureus*, *S. epidermidis*, *S. pyogenes*) and 11–19 mm for Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa*), indicating broad-spectrum antibacterial potential [7]. However, a more recent study also used methanol as the solvent but employed the disk diffusion method at concentrations of 10, 50, 100, and 200 mg/mL, reported no inhibition zones for the same

bacterial strains [8]. This discrepancy highlights the influence of methodological differences—particularly the choice of diffusion technique—on the observed antibacterial outcomes.

**Table 1.** Antibacterial activity of *R. stylosa* extracts against the bacteri pathogens

| Bacteria | Inhibition zone (mm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Concentration Mangrove (mg/ml) | | | | | Aquadest | Gentamicin |
| | 200 | 150 | 100 | 50 | 10 | | |
| *Escherichia coli* | 6,0 ± 0,0 | 6,0 ± 0,0 | 6,6 ± 0,3 | 6,0 ± 0,0 | 6,0 ± 0,0 | 6,0 ± 0,0 | 17,7± 0,5 |
| *Bacillus cereus* | 9,1 ± 1,0 | 8,2 ± 0,6 | 7,4 ± 0,1 | 7,5 ± 0,0 | 7,4 ±0,1 | 6,0 ± 0,0 | 19,3 ± 0,6 |
| *Staphylococcus aureus* | 7,5 ± 0,5 | 8,1 ± 0,4 | 7,1 ± 0,5 | 6,8 ± 0,2 | 6,6 ± 0,3 | 6,0 ± 0,0 | 27,9 ± 1,5 |
| *Pseudomonas aeruginosa* | 6,0 ± 0,0 | 6,0 ± 0,0 | 6,0 ± 0,0 | 6,0 ± 0,0 | 6,0 ± 0,0 | 6,0 ± 0,0 | 19,3 ± 1,0 |
| *Vibrio cholerae* | 7,6 ± 0,1 | 7,6 ± 0,2 | 7,7 ± 0,1 | 9,1 ± 0,2 | 8,1 ± 0,0 | 6,0 ± 0,0 | 17,2 ± 0,2 |

Alkaloid compounds can inhibit bacterial growth through various mechanisms, including inhibition of bacterial nucleic acid and protein synthesis, modification of bacterial cell membrane permeability, damage to cell membranes and cell walls, inhibition of bacterial metabolism, and inhibition of efflux pumps [9]. Flavonoid compounds can also inhibit bacterial nucleic acid synthesis and aggregation through membrane fusion, which then reduces active nutrient uptake, thus preventing bacterial survival [10]. According to Lobiuc et al., phenolic compounds can play an important role in inhibiting bacterial growth by damaging bacterial proteins and cell walls, altering cytoplasmic function and membrane permeability, inhibiting energy metabolism and nucleic acid synthesis in bacterial cells. At the DNA level, phenolics compounds can penetrate the DNA helix during replication, recombination, repair, and transcription [10]. The inhibitory mechanisms of compounds in extracts can vary, depending on the structure of the compounds in the extract and the species of bacteria tested.

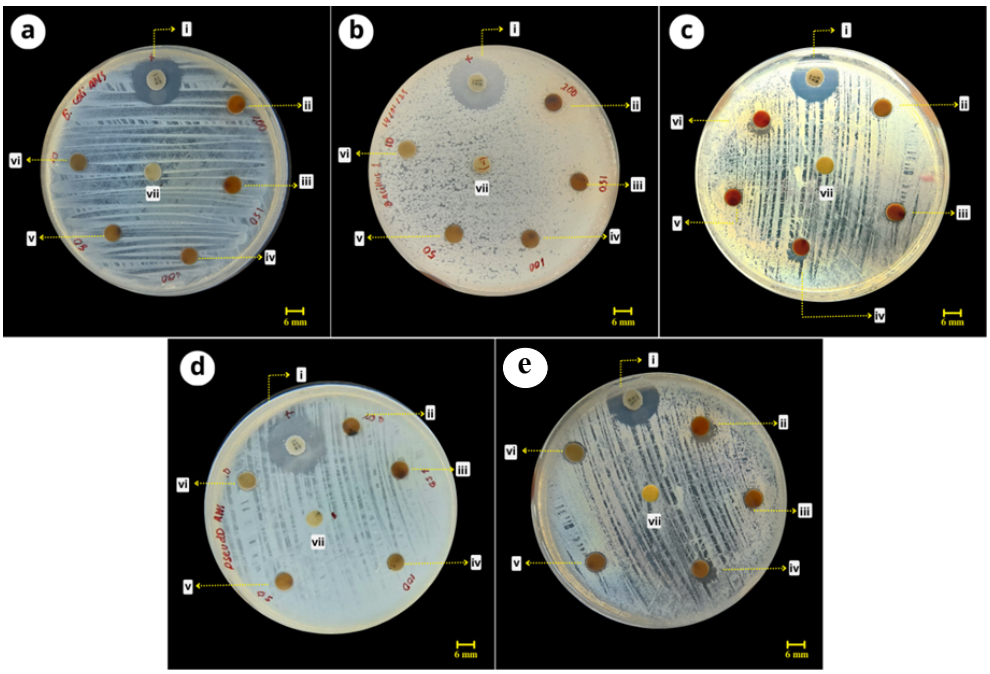


**Fig. 1.** Disk diffusion assay results of R. stylosa extract against bacteria: (a*) Escherichia coli* ATCC 25922; (b) *Bacillus cereus* ATCC 14579; (c) *Staphylococcus aureus* ATCC 25923; (d) *Pseudomonas aeruginosa* ATCC 27853; and (e) *Vibrio cholerae*

**Table 2.** Antibacterial activity of *R. stylosa* extracts against the bacteri pathogens

| Bacteria | Assay | Concentration (mg/mL) |
|---|---|---|
| *Escherichia coli* ATCC 25922 | MIC | 25 |
| | MBC | 100 |
| *Bacillus cereus* ATCC 14579 | MIC | 1,56 |
| | MBC | > 200 |
| *Staphylococcus aureus* ATCC 25923 | MIC | 200 |
| | MBC | > 200 |
| *Pseudomonas aeruginosa* ATCC 27853 | MIC | > 200 |
| | MBC | > 200 |
| *Vibrio cholerae* | MIC | 3,75 |
| | MBC | 6,25 |

As presented in Table 2, *B. cereus* exhibited the highest sensitivity to the ethanol extract of *R. stylosa* leaves, with a minimum inhibitory concentration (MIC) of ≥1.56 mg/mL but a minimum bactericidal concentration (MBC) of >200 mg/mL, indicating strong growth inhibition but limited bactericidal activity. *V.cholerae* also showed notable sensitivity (MIC 3.75 mg/mL; MBC 6.25 mg/mL), while *E.coli* was more resistant (MIC 25 mg/mL; MBC 100 mg/mL). In contrast, *S. aureus* and *P. aeruginosa* were classified as resistant, both showing MBC values >200 mg/mL, suggesting that the compounds in the extract (alkaloids, phenolics, and flavonoids) were ineffective at the tested concentrations.

These findings are consistent with a study by Saad *et al.,* which evaluated the antibacterial activity of *Sonneratia alba* mangrove leaf extract against *S. aureus*, *B. cereus*, *E. coli*, and *P. aeruginosa* using methanol and ethyl acetate solvents [11]. In that study, *P. aeruginosa* showed no inhibitory response, while *S. aureus* and *B. cereus* were more sensitive, with *B. cereus* exhibiting MIC values as low as 1.11 mg/mL. Although Saad *et al.* investigated a

different mangrove species, the results align with the present study on *R.stylosa*, confirming that *B. cereus* and *S. aureus* are generally more sensitive to mangrove-derived compounds, whereas *P. aeruginosa* tends to be resistant. In reference to CLSI (2012), *B. cereus* ATCC 14579 and *V. cholerae* demonstrated strong antibacterial potential (MIC < 4 µg/mL), while *E. coli*, *S. aureus*, and *P. aeruginosa* had MIC values exceeding 32 µg/mL, indicating lower inhibitory effectiveness [12].

This study has several limitations. First, incomplete solubility of the ethanol extract in 96-well microplate assays occasionally resulted in sediment formation, which may have influenced MIC and MBC readings. Second, only five bacterial strains were tested, and the concentration range was limited to 10–200 mg/mL, which restricts broader generalization of the findings. Future studies should expand the range of tested pathogens, include in vivo models, and explore fractionation of active compounds to better understand their antibacterial mechanisms. In conclusion, the ethanol extract of *R.stylosa* leaves demonstrated notable inhibitory activity, particularly against *B.cereus* and *V.cholerae*, highlighting its potential as a natural antibacterial agent while underscoring the need for further investigation.

## 4 Conclusion

The ethanol extract of *R.stylosa* leaves demonstrated notable antibacterial activity, particularly against *B. cereus* and *V.cholerae*, as reflected by low MIC values. However, the extract showed limited bactericidal effects, with high MBC values for most tested strains, indicating that its inhibitory potential does not necessarily translate into strong killing activity. These findings highlight the promise of *R. stylosa* as a natural source of antibacterial agents, while also underscoring the need for further studies to improve extract solubility, expand pathogen coverage, and explore active compound fractionation. Future research should include in vivo validation and mechanistic studies to better assess its therapeutic potential in addressing antibiotic resistance.

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