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In Vitro Antibacterial Bioassay of Methanol Extract Fraction (Rf > 0.5) from *Rhizophora mucronata* L. (Rhizophoraceae) Leaves Against Multidrug-Resistant *Escherichia coli*

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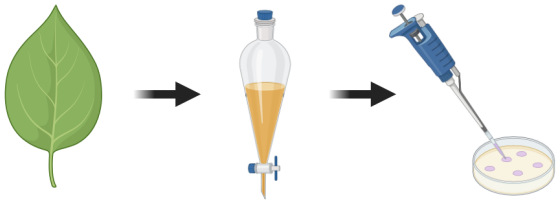

Abstract: A bioactivity study was conducted in January 2018 on the methanolic extract (Rf > 0.5) of mangrove leaves from *Rhizophora mucronata* L. (Rhizophoraceae) targeting resistant *Escherichia coli*. Secondary metabolites were isolated at the Marine Chemical Laboratory, Faculty of Marine and Fisheries, while antibacterial testing was performed at the Microbiology Laboratory, Faculty of Medicine, Syiah Kuala University. Using thin layer chromatography, bioactive fractions were identified, and phytochemical screening confirmed the presence of alkaloids. The extract demonstrated inhibition zones between 7.50–8.50 mm, indicating moderate antibacterial activity against resistant *E. coli*.

Keywords: *Rhizophora mucronata* leaves; alkaloids; bioactivity assay; antibiotic-resistant *Escherichia coli*.

1. Introduction

Escherichia coli is a Gram-negative bacterium that normally resides in the intestines of humans and animals. It becomes pathogenic and capable of causing disease when present in quantities exceeding 10,000 CFU/mL. Strains such as Enteropathogenic *Escherichia coli* (EPEC) are known to cause diarrhea, particularly in individuals with compromised immune systems, including infants, young children, the elderly, and the sick [1]. Transmission of this bacterium is commonly linked to fecal contamination and polluted environments [2]. With an estimated global morbidity and mortality rate of 1.8 billion cases annually, *E. coli* is recognized as a significant public health concern [3].

Medically, diarrhea is commonly treated with synthetic β-lactam antibiotics such as chloramphenicol [4]. However, continuous use of chloramphenicol can result in adverse effects including gastrointestinal disturbances, hypersensitivity reactions, aplastic anemia, and granulocytopenia [5]. Furthermore, prolonged use has been associated with increased bacterial resistance in Enteropathogenic *E. coli* strains [6].

Research has shown that secondary metabolites in the mangrove species *Rhizophora mucronata* possess antibacterial properties against pathogenic bacteria [7]. According to studies, metabolites such as alkaloids, flavonoids, phenols, peptides, saponins, and terpenoids from *R. mucronata* leaf extracts have demonstrated inhibitory effects against bacterial species such as *E. coli, Aeromonas* sp., *Streptococcus* sp., and *Edwardsiella* sp. [8]. Additionally, the extract has shown antifungal activity, notably against *Penicillium digitatum* [9]. However, no studies have yet examined the bioactivity of secondary metabolites in *R. mucronata* leaf extracts specifically with Rf > 0.5. This research aims to evaluate the antibacterial potential of the methanolic leaf extract fraction (Rf > 0.5) of *R. mucronata* against resistant *Escherichia coli strains*.

2. Results

The Leaves of *Rhizophora mucronata* were air-dried for approximately 3–5 days. Once dried, the leaves were chopped into smaller pieces to facilitate solvent interaction and increase the extraction of bioactive compounds [1]. The finely cut leaves were then extracted using 70% methanol for 3x24 hours. The resulting macerate was filtered through filter paper and evaporated at 60°C for approximately 1 hour using a rotary evaporator. A total of 1.98 grams of crude extract (sample code: A17A01) was partitioned using a solvent mixture of chloroform:methanol:water (1:1:1, v/v). This partitioning process yielded two fractions: a polar and a semipolar fraction. Both fractions were subjected to bioactivity testing against resistant *Escherichia coli* strains.

Table 1. Inhibition Zone Diameters of Bioactivity Test.

Sample	Concentration	Inhibition Zone Diameter (mm)
A17A01	100 µg/mL	7.25
DMSO (-)	2%	0
Chloramphenicol (+)	30 µg/mL	7.25

Furthermore, 2.0 grams of sample A17A01 was partitioned using a solvent mixture of chloroform:methanol:water (1:1:1, v/v), resulting in two fractions. The semipolar fraction (approximately 0.02 grams) was labeled F1B16, while the polar fraction (approximately 1.96 grams) was labeled F1B17. As a result, the measurement results for inhibition zone diameters are presented in Table 2.

Table 2. Comparison results of the measurement of the inhibition zone diameter.

Sample	Concentration	Inhibition Zone Diameter (mm)
A17A01	100 µg/mL	7.75
F1B17	100 µg/mL	8.25
DMSO (-)	2%	0
Chloramphenicol (+)	30 µg/mL	8.25
F1B16	100 µg/mL	7.25

Bioactivity testing revealed that fraction F1B17 and the reference antibiotic chloramphenicol both exhibited an identical inhibition zone diameter of 8.25 mm, which was larger than those observed for fraction F1B16 and the crude extract A17A01. These results indicate that fraction F1B17 demonstrated enhanced antibacterial activity following partitioning, compared to its crude form. Given its superior activity, fraction F1B17 was selected for further purification through elution using a methanol:ethyl acetate solvent system (10:90 v/v), followed by compound isolation via Thin Layer Chromatography (TLC).

Elution of fraction F1B17 revealed the presence of compounds with Rf values greater than 0.5. The selection and isolation of compounds with Rf > 0.5 aimed to evaluate their affinity and inhibitory activity against resistant Escherichia coli. The fraction with Rf > 0.5 was subsequently partitioned using a methanol:dichloromethane solvent system (1:1 v/v), resulting in two sub-fractions: a methanol fraction (coded F2B10) and a dichloromethane fraction (coded F2B11). Both F2B10 and F2B11 were then subjected to phytochemical screening using Dragendorff's reagent and cerium sulfate to detect the presence of alkaloids and hydrocarbons, respectively. The phytochemical screening results for F2B10 and F2B11 are presented in Table 3.

Table 3. Phytochemical results of fractions F2B10 and F2B11.

Name of Compound	Reagent	Discoloration	Remark	
			F2B10	F2B11
Hydrocarbons	Cerium sulfate	Blackish color spots	++	-
Alkaloid	Dragendroff	Orange colored spots	++	+

Remark: (++) : Moderate , (+) : Weak, (-) : None

Bioactivity testing against resistant *Escherichia coli* revealed that fraction F2B10 exhibited a larger inhibition zone (8.50 mm) compared to fraction F2B11 (7.50 mm), and also surpassed the inhibition zone of the standard antibiotic chloramphenicol (7.00 mm). These results indicate that F2B10 possesses stronger antibacterial properties than both F2B11 and the positive control. Based on its superior activity, fraction F2B10 was selected for further testing using a range of concentrations: 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, and 100 µg/mL, to evaluate the dose-dependent relationship of its antibacterial effect. The results of measuring the diameter of the inhibition zone of the F2B10 fraction with concentrations of 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL are shown in Table 4.

Table 4. Results of measuring the diameter of the F2B10 inhibition zone

Sample	Concentration	Inhibition Zone Diameter (mm)
F2B10	100 µg/mL	8.50
F2B10	80 µg/mL	7.70
F2B10	60 µg/mL	7.60
F2B10	40 µg/mL	7.55
Chloramphenicol (+)	35 µg/mL	7.75
F2B10	20 µg/mL	7.50
DMSO (-)	2%	0
F2B11	100 µg/mL	7.50

3. Discussion

Based on the initial bioactivity testing, both the crude extract sample A17A01 and the reference antibiotic chloramphenicol produced inhibition zones measuring 7.25 mm in diameter. In contrast, the negative control (2% DMSO) showed no inhibitory effect, confirming that the antibacterial activity observed was attributable to the bioactive compounds in the sample and not the solvent. These findings indicate that A17A01 contains active constituents capable of suppressing the growth of resistant *Escherichia coli*. Potential bioactive compounds in the extract may include hydrocarbons, alkaloids, flavonoids, phenols, terpenoids, peptides, and saponins [2-4]. To further isolate these active components, the extract was fractionated using Thin Layer Chromatography (TLC). The bioactive fraction obtained from *Rhizophora mucronata* leaves was spotted onto a silica gel TLC plate with a capillary pipette and developed in an organic solvent system within a sealed chamber to maintain eluent saturation [7-8]. After separation, the fraction exhibiting an Rf value greater than 0.5 was selected for further analysis. This fraction was re-applied to a TLC plate and treated with cerium sulfate and Dragendorff's reagents, which enabled visualization and chemical characterization of the constituent compounds [5].

Subsequent bioactivity assays on the most promising fraction, F2B10, were conducted across a range of concentrations (20–100 µg/mL) to assess dose-dependent effects [9]. The results revealed a clear positive correlation between concentration and antibacterial efficacy. At 20 µg/mL, F2B10 demonstrated an inhibition zone of 6.00 mm, which gradually increased with concentration, peaking at 8.50 mm at 100 µg/mL (Table 4). This dose-dependent activity suggests that the compounds in F2B10 become increasingly effective at higher concentrations, consistent with pharmacological principles whereby higher doses enhance the interaction between bioactive agents and microbial targets, disrupting bacterial growth more effectively [10-13].

Notably, the inhibition zone at 100 µg/mL exceeded that of chloramphenicol, which previously measured 7.00 mm. This is a significant finding, as it implies that the antibacterial activity of F2B10 may rival, or even surpass, that of conventional antibiotics, particularly important in the context of antibiotic resistance. Furthermore, F2B10 demonstrated greater activity than F2B11, a related fraction, reinforcing the hypothesis that specific compounds within F2B10 are responsible for its superior antibacterial properties [15]. Phytochemical screening results (Table 3) support this conclusion. F2B10 tested positive for alkaloids well-established antimicrobial agents known to interfere with bacterial DNA replication, protein synthesis, and membrane integrity [1]. In contrast, F2B11, which exhibited weaker antibacterial activity, lacked detectable alkaloids and instead contained hydrocarbon compounds. This contrast further suggests that the antibacterial effect observed in F2B10 is likely due to polar, alkaloid-rich constituents soluble in methanol.

These findings are in agreement with previous studies reporting the antibacterial potential of secondary metabolites derived from *Rhizophora mucronata* [2][3]. Specifically, polar compounds with Rf values greater than 0.5 have shown promising activity, further validating their potential as lead candidates for developing novel antibacterial agents. In light of growing concerns over antibiotic resistance, continued isolation and structural elucidation of the active constituents within F2B10 may provide critical insights for future drug discovery.

4. Materials and Methods

4.1. Materials

The equipment used in this study included: an analytical balance (Kern), rotary evaporator (Eyela N-1000), incubator (Memmert Type INB 500), autoclave (Tommy SX-300/500/700), laminar air flow cabinet (Safe Fast Elite 212 SD), UV lamp (UVGL-25), hot plate (Akebono), oven (Jouan), refrigerator (LG), and a thin-layer chromatography (TLC) apparatus. Other laboratory glassware and equipment included: Pyrex Petri dishes, beakers, test tubes, separating funnels, measuring cylinders, Erlenmeyer flasks, dropper pipettes, volumetric pipettes, and cuvettes (1.5 mL). Micropipettes used were Pipetteman P20 (volume range 2–20 µL) and Eppendorf micropipette (100–1000 µL). Additional tools included: caliper, aerator, cotton swabs, inoculation loops, spirit lamp, TLC capillary pipettes, aluminum foil, filter paper, forceps, gauze, tissue paper, gloves, labeling paper, microwave oven, paper discs, sample bottles, and other standard laboratory tools and materials.

4.2.Bacterial Specimen

Escherichia coli O157:H7, obtained from the Regional General Hospital (RSUD) Dr. Zainoel Abidin, Banda Aceh. The bacterial strain was cultured in the Microbiology Laboratory, Faculty of Medicine, Syiah Kuala University.

4.3. Preparation and Standardization of Bacterial Suspension

Bacterial colonies grown on Nutrient Agar (NA) were transferred using an inoculation loop into a sterile tube containing 0.9% NaCl solution. The suspension was then homogenized using a vortex mixer for 15 seconds. Turbidity in the suspension, indicating bacterial growth, was adjusted to match McFarland standard No. 3, which corresponds to approximately 10^9 bacterial cells/mL. This suspension was further diluted using 0.9% NaCl to reach a final concentration of 10^8 cells/mL [14]. The chosen concentration was based on aerobic bacterial sensitivity, which typically ranges from 10^8 to 10^4 cells/mL [14]. A 1.0 mL aliquot of the homogenized suspension was transferred into a cuvette, and the optical density was measured at a wavelength of 625 nm using a spectrophotometer. The absorbance value was considered acceptable between 0.08 and 0.13. Suspensions within this range were deemed ready for use in antibacterial assays [14].

4.4. Bioactivity Assay Procedure

Escherichia coli O157:H7 was applied to sterile Nutrient Agar media using the spread plate method. The bacterial suspension was evenly spread over the surface of the NA plate three times, rotating the Petri dish by 60° between each application to ensure uniform distribution. Sterile discs were impregnated with the F2B10 fraction at concentrations of 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, and 100 µg/mL. Each disc was carefully placed onto the agar surface using sterile forceps, with slight pressure applied to ensure proper adherence. Additionally, discs soaked with 2% DMSO served as the negative control, while 30 µg/mL chloramphenicol discs were used as the positive control [4-5]. All plates were incubated at 37°C for 12–24 hours. Following incubation, the diameter of the inhibition zones was measured using a caliper [6]

5. Conclusions

This study demonstrated that the methanolic extract of *Rhizophora mucronata* leaves and its subsequent fractions possess significant antibacterial activity against resistant *Escherichia coli* O157:H7. Among the tested samples, fraction F2B10, obtained through TLC isolation with Rf > 0.5 showed the highest inhibitory effect, with a dose-dependent increase in inhibition zone diameter, reaching up to 8.50 mm at 100 µg/mL. This activity exceeded that of the reference antibiotic chloramphenicol at 30 µg/mL, indicating the potential of polar compounds, particularly alkaloids as effective antibacterial agents. Phytochemical screening confirmed the presence of alkaloids in F2B10, supporting its bioactivity. These findings suggest that secondary metabolites from *R. mucronata*, especially those with polar characteristics, offer promising potential as alternative antibacterial agents in the fight against antibiotic-resistant pathogens. Further studies involving compound purification and structural characterization are recommended to explore their therapeutic applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.scientifia.com/article/doi.

Author Contributions: N.N. designed the study and was responsible for the methodology, data analysis, laboratory work, data management, visualization, and manuscript preparation. S.A. performed the validation of research findings. All authors have reviewed and approved the final version of the manuscript for publication.

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Conflicts of Interest: The authors declare no conflicts of interest.

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