Phytochemical analysis and antimicrobial activities of
mangrove plant (Rhizophora apiculata) against selected fish
pathogenic bacteria

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Abstract. Rhizophora apiculata leaf extracts were tested on the isolated growth of S.
agalactiae and E. coli and extraction of the bioactive compounds were performed using
Soxhlet extraction technique with methanol (MtOH) as the organic solvent. The presence of
various phytochemicals in bioactive constituents of the plant was detected using standard
chemical procedures. The Kirby-Bauer test was used for the antibacterial assay, and an in
vitro assay was conducted for minimum inhibitory concentration (MIC) and minimum
bacterial concentration (MBC). Brine shrimp lethality assay evaluated the toxicity of R.
apiculata. Antibiotic susceptibility test showed high rates of E. coli resistance to neomycin
and sensitivity to tetracycline and amoxycilin, while S. agalactiae showed sensitivity to all
antibiotics. Inhibition zones of R. apiculata leave extract against the microorganisms
recorded in the range of 2-9 mm. Increasing the inhibition zone is dependent
on increasing plant extract concentrations. The (MIC) testing range of R. apiculata was 6.25 mg/ml to 12.5
mg/ml against S. agalactiae and E. coli, respectively. The MBC/MIC ratio of S. agalactiae
demonstrated to be bactericidal, and the MBC/MIC ratio of E. coli was bacteriostatic. R.
apiculata recorded an LC_{50} value of 81 mg/ml. The findings of this study reveal the valuable
metabolites found in mangrove leaves. The most important secondary metabolites are
alkaloids, terpenoids and tannins as well as saponins and steroids, but not flavonoids. This
study suggests that Rhizophora apiculata has the potential to be a valuable candidate in the
discovery of antimicrobial compounds against fish pathogens.

1. Introduction
Awareness of specific medicinal plant usage has been the result of several thousand years of research
with the intent to prevent, treat or modify disease. Worldwide, various medicinal plants are used
daily in life to help with treating disease [1]. Mangrove plants hold minimal side effects and for this
reason, play a key role in treating disease [2]. Furthermore, the uses of mangrove plants in medicinal
fields have revealed that their extracts possess inhibitory activity against both animal and human
pathogens [3, 4].

Rhizophora apiculata is a mangrove plant commonly used in the practice of traditional medicine
for treating bacterial illnesses [4]. Previous studies have reported the capability of R. apiculata in
inhibiting human immunodeficiency virus (HIV) [5] and their extracts are said to have stimulated the
growth and increased survival rates of Vibrio alginolyticus infected clownfish [6]. Phytochemicals
have been grouped into two groups, primary constituents (proteins, amino acids, chlorophyll, and common sugars) and secondary constituents (phenolic compounds, tannins, saponins, essential oils, alkaloids, flavonoids, terpenoids, and other constituents) [7]. Researchers have shifted their attention towards investigating plant phytochemical constituents on animal and human health. The bioactive constituents that hold the most significant antimicrobial activity are tannins, saponins, alkaloids, flavonoids and terpenoids [8]. Phytoconstituents are bioactive compounds naturally occurring in plants that work together with fibres and nutrients to form an integrated defence system against stress conditions and diseases [1].

Previous research done by [9] reported *R. apiculata* antimicrobial activities by the Minimum Inhibitory Concentration (MIC) test, and their results indicated bacteriostatic activities at low concentration and bactericidal activities at higher concentrations. A study by [4] showed that leaf extract of *R. apiculata* have effects on pathogenic bacteria in fish. *R. apiculata* and *Sonneratia caseolaris* extracts have been examined and found to possess a bioactive compound capable of inhibiting the effects on the microorganism [4]. The present study has been conducted with the objective to explore the antimicrobial activity of the methanolic extract *R. apiculata* against pathogenic bacteria found in fish.

2. Material and Methods

2.1 Taxonomy of *Rhizophora apiculata*

Identification of *Rhizophora apiculata* plant was made at the plant taxonomy laboratory in University Malaysia Terengganu (UMT).

2.2 Preparation of Plant and Extraction

Leaves of *R. apiculata* were gathered from Terengganu, near the beaches of University Malaysia Terengganu (UMT). These leaves were washed thoroughly, air-dried in an oven overnight at 40°C and grinded into a fine powder using a blender. The overall weight of the *R. apiculata* leaves powder was 1 kg. A total of 25 g of plant sample was soxhlet extracted with 250 mL of methanol (MtOH) for 12 h at 60°C [10]. Extracts in methanol solvents were evaporated and concentrated by rotary evaporation at 45°C. The final dry weight after extraction was determined and the extraction yield (%) was calculated [2].

2.3 Preliminary Phytochemical Test

The *R. apiculata* leaves extract were tested for phytoconstituents presence including alkaloids, flavonoids and terpenoids, as well as steroids, saponins, and tannins.

2.3.1 Test for Terpenoids

A sample of 0.8 g was inserted into a test tube with the addition of 10 ml of methanol. The test tube was vigorously shaken and then filtered to produce 5 ml of plant extract sample. Afterwards, 2 ml of chloroform was mixed into the selected plant extract sample. The presence of terpenoids in plants is indicated by the formation of a reddish-brown colour [11].

2.3.2 Test for Flavonoids

For flavonoid confirmation, 0.5 g of extract was mixed with 10 ml of distilled water in a test tube, and a diluted ammonia solution was added. It was filtrated, and 1 ml concentration of sulphuric acid (H₂SO₄) was added. Flavonoid presence is confirmed by a yellow colour [11].

2.3.3 Test for Tannins (Ferric Chloride Test)

Along with 0.5 g of dissolved crude extract, 20 mL of distilled boiling water was added to the test tube and boiled for 1 hour. Ferric chloride was added in drops and allowed to stand. The presence of a blue-black colour indicates the presence of tannins [12].
2.3.4 Test for Saponins (Frothing Test)
To detect the presence of saponins, a sample of 0.5 g of crude extract was inserted into a test tube containing 3 mL of distilled hot water. The mixture was then vigorously shaken for one minute. Development of foam suggests the presence of saponins [12].

2.3.5 Test for Alkaloids
Three millilitres (3 mL) of hexane solution was mixed with 0.2 g of sample in a test tube and shaken well before filtering. Then, 5 mL of 2% Hydrochloric acid (HCl) was added into a test tube, mixed, and filtered. Drops of picric acid were added. Yellow precipitate indicates the presence of alkaloid [11].

2.3.6 Test for Steroids
A sample of 1.0 g of the sample was taken in a test tube and dissolved with 10 mL chloroform. Addition of 10 mL of Sulphuric acid (H$_2$SO$_4$) was done by pouring slowly along the sides of the test tube. When the upper layer turns red, and sulphuric acid layer shows yellow with green fluorescence, the presence of steroids is confirmed [13].

2.4 Microbial Assessment
This study used one Gram-negative Escherichia coli bacterium and one Gram-positive bacterium, both of which were obtained from Fish Disease Laboratory, School of Fisheries and Aquaculture Sciences, University Malaysia Terengganu (UMT), Malaysia.

2.5 Antibiotics Susceptibilities Tests
Determination of antibiotic resistance patterns was carried out using the disk diffusion method of [14]. Isolates were tested in vitro for their sensitivity to 5 different antibiotics, tetracycline (30 µg), ampicillin (10 µg), penicillin (10 µg), amoxicillin (10 µg) and neomycin (3 µg). Concentrations of bacterial cell were adjusted using McFarland 0.5 ($1.5 \times 10^8$CFU/mL$^{-1}$). Using sterile cotton buds, 100 µl of E. coli and S. agalactiae were smeared onto Mueller Hinton Agar (MHA) surface. After 10 min, sterilized forceps were used to place the antibiotic discs on the surface of the agar plates. The plates were incubated for 18 h at 37°C. After the incubation time, the diameter of a zone of inhibition surrounding the discs was measured (mm).

2.6 Antibacterial assay
2.6.1 Disc Diffusion Method
Mueller Hinton Agar (MHA) disk diffusion assay method was carried out following [15]. 1 ml of 5% Dimethyl sulfoxide (DMSO) was mixed with the stock solution in varying concentrations of 5, 10 and 15 mg/ml. Ten µl of different concentrations was used to impregnate the discs before loading on Whatman no one filter paper disk 6 mm in diameter and air-dried in laminar airflow chamber at room temperature. Antibiotic (Tetracycline 30 µg/disc) served as positive control and methanol filter paper as the negative control. The plates were positioned inverted and incubated for 24 hr at 37°C. The diameters of the inhibition zones surrounding the discs were measured and reported in millimetres (mm). The experiment was carried out in triplicates.

2.6.2 Agar Well Diffusion Method
Bacteria concentration was adjusted to achieve turbidity of 0.5 McFarland 1.5 $\times 10^8$ CFU/mL$^{-1}$. The bacteria were isolated on Mueller Hinton Agar (MHA) using sterile cotton swabs. The wells were bored into the agar medium using sterile micropipette tips (1000 µl) and filled with 100 µl solution of various extract concentrations at 5, 10 and 15 mg/ml. 100 µl of 5% Dimethyl sulfoxide (DMSO) served as a negative control and tetracycline (30 µg/ml) as a positive control. Plates were left to
stand between 1-2 h on lamina flow for the proper inflow of solution onto the medium. The plates were incubated for 24 h at 37°C. These plates were observed after the 24 h for their inhibition zone, and the disc was measured. The experiment was carried out in triplicates [16].

2.6.3 Determination of Minimum Inhibitory Concentration (MIC)
Determination of MIC was carried out to the methods of [15] using broth dilution assay technique in sterile 96-wells microtitrter plates. Bacteria were adjusted to achieve a turbidity standard of 0.5 McFarland 1.5 x 10^8 CFU mL^{-1}. Tryptone Soy Broth (TSB) was inserted into each well. For each assay, wells were inoculated with 90 µl of 100 mg/ml crude extracts, continued by two-fold dilution until 0.098 mg/ml. Ten µl bacteria were added directly as inoculants. The microtitre plates were sealed using parafilm to prevent dehydration of bacteria. Plates were further incubated for 24 h at 37°C. After incubation, the turbidity of each well was determined visually, and the optical density (OD) of each well was measured at 540 nm by a micro-titre reader model 680 (Bio-rad, US). Results were confirmed by the addition of 10 µl of 0.1% 2, 3, 5-triphenyltetrazolium chloride (TTC) into each well and incubated for one h to initiate a reaction.

2.6.4 Determination of Minimum Bactericidal Concentration (MBC)
The MBC extract was determined according to [17]. A sample was obtained from plates displaying visible negative growth in MIC assay and subcultured onto a freshly prepared nutrient agar medium to incubate for 24 hr at 37°C. The MBC is determined at the lowest concentration of the extract, where no bacterial growth on the surface of the agar plate was observed. The experiment was performed in triplicates. Results were determined by the ratio of MBC/MIC. When the MBC/MIC ratio is <2 active fractions was deemed bactericidal. Otherwise, the effect is considered bacteriostatic. The fraction is considered ineffective when the ratio is > 16.

2.7 Toxicity Test
Brine shrimp lethality assay tested the general toxicity of R. apiculata extract in accordance to Pisutthanan et al. 2004 [18]. Brine shrimp eggs were hatched into 1000 ml beaker containing seawater and incubated for 24 h at 28°C. Nauplii were collected using 100 µl tips. Serial dilutions were made into dry 96-well microplates using 120 µl of seawater with three replicates. A suspension containing 10-15 nauplii organisms was inserted into each well before incubated for 24 h at 28°C. Dead nauplii in each well were counted using a binocular stereomicroscope. Evaluation of toxicity was expressed by LC_{50} Mortality (%) = Number of dead nauplii / the Initial number of live nauplii x 100.

3. Result and Discussion
The results of the phytochemical screening of R. apiculata leaf extracts are presented in Table 1.

| Table 1. Phytochemical constituent of mangrove plants, Rhizophora apiculata |
|----------------------------------|----------------|---------------|-----------|----------|-----------|-----------|-----------|
| Mangrove Plant                  | Terpenoid | Flavonoids | Saponins | Alkaloids | Tannins | Steroids |
| Rhizophora apiculata            | +         | -           | +         | +        | +        | +         |

Note: + Present, - absent

To determine the presence of secondary metabolites in the plant crude extracts, chemical tests for tannins, alkaloids, flavonoids, terpenoid, saponins and steroid compounds were carried out. The color reaction tests of leaf extracts of R. apiculata chemical constituents are shown in Figure 1.
Figure 1. The chemical colour reaction tests for chemical constituents of leaf extracts of *R. apiculata* 
[A] Steroid test with yellow with green fluorescence (present) [B] Alkaloid test with yellow colour (present) [C] Saponins test (present) [D] Tannin test with green colour (present) [E] Terpenoids test with reddish-brown (present) [F] Flavonoids with the colour does not change (absent)

Figure 2. [A] [B] [C] antibiotic susceptibility of *E. coli* and [D] [E] [F] antibiotic susceptibility of *Streptococcus agalactiae*
Methanolic extracts showed positive results for steroids, tannins, terpenoid, saponins and alkaloids. Flavonoids, on the other hand, were absent. Testing for antibiotic resistance was carried out on gram-positive and gram-negative bacteria. The *E. coli* isolates showed sensitive to amoxicillin and tetracycline but resistant towards neomycin (Table 1). *S. agalactiae* was sensitive to all antibiotics (Table 2). The antimicrobial activity of *R. apiculata* methanolic extract was determined by measuring the inhibition zone via agar well and disc diffusion method (Table 4 and 5). The highest zone of inhibition recorded for *R. apiculata* crude extracts at 15 mg/ml concentration against *S. agalactiae* was 9 mm using agar well and 7 mm using the disc diffusion method (Figure 3).

### Table 2. Antibiotic Susceptibility test of *E. coli* via disc diffusion method

| Antibiotic | Disc potency (mcg) | Bacteria | Zone of inhibition (mm) |
|------------|--------------------|----------|-------------------------|
| Tetracycline | 30                 | S        | 20                      |
| Ampicilin   | 10                 | I        | 20                      |
| Penicilin   | 10                 | I        | 23                      |
| Neomycin    | 10                 | R        | 12                      |
| Amoxycilin  | 10                 | S        | 22                      |

Note: [I] intermediate, [R] resistant, [S] sensitive

### Table 3. Antibacterial susceptibility test of *Streptococcus agalactiae* via agar diffusion method

| Antibiotic | Disc potency (mcg) | Bacteria | Zone of inhibition (mm) |
|------------|--------------------|----------|-------------------------|
| Tetracycline | 30                 | S        | 20                      |
| Ampicilin   | 10                 | S        | 18                      |
| Penicilin   | 10                 | S        | 24                      |
| Neomycin    | 10                 | S        | 19                      |
| Amoxycilin  | 10                 | S        | 20                      |

Note: [I] intermediate, [R] resistant, [S] sensitive

### Table 4. Antibacterial screening of *Rhizophora apiculata* crude extracts using disc diffusion methods

| Bacteria     | Zone of inhibition (Diameter mm) | Concentration |
|--------------|----------------------------------|---------------|
|              | 5 mg/ml  | 10 mg/ml | 15 mg/ml | Tetracycline | DMSO |
| *E. coli*    | 2        | 4        | 6        | 7           | 0    |
| *S.agalactiae* | 3        | 5        | 7        | 9           | 0    |
Table 5. Antibacterial screening of *Rhizophora apiculata* crude extracts using agar well diffusion method

| Bacteria       | Zone of inhibition (Diameter mm) | Concentration |
|----------------|----------------------------------|---------------|
|                |                                  | 5 mg/ml | 10 mg/ml | 15 mg/ml | Tetracycline | DMSO |
| *E. coli*      |                                  | 4       | 5        | 8        | 9           | 0    |
| *S. agalactiae*|                                  | 6       | 7        | 9        | 10          | 0    |

Figure 3. Effect of *Rhizophora apiculata* crude extractson inhibition of *E. coli* [A] and *S. agalactiae* [B] using disc diffusion method and *E. coli* [C] and *S. agalactiae* [D] using agar well diffusion method at concentration of 5 mg/ml [T1] 10 mg/ml [T2] 15 mg/ml [T3] Control [C+] and [C-]

The antimicrobial activity of *R. apiculata* extract was quantitatively assessed by obtaining the MIC and MBC values. The values of MIC recorded for *R. apiculata* methanol extract against *E. coli* and *S. agalactiae* were 12.5 mg/ml and 6.25 mg/ml and the MBC values were 50 mg/ml and 12.5 mg/ml against *E. coli* and *S. agalactiae*, respectively (Table 6). Brine shrimp toxicity study was conducted dependently on data of mortality from concentrations of 50 to 0.39 mg/ml. Figure 4 shows the LC50 value recorded for *R. apiculata* was 81 mg/ml.
Table 6. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentration values of *Rhizophora apiculata* crude extract

| Bacteria     | MIC  | MBC  | MBC/MIC |
|--------------|------|------|---------|
| *E. coli*    | 12.5 | 50   | 4       |
| *S. agalactiae* | 6.25 | 12.5 | 1.92    |

Figure 4. Mortality rate (%) of *Artemia salina* after being exposed to various concentrations of *Rhizophora mapiculata’s* extract

Considerable resistance to commonly used antibiotics has been observed in a large number of bacteria, creating an important public health concern capable of causing serious disease. A new alternative for antibacterial drug preparations for synthetic drugs has been created [19, 2].

Phytochemical analyses of *R. apiculata* leaf extracts show tannins, alkaloid, steroids, saponins and terpenoids, in agreement with [5]. [2] Reported that *R. apiculata* has antimicrobial activity. The antibacterial properties observed from this study are similar to the study of [20]. The antibacterial activity displayed by the mangrove leaf extract may possibly be due to phytochemicals presence, such as that of alkaloids and tannins [21]. Our results indicate that *R. apiculata* contain secondary metabolites allowing a wide variety of biomedical applications, and these results were in agreement with [22]. Secondary metabolites, namely tannins, alkaloids and terpenoids are mainly reported in plant defence mechanism that can be attributed for their antibacterial properties [2, 23, 24]. These findings justify and support the information of the present study regarding the use of phytochemicals isolated from the solvent extract.

Methanol extracts of *R. apiculata* were revealed through agar well diffusion and disc diffusion assay to contain a broad range of antimicrobial activity against gram-positive and gram-negative bacteria strains, in agreement with [25]. Gram-negative *E. coli* susceptibility can possibly be attributed to the effectiveness of the extracts against *E. coli*.

The same effect is capable of killing gram-positive bacteria while inhibiting gram-negative bacteria growth since the cell wall of gram-negative bacteria is thicker cell than gram-positive bacteria, and the outer membrane of gram-negative bacteria contains a high content of lipid-polysaccharide sheet, aiding in the protection against the passage of hydrophobic groups [26].
Antimicrobial activity results showed crude extracts increasing when concentrations increased [27]. Chemical diversity allows some mangroves to have the lower antibiotic ability; however, *R. apiculata* reported potent activity at low levels of concentration [22]. The (MIC) and (MBC) values of *R. apiculata* were used to identify the antimicrobial efficacy of metabolites or synthetic compounds [28]. Values of (MIC) and (MBC) are valuable tools when deciding effective and appropriate concentrations of therapeutic substances [29]. Brine shrimp bioassay was used to identify *R. apiculata* toxicity [30]. This analysis proved useful and straightforward in isolating compounds potentially cytotoxic from plant extracts. Brine shrimp bioassay had been routinely used in the screening of extracts and isolated compounds to assess the toxicity to brine shrimp, indicating possible cytotoxic properties [31]. Medicinal benefits of plant materials can arrive from a phytochemical works. Our results show that antimicrobial compounds are valuable, ecologically safe and economically viable. Further studies using *R. apiculata* extract to isolate bioactive compounds and identify the pure component are encouraged.

### 4. Conclusion

A successful attempt was made in the antimicrobial efficiency and characterization of *R. apiculata* phytochemical works. Our results show that antimicrobial compounds are valuable, ecologically safe and economically viable. Further studies using *R. apiculata* extract to isolate bioactive compounds and identify the pure component are encouraged.

### 5. References

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6. Acknowledgments
The author would like to thank the Institute of Tropical Aquaculture at (UMT) for providing the facilities and funds to carry out this work.