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Antimicrobial Activities of Selected Mangrove Plants on Fish Pathogenic Bacteria

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Abstract: The objective of this study was to conduct *in vitro* study of antimicrobial properties of mangrove plants *Sonneratia caseolaris* and *Rhizophora apiculata* against fish pathogenic bacteria. Leaves of mangrove plant were collected from area nearby University Malaysia Terengganu. Methanol extracts of mangrove plants were obtained by using Soxhlet extractor. The bacterial cultures used were *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *Enterobacter brevis*, *Chryseobacterium indologenes*, *Stenotrophomonas maltophilia* and *Aeromonas hydrophila* isolated from fish. The antimicrobial susceptibility test showed that the bacteria were 87.5% resistant towards colistin sulphate and 62.5% sensitive to fosfomycin. The Minimum Inhibitory Concentration (MIC) of *Sonneratia caseolaris* and *Rhizophora apiculata* ranges from 1.56-6.25 and 12.5-25 mg mL^{-1}, respectively. The lowest Minimum Bactericidal Concentration (MBC) value was 12.5 mg mL^{-1} of *Sonneratia caseolaris* and 25 mg mL^{-1} of *Rhizophora apiculata*. The highest activity with LC_{50} of *Sonneratia caseolaris* was 6.16 mg mL^{-1} and *Rhizophora apiculata* was 0.76 mg mL^{-1}, respectively. Methanol extracts of *Sonneratia caseolaris* was effective on all gram negative bacteria (*Klebsiella pneumoniae*, *Shigella dysenteriae*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *Enterobacter brevis*, *Chryseobacterium indologenes*, *Stenotrophomonas maltophilia* and *Aeromonas hydrophila*) when compared to *Rhizophora apiculata*.

Key words: Antimicrobial activities, mangrove plant, MIC, MBC, LC_{50}, Malaysia

INTRODUCTION

Mangrove plants, *Sonneratia caseolaris* and *Rhizophora apiculata* are normally used in traditional medicine for the treatment of several illnesses caused by microbial and non-microbial origins (Xie *et al*., 2005). Mangrove plant has traditionally been used to treat sores and stings from marine creatures and ulcers as a purgative and an emetic and the smoke from the bark to treat leprosy (Ghani, 2003). Mangrove contains biologically active antibacterial, antifungal and antiviral compounds (Vadlapudi and Naidu, 2009).

Mangroves have been a source of several bioactive compounds. Mangrove plants have been used in folklore medicines and extracts from mangrove species have proven activity against human, animal and plant pathogens. Secondary metabolites like alkaloids, phenolics, steroids and terpenoids have been characterized from mangroves and have toxicological, pharmacological and ecological importance (Kokpal *et al*., 1990). They provide a prosperous source of alkaloids, steroids, triterpenoids and flavonoids (Abeysinghe, 2010). Antibacterial activity of mangroves against fish pathogens had already been studied by many researchers. Lim *et al*. (2006) showed in their study of antimicrobial activities of tannins extracted from mangrove plant *Rhizophora apiculata* barks was significant antibacterial activity. Mishra and Sree (2007) reported the chloroform leaf extract of mangrove plant *Finlaysonia obovata* showed strong antibacterial activity against fish pathogens (Manilal *et al*., 2009).

Three mangrove species (*Avicennia marina*, *Bruguiera cylindrical* and *Acanthus ilicifolius*) collected from the coast was extracted in methanol and tested for different range of biological activities including antimicrobial activity against five species of type cultures (microbial type culture collection) of fish/shrimp Vibrio pathogens, brine shrimp cytotoxic,

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antifouling and ichthyotoxic activities. Antibacterial compounds from natural resources would be the alternative to overcome the resistance problem (Kumar *et al.*, 2009). Antimicrobial activities of 5 Indian mangrove plants (*Rhizophora apiculata*, *Rhizophora mucronata*, *Bruguiera cylindrica*, *Ceriops decandra* and *Avicennia marina*) against UTIs bacterial pathogens studied by Ravikumar *et al.* (2010). The antimicrobial activity of the methanol extracts from leaves of the mangrove plant, *Sonneratia caseolaris* and *Rhizophora apiculata* against fish pathogenic bacteria were studied.

MATERIALS AND METHODS

Plant material: Two plants were used in this study, *Sonneratia caseolaris* and *Rhizophora Apiculata*. The leaves were collected from a rural area of Mengabang Telipot in Terengganu, Malaysia. The plants were identified at the plant taxonomy laboratory in Universiti Malaysia Terengganu.

The weight of the leaves after extraction was weighed using a weighing balance to determine the percentage yield of the crude extract using the equaction:

$$\text{Percentage yield} = \frac{X_1 - X_2}{X_1} \times 100$$

Where:

X_1 = Weight of the dry powdered leaves before extraction (15.0 g)

X_2 = Weight of the dry leaves after extraction

The weight or mass of crude methanolic extract can be calculated using the equaction:

$$\text{Percentage yield} = \frac{X_1}{100}$$

Preparation of extracts: The leaves of plants were washed with both tap and distilled water to remove any epiphytes present and other wastes and they were then air dried under the shade for 3 weeks. The dried plant material was ground to fine powder (15 g dry weight) and was then extracted with 100 mL of methanol for 48 h using a soxhlet extractor according to the methods of (Bele *et al.*, 2009) and then the extract were filtered through a Buchner funnel and using Whatman no. 1 filter paper. This was repeated three times for the complete extraction of methanol-soluble compounds and all the three methanol extracts were pooled. The solvent was evaporated from crude extract by a rotator evaporator. The dried extracts were stored at 4°C until further use. Percentage yield was calculated from the dry extract powder. About 3,250 mg mL^{-1} of the extracts were dissolved in methanol for the antibacterial assays and in Dimethyl Sulfoxide (DMSO) for cytotoxicity assays. The samples in DMSO for cytotoxicity screening were further diluted to 5 mg mL^{-1} with growth medium (DMEM or RPMI 1640) to reduce the percentage of DMSO.

Test organisms: Eight bacterial pathogens namely *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *Enterobacter brevis*, *Chryseobacterium indologenes*, *Stenotrophomonas maltophilia* and *Aeromonas hydrophila* were identified by using a commercial Identification system kit (BBL TM Crystal E/NF, USA). The pathogenic bacteria were cultured individually on Tryptic Soy Broth (TSB) (Merk, Germany) at 37°C for 18 h, before inoculation for assay. Broth culture (100 μL) which contained 108 number of bacteria per mL (the inoculums size of each test strain were standardized at 108 cfu mL^{-1} using McFarland Nephelometer standard). Sterile Tryptic Soy Agar (TSA) (Merk, Germany) plates were seeded with test bacterial strains and allowed to stand at 37°C for 24 h.

***In vitro* anitbiogram of isolates:** Antibiogram tests for the isolate were performed using the disc diffusion technique on Tryptic Soy Agar. NCCLS (2001) guidelines were used for evaluation of the results. All the bacteria isolated were tested *in vitro* for their sensitivity to 10 different antibiotics. These included Nitrofurantion (50 ug), Colistin sulphate (25 ug), Fosfomycin 50 (ug), Lincomycin (15 ug), Tetracycline (30 ug), Spiramycin (100 ug), Oxolinic acid (2 ug), Florfenciol (30 ug), Amoxycilin (25 ug) and Erythromycin (15 ug).

Antibacterial assay

Disk diffusion assay: Disk diffusion assay was carried out on Tryptic Soy Agar (TSA) (Merk, Germany) following the method described by Barker and Kehoe (1995). Briefly from the plant extracts, 350 mg of crude extracts were dissolved in 1 m, 4% Dimethyl Sulphoxide (DMSO) and 0.2 mL of the prepared extracts were loaded on to the filter paper discs (sterilized Whatman no. 1 filter paper discs 6 mm in diameter) to get 20 ug $disc^{-1}$ concentration and allowed to dry at room temperature in laminar air flow chamber. Positive control (Neomycin 10 μg mL^{-1}) and negative control (methanol) disc were allowed to air dry and were then placed equidistantly onto the surface of the pathogen seeded Tryptic Soy Agar plates. The plates were stored in an inverted position and incubated at 37°C for 18 h. The growth inhibition was assessed as the diameter (mm) of the zone of inhibited microbial growth. The experiment was carried out three times.

Agar-well diffusion method: The bacterial isolates were first grown in nutrient broth for 24-48 h before use. The isolates were then subcultured on to Tryptic Soy Agar plates (Oxoid, Ltd.). The wells were then bored into the agar medium using a sterile 6 mm cork borer. The wells were then filled up with solution of the extract and intense care was taken as to not allow the solution to spill on the surface of the medium. The plates were allowed to stand on the laboratory bench between 1-2 h to allow proper inflow of the solution into the medium. Subsequently, the plates were placed in an incubator at 37°C for 24 h (Adegboye *et al*., 2008).

The plates were later observed for their zones of inhibition. The effects of the extract on bacterial isolates were compared with those of standard antibiotics tetracycline at a concentration of 10 ug mL^{-1}. The experiment was carried out in triplicate. Experimental data represent mean of each sample:

$$\text{Antimicrobial index} = \frac{\text{Inhibition zone of sample}}{\text{Inhibition zone of the standard}} \times 100$$

Determination of Minimum Inhibitory Concentration (MIC): Minimum inhibitory concentration was determined by broth micro-dilution assay technique in 96 wells micro-titer plates as described by Eloff (1998) with slight modification. Overnight broth cultures of the each test organism (90 uL) were seeded into the wells and crude metabolite (10 uL) were added in each well at decreasing concentration starting from 1000-75 ug mL^{-1}. The plates were incubated for 24 h at 35±1°C and TTC solution 1%, sterile (Triphenyl Tetrazolium Chloride) were used as microbial growth indicator. MIC was determined as the least concentration of the crude metabolite that inhibited the growth of the test organisms.

Minimum Bactericidal Concentrations (MBC): The MBC of the extract was determined using Ilavenil *et al*. (2010) method with little modifications. Samples were taken from the plates with that displayed negative visible growth in the MIC assay and were subcultured on to a freshly prepared nutrient agar medium and later incubated at 37°C for 48 h. The MBC was determined as the lowest concentration of the extract that did not allow any bacterial growth on the surface of the agar plates. The experiments were carried out in three replicates and the mean readings were recorded.

The results were determined according to the ratio of MBC/MIC. If the ratio of MBC/MIC was ≤ 2 the active fractions were considered to be bactericidal; otherwise, they were considered bacteriostatic. And if the ratio was ≥ 16 the fractions were considered to be ineffective (Manilal *et al*., 2010).

Brine shrimp larvae assay

Brine shrimp egg hatching: Brine shrimp eggs (Artemia Salina, SandersTM Great Salt Lake, Brine Shrimp Company L.C., USA) were hatched in artificial seawater at 20°C and constant illumination. The brine shrimp eggs were incubated in a polypropylene jar with a water height of 1.2 cm. These hatching conditions corresponded to those in the natural environment. After 48 h incubation at room temperature (25-29°C), nauplii (larvae) were collected using a pipette taken from the lighter side whereas their shells were left in another side the shrimp larvae were used for experimental bioassay. During that time, the larvae were still surviving on their own yolk sac and have received no further food during the experimental duration.

Bioassay: Brine shrimp lethality assay, following the modified method of Pisutthanana *et al*. (2004) were carried out to study the general toxicity of the extract of *Sonneratia caseolaris* and *Rhizophora apiculata*. About 10 mg of the extracts were added to 2 mg mL^{-1} of artificial sea water mixture. The mixture contained water insoluble compounds which were dissolved in DMSO 50 μL prior to the addition into the sea water. Serial dilutions were completed using 96 well micro plates and were performed in triplicate in 120 μL sea water. Control wells with DMSO were included in each experiment. A 100 μL suspension of nauplii (containing 10-15 organisms) were added to each well. The plates were then covered and incubated at room temperature (25-29°C) for 24 h. Plates were then examined under binocular steromicroscope and the numbers of dead, non-motile nauplii in each well were counted. Finally, all shrimps were scarified by adding 100 μL of methanol to each well. After 15 min, the total numbers of shrimps in each well were counted. Analysis of the data was performed using PROBIT analysis on a finney computer program. Analysis was executed to determine the lethal concentration required to kill half the population of the test organisms during the period of 24 h (LC_{50}). The toxicity is expressed by the LC_{50}.

$$\text{Mortality}(\%) = \frac{\text{No. of dead nauplii}}{\text{Initial no. of live nauplii}} \times 100$$

Statistical analysis: The experiments were conducted three times and the mean readings were recorded.

RESULTS AND DISCUSSION

The Antimicrobial Susceptibility Test (AST) is an important technique in many disciplines of science. It is used in pathology to determine the resistance of microbial strains to antimicrobials and is used to determine the

Table1: Antibiogram of fish pathogenic bacteria

Antibiotics disc	Potency (mcg)	Isolates 1	2	3	4	5	6	7	8	Percentage R	S
Nitrofurantion	50	S	R	R	R	I	I	I	I	37.50	12.5
Colistin sulphate	25	R	R	R	R	R	R	S	R	87.50	12.5
Fosfomycin	50	R	S	S	S	R	S	I	S	25.00	62.5
Lincomycin	15	S	R	R	S	R	S	R	S	50.00	50.0
Tetracycline	30	S	R	R	S	R	R	S	R	62.50	37.5
Spiramycin	100	R	R	R	I	I	I	S	I	37.50	12.5
Oxolinic acid	2	S	R	R	S	R	S	R	I	50.00	37.5
Florfenciol	30	S	R	R	S	R	S	S	S	37.50	62.5
Amoxycilin	25	I	R	R	R	R	I	R	I	62.50	0.0
Erythromycin	15	I	R	R	S	R	I	R	I	50.00	12.5
MAR	-	-	0.03	0.11	0.11	0.03	0.1	0.02	0.05	0.02	-

R = Resistant; I = Intermediate; S = Sensitive; 1: *Shigella dysenteriae*; 2: *Enterobacter cloacae*; 3: *Klebsiella pneumoniae*; 4: *Enterobacter sakazakii*; 5: *Enterobacter brevis*; 6: *Chryseobacterium indologenes*; 7: *Stenotrophomas maltophilia*; 8: *Aeromonas hydrophila*; MAR: Multiple Antibiotic Resistant

Table 2: Antibiotics references according to National Committee for Clinical Laboratory Standards (CLSI) with *Escherichia coli* ATCC 25922 as quality control

Antibiotics disc	Zone of inhibition (mm) R	I	S
Nitrofurantion	12	13-15	16
Colistin sulphate	8	9-10	11
Fosfomycin	13	14-16	17
Lincomycin	14	15-20	21
Tetracycline	14	15-18	19
Spiramycin	12	13-15	16
Oxolinic acid	14	15-17	18
Florfenciol	14	15-17	18
Amoxycilin	13	14-17	18
Erythromycin	13	14-22	23

efficacy of novel antimicrobials against micro-organisms, essentially those of medical importance. The AST is the first step towards new anti-infective drug development. There are various AST methods that are used by researchers and these could lead to variations in results obtained. The bacterial isolates were subjected to 10 antibiotics for testing their susceptibility (Table 1). The results demonstrate that the isolates were sensitive to fosfomycin (62.5%) and are resistant towards colistin sulphate (87.5%). There was no evidence of Multiple Antibiotic Resistant (MAR) in this study. The range of mean zone of inhibition for concentration 100, 300 and 500 mg mL^{-1} by using well technique was 15, 18 and 19.25 mm for methanol extracts of *Sonneratia caseolaris* and 10.5, 13.12, 14.12 mm, respectively for methanol extracts of *Rhizophora apiculata* while the mean zone of inhibition for positive control tetracycline was 14.62 mm (Table 2).

The mean zone of inhibition for methanol extracts of *Sonneratia caseolaris* and *Rhizophora apiculata* concentration 500 mg mL^{-1} by using disk diffusion method was 13.8 and 9 mm, respectively while the mean zone of inhibition for positive control neomycin was 11.5 mm (Table 3). The antimicrobial activity of the extracts and their potency were quantitatively assessed by determining their MIC and MBC, respectively. The MIC values were between 1.56 and 6.25 mg mL^{-1} for the methanol extracts of *Sonneratia caseolaris* while it was 12.5 and 25 mg mL^{-1} for methanol extracts of *Rhizophora apiculata* (Table 4). The lowest MBC value was 12.5 mg mL^{-1} for *Sonneratia caseolaris* and 25 mg mL^{-1} for *Rhizophora apiculata*.

The toxicity of extracts of *Sonneratia caseolaris* and *Rhizophora apiculata* were tested by brine shrimp bioassay. The average LC 50 for *Sonneratia caseolaris* was 6.16 mg mL^{-1} and *Rhizophora apiculata* was 0.76 mg mL^{-1} (Table 5). The use of plant extracts with medicinal potential represents a valid substitute for the treatment of different ailments and diseases. The search for antimicrobial agents from plants has been a growing interest in the last few decades and plant materials continue to play a chief role in primary health care as therapeutic solutions in many developing countries (Alim *et al*., 2009). *Sonneratia caseolaris* and *Rhizophora apiculata* are known for their antimicrobial activities (Kumar *et al*., 2009).

In the present study, we report the effect of *Sonneratia caseolaris* and *Rhizophora apiculata* extracts on pathogenic fish bacteria *in vitro*. The observation indicates that the methanol extract of the leaves of *Sonneratia caseolaris* were effective against all the bacterial strains used in this study which is in agreement with Devi *et al*. (1997). Desai and Chavan (2010) who reported that the methanolic extracts of leaf, stem, young seed, mature seed and seed coat showed a high antibacterial activity against *Pseudomonas aerunginosa* as compared to ethanolic extracts products.

The disc diffusion (Bauer *et al*., 1966) is a widely accepted *in vitro* investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and

Table 3: *In vitro* screening of crude methanolic extract *S. casiolares* and *R. apiculata* on growth of pathogenic bacteria

	Zone of inhibition (Diameter mm)						
	Sonneratia casiolares (mg)			*Rizophora apiculata* (mg)			Control
Strain	100	300	500	100	300	500	Tetracyclin 10 ug mL^{-1}
Shigella dysenteriae	17	19	21	14	12	14	22
Enterobacter cloacae	12	13	16	10	11	11	10
Klebsiella pneumonia	15	19	18	15	17	18	09
Enterobacter sakazaki	15	18	21	12	14	15	23
Enterobacter brevis	14	20	21	10	12	13	11
Chryseobacterium indologenes	16	19	19	14	14	14	20
Stenotrophomas maltphila	13	17	19	09	13	15	06
Aeromonas hydrophilia	16	19	19	11	12	13	16

Table 4: Antimicrobial activity of methanolic extract of *Sonneratia casiolares* and *Rizophora apiculata* (Disc diffusion method)

Bacteria strain	*Sonneratia casiolares* (500 mg mL^{-1})	*Rizophora apiculata* (500 mg mL^{-1})	*Neomycin* (10 ug mL^{-1})	Methanol MBC (10%)
Shigella dysenteriae	17	11	15	0
Enterobacter cloacae	17	10	09	0
Klebsiella pneumonia	12	09	10	0
Enterobacter sakazaki	11	10	17	0
Enterobacter brevis	18	10	10	0
Chryseobacterium indologenes	14	08	16	0
Stenotrophomas maltphila	09	06	05	0
Aeromonas hydrophilia	14	08	11	0

Table 5: Antimicrobial activity of plant extracts corresponding to their Minimal Inhibitory Concentrations (MIC) and Minimal Bacteriocidal Concentrations (MBC) in mg mL^{-1}

	Sonneratia casiolares		*Rizophora apiculata*	
Bacteria strain	MIC	MBC	MIC	MBC
Shigella dysenteriae	6.25	25.0	25.0	50
Enterobacter cloacae	6.25	12.5	25.0	50
Klebsiella pneumonia	6.25	25.0	25.0	50
Enterobacter sakazaki	1.56	12.5	12.5	25
Enterobacter brevis	1.56	12.5	25.0	25
Chryseobacterium indologenes	6.25	12.5	12.5	25
Stenotrophomas maltphila	6.25	12.5	25.0	25
Aeromonas hydrophilia	6.25	25.0	12.5	50

bacteriocidal activity can be made by this method. The diameters of the zones of inhibition shown by the methanol extract against the pathogenic bacteria are similar to those reported by Chandrasekaran *et al*. (2009). In his study, the methanol extracts of *E. agallocha* showed the highest anti-MRSA activity with a mean zone of inhibition of 22.5 mm and Kumar *et al*. (2009) reported the mangrove leaf extracts of *E. agallocha* showed maximum inhibitory activity of 18 mm against *S. Aureus* and *R. mucronata* exhibited higher activity of 20 mm against *S. lactis*. The difference between the antimicrobial activities of mangroves could be due to the quantity of antimicrobial substances present in each form (Chandrasekaran *et al*., 2009). These might refer to the presence of coumarins, flavonoids and saponins as chemical components of these plants (Bonjar *et al*., 2004) and indicate that the methanol extract of the leaves of *Sonneratia caseolaris* has almost the same activity as the standard antibiotic. The sensitivity of pathogenic bacteria to *Sonneratia caseolaris* and *Rhizophora apiculata* extracts could be attributed due the presence of common bioactive compounds that had inhibitory effects on the microorganism agree with (Manilal *et al*., 2009).

MIC results can indirectly predict the concentration of extract which should be used as minimum dosage. The MIC values were between 1.56 and 6.25 mg mL^{-1} for the methanol extracts of *Sonneratia caseolaris* while it was 12.5 and 25 mg mL^{-1} for methanol extracts of *Rhizophora apiculata*. The lowest MBC value was 12.5 mg mL^{-1} for *Sonneratia caseolaris* and 25 mg mL^{-1} for *Rhizophora apiculata*, the results were in agreement with Lim *et al*. (2006) and Patra (2009). A similar result was reported by Sharma *et al*. (2009) in their studies on the vibriocidal activities of 16 Indian medicinal plants where in 70% of the Vibrio pathogens tested were susceptible to the plant extract at a concentration ranging between 2.5 and 20 mg mL^{-1} and the study result of Chandrasekaran *et al*. (2009) who recorded the MIC value of the methanol extracts of *E. agallocha* which was 0.125 mg mL^{-1} and the MBC was 0.25 mg mL^{-1}. Which agrees with the result of the microorganism test which had a low MIC values and showed low concentrations of MBC.

The results showed that the extract exhibited bacteriostatic (Lim *et al*., 2006). In general gram-positive bacteria are considered to be more sensitive than gram-negative bacteria towards different antimicrobial compounds because of the difference in the structure of

their cell walls (Scherrer and Gerhardt, 1971). The brine shrimp assay is a simple and useful tool for the isolation of potentially cytotoxic compounds from plant extracts (Meyer *et al.*, 1982). The Brine Shrimp Lethality Assay (BSLA) has been used routinely in the primary screening of the crude extracts as well as isolated compounds to assess the toxicity towards brine shrimp which could also provide an indication of possible cytotoxic properties of the test materials.

The results agree with Subhan *et al.* (2008) who found that the extract of mangrove plant *E. agallocha* showed considerable brine shrimp toxicity (LC_{50} = 20 mg mL^{-1}) and the variation in the BSLA results may be due to the difference in the amount and kind of cytotoxic substances (e.g., tannins, flavonoids or triterpenoids) present in the extracts.

CONCLUSION

Based on the screening results, *Sonneratia caseolaris* has been shown possessing antibacterial potential to combat the fish pathogenic bacteria. Bioactive compound of the mangrove plants will be determined in the future studies.

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