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Anti-Vibrio Activity of Mangrove and Mangrove Associates on Shrimp Pathogen, *Vibrio harveyi* VSH5

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Abstract: The petroleum ether, chloroform, acetone, ethyl acetate, methanol and aqueous extracts of six different species of mangrove plants and four mangrove associates were tested in vitro for their anti-vibrio activity for which shrimp pathogen *Vibrio harveyi* VSH5 was used as test pathogen. Out of tested 96 curd extracts, acetone extract of *Acanthus ilicifolius* leaves (16.4 mm) showed more potent anti-vibrio activity than the other leaf extracts. Among the fruit and stem extracts, methanolic extracts of *Rhizophora mucronata* fruit, *Avicennia marina* stem and *Avicennia ofcinalis* stem rendered their maximum activities as 12.1 mm, 10.2 mm and 10.2 mm respectively. Methanol and acetone extracts of *Thespesia populneoides* leaves recorded its maximum activity in mangrove associates. The Minimum inhibitory concentration MIC of acetone extract of *A. ilicifolius* leaves against the test pathogen was calculated as 125 µg/ml. Further, the test extract gave five separated bands on Thin layer chromatography TLC which showed clear zone (anti-vibrio activity) either compounds in individual or in combination through bioautography.

Key words: Anti-Vibrio · Mangroves · Mangrove Associates · MIC · TLC-Bioautography

INTRODUCTION

The prophylactic treatment of shrimp diseases with antibiotics has led to the emergence of antibiotic-resistant microorganisms due to improper administrative practices. Moreover, much interest is now directed towards the vast untapped source of plant-based antimicrobials, many of which reduce the side effects of synthetic antimicrobials [1]. Medicinal plants contain large varieties of chemical substances with important therapeutic properties that can be effectively utilized in the treatment of animal diseases like Vibriosis caused by luminous *Vibrio* pathogens. Mangrove and mangrove associates contain biologically active antibacterial, antifungal and antiviral compounds [2]. The role of mangrove plants in the discovery of drugs has increased notably in recent years due to a substantial improvement in biological screening methods. Therefore, it is also worth to screen the mangrove and mangrove associates for the presence of new antibacterial to combat the disease in shrimp culture. Unfortunately, only a small percentage of the mangroves have been examined and explored thoroughly for their bioactive potential. In this context, the present study was initiated with the aim of screening anti-vibrio activity against shrimp's *Vibrio* pathogen.

MATERIALS AND METHODS

Plant Collection: Six different species of mangrove plants and four mangrove associates were collected from various places of coastal regions of Tamil Nadu. The collected plant species were taxonomically identified as *Acanthus ilicifolius, Avicennia marina, Avicennia ofcinalis*, *Ceriops decandra, Rhizophora apiculata, Rhizophora mucronata, Sesuvium portulacastrum, Suaeda maritime, Clerodendrum inerme* and *Thespesia populneoides* in the Department of Marine Biology, Annamalai University, Tamil Nadu.

Preparation of Solvent Extracts: Sequential solvent extraction method was carried out to obtain crude bioactives. About 50 g of shade dried coarse powder of each selected plants were exhaustively extracted at room temperature for 48 hrs with 300ml of various solvents of

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increasing polarity: petroleum ether, chloroform, acetone, ethyl acetate methanol and water. The extracts were filtered through muslin cloth followed by filter paper (Whatman Filter No.1) and further concentrated by recovering excess solvents to a thick oily natured substance. Stock solution for anti-vibrio activity was prepared by mixing well appropriate amount of dried crude extracts with the respective solvents used for their extraction to obtain a final concentration of 100 mg/ml. About one mg/ml of tetracycline was used as the positive control while the solvents were used as the negative control for each extract.

Anti-Vibrio Assay: The shrimp pathogenic bacteria, *Vibrio harveyi* VSH5 from the stock maintained in Microbiology laboratory of Manonmaniam Sundaranar University, Tirunelveli was used as the target pathogen for anti-vibrio assay. Anti-vibrio activity of the selected plant extracts (each 50 µl from stock) was performed by agar well diffusion method [3]. Simultaneously, both positive (Tetracycline) and negative control (Different solvents) were also made. After 24 hrs of incubation at 30°C, all plates were observed for zone of growth inhibition and the diameter of zones were measured in mm. The inhibition zone of negative control was subtracted from the zone of the plant extracts so as to find the true zone of inhibition of the extract. The experiment was carried out in three replicates and the anti-vibrio activity was expressed as the mean of inhibition diameters (mm) produced. Statistical analysis were performed by using the cluster analysis for data upon average linkage between groups by SPSS statistics 17 software.

Minimum Inhibitory Concentration (MIC) for Anti-Vibrio: Microdilution method using serially diluted plant extract was done to found the MIC for anti-vibrio activity [4]. The extract was diluted into different concentrations viz. 1000, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 µg/ml. Each tube was filled with one ml of sterile TSB (With 1.5% NaCl) and inoculated with 0.1 ml of broth culture of the test organism (Contains $1\text{-}2\times10^7$ cfu/ml). The tubes were incubated at 30°C for 18-24 hrs. A tube without plant extract was maintained as control. Following incubation, the MIC was recorded at the lowest concentration of the extract inhibiting the visible growth of *Vibrio*.

Separation of Active Components by TLC: The crude plant extract spotted on TLC were separated by following solvent systems. Pure solvents of hexane, diethyl ether, petroleum ether, ethyl acetate and water, a mixture of hexane : diethyl ether, petroleum ether : ethyl acetate, ethyl acetate : water, hexane : water were used as mobile phase with different ratios as 9 : 1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8 and 1 : 9. The developed TLC plates were exposed to UV and iodine to visualize separated bands. Using a sterile surgical blade, the solid adsorbent coat (Silica gel) of each bands were scrapped separately and extracted twice with the respective solvents. The obtained residue was redissolved in 50 µl of the same solvents for anti-vibrio assay.

TLC-Bioautography: Chromatograms developed as described above were placed in a sterile Petri plate and overlaid with 1-2 mm layer of soft agar (TSA - 0.7% agar) containing 0.1 % 2,3,5-triphenyltetrazolium chloride (Tetrazolium red) and test bacteria (*V. harveyi* VSH5 at 10^7 cfu/ml) for anti-vibrio assay. TLC-bioautographic plates were covered with a parafilm to prevent the thin agar layer from drying and incubated at 30°C for overnight. Presence of an uncolored area (Inhibition zone) on deep pink-red background surrounding the active spots indicated anti-vibrio activity.

RESULTS AND DISCUSSION

The purpose of the present study was to perform preliminary exploration of potential alternative treatment mode against Vibriosis in aquaculture: anti-vibrio of the leaves, stems and fruits of six mangrove and four mangrove associates. The anti-vibrio activity of the mangrove leaves, stems, fruits and mangrove associates crude extracts (Petroleum ether, chloroform, acetone, ethyl acetate, methanol and water) were screened in vitro by agar well diffusion method against *V. harveyi* VSH5. The mean zone inhibition diameter measured is given in Figs. 1-3. Extracts from different mangrove plants are reported to possess diverse medicinal properties [5, 6]. For example, *A. ilicifolius, A. marina* and *E. agallocha* showed significant analgesic activity [7]. Mangroves and mangrove associates possess novel agrochemical products, compounds of medicinal value and biologically active compounds [8]. Extracts from different mangrove and mangrove associates are active against human and plant pathogens [9].

Out of ninety six crude extracts used in this study, most of the extracts exhibited considerable activity against *V. harveyi* VSH5. The highest anti-vibrio activity was found in acetone extract of *A. ilicifolius* (L) (16.4±0.2 mm) followed by methanol extracts of *A.*

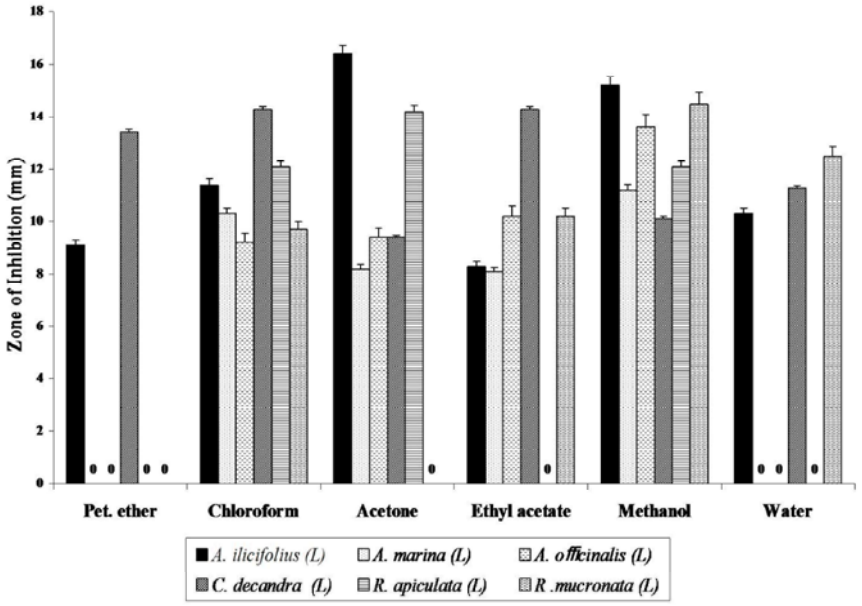

Fig. 1: Anti-vibrio activity of different extracts of mangrove leaves against *V. harveyi* VSH5. Results are expressed as mean ± standard deviation of three replicates. (L)- Leaves.

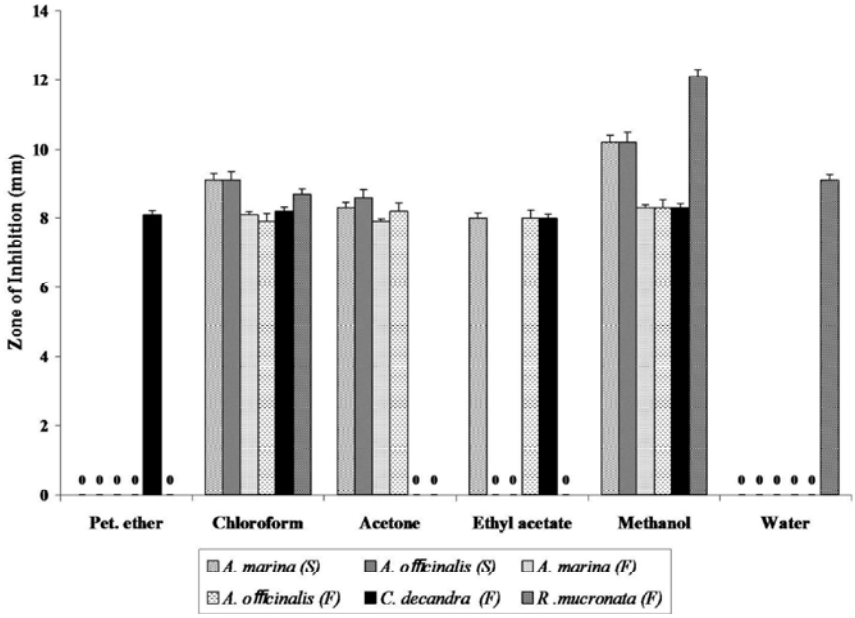

Fig. 2: Anti-vibrio activity of different extracts of mangrove stems and fruits against *V. harveyi* VSH5. Results are expressed as mean ± standard deviation of three replicates. (F)- Fruits.

ilicifolius (L) (15.2±0.0 mm) and *R. mucronata* (L) (14.5±0.1 mm), chloroform and ethyl acetate (14.3±0.1 mm) extract of *C. decandra* (L). Absence of activity was observed in petroleum ether extract of *A. marina* (L), *A. ofcinalis* (L), *R. apiculata* (L) and *R. mucronata* (L), acetone extract of *R. mucronata* (L), ethyl acetate extract of *R. apiculata* (L) and water extract of *A. marina* (L), *A. ofcinalis* (L) and *R. apiculata* (L). Among the stem and fruit extracts, the highest activities were found in methanol extracts viz. *R. mucronata* (F) (12.1 mm), *A. marina* (S) and *A. ofcinalis* (S) (10.2 mm) followed by chloroform extract of *A. marina* (S), *A. ofcinalis* (S) and

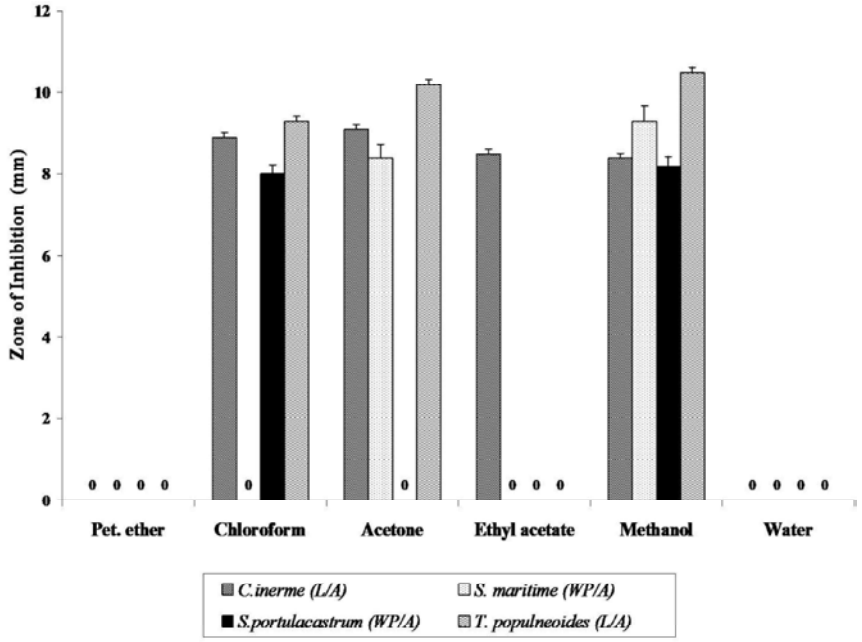

Fig. 3: Anti-vibrio activity of different extracts of mangrove associates against *V. harveyi* VSH5. Results are expressed as mean ± standard deviation of three replicates. (L)-Leaves, (WP)- Whole Plants, (A)- Mangrove Associates.

water extract of *R. mucronata* (F) (Each 9.1 mm), however other extracts showed less (7.9 to 8.9 mm) or no zone of inhibition. Studies regarding mangrove associates demonstrated that the highest anti-vibrio activity was rendered in the methanol and acetone extract of *T. populneoides* (L) as the inhibition zone of 10.5 mm and 10.2 mm respectively, followed by methanol extract of *S. maritime* (WP). Petroleum ether and water extracts of mangrove associates completely rendered no activity on *V. harveyi* VSH5.

Almost all the extracts obtained from methanol and chloroform recorded considerable anti-vibrio activity among the test samples used. In contrast, according to the preliminary study and studies of other people it has been recorded that a number of methanol and ethanol extracts of mangrove plants showed antibacterial activity against pathogenic isolates as well as antibiotic resistant bacteria [9]. Petroleum ether and water extract of all mangrove leaves showed less anti-vibrio activity compared to the extracts obtained from other solvents. Petroleum ether and water extract may contain a low concentration of anti-vibrio compounds or may not extract anti-vibrio compound(s) or all anti-vibrio compounds may have extracted by other solvents during sequential extraction. The positive control (Tetracycline) indicated maximum zone (19.5±0.1 mm) of inhibition rather than the plant extracts.

Hierarchical cluster analysis was applied to find out the similar plant groups based on their anti-vibrio activity with the test pathogen by different solvent extracts (Fig. 4). The dendrogram was obtained using average linkage between groups by SPSS statistics 17 software. At 20% similarity, two clusters were generated. The 1st cluster contained 14 test extracts. The 2nd cluster contained two test extracts namely *A. ilicifolius* (L) and *C. decandra* (L). At 50% similarity, three clusters were generated. The 1st cluster contained extracts of *A. ofcinalis* (F), *C. inerme* (L/A), *A. marina* (L), *A. marina* (S), *A. ofcinalis* (L), *A. ofcinalis* (S), *T. populneoides* (L/A), *A. marina* (F), *R. apiculata* (L), *S. maritime* (WP/A), *R.mucronata* (F), *S. portulacastrum* (WP/A) and *C. decandra* (F). The 2nd and 3rd cluster contained extract of [*R.mucronata* (L)] *and* [*A. ilicifolius* (L) and *C. decandra* (L)], respectively. At 80% similarity, nine clusters were generated. The 1st cluster contained extracts of *A. ofcinalis* (F), *C. inerme* (L/A), *A. marina* (L), *A. marina* (S), *A. ofcinalis* (L) and *A. ofcinalis* (S). The 2nd cluster contained *T. populneoides* (L/A), *A. marina* (F) and *R. apiculata* (L). The 3rd, 4th, 5th, 6th, 7th, 8th and 9th clusters were formed by *S. maritime* (WP/A), *R. mucronata* (F), *S. portulacastrum* (WP/A), *C. decandra* (F), (L) *R. mucronata* (L) *A. ilicifolius* (L) and *C. decandra* (L) respectively. The differences in the anti-vibrio activity of the extracts might be due to the chemical composition of the plants and solvents used for their extractions [10].

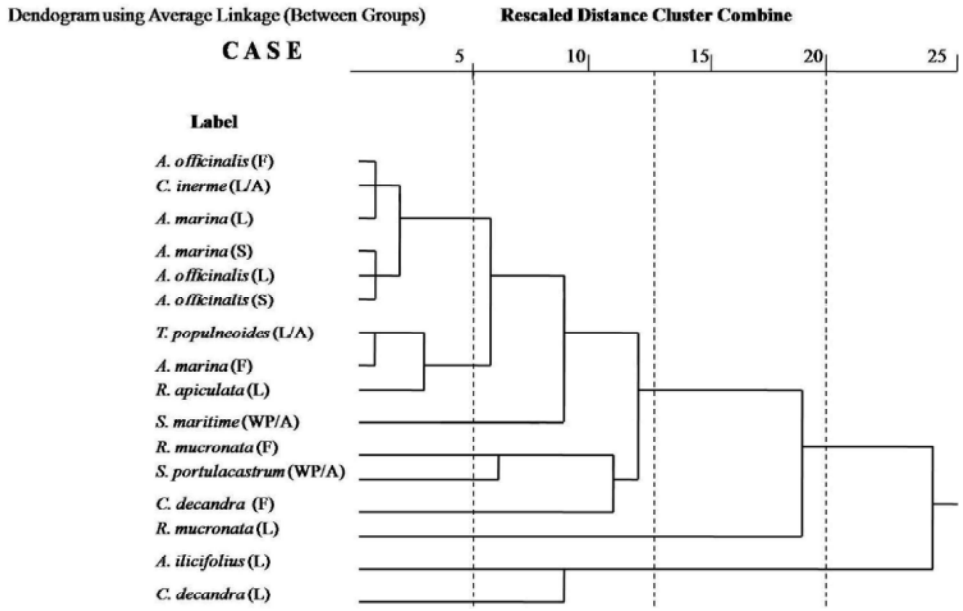

Fig. 4: Cluster analysis showing the similarity groups of different test extracts on their anti-vibrio activity. The dendrogram showing the cluster analysis revealed the response of the tested pathogen to the activity of the different plant extracts. The vertical dashed line (Right to left) indicates 80, 50 and 20% similarity. (L)- Leaves, (S)- Stems, (F)- Fruits, (WP)- Whole Plants, (A)- Mangrove Associates.

Though the leaf extracts of *A. ilicifolius* (L) and *C. decandra* (L) present in a single cluster even at 20% similarity, by considering the individual solvent based activity the methanolic leaf extract of *A. ilicifolius* was selected due to its maximum activity for further studies.

The lowest concentration of acetone extract of *A. ilicifolius* (Leaves) inhibiting the visible growth of *V. harveyi* VSH5 was evaluated through MIC value. The data obtained by the determination of MIC are presented in Table 1. The result revealed that the MIC of the test extract against *V. harveyi* VSH5 was 125 µg/ml. This is in contrast with the study result of Sharma *et al.* [11] who reported that 70% of the tested *Vibrio* pathogens were susceptible to 16 Indian medicinal plants at a concentration ranging between 2.5 and 20mg/ml.

All pure solvents such as hexane, petroleum ether, ethyl acetate and diethyl ether alone did not give a good separation. Combinations of above solvents used as the mobile phase gave well separation. However, when TLC was carried out, the acetone extract of *A. ilicifolius* was separated into five bands (Namely C_1, C_2, C_3, C_4 and C_5) in hexane: diethyl ether (6:4) and petroleum ether: ethyl acetate (7:3). Among the five spots separated in test extract, three spots (C_3, C_4 and C_5) demonstrated high UV active compounds. TLC plates were run in duplicate and one set was used as the reference chromatogram and the other set was assayed for bioautography (Fig. 5A). The bioautography assay for qualitative anti-vibrio detection demonstrated well-defined inhibition zones against *V. harveyi* VSH5 around the active spots. This is in accordance with the study of Sharma *et al*. [11] who reported TLC-bioautography of ethanolic extracts of *Syzygium cumini* and *Lawsonia inermis* exhibiting vibriocidal activity. Bioassays are essential for monitoring the required effects present in the plant samples. In this study TLC based bioautography method was used to detect the active anti-vibrio compounds. Bioautography is a very convenient and simple way of testing curde plant extracts and pure substances for their effects on pathogenic microbes [12]. Many reports revealed that the TLC based bioassay allows in situ detection of active compounds for antibacterials. In the present study, though the bioautography does not give much

Table 1: MIC of acetone extract of *A. ilicifolius* leaves against *V. harveyi* VSH5

		Concentration of the extract (µg/ml)							
Solvent	Control*	7.81	15.62	31.25	62.5	125	250	500	1000
Acetone	+	-	-	-	-	ß	+	+	+

(-) = Resistance (bacterial growth or turbidity), (+) = Concentration showing no turbidity (Bacterial inhibition), (ß) = MIC value, (*) = absence of plant extract.

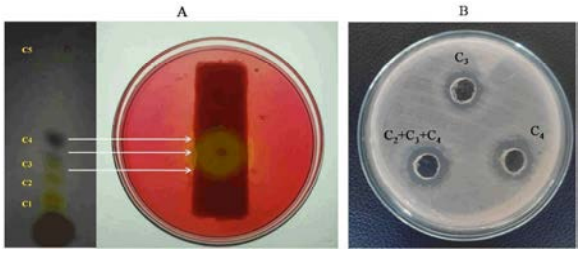

Fig. 5: TLC-Bioautography overlay represents (A) anti-vibrio activity by compounds present in acetone extract of *A. ilicifolius* against *V. harveyi* VSH5. Arrows indicates regions of growth inhibition visualised with tetrazolium red for anti-vibrio with their respective compounds. (B) Semi purified compounds showing anti-vibrio activity by individual (C_3 and C_4) and by combined compounds (C_2+C_3+C_4).

information, studies extended with the semi purified/partially purified compounds (Various spots isolated from TLC) when subjected to analysis individually or in combinations showed interesting results (Fig. 5B). The compounds in individual (C_3 and C_4) and in combination (C_2+C_3+C_4) of acetone extract of *A. ilicifolius* exhibited the anti-vibrio activity with the inhibition zone of 7.1 mm, 7.4 mm and 9.2 mm respectively. In general, application of individual compounds showed poor effectiveness against respective organisms in the assay. Combination treatments such as antibiotic-antibiotic treatments have been discussed by many researchers [13-15]. Plant extract-antibiotic combination studies have also been explored by others [16-18]. This study has demonstrated that the anti-vibrio activity of *A. ilicifolius* acetone extract could be effectively used to control *V. harveyi* in shrimp aquaculture systems with reduced toxicity without the risk of antibiotic resistance, offering a promising alternative to the use of synthetic antibiotics. Further studies are needed to elucidate the active components and mode of action to understand its possibilities and limitations of microbial control in aquaculture.

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