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In Vitro and Experimental Screening of Mangrove Herbal Extract against *Vibrio Alginolyticus in Marine Ornamental Fish*

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Abstract**—**Present study summarizes the control of *Vibrio alginolyticus* infection in hatchery reared Clownfish, *Amphiprion sebae* with the extract of the mangrove plant, *Avicennia marina*. Fishes with visible symptoms of hemorrhagic spots were chosen and the genomic DNA of the causative bacterium was isolated and sequenced based on 16S rDNA gene. The *in vitro* assay revealed that a fraction of *A. marina* leaf extract elucidated with ethyl acetate: methanol (6:4) showed a high activity (28 mm) at 125 µg/ml concentrations. About 4 % of the fraction fed along with live *V. alginolyticus* was significantly decreased the cumulative mortality ($P<0.05$) in the experimental groups than the control group. The responsible fraction was investigated by gas chromatography - mass spectroscopy and found the presence of active compounds. This is the first research in India to control vibriosis infection in marine ornamental fish with mangrove leaf extract.

Keywords**—**Amphiprion seabe, Avicennia marina, Gas Chromatography - Mass Spectroscopy, Vibrio alginolyticus

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I. Introduction

ORNAMENTAL fish keeping is growing in leaps and bounds with an ever-increasing number of hobbyists. In the global scene, fish keeping is considered as the second largest hobby next to photography. Recently, marine ornamental fish has become a significant component in the international aquarium trade due to the technology developments. The importance of this sector in the world trade is one of the sources of income, especially for the coastal rural population in the developing countries. There are approximately 1.5-2 billion people worldwide who keep marine aquaria [1]. Majority of the hobbyists who discontinue fish keeping do so because of the diseases and mortality problems in the aquaria. Infections are found to crop up which can badly affect the profitably of the ventures. Diseases can be caused by variety of factors; the most important one is pathogens. Bacterial disease is the most common problem of ornamental fishes and most infections are caused by gram-negative bacteria. Bacterial fish pathogens are capable of living independently away from the fish host [2]. Pathogenic bacteria cause severe losses in hatchery and grow-out culture systems which are usually related to poor management and water quality. Gram-negative bacteria, especially *Vibrio parahaemolyticus* and *V. alginolyticus,* cause haemorrhagic septicaemia in the hatchery and aquarium systems. Affected fish exhibit signs of weakness, abnormal swimming and occasionally corneal hemorrhage [3].

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Use of antibiotics and chemotherapeutics for prophylaxis and treatment in intensive aquaculture has been widely criticized for their negative impacts like accumulation of drugs in tissues, development of drug resistance and immunosuppression [4]. Alternatively, for some diseases, vaccines against specific pathogens have been developed with varying degree of success. The wide range of pathogens in fish farming also limits the effectiveness of vaccines [5]. Hence, there is an urgent need to look for ecofriendly disease preventative measures to promote sustainable ornamental fish culture. The bark, leaves and fruits of *A. marina* are used in traditional medicine to treat skin diseases [6]. In this context, experiment was conducted to make a treatment trial against the haemorrhagic septicemia infection in the marine ornamental fish, *Amphiprion sebae* using *A. marina* leaf extract.

II. Materials and Methods

A. Infected fish collection and bacteria isolation

Diseased juveniles of common Clownfish, Amphiprion sebae with 90 % mortality were collected from the Marine Ornamental Fish Breeding Hatchery of the Centre of Advanced Study in Marine Biology, Annamalai University. Liver, kidney and spleen of the freshly dead fishes were aseptically removed, homogenized and serially diluted with PBS up to 10^{-5} and plated on zobell marine agar 2216 (ZMA) (Hi Media, Mumbai) with 50% seawater. After incubation at 28°C for 48 hours, the uniform colonies were taken for further culture [7].

B. Biochemical identification of pathogens

The isolated bacteria characterized based on morphology and biochemical characters by AP 20 I E Kit.

C. 16 S rDNA sequence analysis

The genomic DNA of bacterial isolates was extracted from pure cultures of the bacterial isolates according to phenol chloroform method. Universal primers such as, 5′ - AGAGTTTGATCCTGGCTCAG - 3′ (8F) and 5′ - GGTTACCTTGTTACGACTT - 3′ (1492R) were used to amplify 1500 base pairs of 16 S rRNA genes. PCR reaction were carried out with a 25 µl reaction volume containing 13 µl of sterilized distilled water, 0.5 µl deoxynucleoside triphosphate (dNTP 10 mM), 2.5 µl 10 X buffer, 1.5 µl $Mgcl_2$ (50 mM), 2 µl of each primer (10 mM) 1 µl Taq DNA polymerase and 2.5 µl DNA template containing approximately 50 ng DNA. PCR was performed in an Eppendorf Mastercycler Gradient thermal cycler and the thermal cycle profile was 35 cycles of denaturation at 95°C for 1 min, annealing at 48° C for 1 min and extension at 1.5 min with initial denaturation at 95°C 6 min. PCR products were electrophoresed on 1 % agarose gel and confirmed with

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GEL Documentation system (Apha innotech, major EC). Sequencing was performed by automatic sequencing method. The derived sequences were aligned and compared with other bacterial 16 S rDNA sequences available in GenBank of NCBI database.

D. Fish acclimatization and challenging study

Healthy clownfish, *A. sebae* weighing approximately 10 ± 0.5 g were obtained from our marine ornamental fish hatchery unit. After acclimation for 4 days, the fish were challenged with 3 X 10^7 colony forming unit of *V. alginolyticus* of pellet. Control group were exposed to PBS without bacteria. Once the fish were exposed to the bacterial isolates, they were observed up to 90 % mortality.

E. Sample collection and partial purification of mangrove plant leaves

Leaves of *A. marina* were collected from the Vellar estuary, Parangipettai (Lat. 11°29'N, Long. 79°46'E), situated in southeast coast of India. After washing with distilled water, the leaves were shadow dried and powdered. Leaf powder was loaded onto silica gel column pack and elucidated with hexane and chloroform (10:0 to 0:10) followed by ethyl acetate and methanol (10:0 to 0:10). Totally, 40 fractions were collected and each individual fraction was tested for the i*n vitro* potential.

F. Antibacterial activity

Antibacterial susceptibility tests were performed on Muller-Hinton Agar medium. A lawn culture was done (0.5 McFarland) over the surface of the media using sterile cotton swab to ensure the confluent growth of the organism. Sterile discs (5 mm) were prepared with Whatman No.1 paper immersed in 300 µg of individual fractions. Tetracycline (30 µg/disc) was used as positive control to determine the sensitivity of the tested strains and the appropriate solvents were used as negative control. The inoculated plates were incubated at 30 ^{0}C for 24 h and the inhibition zones were observed. All the experiments were carried out in triplicate [8].

G. Assessment of minimum inhibitory concentration (MIC)

A fraction (16th fraction) showing conspicuous results in antibacterial assay was subjected to MIC assay. Then it was dissolved in phosphate buffer saline (PBS) to obtain 2000 µg /ml stock solution. An aliquot of 0.5 ml stock solution was incorporated into 0.5 ml of Muller–Hinton broth to make various concentrations. The stock concentration was serially diluted and analysed for MIC. A 50 µl aliquot of standard suspension of the test organism (10^6 c.f.u./ml) *V. alginolyticus* was transferred to each test tube. The crude mangrove leaf extract and PBS were used as positive and negative control, respectively. The tubes were incubated at 30 ^{0}C for 24 h and the results were recorded at 620 nm in UV–visible spectrophotometer [9].

H. Preparation of mangrove extract coated feed

Partially purified mangrove leaf extract concentrations viz, 0, 100, 200 and 400 mg/kg were mixed with the pellet feed and the experimental animals were fed with 2 % of their body weight throughout the experiment [10].

I. Oral administration of the mangrove leaf extracts coated feed and challenge with V. alginolyticus

All the 4 experimental groups (10 animals/group) were fed with the feed formulation containing 0, 100, 200 and 400 mg/kg of the extract mixture. The water quality parameters were observed every day and on 10th day, the experimental fishes were challenged with the optimized lethal dose (3×10^7 c.f.u) of *V. alginolyticus* and control fish were also exposed with the same dose of PBS buffer. The fishes were observed for pathological changes. All the experimental groups were maintained in triplicate.

J. Gas chromatography and mass spectroscopy (GC-MS) analysis

The GC-MS analysis of the active fraction was performed in a Clarus 500 Perkin Elmer gas chromatography equipped with an Elite-5 capillary column (5 % phenyl 95 % dimethyl poly siloxane) (30mx250µm) and mass detector turbo mass gold of the company which was operated in EI mode. Helium was the carrier gas at a flow rate of 1ml/min. The injector was operated at 290 °C and the oven temperature was programmed as 70 °C for 6min to 150 ^{0}C (5 min), it was gradually increased to 280 °C at 10 min. The identification of components was based on comparison of their mass spectra with those of NIST 2005 Library.

K. Statistics

Data were analyzed using one-way analysis of variance (ANOVA) to find out the significant difference at the 5 % ($P < 0.05$) level.

III. RESULTS

A. Signs of the disease and morphological observation

Infected fishes showed haemorrhage nearer to the caudal fin, erosion of pectoral fins internally (Fig.1). Clinical signs included enlarged spleen and bleeding in the internal organs. In the microscopical examination of the gill and body surfaces of the diseased fish, no parasites and protozoans were observed. As, unique bacterial colonies that grew on the Zobell Marine Agar (ZMA) and Thio-sulfate Citrate Bile salt Sucrose agar (TCBS), a single colony was selected for further characterization and identification. The pure culture of the bacterial isolate exhibited typical morphological characteristics, such as Gram negative, motile and comma shaped nature.

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Fig. 1 Infected Clownfish, *Amphiprion sebae* showing symptoms of haemorrhage near to the caudal fin and erosion of pectoral fins

B. Physiological and biochemical characterization

The bacteria produced yellow colour colonies on the TCBS agar. They showed swarming motility and positive results in methyl red, Voges proskauer, catalase and cytochrome oxidase and negative results in the tests viz, indole production, citrate utilization, H_2S production, urease, tryptophane deaminase, β – galactosidase, lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase. The strain produced acid from glucose, mannitol, sucrose, maltose, saccharose and arabinose and did not produce acid from inositol, sorbitol, rhamnose, lactose, galactose and melibiose. It could also produce enzymes like amylase, lipase and gelatinase. The bacteria produced colonies in 1 %, 2 %, 4 %, 6 %, 8 % and 10 % of NaCl but did not grow in 0 % and 0.5 %).

C. 16 S rDNA sequence analysis

In order to determine the taxonomic positions of the bacteria, the sequence of 16S rRNA gene was amplified (1310 base pairs) and compared with the related 16S rRNA sequence of bacteria in GenBank. By nucleotide BLAST search in NCBI website, the bacterial isolate was identified as *Vibrio alginolyticus* (GenBank accession number - JN820097) (Fig.2).

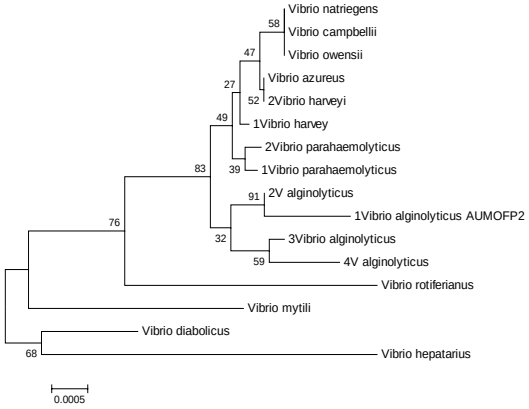


Fig. 2 Phylogenetic tree constructed through ClustalX2 model using the neighborhood – joing method. Bar 0.0005.

D. Induced infection assay

In the challenge experiment, challenge dose was standardized as 3 X 10^7 colony forming units (c.f.u.) to give 90 % mortality in the challenged group. Mortality was recorded on day 2 to 5. The clinical symptoms were noted including hemorrhagic septicemia, skin muscles with faded pigments and lesions on the ventral surface of the body. After the symptoms characterization, the bacterial culture was re-isolated from the infected fishes and confirmed as *V. alginolyticus* based on the colony morphology on TCBS agar medium and conformational biochemical tests.

E. In vitro activity of mangrove leaf extract against the bacterial isolates

Based on the results, 16th fraction elucidated with ethyl acetate: methanol (6:4) expressed maximum activity (28 mm) and the minimum inhibitory concentration (MIC) was 125 µg/ml. It indicated that, the concentration was able to control the bacterial growth, *in vitro*.

F. Disease resistant experiment

In the challenge studies, the control group had highest and fastest mortality (100 %) within 6 days.

Survival was significantly increased ($P < 0.05$) in the groups fed with the diets incorporated with mangrove leaf extract. The maximum survival of 60 % was observed at 400 mg/kg diet followed by 50 % and 30 % at the 200, 100 mg/kg diets, respectively (Fig. 3).

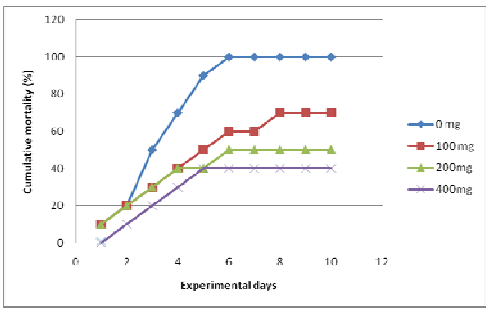


Fig.3. Confirmation of the protective effect of the partially purified extract of *Avicennia marina* and cumulative percent mortality (%) on the challenged *Amphiprion sebae*

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G.Gas chromatography and mass spectroscopy (GC-MS)

In the GC-MS results, 5 bioactive compounds were identified from the partially purified extract of the mangrove plant (Fig. 4). The identification was performed based on the peak area, molecular weight and molecular formula. The n-Hexadecanoic acid was recorded as maximum level at the retention time of 30.94 with 10.7026 % of peak value followed by 2-Cyclohexen-1-one, 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl) (8.6 %), Phytol (8.4 %), Hexadecanoic acid, ethyl ester (6.2 %) and 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (4.9 %) at the retention time of 27.88, 33.27, 31.23 and 28.27 respectively. So, it can be concluded that these five compounds are active molecules in the partially purified fraction, responsible for the inhibition of test bacterium.

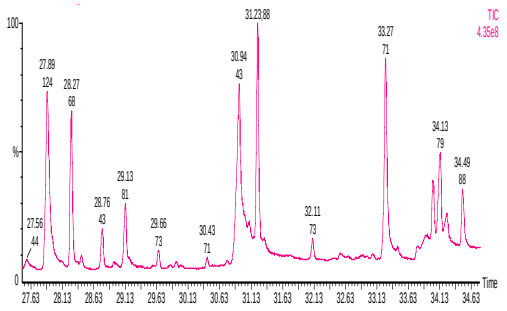


Fig.4. GC-MS chromatogram of the partially purified active fraction of *A. marina* leaf extract

IV. Discussion

Marine ornamental aquaculture is an emerging field in the fishery industry and the development of commercial synthetic sea salt mix, variety of water chemistry monitoring kits and different filtration units that support the fish displays even in non-coastal regions. *Vibrio alginolyticus* is an important aquatic bacterial pathogen, which could be caused heavy mortality in shell fish and finfish [11]. Treatment of these infected fishes has not yet been studied properly.

Mangroves are the ancient Indian systems of health care [12] and their associates have also been used for medical purposes worldwide and 349 metabolites have been recorded. Mangrove contains steroids, diterpenes and triterpenes, saponins, flavonoids, alkaloids and tannins [13]-[17].

Present study, which is first of its kind, focuses attention on the use of mangrove leaf extract to control bacterial infection in Clownfish, the most sought after ornamental fish. Extract of *A. marina* leaves collected from the Vellar estuary was tested against *V. alginolyticus* and used for herbal diet as prophylaxis in experimental model. The partially purified leaf extract of *A. marina* showed a fine antibacterial property in cultured Clownfish, *A. sebae*. The extract elucidated with ethyl acetate and methanol (6:4) was administered along with diet and challenged with *V. alginolyticus.* The experimental groups were protected from the infection mortality than the control group.

Likewise, certain other mangroves like *Rhizophora mucronata*, *R. lamarkii* and *Bruguiera cylindrica* were found to have antibacterial potential against clinical and aquatic bacteria [18]. The methanol extracts of *Excoecaria agallocha* showed the highest mean zone of inhibition (22.5 mm) against MRSA. The MIC of this plant extract was 0.125 mg/mL and the MBC was 0.25 mg/mL. In the present study, partially purified leaf extracts of *A. marina* elucidated with ethyl acetate: methanol (6:4) expressed maximum activity (28 mm) against *V. alginolyticus* and minimum inhibitory concentration (MIC) was 125 µg/ml concentration. In the fish juveniles fed with mangrove leaf herbal diet, survival rate increased (above 60 %) than the fishes fed with the control diet, even after the 10th day of challenging.

Peptidoglycan derived from *Bifidobacterium thermophilum* mixed with pellet feed was significantly improved *Vibrio penaeicidae* compared to the control group [19]. Several researchers have developed an artemia-enriched herbal diet for *Penaeus monodon* with the combination of five herbs, which significantly increased the growth and survival rates during stress conditions [20]. Also, feeding with *Withamia somnifera*, *Ferula asafoetida*, and *Mucuna pruriens* remarkably accelerated the spawning rate, fecundity and larval quality of *P. monodon* in hatcheries [21]. Similarly, several herbal principles have been tested for their growth-promoting activity in aquatic animals [20], [22].

Hitherto, reports on the diseased ornamental fishes treated with herbal dietary are few. Present study used the mangrove leaf extract and the clownfish fed increased the survival rate of the fish, after challenging with the pathogenic bacterium.

Partially purified fraction of the mangrove leaf extract was analyzed by GC-MS and the peaks showed the presence of hexadecanoic acid, Cyclohexen-1-one, 4-hydroxy-3, 5, 5-trimethyl-4-(3-oxo-1-butenyl), Phytol, ethyl ester and 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol from the active fraction. These compounds were previously reported as potential compounds to have antimicrobial properties from mangrove and other plants [23] – [27].

Present study has revealed that the feed incorporated with *A. marina* leaf extracts can increase the survival and control the infection caused by of *V. alginolyticus* in the marine ornamental fish, *A. sebae*. Based on the available literature, this is the first report about controlling *V. alginolyticus* infection in cultured clownfish with mangrove leaf extract. Further research has to include the mode of action for inhibition of the pathogenic bacterium, increase the immunological parameters to disease resistance, etc.

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